Protein kinase IKKβ-catalyzed phosphorylation of IRF5 at Ser462 induces its dimerization and nuclear translocation in myeloid cells

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The siRNA knockdown of IFN Regulatory Factor 5 (IRF5) in the human plasmacytoid dendritic cell line Gen2.2 prevented IFNβ production induced by compound CL097, a ligand for Toll-like receptor 7 (TLR7). CL097 also stimulated the phosphorylation of IRF5 at Ser462 and stimulated the nuclear translocation of wild-type IRF5, but not the IRF5[Ser462Ala] mutant. The CL097-stimulated phosphorylation of IRF5 at Ser462 and its nuclear translocation was prevented by the pharmacological inhibition of protein kinase IKKβ or the siRNA knockdown of IKKβ or its “upstream” activator, the protein kinase TAK1. Similar results were obtained in a murine macrophage cell line stimulated with the TLR7 agonist compound R848 or the nucleotide oligomerization domain 1 (NOD1) agonist KF-1B. IKKβ phosphorylated IRF5 at Ser462 in vitro and induced the dimerization of wild-type IRF5 but not the IRF5[Ser462Ala] mutant. These findings demonstrate that IKKβ activates two “master” transcription factors of the innate immune system, IRF5 and NF-κB.

IKKβ | interferon β | IRF5 | plasmacytoid dendritic cell | TLR7

The transcription factor IFN Regulatory Factor 5 (IRF5) has a critical role in the production of proinflammatory cytokines. The secretion of interleukin 12 (IL-12), IL-6, and TNFα by ligands that activate Toll-like receptor 3 (TLR3), TLR4, TLR5, and TLR9, is greatly impaired in macrophages, conventional dendritic cells (cDCs), and plasmacytoid dendritic cells (pDCs) of mice that do not express IRF5 (1). However, the role of IRF5 in type 1 IFN production in pDCs has been less clear. The TLR9-stimulated secretion of IFNα was initially reported to be similar in pDCs from IRF5-deficient and wild-type mice (1), but a later study found that the TLR7- or TLR9-stimulated production of IFNα was reduced in pDCs from IRF5-deficient mice (2). Subsequently, some IRF5-deficient mouse lines were shown to carry a second mutation in the gene encoding the guanine nucleotide exchange factor (dedicator of cytokinesis 2 (Dock2), and it was reported that TLR9-stimulated IFNα secretion was largely intact in pDCs from mice where this secondary mutation had been eliminated (3). A further study using pDCs from IRF5-deficient mice, carrying or not carrying the Dock2 knockout, confirmed that IRF5 had little effect on TLR9-stimulated IFNα secretion, but indicated an important role for IRF5 in the secretion of IFNβ (4). IRF5 was also required for the TLR9-stimulated production of IFNβ in the human pDC line CAL-1 (5) and for the production of IFNβ induced by streptococcal RNA in cDCs (6) and by the fungal pathogen Candida albicans, in a pathway dependent on the Dectin 1 and Dectin 2 receptors (7). IRF5 was also found to be needed for the production of IFNβ by a small subset of viruses (8, 9). Thus, IRF5 does appear to be a key regulator of IFNβ production by several pathogens.

IRF5 is present in a latent form in the cell cytosol but accumulates in the nucleus to stimulate gene transcription following viral infection (10, 11) or stimulation with the TLR7/TLR8 agonist R848 (9). The nuclear export signal (NES) (12) of IRF5 therefore appears to be dominant over the two nuclear localization signals (8) in uninfected/unstimulated cells. R848 also stimulated the transcription of an IRF5 reporter gene in HEK293 cells overexpressing TLR7 or TLR8, and this was accompanied by the translocation of an IRF5–GFP fusion protein from the cytosol to the nucleus (9). Taken together, these findings indicated that IRF5-dependent gene transcription requires the translocation of the transcription factor to the nucleus.

The production of IFNβ triggered by ligands that activate TLR3 and TLR4, or by viruses that form double-stranded (ds) RNA during their replication, does not depend on IRF5, but instead requires the phosphorylation of IRF3 catalyzed by the IkB kinase (IKK)-related kinase TANK-binding kinase 1 (TBK1) (13–15). TBK1 was reported to phosphorylate a GST–IRF5 fusion protein in vitro, whereas IKKβ did not (9), and the TLR7-stimulated activation of a Gal4–IRF5 reporter gene was inhibited by the overexpression of a catalytically inactive mutant of TBK1 or the related IKKe. Based on these experiments, it was suggested that TBK1/IKKε might activate IRF5 as well as IRF3. Two serines in IRF5, Ser158 and Ser309, were subsequently identified as amino acid residues that became phosphorylated when DNA vectors encoding IRF5 and TBK1 were coexpressed in cells (16).

Here we demonstrate that the TLR7 agonist CL097 induces a striking increase in the phosphorylation of the endogenous IRF5 at Ser462 in the human pDC cell line Gen2.2 and establish that the phosphorylation of this site is required for the dimerization and nuclear translocation of IRF5. We also show that phosphorylation of Ser462 is needed for the nuclear translocation of IRF5 in the macrophage cell line RAW264.7 by the TLR7 agonist R848 or the siRNA knockdown of IKKβ or its “upstream” activator, the protein kinase TAK1. Similar results were obtained in a murine macrophage cell line stimulated with the TLR7 agonist compound R848 or the nucleotide oligomerization domain 1 (NOD1) agonist KF-1B. IKKβ phosphorylated IRF5 at Ser462 in vitro and induced the dimerization of wild-type IRF5 but not the IRF5[Ser462Ala] mutant. These findings demonstrate that IKKβ activates two “master” transcription factors of the innate immune system, IRF5 and NF-κB.

