UNIVERSITY OF CALIFORNIA, SAN DIEGO

The role of reactive oxygen species and calcium in the interferon regulatory factor 3 mediated toll like receptor 4 signaling

A dissertation submitted in partial satisfaction of the requirements for the Doctor of Philosophy

in

Biology

by

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2007

The Dissertation of Chih-Yuan Chiang is approved, and it is acceptable in quality and form for publication on microfilm:

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DEDICATION

This work is dedicated to my parents, Tieh-Sheng and An-Ling Chiang and my brother Cheng-Yuan Chiang. Thank you for you encouragement and support throughout my life. Without your loving and caring, I wouldn't have such accomplishment.

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ACKNOWLEDGEMENT

I thank my mentors, Dr. Michael David and Dr. Federick Bushman, for taking me as a graduate student. Thank you for carrying me through the most difficult time of my life. I thank my committee members, Dr. Ananda Goldrath, Dr. Daniel Donoghue, Dr. Alexander Hoffmann and Dr. Suresh Subramani for giving me advises and guiding my research projects. I thank the David Lab members, Richard, Dennis, Irene, Kirsten and Hiro for creating a wonderful working atmosphere. I thank Jennifer Wingrove for her dedication on the project.

Chapter 2, in full, is a reprint of the material as it appears in Journal of Immunology, 2006, Chiang Chih-Yuan Edward; Dang, Oanh; Anderson, Keith; Matsuzawa Atsushi; Ichijo, Hidenori; David, Michael, 2006. The author of the dissertation is the primary investigator and author of this paper.

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Chiang, E., Wingrove, J., and David, M. Calcium is required for the Activation of IFN regulatory factor 3 by lipopolysaccharide. (Manuscript In preparation)

ABSTRACT OF THE DISSERTATION

The role of reactive oxygen species and calcium in the interferon regulatory factor 3 mediated toll like receptor 4 signaling

by

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Doctor of Philosophy in Biology

University of California, San Diego, 2007

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A key component of the innate immune system is the Toll-like Receptors (TLR), which identify invading microorganisms by recognizing pathogen associated molecular patterns. Toll like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS) that is expressed on the cell wall of gram negative bacteria. TLR4 is unique in utilizing four adaptor proteins to specify the downstream transcriptional events. The MyD88 dependent pathway utilizes both MyD88 and TIRAP (TIR-associated protein) which lead to NF-κB activation. This pathway is critical in mediating the induction of pro-

inflammatory cytokines. The MyD88 independent pathway signals though TRIF (TIR-doimain-containing adaptor protein-inducing IFN-β) and TRAM (TRIF-related adaptor molecule) which activate IKKε/TBK1 and this lead to Interferon regulatory factor 3 (IRF-3) activation. IRF-3 plays a pivotal role in up-regulating interferon stimulated genes in response to bacterial, viral infection and DNA damaging agents.

In the present study we show that interferon stimulated gene 54 (ISG54) induction by LPS, which occurs in an IRF-3-dependent manner, requires the generation of ROS by the NADPH oxidase 4 (NOX4). Subsequent activation of ASK1 links LPS-induced ROS production to the activation of MKK6 and p38, two kinases that were previously identified as components of the LPS-induced IRF-3 activation cascade. Production of ROS occurs frequently concomitant with an increase in cytosolic calcium. Here, we reported that calcium mediates IRF-3 activation in response to LPS. In most eukaryotic cells, the release of calcium from the endoplasmic/sarcoplasmic to the cytosol is mediated by two receptors: inositol-1,4,5-triphosphate receptors (IP_3R) and Ryanodine receptors (RYRs).Phospholipase C gamma 2 (PLCγ2) specifically mediates the cleavage of PIP_2 into IP_3 which regulates the release of calcium from ER into cytosol through the IP_3Rs. The calcium dependence is specific to LPS mediated ISG54 induction, and is not common to ISG54 induction by IFN-β. The expressions of two other IRF3 dependent genes, TRAIL and RANTES, were also abrogated at the presence of PLCγ2 specific siRNA and the IP_3R antagonist. However, the expression of NFκB dependent genes such as TNFα and IκB remained intact. This further suggests that calcium is required for MyD88 independent but not MyD88 dependent pathway.

CHAPTER 1:

Background

THE INNATE IMMUNITY:

The mammalian immune system is comprised of both adaptive and innate immunity (Table I). The adaptive immunity includes two types of lymphocytes: T cells and B cells. Both lymphocytes express a diverse repertoire of receptors. It is estimated that 10^{14} and 10^{18} different B cell and T cell receptors respectively are generated somatically. T cells use their antigen receptors to recognize the peptides bound to MHC class II molecules on the surface of antigen-presenting cells (APCs). However, these peptides can be either self peptides or peptides derived from microbial pathogens. Because the antigen receptor is randomly generated, T cells cannot distinguish self from non-self on the basis of peptide recognition alone. T cell activation further requires the interaction of CD28 with the co-stimulatory molecule, CD80 and CD86, which are expressed on the surface of the APCs. The induction of CD80 and CD86 molecules on the surface of the APC is contributed by the innate immune system (1). APCs express groups of pattern recognizing receptors (PRRs) which detect the invasion of foreign pathogens. PRRs include the cell surface expressed toll like receptors (TLRs) -1, -2, -4, -5 and -6; the endosome expressed TLR-3, -7, -8, -9 (2); the cytosolic expressed nucleotide binding oligomerization domain (NOD) proteins(3-5), Retinoic-acid-inducible protein I (RIG-I) and Melanoma differentiation associated gene 5 (MDA5) (6-8). For the scope of the dissertation, only the toll like receptor signaling event will be discussed in detail.

PRRs recognize microbial components, known as pathogen associated molecular patterns (PAMPs), which are essential for the survival of the microorganism and therefore difficult for the microorganism to alter. This evolutionary strategy prevents the

generation of microbial escape mutants and allows a limited number of germ-line encoded PRRs to recognize a great variety of molecular structures associated with pathogen. The ligation of TLR ligands to their cognate receptors causes dentritic cell (DC) maturation. DC maturation is characterized by the induction of co-stimulatory molecules and the production of cytokines such as IL-12, IL-18 and TNF-α. Matured DCs show increased antigen-presenting capacity and migrate from the peripheral tissues to draining lymph nodes, where they activate naïve T_H cells. Recognition of an antigen in the absence of CD80 or CD86 molecules leads to permanent inactivation of the T cells (2, 9, 10).

The innate immune system is also important in modulating the naïve T_H cell development. Naïve T_H cells differentiate into two subsets: T_H1 and T_H2. T_H1 cells secrete interferon-γ (IFN-γ) and promote mainly cellular immunity, whereas T_H2 cells produce interleukin 4 (IL-4), IL-5, IL-10 and IL-13 and primarily promote humoral immunity. The cytokines produced by matured APCs provide external cues in this step. IL-12 drives T_H1 differentiation, whereas IL-4 induces T_H2 differentiation. These "conditional" cytokines are produced in the early phase of infection. Infection by intracellular pathogens induces primarily a T_H1-dominant response that protects against the majority of microorganism, whereas helminth infection induces a T_H2 response (Figure 1-1) (10).

