MECHANISM OF IRF5 ACTIVATION AND REGULATION OF Cgas activity

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| Zhijian J. Chen, Ph.D. |
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dedication

To My Grandparents, Parents and Significant Other

MECHANISM OF IRF5 ACTIVATION AND REGULATION OF Cgas activity

(double spaced in inverted pyramid form if more than one line long)

by

Junyao ren

Dissertation

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

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The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

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MECHANISM OF IRF5 ACTIVATION AND REGULATION OF Cgas activity

JUNYAO REN, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2018

Supervising Professor: Zhijian J. Chen, Ph.D.

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Prior Publications

Ren, J., Chen, X. and Chen, Z.J., 2014. IKK2 is an IRF5 kinase that instigates inflammation. Proceedings of the National Academy of Sciences, 111(49), pp.17438-17443.

Liu, S., Cai, X., Wu, J., Cong, Q., Chen, X., Li, T., Du, F., Ren, J., Wu, Y.T., Grishin, N.V. and Chen, Z.J., 2015. Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. Science, 347(6227), p.aaa2630.

Yang, H., Wang, H., Ren, J., Chen, Q. and Chen, Z.J., 2017. cGAS is essential for cellular senescence. Proceedings of the National Academy of Sciences, p.201705499.

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CHAPTER ONE  
Introduction

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CHAPTER Two

IKKβ is an IRF5 kinase that instigates inflammation

Results

The transcription factor interferon regulatory factor 5 (IRF5) is essential for the induction of inflammatory cytokines, but the mechanism by which IRF5 is activated is not well understood. Here we present evidence that the kinase IKKβ phosphorylates and activates IRF5 in response to stimulation in several inflammatory pathways, including those emanated from Toll-like receptors and retinoic acid-inducible gene I–like receptors. IKKβ phosphorylates mouse IRF5 at specific residues, including serine 445 (S446 in human IRF5 isoform 1), as evidenced by mass spectrometry analysis and detection with a phosphospecific antibody. Recombinant IKKβ phosphorylated IRF5 at Ser-445 in vitro, and a point mutation of this serine abolished IRF5 activation and cytokine production. Depletion or pharmacologic inhibition of IKKβ prevented IRF5 phosphorylation. These results indicate that IKKβ is an IRF5 kinase that instigates inflammation.

IRF5 forms a dimer and is essential for cytokine induction by multiple innate immunity pathways.

To investigate the function and active form of IRF5, we depleted endogenous IRF5 in THP-1 cells by shRNA and reconstituted with mouse or human IRF5 (Figure 1-1A). Then we measured cytokine induction by LPS in these cells. The expression of CXCL10 and IL-12 was largely abolished when IRF5 was knocked down but strongly induced when either human or mouse IRF5 was ectopically expressed (Figure 1-1B); the higher induction levels in the IRF5 reconstituted cells were likely due to the higher levels of IRF5 (Figure 1-1A). Similarly, LPS induction of IFN-β and several interferon-stimulated genes (ISGs), including IFIT3, RSAD2 and ISG15, was inhibited in the absence of IRF5 but restored when IRF5 was expressed (Figure 1-1C).

To test whether activated IRF5 forms a dimer, we stimulated THP1 cells stably expressing HA-tagged IRF5 with LPS as well as other stimuli, including poly(dA:dT) and herring testis DNA (HT-DNA), both of which are known to activate the cGAS cytosolic DNA sensing pathway(Sun, Wu et al. 2013, Wu, Sun et al. 2013). Poly(dA:dT) also activates the RIG-I pathway through RNA polymerase III(Ablasser, Bauernfeind et al. 2009, Chiu, Macmillan et al. 2009). We also transfected the cells with the double-stranded RNA analogue poly(I:C), which is known to stimulate the RIG-I and MDA5 pathways (Yoneyama, Kikuchi et al. 2004). In each case, analyses by native polyacrylamide gel electrophoresis (PAGE) followed by immunoblotting showed that stimulation of the cells led to the formation of a slower migrating band that likely represents an IRF5 dimer, much like IRF3 dimerization following virus infection (Figure 1-2A). We have not been able to detect dimerization of endogenous IRF5 in THP1 cells, because the commercially available IRF5 antibody detected a strong non-specific band at the expected IRF5 dimer position on the native gel. Thus, for the remainder of this project, we measured IRF5 activation using IRF5 dimerization assay in THP1-HA-IRF5 stable cells or by immunoblotting with a phospho-IRF5 specific antibody (see below).