Significance

NF-κB and IFN Regulatory Factor 5 (IRF5) are required for the transcription of many proinflammatory cytokines in myeloid cells. The protein kinase IKKβ is the major activator of NF-κB but how IRF5 is activated has been unclear. This paper demonstrates that IKKβ also activates IRF5 by catalyzing the phosphorylation of Ser462. The phosphorylation of this serine induces the dimerization of IRF5 and its translocation to the nucleus. The activation of the master transcription factors of the innate immune system by the same protein kinase provides a mechanism for the coordinated control of IRF5 and NF-κB in response to inflammatory stimuli.

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Results

IFNβ Is Required for IFNβ Production in Gen2.2 Cells. It is widely accepted that the high level of expression of the transcription factor IRF7 in pDCs underlies the ability of these cells to produce large amounts of type 1 IFNs in response to ligands that activate TLR7 or TLR9 (17). In view of emerging evidence that IRF5 may be important for the production of IFNβ (see Introduction) we decided to reinvestigate the relative importance of IRF5 and IRF7 in stimulating transcription of the IFNβ and IFNα1 genes in Gen2.2 cells, which is triggered by stimulation with the TLR7 ligand CL097 (18). We found that the siRNA “knockdown” of IRF5 (Fig. S1A) prevented the CL097-stimulated production of IFNβ mRNA (Fig. 1A) or IFNβ secretion (Fig. 1B). In contrast, the CL097-stimulated production of IFNα1 mRNA in human Gen2.2 cells was consistently enhanced by the siRNA knockdown of IRF5 (Fig. 1C). The secretion of IFNα1 could not be determined as it was below the level that can be detected by ELISA. These findings contrast with earlier reports that the TLR9-stimulated secretion of IFNα1 from IFNα1-deficient mice (1, 3, 4). Control experiments showed that the siRNA knockdown of IRF5 in Gen2.2 cells greatly decreased the production of IL-12 mRNA (Fig. S1B and C), in line with earlier studies performed in other immune cells (see Introduction). However, the TLR7-stimulated production of IL-6 mRNA was unaffected by the knockdown of IRF5 (Fig. 1D). This result also differs from an earlier report in which the TLR9-stimulated production of IL-6 mRNA was suppressed in spleen-derived pDCs from IRF5 knockout mice (1).

In contrast to IRF5, the siRNA knockdown of IRF7 (Fig. S1A) prevented the induction of IFNα1 mRNA (Fig. 1C) and reduced the production of IFNα1 mRNA significantly (Fig. 1A). Consistent with the latter finding, the secretion of IFNα1 was decreased by 50% at each time point examined (Fig. 1B). The knockdown of IRF7 did not affect the production of IL-6 (Fig. 1D), IL-12p40 (Fig. S1B), or IL-12p70 significantly (Fig. S1C). Taken together, our results support the emerging consensus (see Introduction) that IRF5 has a critical role in the production of IFNβ, whereas IRF7 is critical for the production of IFNα1. In overexpression experiments IRF5 and IRF7 form heterodimers and the IRF5/IRF7 heterodimer is reported to be less active than the IRF7 homodimer in stimulating IFNα1 gene transcription (19). This could explain why the knockdown of IRF5 enhances the CL097-stimulated production of IFNα1 mRNA. Heterodimer formation might also explain why the IRF5-dependent production of IFNβ is enhanced by IRF7, if the IRF5/IRF7 heterodimer is more efficient than the IRF5 homodimer in stimulating IFNβ gene transcription. The knockdown of IRF3 (Fig. S1A) did not affect the CL097-stimulated production of IFNα1, IFNβ, or any other cytokine measured significantly (Fig. 1 and Fig. S1).

The CL097-Stimulated Phosphorylation of IRF5 at Ser462 Is Catalyzed by IKKβ. The results presented in Fig. 1 raised the question of how IRF5 might be activated to stimulate IFNβ gene transcription. We therefore used SILAC in conjunction with mass spectrometry as an unbiased approach to identify proteins that became phosphorylated when Gen2.2 cells were stimulated with CL097 (Fig. S2A). A tryptic peptide with a molecular mass corresponding to amino acid residues 459–467 plus one phosphate group (the numbering corresponds to the longest alternatively spliced variant of IRF5) was detected in every experiment, and analysis of the fragment ions generated identified Ser462 as the site of phosphorylation, the only Ser/Thr residue in this peptide. The phosphorylation of Ser462 increased 27-fold ± 7.5-fold (±SEM for 10 independent experiments) after stimulation for 30 min with CL097 (Fig. 2A). No other phosphorylation site in the endogenous IRF5 was detected in these experiments either before or after stimulation with CL097.

We have reported that the CL097-stimulated production of IFNβ mRNA and IFNβ secretion in Gen2.2 cells is prevented by the siRNA knockdown or pharmacological inhibition of IKKβ (18). The molecular mechanism(s) was not identified in these studies, but was largely independent of the activation of the transcription factor NF-κB. We therefore performed additional SILAC mass spectrometry experiments to investigate whether the phosphorylation of IRF5 at Ser462 was dependent on IKKβ activity. We found that the CL097-stimulated phosphorylation of IRF5 was prevented by compound BI605906 (Fig. 2A and Fig. S2 B and D), the most specific inhibitor of IKKβ so far identified, which does not inhibit IKKα or the IKK-related kinases, termed TBI1 and IKKe (20). Consistent with this observation, the phosphorylation of the endogenous IRF5 was also suppressed by compound NG-25 (Fig. 2A and Fig. S2 C and D), a potent and relatively specific inhibitor of TAK1, the protein kinase that activates IKKβ.