TOLL LIKE RECEPTORS:

The Molecular structure of the Toll like receptors:

Toll receptors, which play a critical role in defining the polarity of the dorsal-ventral pattern, are first discovered in *Drosophila* (11). Subsequent studies reveal that Toll also has an essential role in the insect innate immune response against fungal infection (12). Sequence and pattern analysis identified a human homologue of *Drosophila* toll protein, which is referred to Toll like receptors (TLRs). TLRs and IL-1 receptors (IL-1R) have a conserved region of ~200 amino acids in their cytoplasmic tails, which is known as the Toll/IL-1R (TIR) domain. TIR domain comprises three conserved boxes which are crucial for signaling. By contrast, the extracellular region of the TLRs and IL-1Rs differs markedly: the extracellular region of TLRs contains leucine-rich repeat (LRR) motifs, whereas the extracellular region of IL-1Rs contains three immunolglobulin-like domains. The LRR domains of the TLRs form a horseshoe structure, and it is thought that the concave surface of the LRR domains is involved directly in the recognition of various pathogens. Twelve TLRs are identified to date (Table II). Based on their primary sequences, TLRs can be further divided into several subfamilies, each of which recognizes related PAMPS: TLR-2 heterodimerizes with either TLR-1 or TLR-6 and mediates the response to peptidoglycan (PGN) and lipoproteins, which are presented in Gram-negative, Gram-positive bacteria and mycoplasmas; TLR-3, TLR-7 and TLR-9 recognize nucleic acids which are the genetic material of double stranded RNA viruses (dsRNA), singles stranded RNA viruses (ssRNA) and CpG islands, respectively. However, the TLRs are unusual in that some can recognize several structurally unrelated ligands. For example, TLR-4 recognizes a very divergent collection of ligands such as lipopolysaccharide (LPS), the plant diterpene

paclitaxel, the fusion protein of respiratory syncytial virus (RSV), fibronectin, and heat shock proteins.

Differential adapter utilization by TLRs mediates TLR-specific gene expression:

Toll like receptors directly interact with adaptor molecules which are critical in mediating and specifying downstream TLR signaling cascade. The four adaptors identified are MyD88 (Myeloid differentiation factor 88) (13, 14), Mal (MyD88-adaptor-like)/TIRAP (TIR domain containing adaptor protein) (15-17), TRIF/TICAM-1(TIR containing adaptor molecule-1) (18, 19) and TRAM (TRIF-related adaptor molecule) (20). All four adaptors have conserved TIR domain whereas MyD88 is the only adaptor contains an extra death domain (DD). TLR-4 is unique in utilizing all four adaptors to activate two downstream pathways: the MyD88-dependent and –independent pathways. The MyD88 dependent pathway is composed of both MyD88 and Mal/TIRAP. MyD88- or TIRAP- deficient mice have impaired pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) production. Surprisingly, delayed activations of JNK, p38, ERK and NF-κB remain intact in either MyD88-deficinet or TIRAP deficient mice in response to LPS (14, 17). This suggests that MyD88 and TIRAP have compensatory function in response to LPS.

The MyD88 independent pathway is composed of both TRIF and TRAM. TRIF-deficient and TRAM-deficient mice have impaired expression of IFN-β and IFN inducible genes (ISGs) in response to LPS. Furthermore, LPS-induced augmentation of CD69 and CD86 expression is severely reduced in TRIF-deficient and TRAM-deficient $B220^{+}$ cells **(18, 20)**. The NF-κB activation of the MyD88 independent pathway is thirty minutes delayed than that of the MyD88 dependent pathway in murine embryonic

fibroblasts (mEF). It is reported that IRF-3 mediates TNF-α production which signals through TNF receptors in an autocrine manner . Activation of TNF-α pathway is responsible for the delayed NF-κB activation (21, 22)

In summary, MyD88 is a common adaptor that's recruited to IL-1β receptors, IL-18 receptors and all TLR receptors (except TLR-3) in response to their cognate ligands (10, 13, 14). MyD88 and Mal/TIRAP take part in TLR-4 mediated MyD88-dependent pathway whereas TRAM and TRIF/TICAM-1 is responsible for the MyD88-independent pathway. Mal/TIRAP also interacts with MyD88 in specifying TLR-2 downstream signaling cascade (16, 17). TRIF/TICAM-1, however, is the only adaptor molecule that is associated with TLR-3 receptor in response to dsRNA stimulation (Figure 1-2). The interaction of the different TLRs with distinct combinations of adaptor molecules creates a platform to which additional kinases, transacting factors, and possibly other molecules are recruited, events that lead, ultimately, to gene expression (23).

The signal transduction pathway of the TLR:

The MyD88-dependent pathway is shared between all TLRs except TLR-3. Stimulation of TLRs triggers the association of MyD88, which in turn recruits IRAK4 (IL-1R associated kinase 4), which then induces the phosphorylation of IRAK1. TRAF6 (tumor-necrosis-factor-receptor-associated factor 6) is also recruited to the receptor complex, by associating with phosphorylated IRAK1. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with TAK1 (transforming-growth-factor-β-activated kinase), TAB1 (TAK1-binding protein1) and TAB2 at the plasma membrane, which induces the phosphorylation of TAB2 and TAK1. IRAK1 is

degraded at the plasma membrane, and the remaining complex (consisting of TRAF6, TAK1, TAB1 and TAB2) translocates to the cytosol, where it associates with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1). This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1. TAK1, in turn, phosphorylates both mitogen activated protein kinases (MAPK) and the IKK complex (inhibitor of nuclear factor-κB (IκB) kinase), which consists of IKK-α, IKK-β and IKK-γ. IKK complex phosphorylates IκB and leads to its ubiquitylation and subsequent degradation.

The MyD88-independent pathway is unique to both TLR-3 and TLR-4. MyD88 independent pathway transduces its signal through TRIF/TICAM-1. IKKε and TBK1 (TRAF-family-member-asociated NF-κB activator (TANK)-binding kinase 1) are downstream kinases of TLR-3 and -4. IKKε and TBK1 activate IRF-3 which is an essential component of the IFN-β enhanceosome (24, 25) (Figure 1-2). In addition, p65 is also reported to associate with IRF-3 in mediating TLR4 but not TLR3 gene transcription (26, 27).

INTERFERON REGULATORY FACTOR 3 (IRF-3):

IRF-3 mediates type I interferon and interferon stimulated genes production:

The promoter region of the IFN- β gene contains several regulatory cis-elements. The positive elements constitute a virus-inducible enhancer, designated as PRDI, -II, -III, -IV. PRD II and –IV elements were bound by NF-κB and ATF-2/c-Jun, respectively to

cooperate with PRDI and –III in the induction of the IFN-β gene in virus-infected cells. As for the IFN-α gene family, which consists of more than a dozen members, sequences similar to PRDII and –IV have not been identified, but sequences similar to PRD-I and –III have been found within each of their promoters. The PRDI and –III elements are known to bind members of a family of transcription factors, termed interferon regulatory factors (IRFs). IRFs include nine family members. Four out of nine IRFs members have been implicated in the transcriptional induction of the IFN α/β genes. These four members are IRF-1, IRF-3, IRF-7 and IRF-9/ISGF3. However, the production of IFN α/β in IRF-1 deficient cells remains intact in response to Newcastle disease virus infection (28). This suggests that either IRF-1 is not involved or that redundant mechanisms may be operating in the gene induction. Evidence has also been provided that an IFN activated transcription factor, ISGF3, is involved in IFN α/β gene induction. However, it is unclear whether ISGF3 directly acts on the IFN α/β genes. Recently, much attention has been focused on two structurally related members, IRF-3 and IRF-7 (29) .

IRF-3 is a 55 kDa protein that's constitutively expressed in various tissues. No increase in the relative steady-state levels of IRF-3 mRNA was observed in response to stimuli. Once IRF-3 is activated, it homodimerizes and translocates into the nucleus where it interacts with the transcriptional co-activator, CBP/p300 (30, 31). The presence of an NES element ultimately may shuttle IRF-3 from the nucleus and terminate the initial activation of IFN-responsive promoters. The phosphorylated form of IRF-3 exported form the nucleus may now be susceptible to proteasome-mediated degradation (32). The IRF-3 is critical in mediating IFN-α/β production through biphasic mechanism.

In the early phase, IRF-7 is expressed only at very low levels by spontaneous IFN α/β signaling. Activation of IRF-3 results in efficient and weak activation of IFN-β and IFN-α4 genes, respectively. In the late phase, interferons activate the JAK/STAT pathway in which the ISGF3 complex up-regulated IRF-7 production. IRF-3 and IRF-7 cooperate with each other for amplification of IFN-α/β gene induction, result in the full procurement of the normal mRNA induction profile of the IFN-α gene subfamily (29).