To further investigate the role of IRF5 activation in inflammatory cytokine induction, we stably expressed IRF5 in HEK293T cells, which do not have detectable expression of endogenous IRF5, and then stimulated the cells by poly(I:C) transfection or infection with Sendai virus, an RNA virus known to activate the RIG-I – MAVS pathway. In both cases, the induction of TNF-α and IFN-β was strongly enhanced in 293T-IRF5 cells when compared to the parental cells. Sendai virus infection was capable of inducing IFN-β in the parental 293T cells because these cells express IRF3. Thus TNF-α induction by cytosolic RNA or RNA viruses is critically dependent on IRF5, whereas IFN-β induction was largely dependent on IRF3 but can be further enhanced by IRF5. These results suggest that the RIG-I pathway can activate both IRF3 and IRF5. Indeed, overexpression of MAVS led to the induction of both TNF-α and IFN-β (Figure 1-2B, right panels), as well as the dimerization of IRF5 (Figure 1-2C).

Interestingly, overexpression of IKKβ strongly induced TNF-α expression and IRF5 dimerization but only weakly induced IFN-β (Figure 1-2B and 1-2C). The weak induction of IFN-β by IKKβ overexpression can be explained by the fact that IRF3 is phosphorylated by TBK1 and IKKε but not IKKβ(Fitzgerald, McWhirter et al. 2003, Sharma, tenOever et al. 2003). These results raise the interesting possibility that IKKβ may play an important role in IRF5 activation.

IKKβ activates IRF5 in vitro and is important for IRF5 activation in cells

To obtain further biochemical evidence for the role of IKKβ in IRF5 activation, we prepared cytosolic extracts from HEK293T cells stably expressing Flag-mIRF5-HA and incubated the extracts with recombinant IKKβ or TBK1 protein together with ATP. Native PAGE analyses of the reaction mixtures revealed that IKKβ caused the dimerization of IRF5 but not endogenous IRF3, whereas TBK1 had the opposite effects (Figure 1-3A). We also incubated *in vitro* translated, 35S-labelled IRF5 with IKKβ and observed IRF5 dimerization (Figure 1-3B).

To test which kinase is important for IRF5 activation in cells, we treated THP1 cells stably expressing Flag-mIRF5-HA with the IKKβ inhibitor TPCA-1 or TBK1 inhibitor BX-795, and then stimulated the cells with LPS. TPCA1 but not BX-795 inhibited IRF5 dimerization, suggesting that IKKβ was responsible for LPS-induced dimerization of IRF5 (Figure 1-3C). At the same time, we transiently overexpressed IKK2, TBK1, MAVS and TRIF protein to activate IKK2 and tested IRF5 activation in presence or absence of TPCA-1 and BX-795 (Figure 1-3D and E). These results proved that IKK2 was the exclusive kinase for IRF5. Surprisingly, IKK2 alone could activate IRF3 and this activation was independent of TBK1 kinase activity (Figure 1-3D, lane 3). Similarly, MAVS mediated IRF3 activation could not be blocked by TBK1 inhibitor alone while in TRIF mediated activation of IRF3, the kinase activity of TBK1 was essential, further indicating that IKK2 may also play some role in the activation of IRF3 in certain pathways.

To further examine the role of IKKs and other signaling molecules in IRF5 activation, we used shRNA to stably knock down the expression of IKKα, IKKβ, TRAF6 or NEMO in HEK293T cells stably expressing Flag-mIRF5-HA. These cells were transfected with IKKβ or MAVS, followed by analysis of IRF5 dimerization by native PAGE. The results showed that IKKβ, TRAF6 and NEMO, but not IKKα, were required for IRF5 dimerization induced by MAVS (Figure 1-4A). And the requirement for TRAF6 in IRF5 activation was validated by the dimerization of IRF5 after overexpressing TRAF6 in TRAF6 knockdown cells (Figure 1-4B). IKKβ knockdown partially inhibited IRF5 dimerization induced by IKKβ overexpression, presumably because the shRNA only partially reduced the IKKβ level. Knocking down other proteins, including IKKα, TRAF6 and NEMO, had little effects on IRF5 activation by IKKβ. One more interesting observation was that though TNF-a treatment could potently activate IKK2, the treatment could not activate IRF5 (Figure 1-4C), suggesting that certain adapter protein, such as MAVS, TRIF, etc. might be needed in the activation process. Together, these results suggest that TRAF6, NEMO and IKKβ mediate IRF5 activation by MAVS.