The suppression of IRF5 phosphorylation at Ser462 by inhibitors of IKKβ or TAK1 did not establish that IKKβ catalyzed the phosphorylation of Ser462 directly, because IKKβ might have exerted its effect indirectly by phosphorylating and activating another protein kinase, which then catalyzed the phosphorylation of Ser462. Indeed IKKβ was reported to be unable to phosphorylate IRF5 in vitro (9). In contrast, we found that purified IKKβ and, to a lesser extent, IKKe did phosphorylate IRF5 in vitro (Fig. 2B). Moreover, IKKβ and IKKe phosphorylated IRF5 at Ser462 (Fig. 2C), as judged by immunoblotting with an antibody that recognizes IRF5 phosphorylated at Ser462, but not the IRF5[S462A] mutant (Fig. 2D, Upper). Purified TBI1 phosphorylated purified IRF5 more robustly than IKKβ or IKKe in vitro (Fig. 2B), but did not phosphorylate IRF5 at Ser462 (Fig. 2C). The IKKβ-catalyzed phosphorylation of IRF5 was suppressed by BI605906 and the TBI1-catalyzed phosphorylation of IRF5 by compound

Fig. 1. IRF5 is required for CL097-stimulated IFNβ production but not for IFNα1 or IL-6 production in Gen2.2 cells. (A–D) Gen2.2 cells were transfected with siRNA against IRF3 (hatched bars), IRF5 (white bars), IRF7 (gray bars), or a control siRNA (black bars). After 72 h, the cells were stimulated for the times indicated with 1 μg/ml CL097. The mRNA encoding IFN-β (A), IFN-α1 (C), and IL-6 (D) was then measured in duplicate by qRT-PCR. Graphs show the fold increase in mRNA production relative to that measured in cells treated with control siRNA and not stimulated with CL097 and are presented as the mean ± SD for one representative experiment. The mRNA levels were normalized to 18S rRNA. (B) As in A, except that IFNβ secreted into the culture medium was measured by ELISA. All experiments (A–D) were repeated three times with similar results.
The Phosphorylation of IRF5 at Ser462 Is Required for Nuclear Translocation. To study the role of Ser462 phosphorylation, we initially expressed DNA encoding an IRF5–GFP fusion protein in Gen 2.2 cells. The IRF5–GFP was excluded from the nucleus but underwent partial translocation to the nucleus within 30 min of stimulation with CL097. In contrast, CL097 did not stimulate the nuclear translocation of the IRF5[S462A] mutant, suggesting a critical role for Ser462 phosphorylation in the nuclear accumulation of IRF5 (Fig. 3A and Fig. S3A).

The Nuclear Translocation of IRF5 Is Prevented by IKKβ or TAK1 Inhibitors. Because IKKβ phosphorylated IRF5 at Ser462 in Gen 2.2 cells (Fig. 2C) and in vitro (Fig. 2B and C), and the CL097-stimulated phosphorylation of Ser462 was required for the nuclear translocation of IRF5 in Gen 2.2 cells (Fig. 3A and Fig. S3A), we investigated whether nuclear translocation of IRF5 was prevented by the inhibition of IKKβ or its upstream activator TAK1. We found that the CL097-stimulated nuclear translocation of IRF5 was prevented by two structurally unrelated inhibitors of IKKβ, BI605906 and compound PS1145 or by the TAK1 inhibitor NG-25 (Fig. 4A and Fig. S3B). In contrast, the TBK1/IKKε inhibitor MRT67307 did not block the nuclear translocation of IRF5 (Fig. 4A and Fig. S3B) at a concentration that inhibited the

MRT67307 (Fig. 2B and C), a potent inhibitor of TBK1 and IKKe that does not inhibit IKKβ or IKKe (20). These control experiments demonstrated that the phosphorylation of IRF5 was catalyzed by IKKβ and TBK1 and not by another kinase(s) that might have been present in the preparations as a contaminant.

The cotransfection of FLAG–IRF5 with HA–IKKβ, but not the catalytically inactive mutant IKKβ[D166A], induced the phosphorylation of IRF5 at Ser462 (Fig. 2D, Top) and the dimerization of IRF5 (Fig. 2D, Bottom). Importantly, only the dimeric IRF5 was phosphorylated at Ser462 (Fig. 2D, Middle), strongly suggesting that dimerization was triggered by the phosphorylation of Ser462. This was confirmed by the finding that the IRF5[S462A] mutant did not dimerize when cotransfected with IKKβ (Fig. 2D, Bottom). These experiments also established the specificity of the antibody for the Ser462 phosphorylation site on IRF5 (Fig. 2D, Top).

Fig. 2. IRF5 is phosphorylated by IKKβ at Ser462 in Gen2.2 cells and in vitro. (A) SILAC-labeled Gen2.2 cells were incubated without (−) or with (+) BI605906 or NG-25 stimulated for 30 min without (−) or with (+) 1.0 μg/mL CL097 and the fold increase in the abundance of the trypic phosphopeptide LQISpNPDLK (where Sp is phosphoserine) corresponding to amino acid residues 459–467 of IRF5 was quantified as described in Materials and Methods, relative to the level in unstimulated cells (mean ± SEM). The number of independent experiments performed is indicated in parentheses. (B and C) Flag–IRF5 was phosphorylated with His6–IKKβ, GST–IKKα, or GST–TBK1 in the presence or absence of BI605906 or MRT67307 using [γ32P]ATP (8) or unlabeled ATP (C) and the incorporation of 32P-radioactivity into proteins or phosphorylation of Ser462 of IRF5 examined by autoradiography (B, Upper) or immunoblotting (C). The gel in B was also stained with Coomassie blue. (D, Lower) HEK293T cells were transfected with DNA encoding wild-type (WT) HA–IKKβ or the catalytically inactive HA–IKKβ[D166A] mutant and either Flag–IRF5[WT] or the Flag–IRF5[S462A] mutant. The cell extracts were subjected to SDS/PAGE (Upper) or native gel electrophoresis (Lower two panels) and immunoblotted with the antibodies indicated. Similar results were obtained in three independent experiments.