Multiple signaling pathways activate IRF-3:

IRF-3 contains N-terminal DNA Binding Domain (DBD) which is characterized by a conserved tryptophan repeats. The C-terminal of IRF-3 contains IRF association domain (IAD) that mediates homo- and hetermoeric interactions among IRFs and interacts with other transcriptional co-modulators. The activation of IRF-3 is characterized by the phosphorylation of either the threonine residue at the N-terminus (T135) or the serine/threonine cluster located at the C-terminus (S385, S386, S396, S398, S402, T404 and S405) (Figure 1-3). The presence of multiple phosphorylation sites in IRF-3 suggests more than one signaling pathways are involved in shaping the activation of IRF-3 through unique mechanisms.

Hiscott *et al.* reported that intramolecular association between the C terminus and the DBD maintains IRF-3 in a latent state by masking both DBD and IAD regions of the protein. Initial phosphorylation at Ser385 and Ser 386 partially unfold the structure and facilitate phosphorylation at the rest of the S/T residues located from amino acid 396 to 405. The conformational change in IRF-3 relieves C-terminal auto-inhibition and exposes both DBD and IAD regions. The “auto-inhibition” model is further supported by

the X-ray crystal structure of the C-terminal domain of IRF-3. In the un-stimulated state, the N-terminal α-helix makes direct contacts with the C-terminal helices, creating a compact, largely hydrophobic structural unit. Activation of IRF-3 causes negatively-charged phosphate groups to serine and threonine residues in the helical bundle is predicted to induce a dramatic conformational change that would disrupt the auto inhibited state (33).

How IRF-3 is activated in response to various stimuli remains controversial. Karpova, AY *et al.* demonstrated that DNA-PK (DNA dependent protein kinase) is activated upon Sendai virus (SV) infection. DNA-PK subsequently binds to IRF-3 and specifically phosphorylates it at threonine 135 (34). On the contrary, Servant, MJ *et al*. reported a different mechanism in which SV requires C-terminal located serine threonine cluster to achieve IRF-3 activation (35). In parallel to Servant, MJ *et al*., Yoneyama, M *et al.* reported a similar mechanism in which phosphorylations at the C terminal serine threonine cluster is important for the activation of IRF-3 in response to Newcastle Disease virus (30). DNA damaging agents such as UV and doxorubicin also activate IRF-3. Servant, MJ *et al*. reported that MEKK1-MKK4/SEK1-JNK of the MAPK axis is activated upon doxorubicin stimulation and this leads to the phosphorylation of N-terminus located phosphorylation sites (35). On the contrary, Kim et al reported that the two C terminus located serine residues, Ser385 and Ser386, are critical in UV/doxorubincin mediated IRF-3 activation (36).

Given the pivotal role of IRF-3 in mediating the production of antiviral and anti-bacterial cytokines, it is not surprising that IRF-3 is the target of various pathogens. For example, the binding of E6 protein of Human papillomavirus (HPV) to IRF-3 results in a

marked reduction of the IRF-3 transactivation function in vivo (37). Human Herpes virus 8 (HHV-8) encodes vIRF-3, a dominant-negative mutant of both IRF-3 and IRF-7. vIRF-3 inhibits virus-mediated transcriptional activity of the IFN-α promoter (38). Furthermore, the double stranded RNA (dsRNA) binding protein NS1 of influenza virus is shown to prevent the potent antiviral interferon response by inhibiting the activation of IRF-3 (39).

THE CALCIUM SIGNALING NETWORK:

The calcium signaling is used throughout the life history of an organism. Life begins with a surge of Ca^{2+} at fertilization and this versatile system is then used repeatedly to control many processes during development such as cell differentiation, cell proliferation, transcription factor activation and apoptosis.

Upon stimulations, cells employ various mechanisms to keep the intracellular Ca^{2+} level at a desired state. Cells obtain Ca^{2+} from either extracelluar or intracellular sources (40, 41). Three pathways have been characterized by which Ca^{2+} ions can enter cells from the exterior. First, nerve and muscle cells possess voltage-operate calcium channels (VOCCs) which open in response to a membrane depolarization and allow Ca^{2+} entry. VOCCs are characterize by it speed, brevity and intensity of the observed Ca^{2+} transients. Secondly, in some neuronal cells, receptor operated channels (ROCs) on the plasmas membrane may open as a consequence of activation and allow Ca^{2+} to enter. Thirdly, in cells stimulated by growth factors or cytokines, there maybe a need for a protracted period of Ca^{2+} elevation of cytosol Ca^{2+}

must lead to depletion of the Ca^{2+} stores. This is overcome by allowing extracellular Ca^{2+} to enter through plasma membrane cation channels, called store-operated channels (SOCs). This allows the stores to be replenished.

The other principal source of Ca^{2+} for signaling is the internal stores that are located primarily in the endoplasmic/sarcoplasmic reticulum (ER/SR), in which Ryanodine receptors (RYR) or Inositol 1,4,5-triphosphate receptors (IP_3Rs) regulate the release of Ca^{2+}. IP_3Rs are virtually universal, whereas RyRs are most evident in excitable cells such as skeletal and cardiac muscles. In skeletal muscle cells, the RYRs are located in the membrane of the SR and associate with the cytoplasmic domain of the dihydropyridine receptor, a voltage-sensing protein in the plasma membrane. A change in potential across the plasma membrane induces a conformational change in the RYRs, so that Ca^{2+} ions are released from the SR into the cytosol (42). On the other hand, IP_3Rs are under the regulation of IP_3, the cleavage products of PIP_2 by the enzymatic activity of PLC (phospholipase C) (Figure 1-4). Eleven PLC isozymes have been classified into five families based on their structure and sequence relationships. Namely, the eleven PLCs are PLCβ (1–4), PLCγ (1 and 2), PLCδ (1, 3 and 4), PLCε (1) and PLCζ (1). All PLCs contain the PH (Pleckstrin homology) domain and catalytic X and Y domain, in addition to other regulatory domains including the C2 domain and the EF-hand domain (43). PLC β is activated by G protein mediated mechanisms involving members of the Gq, Gi or Go families. PLCγ is unique in possessing one SH_3 domain and two SH_2 domains, inserted between the X and Y sequences of the catalytic domain. PLCγ is therefore able to interact with either receptor protein tyrosine kinase or non-receptor protein tyrosine kinase to convey the signal cascade. PLCζ is characterized to be

the physiological stimulus for egg activation and development at mammalian fertilization (44, 45). In contract to PLCβ and PLCγ, the mechanisms for the activation of PLCδ and PLCε require substantial further study (7, 42).

The approximate concentration of free calcium in the cytosol of the resting cell ranges between 10~100 nM whereas the free calcium in the endoplasmic reticulum is maintained at 30~300 μM. Various mechanisms are employed to maintain the Ca^{2+} in the homeostasis state. These mechanisms depend on pumps that remove the Ca^{2+} signal during the recovery from stimulation. The plasma membrane Na^{+}/Ca^{2+} exchanger and Ca^{2+} ATPase (PMCA) pumps extrude Ca^{2+} to the outside whereas the ER/SR Ca^{2+} ATPase (SERCA) pumps bring the cytosolic Ca^{2+} back to the SR/ER (Figure 1-4).

REACTIVE OXYGEN SPECIES SIGNALING:

Reactive oxygen species (ROS) include superoxide ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\bullet}$) and a variety of their reaction products. Two molecules of superoxide can react to generate hydrogen peroxide in a reaction known as dismutation, which is accelerated by the enzymes superoxide dismutase (SOD). In the presence of iron, superoxide and hydrogen peroxide react to generate hydroxyl radicals (Figure 1-5). ROS are historically been viewed as a harmful but unavoidable consequence of an aerobic lifestyle. The short lived ROS reacts non-specifically and rapidly with biomolecules, including DNA, proteins, lipids and carbohydrates. This can lead to the DNA mutation, protein oxidations and lipid peroxidation. However, the discovery of ROS producing enzymes suggests their important roles in mediating cellular processes.