Phosphorylation of IRF5 at Ser-445 by IKKβ is important for cytokine induction

To map the phosphorylation site(s) of IRF5, we incubated Flag-mIRF5-HA, which was partially purified from HEK293T cells stably expressing the protein, with IKKβ or with BSA (as a control) in the presence of ATP and Mg2+ at 30°C for 1 hour. IKK but not BSA caused IRF5 dimerization in this reaction (Figure 1-5A). The IRF5 protein from these reaction mixtures was further purified and analyzed by mass spectrometry, which revealed that peptides containing phosphorylated Ser-445 and Ser-434 of mIRF5 were greatly enriched in the reactions that contained IKKβ, whereas the total counts of mIRF5 peptides were similar in both reactions (Table 1-1 and 1-2, Figure 1-5 B, C and D). In addition, we also detected mIRF5 peptides containing phosphorylation at Ser-430 and 436 (Table 1-1).

To further confirm the phosphorylation sites identified, we incubated the partially purified Flag-mIRF5-HA as mentioned above with BSA or recombinant MAVS protein (in the same condition as above). Similarly, MAVS but not BSA activated and dimerize IRF5 (Figure 1-5E). The subsequent IP-Mass spectrometry analysis detected exclusive phosphorylation modification on Serine 445 site and about 10% of the peptide detected were phosphorylated (Table 1-3).

To test which serine residues are important for IRF5 activation by IKKβ, we mutated each serine residue identified above to alanine, *in vitro* translated the mutant proteins in the presence of 35S-methionine, and used the proteins in reactions that contained IKKβ or BSA (Figure 1-6A). Among the mutants tested, the S445A mutation completely inhibited, and S434A mutation partially inhibited, IRF5 dimerization, whereas the other mutations had little inhibitory effect. Interestingly, Ser-434 and Ser-445 are the most conserved residues among IRF5 proteins from different species and they are homologues to Ser-385 and Ser-396, respectively, of human IRF3 (Figure 1-6B), which are known to be critical phosphorylation sites essential for type-I interferon induction(Hiscott, Lin et al. 2006).

A previous study showed that a S480A mutation in human IRF5 (equivalent to S439A of mouse IRF5) impaired its ability to induce IFN-α (Barnes, Kellum et al. 2002). When this serine was mutated to aspartic acid, (S380D in the version of human IRF5 used in the study), IRF5 formed a dimer whose crystal structure was solved (Chen, Lam et al. 2008). We therefore mutated this residue (S439A in mouse IRF5) as well as other serine residues (S430A and S445A) and transfected them into HEK293T cells together with IKKβ or MAVS. IRF5-S445A failed to dimerize in response to stimulation by IKKβ or MAVS, whereas the S430A and S439A mutations had no effect (Figure 1-6C). Immunoblot analysis showed that the IRF5 serine to alanine mutants were expressed at similar levels to that of WT IRF5 (Figure 1-6C, Lower).

Importantly, the S445A mutation abrogated the ability of IRF5 to stimulate the induction of TNF-α in response to IKKβ, MAVS or Sendai virus infection (Figure 1-7A), whereas mutations at other serine residues did not have significant inhibitory effects (Figure 1-7C). The S445A mutation also partially inhibited IFN-β induction by MAVS but did not significantly affect IFN-β induction by Sendai virus (Figure 1-7B), presumably because IRF3 plays a dominant role in IFN-β induction in response to Sendai virus infections.

We also tested the IRF5 S445D mutant and found that this mutation largely inhibited IRF5 dimerization (Figure 1-7D) and abrogated the ability of IRF5 to boost TNF-α induction by IKKβ (Figure 1-7E). Thus, the S445D mutation does not appear to mimic the effect of phosphorylation. And also, S445D mutant protein was not stable in cells. As shown in Figure 1-6C, lane 22 to 25, the protein was almost undetectable in SDS-PAGE analysis while its mRNA level was comparable to other mutants (Figure 1-6D).

As shown previously, IKKβ only weakly induced IFN-β in a manner independent of IRF5, again consistent with a dominant role of IRF3 in IFN-β induction (Figure 1-7B). To determine the role of IRF5 phosphorylation in TLR signaling, we established a THP-1 stable cell line depleted of endogenous IRF5 and reconstituted with WT IRF5 or the S445A mutant. The S445A mutation largely abrogated the ability of IRF5 to induce IL-12 in response to LPS stimulation (Figure 1-7F). Taken together, these results suggest that IKKβ phosphorylates mIRF5 at Ser-445, and that this phosphorylation is important for inflammatory cytokine induction.