Fig. 3. The mutation of Ser462 to Ala prevents the nuclear translocation of IRF5 induced by TLR7 agonists. (A) Gen2.2 cells were transfected with IRF5–GFP or IRF5[S462A]–GFP. After 48 h, the cells were stimulated for 30 min with 1.0 μg/mL CL097 or left unstimulated. The cells were fixed, centrifuged onto precoated slides, permeabilized, and stained with anti-GFP or DAPI to reveal nuclei. (B) Same as A, except that RAW264.7 cells were stimulated with R848 (1.0 μg/mL).
lipopolysaccharide/TLR4-stimulated phosphorylation of IRF3 at Ser396 in macrophages (Fig. S4), which is dependent on TAK1/IKKβ catalytic activity (20).

Overexpression studies can sometimes cause the specificity of signaling to break down and we therefore also studied the nuclear accumulation of the endogenous IRF5. These experiments showed that a small proportion of the endogenous IRF5 underwent nuclear translocation in response to CL097, which was decreased by the IKKβ inhibitors BI605906 and PS1145, and even more strongly by TAK1 inhibitor NG-25, whereas the TAK1 inhibitor MRT67307 was without effect (Fig. 5A). The CL097-stimulated nuclear translocation of IRF5 was also suppressed by siRNA knockdown of IKKβ (Fig. 5B). Interestingly, the knockdown of IKKα did not affect the CL097-stimulated nuclear accumulation of IRF5 significantly, but in combination with the IKKβ inhibitor BI605906 completely prevented the nuclear accumulation of IRF5 (Fig. 5C). Similar observations were made when the nuclear accumulation of p65/RelA subunit of NF-κB was studied (Fig. 5B and C). These experiments suggest that IKKα may make a minor contribution to the nuclear accumulation of IRF5 or p65/RelA in Gen2.2 cells, if IKKβ is inhibited. The knockdown of TAK1, also abolished the nuclear accumulation of IRF5 (Fig. 5D). This is as expected because TAK1 is required for the activation of IKKα and IKKβ.

The TLR7-Stimulated Nuclear Translocation of IRF5 at Ser462 in RAW264.7 Cells Requires IKKβ Activity. IRF5 is required for the production of proinflammatory cytokines, such as IL-12 and TNF in macrophages and conventional dendritic cells (1). We found that the TLR7 agonist R848 stimulated the nuclear translocation of IRF5–GFP, but not the IRF5[S462A]–GFP mutant, in the murine RAW264.7 macrophage-like cell line (Fig. 3B and Fig. S3C). Moreover, the IKKβ inhibitors BI605906 and PS1145 or the TAK1 inhibitor NG-25 prevented the nuclear translocation of IRF5–GFP, whereas the TKB1/IKKα inhibitor MRT67307 (Fig. 4B and Fig. S3D), the p38 MAP kinase inhibitor compound BIRB0796 (21), the mitogen-activated protein kinase or extracellular signal regulated kinase 1/2 (MEK1/2) inhibitor compound PD0325901 (22), or the combination of both BIRB0796 and PD0325901 (Fig. S5A) did not. In control experiments BIRB0796 blocked the phosphorylation of MAPKAP kinase-2 (MK2), a substrate of p38α MAP kinase, whereas PD0325901 suppressed the phosphorylation of extracellular signal regulated kinases 1 and 2 (ERK1 and ERK2), as expected. The phosphorylation of mitogen- and stress-activated kinases 1 and 2 (MSK1/MSK2), which is catalyzed by both p38α MAP kinase and ERK1/2 (23), was prevented by a combination of BIRB0796 and PD0325901 (Fig. S5B).

**Discussion**

In this study, we have established that the IKKβ-catalyzed phosphorylation of Ser462 is required for its dimerization and nuclear localization of IRF5 by ligands that activate TLR7 in myeloid cells. The mutation of Ser462 to Ala or the suppression of Ser462 phosphorylation with inhibitors of the activity or activation of IKKβ blocked the nuclear entry of IRF5 in response to ligands that activate TLR7. The results provide a molecular explanation for our earlier finding that the TLR7-stimulated nuclear translocation of IRF5 at Ser462 in macrophages (Fig. S4), which is dependent on TAK1/IKKβ catalytic activity (20).
The production of IFNβ in the human pDC line Gen2.2 is dependent on IKKβ activity, but independent of the activation of NF-κB (18). Ser462 of IRF5 is conserved in all vertebrate orthologs of this protein (Fig. 6A). Interestingly, this serine is situated at a position equivalent to Ser396 of IRF3 (Fig. 6B), whose phosphorylation by TBK1 is important for the nuclear translocation of IRF3. Ser462 of IRF5 is positioned within hydrogen-bonding distance of Arg354, an arginine residue that is conserved in IRF3 (24). The phosphorylation of Ser462 of IRF5 or Ser396 of IRF3 may therefore induce an interaction between the phosphoserine and arginine residues that stabilizes the dimeric form of IRF5 and permits its nuclear translocation and the stimulation of gene transcription. Consistent with this notion, we observed that IKKβ induced the dimerization of wild-type IRF5, but not the IRF5[S462A] mutant in cotransfection experiments, and that only the dimeric form was phosphorylated at Ser462 (Fig. 2D). The IKKβ-catalyzed phosphorylation of IRF5 at Ser462 may underlie the activation of this transcription factor by many agonists, because compound KF-1B, a ligand that activates the cytosolic NOD1 receptor, also stimulated the nuclear translocation of IRF–GFP, but not the IRF5[S462A]–GFP mutant in RAW264.7 cells, and translocation was suppressed by the inhibition of IKKβ or TAK1, but not by the inhibition of TBK1/IKKa (Fig. S6).