ROS are deliberately generated by various enzymes such as xanthine oxidase, cyclo-oxygenases, lipoxygenases and NADPH oxidase (NOX). NOX is a well-characterized enzyme in phagocytes that generates high levels of superoxide in phagocytes as part of the anti-microbicidal mechanisms (46). The NOX enzymes are classified into three main groups, according to the presence of domains in addition to the gp91 phox (NOX2) domain. The first group includes NOX1, NOX3, and NOX4. They are nearly identical in size and structure to gp91 phox. Similar to gp91phox, they contain the electron transfer centers that are required to pass electrons from NADPH to molecular oxygen to form superoxide. According to the predicted topology of this sub-group, the enzyme oxidizes NADPH on cyotosolic side of the membrane and reduces oxygen across the membrane to generate superoxide. The second group include NOX5. Building on the basic structure of gp91phox, NOX5 has an amino terminal calmodulin like domain that contains four binding sites for calcium. In calmodulin, calcium binding results in a conformational change, exposing hydrophobic resides that bind to and regulate target enzymes. The third group, the DUOX enzymes, further extends the Nox5 structure, containing an amino-terminal peroxidase-homology domain. An extra predicted transmembrane α-helix between the peroxidase homology domain and the calcium binding domain places the peroxidase domain on the extracellular face of the plasma membrane (47).

The production of ROS occurs frequently concomitant with an increase in cytosolic calcium. Singh *et al.* identified the interdependent relationship of calcium and ROS in regulating B cell receptor (BCR) signaling (48). The ROS produced by DUOX1 plays a critical role in the early event of BCR activation. ROS directly

involves in the transient inhibition of BCR-associated protein tyrosine phsophatase (PTP) activity. Inhibition of PTP activity shifts the equilibrium in favor of kinase activation. However, the BCR dependent ROS production is significantly inhibited in the presence of either EGTA (extracellular calcium chelator) or TMB-8 (inhibits the release of calcium from the endoplasmic reticulum). Intriguingly, suppression of BCR-initiated ROS generation also results in an attenuated calcium response. The evidence that inhibition of one leads to a concomitant inhibition of the other suggests a positive cooperativity between the BCR-dependent calcium and BCR-dependent ROS pathways. While both the BCR-dependent calcium and ROS pathways influence each other, it was the ROS that ultimately affected BCR signaling. This was determined from experiments indicting that, whereas the inhibitory effect of calcium could be overcome by independent elevation in ROS levels, the reverse was not true.

MITOGEN-ACTIVATED PROTEIN KINASES (MAPKS) IN THE INNATE IMMUNE RESPONSE:

In mammalian cells, the four best characterized mitogen-activated-protein kinase (MAPK) pathways are the ERK/MAPK1/2, the p38, the c-Jun amino-terminal kinase (JNK) and ERK5 pathways. Studies have shown that, in general, the ERK pathway is activated by growth factors, mitogenic stimuli and tumor promoters, whereas environmental stress and inflammatory cytokines stimulate the p38 and JNK pathways. MAPKs are regulated by phosphorylation and are substrates of dual specificity MAPK kinases (MAPKK) that phosphorylate at the tripeptide motif Thr-Xaa-Try. The ERK MAP kinases are activated by the MAP kinases kinases (MKK) MKK1 and MKK2. The p38 MAP kinases are activated by MKK3, MKK4, and MKK6. The JNK pathway is

activated by MKK4 and MKK7. The MKKs must also be activated by phosphorylation within their activation loops. This is accomplished by a group of serine/threonine kinases known as the MAPK kinase kinases (MKKKs) (49).

It was long thought that invertebrate animals could not mount a meaningful defense against pathogens. Although *C. elegans* appear to lack a Toll receptor signaling based defense mechanism, recent work suggested a role of MAPK signaling pathway in defense toward infection and death caused by bacterial pathogens. A genetic screen for Caenorhabditis elegans mutants with enhanced susceptibility to killing by *Pseudomonas aeruginosa* led to the identification of two genes required for pathogen resistance: sek-1, which encodes a MAPKK, and nsy-1, which encodes a MAPKKK. Furthermore, RNA interference assays and biochemical analysis established that a p38 ortholog, pmk-1, functions as the downstream MAP kinase required for pathogen defense (50). The role of the MAPK signaling in the innate immunity is conserved in vertebrate animals. Recent studies suggested that stimulation with LPS through TLR4 causes the association of a MAPKKK, Apopotosis signal-regulating kinase 1 (ASK1), with TRAF6. In unstimulated cells, ASK1 activation is inhibited by binding to its inhibitor, thioredoxin. The binding of ROS to thioredoxin causes its conformation changes and therefore can no longer exert its inhibitory effect against ASK1 activation (51). ROS is produced in response to LPS and this leads to ASK1 activation. ASK1 activation results in the activation of both NF-κB and IRF-3 pathway. Furthermore, ASK1 deficient mice have impaired pro-inflammatory cytokine production and are resistant to LPS induce septic shock (52).

Pathogens have developed various mechanisms impairing the MAP kinase signal transduction pathway. Anthrax toxin, which is produced by *Bacillus anthracis*, interrupts

several MAPK signaling pathways by proteolytically degrading all MAPKKs except MAPK5. It is thus discovered that the cleavage of MKK6 by the lethal toxin of *B. anthracis* impairs the activation of IRF-3 in response to LPS (53). Furthermore, *Yersinia pseudotuberculosis* disrupts MAPK signaling pathway by secreting bacterially encoded cysteine protease YopJ protein into infected macrophages. YopJ binds specifically to MAPKKs and inhibits kinase activity by preventing phosphorylation (54).

Table 1-1: Innate and Adaptive Immunity (9)

Property	innate immune system	Adaptive immune system
Receptors segments segments	Fixed in Genome Rearrangement is not necessary	Encoded in gene Rearrangement necessary
Distribution	Non-clonal All cells of a class identical	Clonal All cells of a class distinct
Recognition	Conserved molecular patterns (LPS, LTA, mannans, glycans)	Details of molecular structure (proteins, peptides, carbohydrates)
Self-Nonself Discrimination	Selected over evolutionary time	Selected in individual somatic cells
Action time	Immediate activation of effectors	Delayed activation of **effectors**
Response	Co-stimulatory molecules Cytokines: IL-1β, IL-6 Chemokines: IL-8	Clonal expansion or anergy IL-2 Effector cytokines: IL-4, IFN-γ

Table 1-2: TLR Recognition of Microbial Component(2):

MicrobialComponents	Species	TLR usage
Bacterial		
LPS	Gram-negative bacteria	TLR-4
Diacyl lipopetides	*Mycoplasma*	TLR-6/TLR-2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR-1/TLR-2
LTA	Group B *streptococcus*	TLR-6/TLR-2
PG	Gram-positive bacteria	TLR-2
Porins	*Neisseria*	TLR-2
Lipoarabinomannan	Mycobacteria	TLR-2
Flagellin	Flagellated bacteria	TLR-5
CpG-DNA	bacteria and mycobacteria	TLR-9
Fugus		
Zymosan	*Saccharomyces cerevisiae*	TLR-6/TLR-2
Phospholipomannan	*Candida albicans*	TLR-2
Mannan	*Candida albicans*	TLR-4
Glucuronoxylomannan	*Cryptococcus neoformans*	TLR-2 and TLR-4
Parasites:		
tGPI-mutin	*Trpanosoma*	TLR-2
Glycoinositolphospholipids	*Trpanosoma*	TLR-4
Hemozoin	*Plasmodium*	TLR-9
Profilin-like molecule	*Toxoplasma gondii*	TLR-11
Viruses		
DNA	Viruses	TLR-9
dsRNA	Viruses	TLR-3
ssRNA	RNA Viruses	TLR-7 and TLR-8
Envelope proteins	RSV, MMTV	TLR-4
Hemagglutinin protein	Measles virus	TLR-2
Host		
Heat-shock protein 60, 70		TLR-4
Fibrinogen		TLR-4