Detection of IRF5 phosphorylation at Ser-445 with a phospho-specific antibody

To further investigate IRF5 phosphorylation in cells, we developed an antibody that recognizes IRF5 phosphorylated at Ser-445 by immunizing rabbits with a synthetic phosphopeptide (IRLQIpS445NPDLC) corresponding to amino acids 440-450 of mouse IRF5 (441-451 of human IRF5). To test the specificity of this antibody, 293T cells stably expressing WT or S445A mutant of Flag-mIRF5-HA were transfected with an expression vector encoding IKKβ or MAVS, both of which stimulated dimerization of WT but not S445A IRF5. Immunoprecipitation with the HA antibody followed by immunoblotting with the pIRF5 antibody showed that the antibody selectively detected WT but not S445A IRF5 after stimulation (Figure 1-8A), confirming that this antibody is specific for IRF5 phosphorylated at Ser-445.

To determine if IRF5 is phosphorylated at Ser-445 in response to physiological stimuli, we infected 293T cells stably expressing WT or S445A Flag-mIRF5-HA with Sendai virus. Immunoblotting with the p-IRF5 antibody confirmed that WT but not S445A IRF5 was phosphorylated in the virus-infected cells and that this phosphorylation was abolished by the IKK inhibitor TPCA1 (Figure 1-8B, top panel). Sendai virus-induced dimerization of endogenous IRF3 was not affected by overexpression of WT or S445A IRF5 and was only partially inhibited by TPCA1 (Figure 1-8B, bottom). LPS stimulation of the macrophage cell line Raw264.7 stably expressing Flag-mIRF5-HA also led to IKK-dependent phosphorylation of IRF5 at Ser-445 (Figure 1-8C).

Finally, to determine if endogenous IRF5 is phosphorylated at Ser-445, we stimulated THP1 cells with LPS and then immunoprecipitated IRF5 with the p-IRF5 antibody followed by immunoblotting with the same antibody (Figure 1-8D). We also tested the effect of several kinase inhibitors on IRF5 phosphorylation and found that only IKKβ inhibitors (TPCA-1 and PS1145), and not TBK1 inhibitor (BX-795), could inhibit the phosphorylation of IRF5 at Ser-445 in response to LPS (Figure 1-8D). Finally, we performed immunofluorescence analyses in THP1 cells using IRF5 and p-IRF5 antibodies. Consistent with previous reports (27), IRF5 translocated into the nucleus in response to LPS stimulation (Figure 1-9A). Importantly, p-IRF5 signal was barely detectable in the absence of stimulation, and LPS stimulation led to accumulation of p-IRF5 in the nucleus (Figure 1-9B). These experiments demonstrate that LPS stimulates the phosphorylation of endogenous IRF5 at Ser-445 and its subsequent translocation to the nucleus.

Conclusions and discussion

In this part, we presented evidence that IKKβ is an IRF5 kinase and we identified Ser-445 of mouse IRF5 (Ser-446 of human IRF5) as a critical phosphorylation site that is essential for IRF5 to induce cytokines. We also developed an antibody specific for IRF5 phosphorylated at Ser-445 and we used this antibody to demonstrate that IRF5 is phosphorylated at Ser-445 in an IKKβ-dependent manner in response to LPS stimulation or Sendai virus infection. Our results suggest that IKKβ plays a crucial role in activating both NF-kB and IRF5, two master regulators of pro-inflammatory cytokines.

IKKβ is activated by a variety of stimulatory agents, including inflammatory cytokines and microbial pathogens that activate different pattern recognition receptors(Israel 2010, Liu, Xia et al. 2012). Consistent with the pleiotropic functions of IKKβ, we found that IRF5 is activated by multiple pathways including those that engage TLRs and cytosolic DNA and RNA sensors. However, not all stimuli that activate IKKβ are capable of activating IRF5. For example, we found that TNF-α, which is known to strongly stimulate IKKβ, could not activate IRF5 (data not shown). Thus, IRF5 activation requires additional signals in addition to IKKβ. A similar scenario has recently been reported in the cytosolic DNA sensing pathway, which employs the adaptor protein STING to not only activate TBK1 but also recruit IRF3, thereby specifying the phosphorylation of IRF3 by TBK1 (Tanaka and Chen 2012). It is possible that similar adaptor proteins may be engaged by TLR and other pathways to recruit IRF5 for phosphorylation by IKKβ.