Ser462 was the only phosphorylation site in the endogenous IRF5 that we detected in 10 independent SILAC-mass spectrometry experiments performed in TLR7-stimulated Gen2.2 cells. In contrast, other investigators identified six phosphorylation sites when IRF5 was overexpressed in HEK293 cells with either TBK1, TRAF6, or Receptor-Interacting Protein Kinase 2 (RIPK2) (16). Cotransfection with TBK1 induced the phosphorylation of IRF5 at Ser158 and Ser309, whereas four other sites (Thr10, Ser317, Ser451, and Ser462) were phosphorylated after cotransfection with TRAF6 or RIPK2. Mutagenesis studies indicated that Ser462 was the most critical phosphorylation needed for IL-12p40 gene transcription. A further reduction in IL-12p40 gene transcription occurred when both Ser451 and Ser462 were mutated to Ala. Conversely, the mutation of Ser462 and Ser451 to Asp (to mimic the effect of phosphorylation by introducing a negative charge) induced both nuclear translocation and IL-12p40 reporter gene expression (16). These studies are consistent with an important role for Ser462 phosphorylation in permitting IRF5 to undergo nuclear translocation and stimulate IL-12p40 gene transcription (16).

The expression of RIPK2 is essential for activation of the NOD1/2 signaling network and can activate NF-κB in overexpression experiments. However, its kinase activity is not required for activation and a catalytically inactive mutant is even more efficient than wild-type RIPK2 in inducing NF-κB and MAP kinase activation (25). The overexpression of TRAF6 can also activate NF-κB (e.g., ref. 26). However, our results suggest that cell transfection with RIPK2 or TRAF6 induces IRF5 phosphorylation at Ser462 indirectly by activating IKKβ.

TBK1 has been implicated in the TLR7-stimulated activation of IRF5 (9), but we found that the nuclear translocation of IRF5 was unaffected by MRT67307, a potent inhibitor of TBK1 and the related kinase IKKε (Figs. 4 and 5 and Fig. S3B and D). Moreover, TBK1 does not phosphorylate IRF5 at Ser462 in vitro (Fig. 2C) and we have failed so far to detect phosphorylation of the endogenous IRF5 at Ser158 and Ser309 that are targeted by TBK1 in vitro. Therefore, whether phosphorylation of the serine residues that can be forced by overexpression with TBK1 (16) has physiological relevance, is unclear.

In summary, our results establish that IKKβ activates two “master” transcription factors of the innate immune system, IRF5 and NF-κB (Fig. 6C). The phosphorylation induces the nuclear translocation of these proteins stimulating the transcription of IL-12 and other inflammatory cytokines in macrophages and IFNβ in pDCs. Further research is required to understand how IKKβ mediates the TLR7-stimulated production of IFNα (18) and why IKKα is needed for IFN production in pDCs (18, 27).

Materials and Methods

Agonists and Inhibitors. The TAK1 inhibitor NG-25 (28), the IKKβ inhibitor BI655906 (20), KF-1B (29, 30), MRT67307 (31), BIRB796 (32), and PD0325901 (33) were synthesized as described. The TLR7 agonists, CL097 (catalog no. tlr7-c97) and R848 (tlrl-r848) were purchased from Invivogen. LPS (lipopolysaccharide; Escherichia coli 055:B5) was from Alexis Biochemicals (ALX-581-001) and PS1145 (34) from Sigma.

Immunofluorescence Studies. The Gen2.2 cells are difficult to transfect with cDNA and several transfection protocols tested did not result in any transfection. The successful procedure, which consistently produced transfection of 10% of the cells is outlined below. The 2 x 10⁶ Gen2.2 cells were nucleofected with 1.0 μg IRF5–GFP or IRF5[S462A]–GFP cDNA using the Amaxa nucleofector, A-033 or X-001 programs. After 24 h, cells were replated at a density of 10⁶ cells/mL. After 24 h, cells were incubated for 60 min with inhibitors and then stimulated with ligands, as specified in the figure legends. The cells were fixed for 10 min with 4% (vol/vol) formaldehyde and 50,000 cells were centrifuged into precoated slides (Thermo Scientific). The cells were permeabilized by incubation for 10 min with methanol at −20°C. RAW264.7 cells were treated similarly, except that 2.0 μg IRF5–GFP or IRF5[S462A]–GFP cDNA were nucleofected using the Amaxa nucleofector D-032 program. After 24 h, 10⁶ cells were plated on coverslips, inhibitors were added after 48 h rather than 24 h, and after fixing in formaldehyde, the cells were permeabilized for 10 min with 0.2% (vol/vol) Triton X-100.

