Figure S1 Screening for PtdIns5P, which enhances IRF3 phosphorylation by TBK1. (A) Several reagents, isolated fractions or reconstituted proteins were tested in an in vitro kinase assay by incubation with recombinant IRF3 and TBK1, and IRF3 phosphorylation by TBK1 was detected by an anti-pS394IRF3 antibody. (B) HEK293T cells were transfected with the indicated expression plasmids and insoluble fractions were subjected to in vitro kinase assay. (C) in vitro kinase assay was performed using endogenous TBK1 which precipitated from RAW264.7 cells, and TBK1 and IRF3 phosphorylation was detected. Figure S1, related to Figure 1.

Figure S2 PIKfyve activates the ISRE promoter and its expression increases after stimulation. (A) HEK293 cells were transfected with the indicated expression plasmids together with ISRE-Luc plasmid and luciferase activity was measured. (B) Expression of PIKfyve mRNA and proteins were analyzed after the indicated stimulation in MEF cells. Data are represented as mean +/- SD. Figure S2, related to Figure 2.

Figure S3 PIKfyve kinase inhibitor does not suppress NF-κB-dependent cytokine production. (A) IFNβ and IP-10 production in MEF cells was measured by ELISA during YM-201036 treatment. (B) TNFα production by GM-DC was measured after stimulation with LPS or R837 during YM-201636 treatment. (C) GM-DCs were stimulated with R837 during YM-201636 treatment and immunoblotting was performed with the indicated antibodies. Data are represented as mean +/- SD. Figure S3, related to Figure 3.

Figure S4 PIKfyve knockdown suppresses IRF3-dependent cytokine production. (A)
PIKfyve expression in MEF cells was detected after siRNA electroporation. (B) IP-10 and RANTES expression after poly I:C transfection in MEF cells was measured by qPCR. (C) siRNA-electroporated RAW 264.7 cells were stimulated with LPS or poly I:C transfection. IRF3 phosphorylation was detected after SDS-page (top). TBK1 was precipitated with anti-TBK1 with protein A agarose beads and bound proteins were blotted with anti-pTBK1 (bottom). (D) MEF cells after PIKfyve knockdown were stimulated by poly I:C, and JNK and p38 phosphorylation were detected. (E) PIKfyve expression in GM-DCs was suppressed by siRNA electroporation, and IFNβ or IL-6 expression was measured after NDV infection. (F) IRF3 phosphorylation was detected after NDV infection in GM-DCs. (G) PIKfyve expression in HEK293 cells was measured after siRNA electroporation. (H) ISRE and NF-κB promoter activation was measured by luciferase assay. siRNA-treated HEK293 cells were stimulated with LPS or transfected with poly I:C or poly dA:dT. HEK293 cells expressing TLR4/MD2/CD14 were used for LPS stimulation. (I) ISRE promoter activation by TBK1 was measured in siRNA-treated HEK293 cells. Data are represented as mean +/- SD. Figure S4, related to Figure 4.

**Figure S5** LHR pocket binding of IRF3 is important for cytokine expression. (A) Wild type MEF cells were infected with control retrovirus and IRF3<sup>+/−</sup> MEF cells were infected with retrovirus expressing wild type or K352A/R353A IRF3. Gene expression of IFNβ after LPS and poly dA:dT transfection or IP-10 and RANTES expression after poly I:C transfection was measured by qPCR. (B) Overall structure for hIRF3 is shown. (C) Docking simulation with inositol1,5P2 and IRF3 by Autodock4 is shown around the LHR pocket. **Left;** inositol1,5P2 with the molecular surface of IRF3. Side chain of
Arg361 is shown in blue. **Right; inositol1,5P$_2$** and side chain of binding amino acids with predicted hydrogen binding are shown. (D) Membrane association of IRF3 is predicted. Data are represented as mean +/- SD. Figure S5, related to Figure 6.

**Figure S6** C8-PtdIns5P induces cytokine production in GM-DCs. (A) MEF cells were stimulated with the indicated C8-lipids and IL-1β production was measured by ELISA. (B) MEF cells were stimulated with the indicated C8-lipids, poly I:C or LPS, and the production of RANTES and IP-10 was measured by ELISA. (C) GM-DCs were stimulated with C16-PtdIns containing PC/PE liposomes and IP-10 production was measured by ELISA. (D) GM-DCs stimulated with C8-PtdIns5P and poly I:C, and IRF3 and Rel A phosphorylation were detected by immunoblotting with the indicated antibodies. (E) IRF3 dimer formation was detected by native page after C8-PtdIns5P stimulation. (F) MEF cells from wild-type or TBK1$^{+/−}$/IKKi$^{−/−}$ mice were stimulated with C8-PtdIns5P and poly I:C, and expression of IFNα/β was measured by qPCR. (G) Activation model of TBK1-IRF3 signal. PtdIns5P production is increased by virus infection through PIKfyve, and increased PtdIns5P cases conformational change of IRF3 to expose phosphorylation site. Then, exposed phosphorylation site in IRF3 is phosphorylated by TBK1. Data are represented as mean +/- SD. Figure S5, related to Figure 7.