Through mass spectrometry, we identified several serine residues on mIRF5 that are phosphorylated by IKKβ, and these include Ser-430, 434, 436 and 445. Our functional analyses showed that Ser-445, and to a lesser extent Ser-434, is required for IRF5 dimerization, whereas mutations of other serine residues had no effect. These results are different from those of a previous report which showed that Ser-436 and Ser-439 (equivalent to Ser-477 and Ser-480 in the human IRF5 used in the study) were important for IFN-α induction (Barnes, Kellum et al. 2002). Importantly, Ser-434 and 445 of mIRF5 are homologous to Ser-385 and 396 of human IRF3 and they reside in a highly conserved region containing a cLxISN motif (Hiscott, Lin et al. 2006). The p-IRF5 antibody that we have developed clearly detected the phosphorylation of IRF5 at Ser-445 in cells stimulated with LPS or infected with Sendai virus, consistent with the phosphorylation of IRF3 at Ser-396 in response to RNA virus infection. Collectively, our results demonstrate that Ser-445 is phosphorylated by IKKβ in cells in response to stimulation and that this phosphorylation is critical for IRF5 activation.

It is interesting that despite homologous domain structures and considerable sequence similarities between IRF5 and IRF3, these proteins are phosphorylated by distinct but homologous kinases, which are IKKβ and TBK1, respectively. It has also been reported that IKKα is responsible for the phosphorylation of IRF7 in response to stimulation of endosomal TLRs such as TLR7 and TLR9 (Hoshino, Sugiyama et al. 2006). Thus, IKKβ and IKK-like kinases may be largely responsible for the activation of IRFs, and further work is needed to identify the kinase specific for each IRF. Future work should also delineate the biochemical basis for the specificity of IRF phosphorylation by a cognate IKK or IKK-like kinase. In the case of IRF5, which is essential for the production of inflammatory cytokines and has been closely linked to human autoimmune diseases(Lazzari and Jefferies 2014), the work reported here, which includes the discovery of IKKβ as an IRF5 kinase, the identification of Ser-445 of mIRF5 (Ser-446 of human IRF5) as a critical phosphorylation site, and the development of antibody that recognizes phosphorylated IRF5 at Ser-445, should facilitate further research on the mechanism of IRF5 activation and its role in human diseases.

Material and methods

Antibodies and Other Reagents

The antibodies used in this study and their sources are listed as follows: Santa Cruz Biotech: IRF3, IKKα, TRAF6 and NEMO; Cell Signaling: phospho-IKKα/β, phospho-TBK1, IBα and phospho-IkBα; Sigma-Aldrich: Flag antibody (M2), Tubulin, M2-conjugated agarose and anti-HA-conjugated agarose; Thermo Scientific: HA; Abcam: IRF5. The antibody against phosphor-Ser445 IRF5 was generated by immunizing rabbits with a synthetic peptide (IRLQIpS445NPDLC). LPS, HT-DNA, poly(dA:dT), and poly(I:C) were from Sigma. Plasmid and DNA or RNA ligands were transfected into cells using lipofectamine 2000 (Life Technologies). The kinase inhibitors were dissolved in DMSO and used at the following final concentrations: TBK1 inhibitor (BX795, Selleckchem), 10μM; IKKβ inhibitor (TPCA-1, Sigma), 20μM. GST-IKKβ and GST-TBK1 recombinant proteins were expressed and purified from Sf9 cells.

Expression Constructs

For expression in mammalian cells, cDNA encoding N-terminal Flag- or HA-tagged mouse IRF5 S430A, IRF5 S434A, IRF5 S436A and IRF5 S439A were cloned into pcDNA3; HA-tagged mouse IRF5 WT, IRF5 S445A and human IRF5 WT were cloned into pcDNA3 and pTY-EF1a-GFP-IRES-hygroR lenti-viral vectors. Mutants were constructed with the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

Viruses, Cell culture and Transfections

Sendai virus (Cantell strain, Charles River Laboratories) was used at a final concentration of 100 hemagglutinating unit/ml. All cells were cultured at 37°C in an atmosphere of 5% (v/v) CO2. HEK 293T cells and Raw 264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) cosmic calf serum with penicillin (100 U/ml) and streptomycin (100 μg/ml). THP-1 cells were cultured in RPMI Media 1640 supplemented with 10% (v/v) fetal bovine serum with penicillin (100 U/ml), streptomycin (100 μg/ml) and 0.05 mM 2-mercaptoethanol.