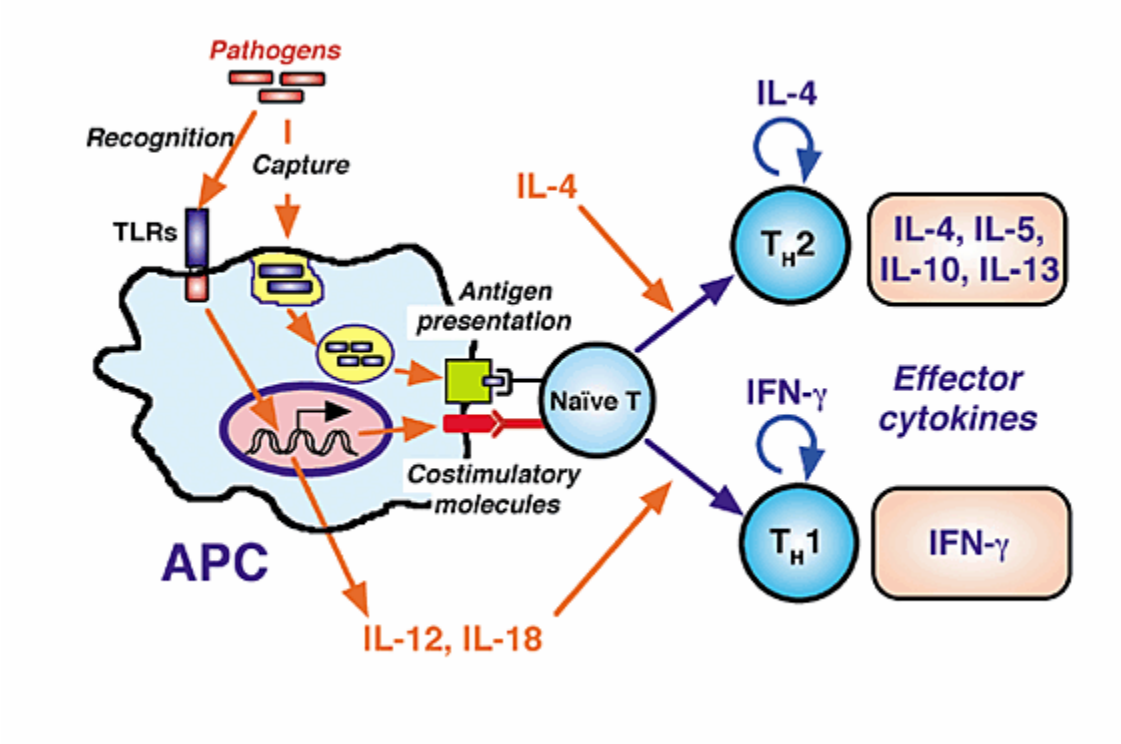

Figure 1-1: APCs mediate the activation and development naïve T cell (54). Pathogen associated molecular patterns (PAMPs) are the ligands of TLR receptors. Activation of TLR pathway leads to the upregulation of co-stimulatory molecules, CD80 and CD86, which are required for Naïve T cell activation. The differentiation of Naïve T cells is also modulated by cytokines produced by matured APCs.

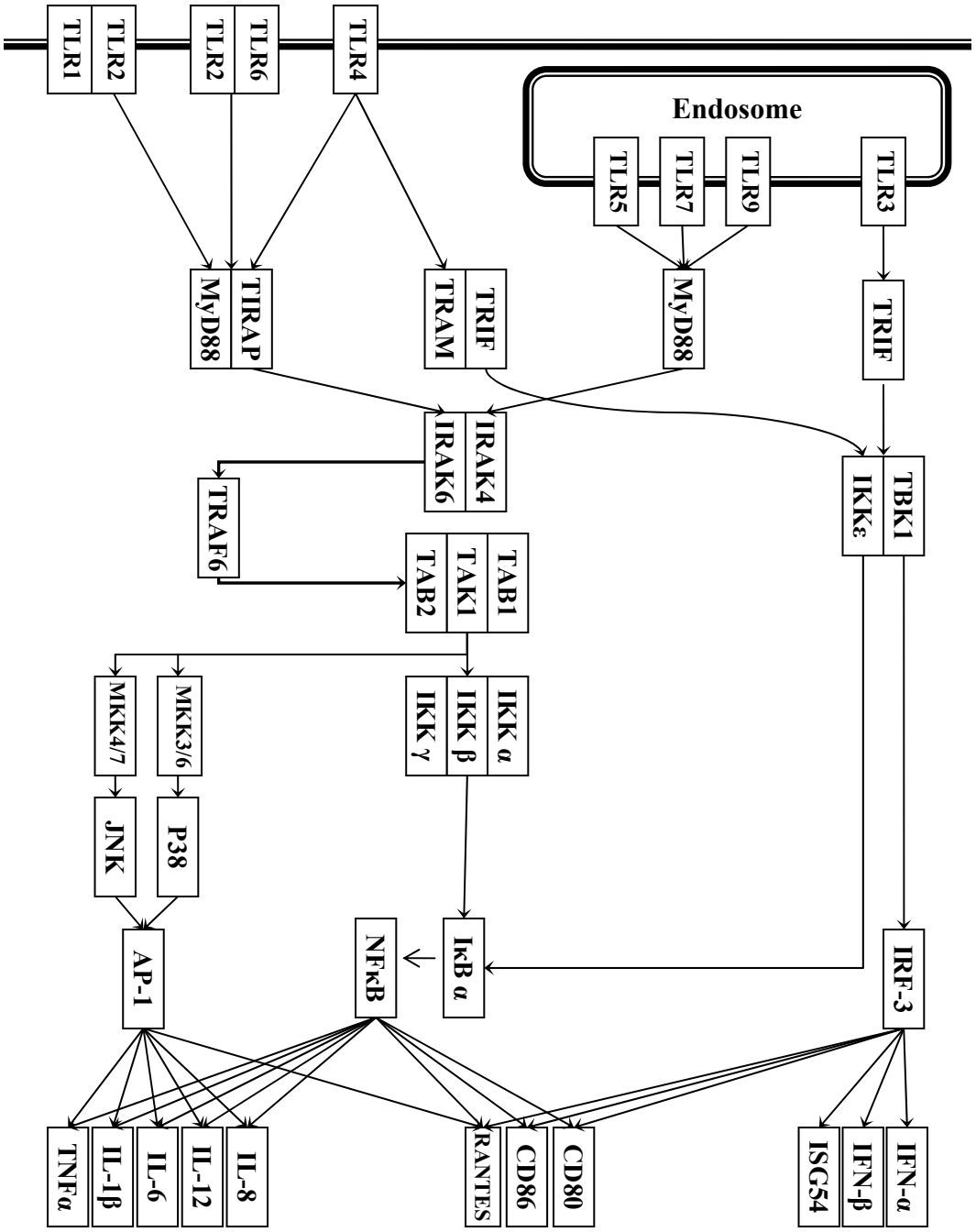

Figure 1-2: Toll Like Receptors signal transduction pathway (33):

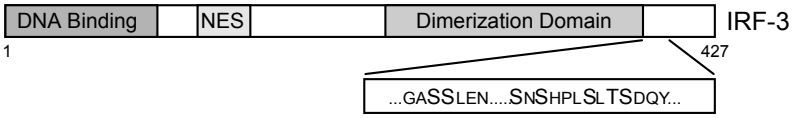

Figure 1-3: The molecular structure of IRF-3: IRF-3 contains a nuclear export sequence (NES) that maintains the protein in the cytoplasm until appropriately phosphorylated in response to viral infection. Activation of IRF-3 was reported to require phosphorylation of several serine residues located in two clusters at the carboxy-terminus of the protein. Phosphorylation of two residues, Ser385 and Ser386, seems to be required for the subsequent phosphorylation of additional serines in the serine-rich region between aa. 396 and 405

