**Figure 1-1. IRF5 is essential for cytokine induction by multiple innate immunity pathways.**

(A) Efficiency of IRF5 knockdown and rescue in THP-1 cells. Cell extracts from WT THP1 (lane 1) and the cells stably expressing an shRNA against IRF5 with (lane 3) or without (lane 1) reconstitution with mIRF5-HA were immunoblotted with an IRF5 antibody.

(B) Depletion of IRF5 abolishes LPS-induced cytokine production in THP-1 cells. Cells used in this experiment include: Wild-type (THP-1 WT), IRF5 knockdown (THP-1 shIRF5), IRF5 knockdown and rescued with mouse IRF5 (THP-1 shIRF5+Flag-mIRF5-HA, labeled as THP-1 shIRF5+mIRF5) and IRF5 knockdown and rescued with human IRF5 (THP-1 shIRF5+HA-hIRF5, labeled as THP-1 shIRF5+hIRF5). These cells were stimulated with 5µg/mL LPS for the indicated time before total RNA was isolated. CXCL10 and IL-12 (p40 subunit) mRNA levels were analyzed by q-RT-PCR. Unless indicated otherwise, error bars represent standard deviations of triplicate assays.

(C) IRF5 is essential for LPS-induced expression of cytokines and interferon-stimulated genes (ISGs). THP-1 cell lines as described in (A) were stimulated with LPS (5µg/mL) for the indicated time. Total RNA was isolated to measure the expression of the indicated genes by q-RT-PCR.

**Figure 1-2. IRF5 forms a dimer in response to innate immune stimulus**

(A) IRF5 forms dimer upon activation. THP-1 shIRF5+Flag-mIRF5-HA cell line as described in (1-1B) was left untreated (Control, Ctl) or stimulated by incubation with LPS (5µg/mL) or transfection with poly(dA:dT) (2µg/mL), HT-DNA (2µg/mL) or poly(I:C) (2µg/mL) for the indicated time. The formation of IRF5 dimer was analyzed by native gel electrophoresis, followed by immunoblotting with the HA antibody.

(B) IRF5 promotes cytokine induction in 293T cells. Wild type 293T cells and those stably expressing Flag-mIRF5-HA were stimulated with Sendai virus (SeV) or poly(I:C) (2µg/mL) for the indicated time followed by measurement of TNF-α and IFN-β RNA levels by q-RT-PCR. Right panels: the cells were transfected with empty pcDNA vector, pcDNA-Flag-MAVS (MAVS) or pcDNA-Flag-IKKβ (IKKβ) for 24 hr before total RNA was isolated for analyses by q-RT-PCR.

(C) Over-expression of IKKβ or MAVS activates IRF5 in cells. 293T Flag-mIRF5-HA cell line as described in (C) was transiently transfected with empty pcDNA vector or the vector containing Flag-MAVS or Flag-IKKβ for 24 hr. Dimerization of IRF5 was analyzed by native PAGE followed by immunoblotting with the HA antibody.

**Figure 1-3. IKKβ activates IRF5 in vitro and is important for IRF5 activation in cells**

(A-B) IKKβ activates IRF5 *in vitro*. (A) Cytosolic fraction (S20) from 293T Flag-mIRF5-HA cell line was incubated with purified IKKβ or TBK1 protein in the presence of ATP. Dimerization of IRF5 or IRF3 was analyzed by native PAGE followed by immunoblotting. (B) *In vitro* translated 35S-IRF5 or 35S-IRF3 protein was incubated with BSA, IKKβ or TBK1 in the presence of ATP. Dimerization of IRF5 or IRF3 was analyzed by native PAGE followed by autoradiography. Ctl: control cytosolic fraction without kinase.

(C) IKKβ inhibitor blocks IRF5 activation by LPS. THP-1 shIRF5 cells stably reconstituted with Flag-mIRF5-HA were treated with IKKβ inhibitor (TPCA-1, 20µM) or TBK1 inhibitor (BX-795, 10µM) for 2 hr before stimulation with LPS (5µg/mL) for 2 hr. IRF5 activation was analyzed by native PAGE and immunoblotting. Ctl: DMSO control.