The Gen2.2 and RAW264.7 cells were incubated for 1 h with 2% (wt/vol) BSA, incubated for 16 h at 4°C with anti-GFP (Abcam 1:2,000),
washed with 0.2% (vol/vol) Tween in PBS at ambient temperature, incubated for 1 h at 21 °C with the secondary antibody Alexa 448 (Life Technologies 1:5,000), and counterstained with DAPI (0.2 μg/mL) to reveal nuclei. Images were acquired using a Delta Vision DV3 deconvolution microscope with an immersion-oil 63x objective lens and images were processed using OMERO. Images presented correspond to one tack from deconvolved 3D images.

All other materials and methods are described in SI Materials and Methods.

Supporting Information

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SI Materials and Methods

DNA Constructs. Several forms of IRF5 are produced by alternative splicing of human IRF5. The variant used in the present study lacks amino acid residues 161–176 of the longest form, but is otherwise identical. Human IRF5 (National Center for Biotechnology Information NM_001098627) was amplified from IMAGE EST 2963923 using KOD Hot Start DNA Polymerase (Merck-Millipore), cloned into pSC-c (Agilent) and sequenced. This was subcloned into the BamHI site in pCMV3 FLAG1 or the BglII EcoRI sites in pEGFPN1. Ser448 of IRF5 (which is Ser462 in the longest form of IRF5) was mutated to alanine. The mutation was introduced using the Quickchange method, but with KOD Hot Start DNA Polymerase. DNA vectors expressing wild-type IKKβ and the catalytically inactive IKKβ[D166A] mutant have been described (1).

Antibodies. Antibodies recognizing IRF5 phosphorylated at Ser462, or that recognize all forms of IRF5 equally well and that recognize GST were raised in sheep and purified by affinity chromatography by the Antibody Production Team of the MRC Protein Phosphorylation and Ubiquitylation Unit, Dundee, United Kingdom (coordinated by Dr. James Hastie). The phospho-specific antibody recognizing IRF5 phosphorylated by IKKβ in vitro, was not sufficiently sensitive to detect phosphorylation of the endogenous IRF5 in Gen2.2 or RAW264.7 cells. Antibodies recognizing all forms of TAK1 (4505), p38α MAP kinase (9212), MAPKAP-K2 (also called MK2) (3042), and GADPH (2118), as well as phospho-specific antibodies recognizing the p65 subunit of NF-kB phosphorylated at Ser536 (3031), p105/NF-kB1 phosphorylated at Ser933 (4806), ERK1 and ERK2 phosphorylated at Thr202/Tyr204 (9101), MSK1 phosphorylated at Thr381 (9595), and IRF3 phosphorylated at Ser396 (4947), were from Cell Signaling Technologies. The antibodies recognizing all forms of the p65 subunit of NF-kB (sc-372) and IRF7 (sc-74472) was purchased from Santa Cruz Technologies, the SMC-1 antibody was from Bethyl Laboratories (A300-055A), the antibody recognizing all forms of IKKβ was from Merck-Millipore (DAM1774677), the FLAG antibody was from Sigma (A8592), the HA antibody was from Roche (12 013 819 001), the His antibody was from Abcam (ab1187), and the anti-IRF3 antibody was from Invitrogen (51-3200).

Cell Culture. Gen2.2 cells were cultured as described (2). RAW264.7 and HEK293T cells were maintained in Dulbecco’s modified eagle’s medium supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin, 1 mM sodium pyruvate, 2 mM l-glutamine, nonessential amino acids, and 10% heat-inactivated FCS (Life Technologies).

Cell Stimulation, Lysis, and Immunoblotting. Gen2.2 or RAW cells were incubated for 1 h with or without inhibitors and then treated with ligands as detailed in the figure legends. The cells were rinsed with ice-cold PBS, lysed in 50 mM Tris-HCl pH 7.5, 1.0 mM EDTA, 1.0 mM EGTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium 2-glycerophosphate, 0.27 M sucrose, 1% (vol/vol) Triton X-100, 1.0 mM DTT, 1 mM phenylmethylsulphonyl fluoride (PMSF), containing 1.0 mM benzamidine, and a protein inhibitor mixture (1 tablet per 50 mL buffer) (Roche; 11 873 580 001). After centrifugation for 15 min at 13,000 × g at 4 °C, the supernatant, termed cell extract, was removed and its protein concentration quantified by the Bradford method. Aliquots of the cell extract (20 μg protein) were denatured in SDS, separated by SDS/PAGE, transferred to PVDF membranes, and immunoblotted with the antibodies specified in the figure legends.

Where indicated, the cells were lysed using the CellLytic NuCLEAR Extraction kit (Sigma-Aldrich) and the cytosol and nuclear fractions were separated according to the manufacturer’s instructions.