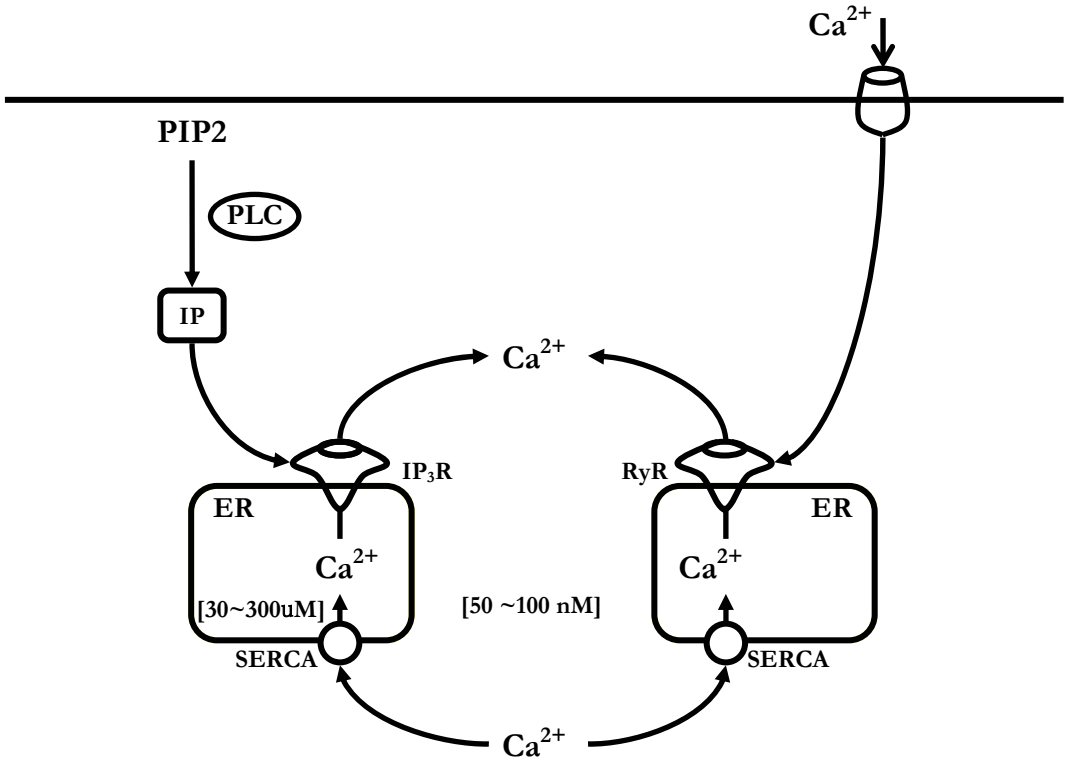

Figure 1-4: Calicium signaling (41): Cytosolic calcium concentration is ranges between 10~100 nM whereas the free calcium in the endoplasmic reticulum is maintained at 30~300 μM. IP_3Rs and RYRs mediate the release of calcium from ER/SR into the cytosol. IP_3R is regulated by IP_3, the cleavage product of PIP_2 by the enzymatic action of phospholipase C. RYRs are associated with the cytoplasmic domain of the dihydropyridine receptor, a voltage-sensing protein in the plasma membrane. A change in potential across the plasma membrane induces a conformational change in the RYRs, so that Ca^{2+} ions are released from the SR into the cytosol. Cytosolic Ca^{2+} is brought back to the SR/ER through ER/SR Ca^{2+} ATPase (SERCA) pumps.

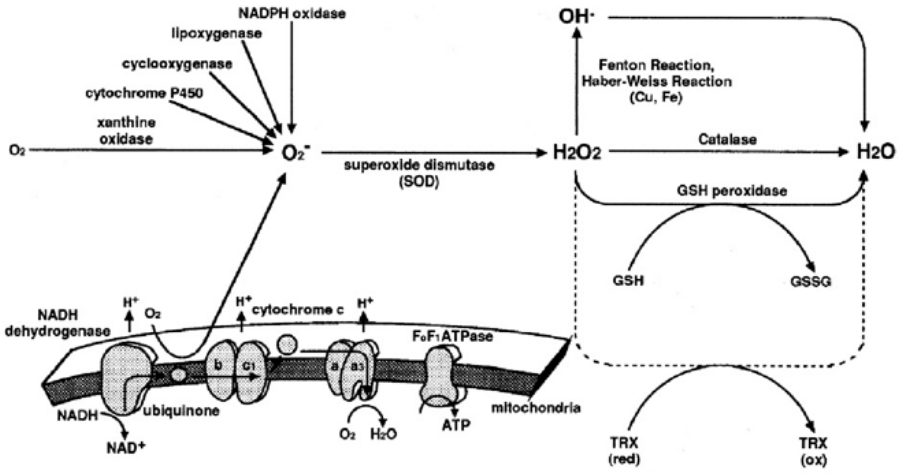

Figure 1-5: Source of Reactive oxygen species: ROS include radicals such as superoxide anion (O_2-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH-). Superoxide anions are generated by several pathways in the cells such as the mitochondria respiratory chain, NADPH-oxidases, xanthine oxidase, cyclo-oxygenases or lipoxygenases. O_2- can be readily converted into H_2O_2 and OH- by superoxide dismutase (SOD) and the Fenton/ Haber-Weiss reaction.

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CHAPTER 2

Apoptosis-Regulating Signal Kinase 1 Is Required for Reactive Oxygen Species-Mediated Activation of IFN Regulatory Factor 3 by Lipopolysaccharide

ABSTRACT:

Interferon Regulatory Factor 3 (IRF-3) participates in the transcriptional induction of interferon (IFN) α and IFNβ, as well as a subset of IFN-stimulated genes (ISGs), as a result of viral infection. In addition, bacterial cell wall components such as lipopolysaccharide (LPS) or lipoteichoic acid also activate IRF-3 in a p38-dependent manner. Here we show that IRF-3-mediated ISG induction by LPS requires the production of reactive oxygen species (ROS) by the NADPH-dependent oxidase, NOX4. Furthermore, we present evidence that LPS-mediated ROS production leads to activation of apoptosis regulating-signal kinase 1 (ASK1), a mitogen-activated protein kinase kinase kinase (MAPKKK) family member capable of activating the MKK6/p38 axis. ASK1 kinase activity proved essential for IRF-3-mediated ISG induction by LPS. Thus, our results presented here suggest a novel role for ROS and ASK1 in the innate immune response as signaling intermediates in the IRF-3 activation pathway.

INTRODUCTION:

Innate immune recognition is mediated by germline-encoded Toll-like receptors (TLRs) that recognize conserved pathogen-associated molecular patterns (PAMPs) (1). In humans, nine TLRs have been identified. TLR4, in the presence of CD14, mediates the host cell responses towards LPS. Ectopic expression of TLR4 can confer sensitivity towards LPS in otherwise LPS unresponsive cells (2-4). Other TLRs are involved in the recognition of PAMPs such as LTA, dsRNA, glucans or peptidoglycans (1). Significant progress had been made in the identification of the

signaling molecules involved in TLR induced gene expression, however, much of the work focused on the MyD88-dependent activation of NF-κB.