(D-E) 293T Flag-mIRF5-HA cells were first treated with TPCA-1 or BX-795 as described in (C), at the same time, the cells were also transfected with pcDNA-Flag- IKKβ, pcDNA-Flag-TBK1, pcDNA-Flag-MAVS or pcDNA-Flag-TRIF as indicated for 24 hours. IRF5 and IRF3 activation was analyzed by native PAGE and immunoblotting. p-IKK, p-TBK1 and p-ikBa were also blotted.

**Figure 1-4. TRAF6, NEMO and IKKβ mediate IRF5 activation by MAVS**

(A) Knockdown of IKKβ or TRAF6 abolishes IRF5 activation by MAVS. IKKα, IKKβ, TRAF6 or NEMO was stably knockdown in 293T Flag-mIRF5-HA cells using lentiviral shRNA as indicated. These cells were transfected with empty pcDNA vector, pcDNA-Flag-MAVS or pcDNA-Flag-IKKβ for 24 hr. Activation of IRF5 was analyzed by native PAGE and immunoblotting. Lower panel: the knockdown efficiency for each gene was analyzed by immunoblotting.

(B) In 293T Flag-mIRF5-HA with TRAF6 knocked down cells, pcDNA-IKKβ, pcDNA-MAVS, or (and) pcDNA-TRAF6 were transfected for 24 hours. IRF5 activation was analyzed by native PAGE and immunoblotting.

(c) 293T Flag-mIRF5-HA cells were transfected with pcDNA-Flag-IKKβ (IKKβ) or pcDNA-Flag-MAVS (MAVS) or treated with recombinant TNF-α protein (TNF-α) for the indicated time. Activation of IRF5 was analyzed by native PAGE followed by immunoblotting with an IRF5 antibody. Activation of IKKβ was monitored by immunoblotting with a phospho-IκBα antibody.

**Figure 1-5. IKKβ Phosphorylates IRF5 at Ser-445**

(A) IKKβ activates IRF5 in vitro. IRF5 partially purified from 293T Flag-mIRF5-HA cells was incubated with IKKβ or BSA in the presence of ATP. Activation of IRF5 was analyzed by native PAGE and immunoblotting.

(C) A representative tandem mass (MS2) spectrum after HCD fragmentation of the ion with m/z = 1061.50 (z = 2+) indicates phosphorylation at S445. “b” and “y” ions with or without neutral loss are labeled in blue. Diagnostic ions for phosphorylation are highlighted in red.

(C) Tandem mass (MS2) spectrum after HCD fragmentation of the ion with m/z = 554.28 (z = 2+), indicating S445 phosphorylation.

(D) Tandem mass (MS2) spectrum after HCD fragmentation of the ion with m/z = 1067.50 (z = 2+), indicating S434 phosphorylation.

(E) In vitro activation of IRF5 similar to (A), but recombinant MAVS protein instead of IKKβ was used.

**Figure 1-6. Phosphorylation of IRF5 at Ser-445 is essential for its dimerization**

(A) Serine 445 is essential for IRF5 activation by IKKβ *in vitro*. Wild type or mutant 35S-IRF5 proteins were translated *in vitro* and incubated with IKKβ or BSA in the presence of ATP. Dimerization of IRF5 was analyzed by native gel electrophoresis, followed by autoradiography.

(B) Sequence alignment of the C-termini of IRF5 and IRF3 from different species as indicated. Conserved serine residues in IRF5 and IRF3, including S434 and S445 of mouse IRF5, are highlighted.

(C) S445A mutation of IRF5 prevented its activation by IKKβ and MAVS in cells. 293T cells were transfected with the expression vectors for WT or mutant Flag-mIRF5 as indicated for 12 hr before another transfection with the expression vector encoding IKKβ or MAVS for 24 hr. IRF5 dimerization (top) and expression (bottom) were analyzed by immunoblotting after native- and SDS-PAGE, respectively.