Phosphoproteomics. Gen2.2 cells were labeled using stable isotope labeling of amino acids in cell culture (SILAC) with Arg0Lys0, Arg6Lys4, or Arg10Lys8 in RPMI medium (Thermo; 89984). The 2.4 × 10^7 cells were incubated for 1 h in the presence or absence of 2 μM NG-25 or 5 μM BI605906, stimulated or not for 30 min with 1.0 μg/mL CL097, and then lysed in 50 mM Tris-HCl pH 8.2, 10 mM glycerol 2-phosphate, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM DTT, and 1.0 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 8 M urea. The cell lysates were clarified by centrifugation, the supernatant (cell extract) removed, and protein concentrations determined by the Bradford procedure. Cell extracts (1.0 mL, 2.0 mg protein) from each experimental condition were mixed in a 1:1:1 ratio and cysteine residues alkylated by incubation for 30 min at ambient temperature with 50 mM iodoacetamide. The sample (3 mL) was diluted to 16 mL with 0.1 M ammonium bicarbonate to a final urea concentration of 1.5 M, and proteins were digested for 16 h at 37 °C with 100 μg of trypsin. The digestes were made 1% (vol/vol) in trifluoroacetic acid and desalted by passing through a C18 Sep-Pak cartridge (Waters). The peptides were dried, dissolved in 0.5 mL of 0.2% (vol/vol) formic acid 80% (vol/vol) acetonitrile, fractionated by hydrophilic (HILIC) chromatography, and the phosphopeptides eluting between 24 and 70 min were dried, resuspended in 5% (vol/vol) trifluoroacetic acid containing 70% (vol/vol) acetonitrile, and 250 mM lactic acid, and enriched using 1.25 mg of immobilized TiO2. The phosphopeptides bound to the TiO2 columns were eluted with 1% (vol/vol) NH4OH pH 10.5, trifluoroacetic acid added to a final concentration of 2% (vol/vol), and the samples subjected to mass spectrometry. The data were analyzed using the MaxQuant program.

Phosphorylation of IRF5. FLAG-tagged IRF5 was expressed in HEK 293T purified by immunoprecipitation with anti-FLAG M2 affinity gel (Sigma-Aldrich; A2220) and eluted with FLAG peptide (Sigma-Aldrich; F3290). His6-tagged IKKβ and GST-TBK1 were expressed from baculovirus vectors in insect Sf21 cells. FLAG–IRF5 (0.5 μg) was then incubated for 30 min at 30 °C with 0.5 μg IKKα, IKKβ, or TKB1, 10 mM magnesium acetate and 0.2 mM [γ-32P]ATP (specific radioactivity 5,000 cpm/pmol) in 50 mM Tris-HCl pH 7.5, 0.1 mM EGTA, 2 mM DTT in a total volume of 30 μL. The samples were denatured in SDS, subjected to SDS/PAGE, and phosphorilation was monitored by autoradiography and by immunoblotting with a phospho-specific antibody recognizing IRF5 phosphorylated at Ser462.

DNA encoding Flag–IRF5 or Flag–IRF5[D166A] were co-transfected into HEK 293T cells with HA–wild-type IKKβ or the catalytically inactive HA–IKKβ[D166A] mutant. After 48 h, the cells were lysed and the extracts subjected to electrophoresis on native or SDS/polyacrylamide gels.

Dimerization of IRF5. To study the dimerization of IRF5, 8% native gels were prerun for 30 min at 40 mA at 0 °C in 25 mM Tris-192 mM glycine pH 8.4 with 1% (wt/vol) deoxycholate (DOC) in the
cathode chamber only. Cell extract (10 μg protein) containing 1% (wt/vol) DOC was electrophoresed at 0 °C for 60 min at 25 mA. The gels were incubated for 30 min at 21 °C with 25 mM Tris-192 mM glycine pH 8.4, 0.1% (wt/vol) SDS, transferred to PVDF membranes, and immunoblotted with the antibodies specified in Results.

RNA Interference and qPCR. The 5 × 10⁶ cells were transfected with 0.4 nmol siRNA in Amaza Nucleofector (Lonza) with the Amaza Cell Line Nucleofector V kit (Lonza), using program A033. The siRNAs encoding IRF3, IRF5, IRF7, IKKα, IKKβ, and Cy3-labeled negative control were purchased from Ambion (Life Technologies). The siRNA encoding TAK1 was from Dharmacon. After transfection for 24 h, the cells were replated in duplicate at 10⁶ cells/mL per condition. After a further 48 h, the cells were stimulated with CL097 (1.0 μg/mL) or left untreated for the times indicated in the figure legends. Total RNA extracted from the cells using the OMEGA kit was reverse transcribed using qScript cDNA SuperMix from Quanta Biosciences. Then cDNA (50 ng) was incubated with primers (100 nM) and PerfeCT Syber Green Fast (Quanta Biosciences). The cDNA corresponding to the amplified mRNA was measured using the ΔΔ cycle threshold (CT) method normalizing to 18S rRNA. The following primers were used in this study: ifnb1 forward, CTTTGCTATTTTCA-GACAAGATTCA and ifnb1 reverse, GCCAGGAGTTCT-CAACAAT; ifna1 forward, CCCTCTCTTTATCAACAAAC- TTGC and ifna1 reverse, TTGTGTATGTGACCAGCG; il-12p35 forward, GCCGCCTCGGGGACAATTAT and il-12p35 reverse, CCACAAGGAGGCTGCACG; il-12p40 forward, AGCAGGGTCTTGGCTCTG and il-12p40 reverse, CCA-TCTCTCTTTCTTGTGAAGCA; and il-6 forward, TCGAGCACCACGGGAACGAA and il-6 rev, TGGACGGAAGGCACCTTGGG.

ELISA. The IFNβ concentrations in the cell culture medium were measured by ELISA using the Verikine IFN-β ELISA kit (41410).

Statistical Analysis. Data were expressed either as SEM or SD. Statistical significance was calculated using the unpaired, two-tailed, Student t test.