The ubiquitously expressed transcription factor IRF-3 emerged as an important response factor to viral infection. Infection with cytomegalovirus, Newcastle Disease or Sendai Virus causes nuclear translocation of IRF-3 and cooperative DNA binding with the transcriptional co-activator CBP/p300 (5-8). In addition, we have previously shown that IRF-3 activation by LPS occurs in a p38-dependent manner (9). This is followed by the induction of a distinct subset of ISRE containing genes. In the last few years, several components of LPS-mediated IRF-3 activation have been identified. IRF-3 activation by LPS occurs in a MyD88-independent manner, however, cells deficient in the adaptor molecules TRIF or TRAM lack IRF-3 activation after TLR4 engagement (10-13). Two IKK-related proteins - IKKε and TBK1 - were identified as kinases that phosphorylate IRF-3 in response to viral infection or TLR3 stimulation (12,14). Overexpression of IKKε or TBK1 induces the phosphorylation of IRF-3 and activates IFN-inducible genes. Furthermore, analysis of TBK1-/- MEFs confirmed the role of TBK1 in IRF-3-dependent gene expression.

Reactive oxygen species (ROS) include hydrogen peroxide (H_2O_2), and radicals such as superoxide anion ($O_2^{-\cdot}$), and hydroxyl radical ($OH^{\cdot}$). Superoxide anions are generated by several pathways such as the mitochondrial respiratory chain, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, xanthine oxidase, cyclo-oxygenase or lipoxygenase (15). Once formed, $O_2^{-\cdot}$ can be readily converted into H_2O_2 and $OH^{\cdot}$ by superoxide dismutase (SOD) and the Fenton/ Haber-Weiss reaction. ROS production (“respiratory burst”) in response to LPS has long been implicated in the

actericidal actions of macrophages. Recently, ROS also received attention as intracellular signaling intermediates due to their ability to alter cytokine expression, modulate cellular proliferation and induce apoptosis (16,17).

ASK1 is a MAPKKK that actives the MKK3/MKK6-p38 and MKK4/MKK7 JNK kinase pathways in response to oxidative stress, anti-cancer drugs, growth factor deprivation or TNFα. In resting cells, ASK1 is bound to thioredoxin (TRX) preventing its activation (18). Under oxidizing conditions, TRX forms a disulfide bridge and dissociates from ASK1, allowing ASK1 to autophosphorylate and become activated. ASK1 regulation by TRX denotes that it is regulated by ROS.

This association, in addition to being an upstream activator of p38, led us to hypothesize that LPS-stimulated ROS production could activate ASK1, leading to the activation of p38, IRF-3 and subsequent ISG induction.

MATERIAL AND METHODS:

Cells – Human U373 astrocytoma cells (U-373 MG) stably transfected with human CD14 were generously provided by Dr.Tobias. Peritoneal macrophages were collected 5 days after intraperitoneal administration of thioglycolate,

Reagents – LPS and allopurinol were from Sigma. L-NAC, MK886, Rotenone, Antimycin A3, and DPI were obtained from Calbiochem. IFNβ was a generous gift from Biogen, Inc.

Mice – iNOS, eNOS, and gp91phox-deficient mice were purchased from Jackson Labs. IRF-3-/- mice were generously provided by Dr. Taniguchi.

RNAse Protection Assay (RPA) – Total RNA was isolated using TRIzol Reagent (Gibco BRL). 10 μg RNA and [^{32}P]-labeled riboprobes for ISG54, RANTES and GAPDH were incubated in buffer (4:1 formamide and 5x stock (=200mM PIPES, pH 6.4, 2 M NaCl, 5mM EDTA)) overnight at 56°C before digestion with T1 RNase (Gibco BRL). Protected fragments were solubilized in 98% formamide/10mM EDTA and subject to electrophoresis on a 4.5% polyacrylamide/urea gel.

Electrophoretic Mobility Shift Assay (EMSA) - EMSAs were performed using a ^{32}P-labeled probe corresponding to the ISG15-ISRE (5'GATCCATGCCTCGGGAAAGGGAAA-CCGAAACTGAAGCC3'). Extracts were incubated with poly-(dI-dC) and labeled probes in buffer (40 mM KCl, 20 mM Hepes, pH 7.0, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, 4% Ficoll, 0.02% NP-40), and resolved on 6% TBE-PAGE.

Western Blotting – Cells were lysed in buffer containing 20mM Hepes pH 7.4, 1% TX100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1mM Na-vanadate and 1mM PMSF. Cell lysates were resolved by SDS-PAGE, and blots were immunoblotted with the indicated antibodies. All blots were developed with HRP-conjugated secondary antibodies and ECL (Amersham).

Luciferase Assay – Cells were transfected with 5x-ISRE-luciferase and renilla-luciferase under the control of the thymidine kinase promoter using Superfect (Qiagen). 16h after transfection, cells were stimulated with 1μg/mL LPS for 24h. Luciferase activity was measured using a Dual-Luciferase Assay Kit (Promega) according to the manufacturer's instructions.

Immunofluorescence – Slides were washed with PBS, fixed with 4%

paraformaldehyde, permeabilized with PBS/0.2% TX100 and blocked with 10% goat serum. After rinsing, and incubation with anti-IRF-3 serum in PBS/0.05% Tween20/3% BSA, cells were incubated with goat anti-rabbit immunoglobulin G-TRITC antibody conjugate (Jackson Immunoresearch) and after rinsing overlaid with Vectashield (Vector labs).

Real-time PCR - ISG54 expression was assayed by Real-time PCR using Omniscript (Qiagen), SYBR green (Applied Biosystems), and the primers: ISG54 (5'-TCTGATTCTGAGGCCTTGCA-3') and (5'-CTTGCTGACC-TCCTCCATTCTC-3'); β-actin (5'-ACGGCCAGGTCATCACTATTG-3') and (5'-CAAGAAGGAAGGCTGGAAAAGAG-3')

siRNA transfection - siRNA against murine NOX4 was purchased from Dharmacon (catalog # M-058509-00). Cells were transfected with 20μM siRNA using lipofectamine 2000 (InVitrogen), and stimulated with LPS 48h later.

RESUTLS AND DISCUSSION:

The ISG54 gene is under the sole control of an ISRE in its promoter region, therefore, induction of ISG54 by IFNα/β occurs strictly in a STAT1/STAT2/IRF9-dependent manner, whereas its activation as a consequence of viral infection or LPS stimulation requires the activation of IRF-3.

In our earlier studies (9) we noticed that LPS-induced, IRF-3-mediated ISG54 induction is substantially delayed when compared to the rapid response elicited by the IFNα/β-stimulated Jak/STAT pathway (Figure 1A). This delayed activation kinetics is reminiscent of the slow activation of hypoxia-inducible factor 1 (HIF-1), a transcription factor that is activated by ROS (19). Intriguingly, most stimuli (virus infection, bacterial cell surface contact, or environmental stresses such as DNA damage) that activate IRF-3 also induce the production of ROS (20). Thus, we hypothesized that ROS might play a role in the TLR4 pathway leading to IRF-3 activation.

To address this possibility, we exposed cells to the antioxidant L-N-Acetylcysteine (L-NAC) prior to stimulation with LPS or IFNβ. As shown in Fig.1B, pretreatment of cells with L-NAC completely inhibited the LPS-mediated induction of ISG54. To exclude that L-NAC was acting in a non-specific, potentially cytotoxic manner, we also examined its effect on ISG54 mRNA levels upon IFNβ stimulation. As anticipated, L-NAC failed to exert an inhibitory effect upon the IFN-induced, Jak/STAT-mediated expression of ISG54 (Figure 2-1B, lanes 5 and 6), demonstrating its specificity towards LPS-induced IRF-3 activation. Similar results were seen when other antioxidant such as BHA or NDGA were used. Thus, our results support the notion of a critical role of ROS in the LPS-induced activation of IRF-3 and subsequently ISGs.

Numerous enzymatic processes lead to the production of ROS. To define the source of ROS generated after LPS stimulation, we tested a wide array of specific inhibitors directed against different ROS sources such as xanthine oxidase, lipoxygenase, cyclo-oxygenase, mitochondrial respiratory chain complex I and II, for their potential effect on LPS-induced ISG54 expression. However, none of these compounds were able to prevent LPS-mediated ISG induction (Figure 2A and B; not shown).