(D) Total RNA from cells used in (C) were isolated for the measurement of IRF5 mRNA levels by q-RT-PCR.

**Figure 1-7. Phosphorylation of IRF5 at Ser-445 is important for cytokine induction**

(A-B)293T cell lines stably expressing WT or S445A IRF5 were transfected with expression vectors for IKKβ or MAVS for 24 hr, or infected with Sendai virus for the indicated time. Total RNA was isolated for the measurement of TNF-α and IFNβ RNA levels by q-RT-PCR.

(C) S445A mutation of IRF5 significantly inhibited TNF-α induction. Total RNA was isolated from cells described in (A) to measure TNF-α levels by q-RT-PCR.

(D) S445D mutation also inhibited IRF5 dimerization. Wild type and mutant 35S-IRF5 (S445D and S445A) proteins were translated *in vitro* and incubated with IKKβ or BSA in the presence of ATP. Dimerization of IRF5 was analyzed by native gel electrophoresis, followed by autoradiography.

(E) S445D mutation of IRF5 inhibited TNF-α induction. Similar to (B) except that TNF-α RNA was measured by q-RT-PCR.

(F) Wild-type (THP-1 WT), IRF5 knockdown (THP-1 shIRF5), IRF5 knockdown and rescued with wild type or S445A mouse IRF5 (THP-1 shIRF5+mIRF5 WT or THP-1 shIRF5+mIRF5 S445A) THP-1 cell lines were stimulated with 5µg/mL LPS for 6 hr before total RNA was isolated. IL-12 p40 mRNA levels were analyzed by q-RT-PCR. \*, P < 0.05, statistically significant difference.

**Figure 1-8. Detection of IRF5 phosphorylation at Ser-445 with a phospho-specific antibody**

(A) 293T cells stably expressing WT or S445A Flag-mIRF5-HA were transfected with expression vectors for IKKβ or MAVS for 24 hr. Aliquots of the cell extracts were analyzed for IRF5 dimerization by native PAGE, whereas other aliquots were immunoprecipitated with the HA antibody followed by immunoblotting with an antibody against IRF5 or phosphorylated IRF5 at Ser-445. Expression of IKKβ and MAVS was examined by immunoblotting with the Flag antibody (bottom).

(B) 293T cell lines as described above were treated with or without 20 µM TPCA-1 for 2 hr before infected with Sendai virus for 24 hr. IRF5 was immunoprecipitated with an HA antibody followed by immunoblotting with an antibody against IRF5 or phosphorylated IRF5. Dimerization of IRF3 was detected by native PAGE and immunoblotting (bottom).

(C) Raw 264.7 cell stably expressing Flag-mIRF5-HA was treated with or without TPCA-1 (20 µM) for 2 hr before stimulated with LPS (5µg/mL) for 2 hr.; IRF5 was immunoprecipitated with an HA antibody followed by immunoblotting with an antibody against IRF5 or phosphorylated IRF5. Dimerization of IRF5 was detected by immunoblotting of cytosolic extracts.

(D) THP-1 cell was treated with or without the IKKβ inhibitors (TPCA-1 and PS1145) or TBK1 inhibitor (BX-795) for 2 hr before stimulation with LPS (5µg/mL) for 2 hr. Phosphorylated IRF5 was immunoprecipitated with an IRF5 antibody followed by immunoblotting with the same antibody or the phospho-IRF5 (S445) antibody

**Figure 1-9. Immunofluorescence study shows that IRF5 translocates into the nucleus after** **phosphorylation**

(A-B) Phosphorylated IRF5 accumulates in the nucleus. Differentiated THP-1 cells were stimulated with LPS for 2 hr. Nuclear translocation and phosphorylation of IRF5 were monitored by confocal immunofluorescence using antibodies against IRF5 (A) or p-IRF5 (B)