Fig. S1. The knockdown of IRF5 suppresses the CL097-stimulated production of IL-12p35 and IL-12p40 mRNA in Gen2.2 cells. (A–C) Gen2.2 cells were transfected with siRNA against IRF3 (hatched bars), IRF5 (white bars), IRF7 (gray bars), or a control siRNA (black bars). After 72 h, the cells were stimulated for the times indicated with 1.0 μg/mL CL097. (A) The cell lysates were subjected to SDS/PAGE and immunoblotted with the antibodies indicated. The mRNAs encoding IL-12p35 (B) or IL-12p40 (C) were measured in duplicate by qRT-PCR and the graphs show the fold increase in mRNA expression (mean + SD) relative to the levels in unstimulated cells treated with control siRNA for one representative experiment. The mRNA levels measured at each time point were normalized to 18S rRNA. Similar results were obtained in three independent experiments.
Pull lysates 1:1:1

Trypsin digestion

Fractionation by HILIC

Enrichment of phosphopeptides with TiO$_2$

LC-MS/MS

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Fig. S2. The CL097-stimulated phosphorylation of IRF5 at Ser462 in Gen2.2 cells is prevented by the inhibition of IKKβ or TAK1. (A) Outline of how the SILAC/mass spectrometry experiment shown in Fig. 2 was carried out. (B and C) The abundance of the IRF5 phosphopeptide LQISpNPDLK (where Sp denotes phosphoserine) corresponding to amino acid residues 459–467 is shown in the absence and presence of BI605906 (B) and NG-25 (C) for one representative experiment. (D) The raw SILAC/mass spectrometry for the IRF5 phosphopeptide (uniprot A8DUA8) (two replicate experiments) is shown in the absence and presence of BI605906 or NG-25. The ratios are normalized (L, light; M, medium; and H, heavy labeled).
Fig. S3. Quantitation of the nuclear translocation of IRF5–GFP induced by ligands that activate TLR7 (A) Gen2.2 cells were transfected with IRF5–GFP (black bars) or IRF5[S462A]–GFP (white bars). After 48 h, the cells were stimulated for 30 min with 1.0 μg/mL CL097 or left unstimulated. (B) As in A, except that Gen2.2 cells transfected with IRF5–GFP were incubated for 1 h without or with the IKKβ inhibitors BI605906 (5.0 μM) or PS1145 (15 μM) or the TAK1 inhibitor NG-25 (2.0 μM) or the TBK1/IKKe inhibitor MRT67307 (2.0 μM) and then stimulated without or with CL097 (1.0 μg/mL). (C) Same as in A, except that RAW264.7 cells were stimulated with R848. (D) Same as in B, except that RAW264.7 cells were stimulated with R848. (A–D) The percentage of cells containing nuclear IRF5–GFP was compared with the total number of GFP-positive cells and the data quantified from 20–30 fields of GFP-positive cells. The data were compiled from three independent experiments performed in duplicate. The graphs show the percentage of cells with GFP in the nucleus for each condition (mean ± SD). Values were taken to be statistically significant at P < 0.05 (**P < 0.01, ***P < 0.001; ns, not significant).

Fig. S4. MRT67307 blocks the TLR3-stimulated phosphorylation of IRF3 in RAW264.7 macrophages. RAW cells were incubated without (−) or with (+) MRT67307 (2.0 μM), then stimulated for 30 min with 100 ng/mL lipopolysaccharide (LPS). After denaturation in SDS, SDS/PAGE and transfer to PVDF membranes, immunoblotting was performed using antibodies that recognize IRF3 phosphorylated at Ser396, p105 phosphorylated at Ser933, and p38α MAP kinase (p38α) as a loading control.
Inhibitors of the activity or activation of MAP kinases do not affect the R848-stimulated nuclear translocation of IRF5–GFP. (A) RAW264.7 cells were transfected with IRF5–GFP as in Fig. 3, incubated for 1 h without or with the p38 MAP kinase inhibitor BIRB0796 (1.0 μM), the MEK1/2 inhibitor PD0325901 (0.1 μM), or both inhibitors, and then stimulated for 30 min with R848 (1.0 μg/mL). The distribution of IRF5–GFP between the cytosol and nucleus was analyzed by deconvolution microscopy. (B) Same as in A, except that transfection with IRF5–GFP was omitted. After stimulation with R848, the cell extracts (20 μg protein) were denatured in SDS, subjected to SDS/PAGE, transferred to PVDF membranes, and immunoblotted with antibodies that recognize the active phosphorylated forms of ERK1/2, or MSK1/MSK2, or with antibodies that recognize all forms of MK2 and p38α MAP kinase (p38α). The phosphorylation of MK2 was detected by a small decrease in its electrophoretic mobility after stimulation with R848.
Fig. S6. The nuclear translocation of IRF5–GFP induced by the NOD1 agonist KF-1B is prevented by the mutation of Ser462 to Ala or by the inhibition of IKKβ or TAK1 in RAW264.7 cells. (A and B) RAW264.7 were transfected with IRF5–GFP or IRF5[S462A]–GFP. After 72 h, the cells were stimulated for 30 min with 25 μM KF-1B or left unstimulated. In A, the distribution of IRF5–GFP between the cytosol and nucleus was analyzed by deconvolution microscopy. In B, the percentage of cells with nuclear IRF5–GFP (black bars) or IRF5[S462A]–GFP (white bars) of the total number of GFP-positive cells were quantified in 20–30 fields with GFP-positive cells for three independent experiments performed in duplicate. The graphs show the mean ± SD of the percentage of nuclear GFP-positive cells in each condition. Values were taken to be statistically significant at \( P < 0.05 \) (*** \( P < 0.001 \); ns, not significant). (C and D) As in A and B, respectively, except that RAW264.7 cells transfected with IRF5–GFP were incubated for 1 h without or with the IKKβ inhibitors BI605906 (5.0 μM) or PS1145 (15 μM), the TAK1 inhibitor NG-25 (2.0 μM), or the TBK1/IKKe inhibitor MRT67307 (2.0 μM) and then stimulated without or with 25 μM KF-1B.