RNAi and Rescue with Transgenes

The lentiviral shRNA vector, pTY-shRNA-EF1a-puroR-2a-GFP-Flag, was provided by Dr. Yi Zhang (Harvard Medical School). The original vector was modified to pTY- shRNA-EF1a-GFP-IRES-puroR and pTY- shRNA-EF1a-GFP-IRES-hygroR by replacing the 2A peptide sequence with IRES sequence and also by replacing puromycin resistant gene with hygromycin resistant gene. The shRNA sequences were cloned into the vectors with U6 promoter. RNAi-resistant cDNA sequences were cloned into the vectors to replace GFP. Lentiviral infection and establishment of stable cell lines were described previously (Tanaka and Chen 2012). The shRNA sequences are as follows (only the sense strand is shown): human IRF5, 5’-GAGGAAGAGCTGCAGAGGAT-3’; mouse IRF5, 5’-GCAGAGAATAACCCTGATTTA-3’; human IKKβ, 5’-GGGAGAACGAAGTGAAACT-3’; human IKKα, 5’-GTACCAGCATCGGGAACTT-3’; human NEMO, 5’-GGACAAGGCCTCTGTGAAA-3’; human TRAF6, 5’-GGAGAAACCTGTTGTGATT-3’.

Quantitative RT-PCR

Total cellular RNA was isolated using TRIzol. 0.1-1 μg total RNA was used for reverse transcription (RT) using iScript Kit (Bio-Rad). The resulting cDNA served as the template for Quantitative-PCR analysis using iTaq Universal SYBR Green Supermix (Bio-Rad) and ViiTM7 Real-Time PCR System (ABIApplied Biosystems Inc., Foster City, CA). Primers for specific genes are listed as follows: human CXCL10, 5’-GTGGCATTCAAGGAGTACCTC-3’ and 5’-TGATGGCCTTCGATTCTGGATT-3’; human TNF-α, 5’-CCTCTCTCTAATCAGCCCTCTG-3’ and 5’-GAGGACCTGGGAGTAGATGAG-3’; human IFN-β, 5’-ACTGCAACCTTTCGAAGCCTTT-3’ and 5’-TGGAGAAGCACAACAGGAGAGC-3’; human GAPDH, 5’-ATGACATCAAGAAGGTGGTG-3’ and 5’-CATACCAGGAAATGAGCTTG-3’.

Partial Purification of IRF5 for in vitro Reaction

As IRF5 would spontaneously form dimer when the protein was affinity purified with a purification tag (e.g, Flag or GST), we attempted to partially purified IRF5 from the 293T FG-mIRF5-HA stable cell line. Cytosolic extracts from these cells were first fractionated using HiTrap Heparin HP column (GE Healthcare). Fractions containing IRF5, as judged by immunoblotting, were concentrated and buffer exchanged for 3 times with hypotonic buffer (20 mM Tris-HCl, pH 7.4; 10 mM NaCl; 3 mM MgCl2) using Amicon Ultra-0.5mL centrifugal filters (Millipore). The partially purified IRF5 was used for in vitro assays.

Purification of IRF5 for Mapping Phosphorylation Site(s)

To determine the phosphorylation site(s) induced by IKKβ, reaction mixture (60 µL) containing 20 mM HEPES-KOH (pH 7.0), 2 mM ATP, 5 mM MgCl2, 40µL of partially purified Flag-mIRF5 from 293T stable cell line and 2µg Flag-IKKβ or BSA was incubated at 30°C for 1 hr followed by incubation with M2-conjugated agarose at 4°C for 4 hr. The beads were washed 3 times with lysis buffer containing 150mM NaCl and 1% Triton X100. Bound proteins were then eluted by boiling in 2x Laemmli Sample Buffer before SDS-PAGE and silver staining. Gel slices from each lane were excised and digested with trypsin in situ. Digested samples were subjected to mass spectrometry using Q Exactive and raw data were analyzed by the search engine MASCOT (MATRIX SCIENCE).

Confocal Microscopy

THP-1 cells (4 × 105) were seeded and differentiated with 50 nM phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) for 48 h and then cultured for another 48 h by replacing the PMA-containing media with fresh media without PMA. The differentiated cells were left unstimulated or stimulated with LPS for 2 h. The cells were immunostained with IRF5 antibody (Abcam; ab21689) or phosphospecific IRF5 antibody. The images were acquired and processed with the Zeiss LSM 700 confocal laser scanning microscope system.

CHAPTER three  
Results

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CHAPTER Four  
Conclusions and Recommendations

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APPENDIX A  
Appendix A Title

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Bibliography

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