As the nitric-oxide synthases eNOS and iNOS are also capable to produce ROS, we tested peritoneal macrophages deficient in these enzymes for LPS-induction of ISGs. As shown in Figure 2C, both cell types facilitated expression of RANTES, another IRF-3-dependent ISG (21), to a similar extent as observed in WT cells. The 7 members of the NADPH-oxidase family (Nox/Duox) share significant homology with gp91phox (22). Diphenylene-Iodonium chloride (DPI) has been widely used to inhibit NADPH-oxidase-dependent, inducible ROS production in phagocytes (23,24). As shown in Figure 3A, pretreatment of cells with DPI prior to stimulation with LPS completely abrogated the induction of ISG54. Importantly, DPI had no effect on the Jak/STAT-dependent activation of ISG54 by IFNβ (Figure 3A).

To confirm that DPI was indeed acting through inhibition of IRF-3 activation, we assessed IRF-3 DNA-binding and nuclear translocation in cell stimulated in the presence of DPI. Indeed, DPI pretreatment efficiently prohibited LPS from inducing IRF-3 DNA-binding (Figure 3B), or its nuclear accumulation (Figure 3C). The identity of the LPS-induced band was verified as IRF-3 by supershift with anti-IRF-3 antibody (not shown). Thus, prevention of IRF-3-binding to the ISRE and lack of IRF-3 nuclear accumulation provide additional evidence that an NADPH-oxidase family member is required for the

activation of IRF-3 by LPS. To determine whether the NADPH-oxidase was required upstream or downstream of the adaptor protein TRIF, which has been previously shown to mediate LPS-induced IRF-3 activation (10,11,13), we tested the effect of DPI on the transcription of the ISRE-luciferase when it was activated via overexpression of TRIF rather than LPS stimulation. As shown in Figure 3D, DPI is still able to inhibit transcription of this reporter, suggesting that the NADPH-oxidase acts downstream of TRIF. Surprisingly, NADPH-oxidase gp91phox-deficient mice showed no defect in IRF-3-mediated transcription (data not shown). However, during the preparation of this manuscript Park et al. (25) reported the association of another NADPH-oxidase family member, Nox4, with TLR4. To test whether this enzyme was required for IRF-3-mediated responses, we employed siRNA against NOX4 as described in (25), and tested its effect on LPS-mediated induction of ISG54. Indeed, a >70% inhibition of ISG54 transcription in response to LPS resulted from the abrogation of NOX4 expression (Figure 3E), demonstrating that NOX4 is responsible for the generation of the ROS required for LPS-induced, IRF-3-mediated transcription.

Next, we decided to explore the connection between free ROS and the phosphorylation events that lead to IRF-3-mediated ISG-induction. LPS-induced activation of p38 was reported to require ROS production (26) as well as ASK1 (27). We had previously found that IRF-3 activation by LPS requires the activity of MKK6 and p38 (9, 28), and therefore hypothesized that ASK1 might be the link between LPS-induced ROS production and activation of the MKK6/p38/IRF-3 cascade.

To examine a potential role of ASK1 in LPS-induced IRF-3-mediated transcription, we first tested whether ectopic expression of the ASK1 inhibitor TRX

would attenuate the LPS-induced activation of an ISRE-luciferase reporter. Indeed, ectopic expression of TRX caused a dose-dependent decrease in the induction of ISRE-luciferase by LPS (Figure 4A), however, no such inhibition was seen when the constitutively active IRF-3-5D mutant was used to activate the reporter construct (data not shown). While these results were indicative of an ASK1 requirement in LPS-induced ISG induction, we also decided to test whether ASK1 was sufficient to induce the ISRE-luciferase. Ectopic expression of WT ASK1 resulted in a dose-dependent increase in luciferase production after LPS stimulation, whereas the kinase dead ASK1(K709M) mutant failed to promote such a response (Figure 4B). These findings further indicated a role of ASK1 in the LPS signaling pathway leading to the induction of ISGs.

ASK1 activation in response to oxidative stress requires phosphorylation of T845 in its activation loop (29). We therefore employed phospho(T845)-ASK1 antibodies to examine the phosphorylation state of ASK1 after LPS stimulation. ASK1 was clearly phosphorylated on T845 for an extended period of time in response to LPS to a similar extent as observed after TNFα treatment (Figure 5A).

In order to definitively determine whether ASK1 is essential in the LPS induced transcription of ISGs, we analyzed LPS responses in ASK1-/- peritoneal macrophages. While LPS stimulation of WT macrophages yielded a clear induction of ISG54, a substantially weaker response was observed in their ASK-/- counterparts (Fig5B). In order to determine where ASK1 was required in the activation cascade relative to TRIF, we ectopically expressed TRIF, IRF-3 and the ISRE-luciferase in WT and ASK1-/- murine embryonic fibroblasts (MEFs). As shown in Figure 5C, expression of TRIF together with IRF-3 in WT MEFs is sufficient to strongly activate the ISRE-Luciferase

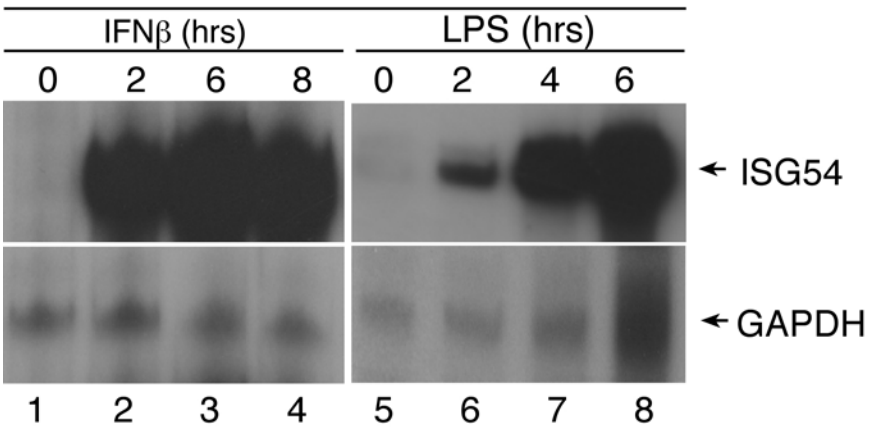
independent of LPS. However, when ASK1-/- MEFs were used for transfection, no induction of the reporter construct could be seen, regardless of LPS stimulation, indicating that ASK1 in required downstream of TRIF.

IRF-3 activation is a crucial part of the innate immune response as evidenced by the fact that IRF-3-/- mice fail to produce type I IFNs, as well as other proinflammatory cytokines and chemokines in response to TLR ligation (30,31). Recent reports demonstrated a role for TBK1 and IKKε in IRF-3 activation in response to viral infection or dsRNA (12,14), which requires the phosphorylation of IRF-3 on S396 (32). Whether phosphorylation of this residue occurs in response to LPS is somewhat controversial, offering the possibility that a distinct pathway mediates IRF-3 activation via TLR2 and TLR4 ligands. In the present study we show that ISG induction by LPS, which occurs in an IRF-3-dependent manner, requires the generation of ROS by the NADPH-dependent oxidase NOX4. Subsequent activation of ASK1 links LPS-induced ROS production to the activation of MKK6 and p38, two kinases that we had previously identified as components of the LPS-induced IRF-3 activation cascade. It remains to be determined whether ASK1 is also a contributor to the activation of IRF-3 in response to other stimuli such as viral infection or DNA damaging agents.

ACKNOWLEGEMENT:

Chapter 2, in full, is a reprint of the material as it appears in Journal of Immunology, 2006, Chiang ChihYuan Edward; Dang, Oanh; Anderson, Keith; Matsuzawa Atsushi; Ichijo, Hidenori; David, Michael, 2006. The author of the dissertation is the primary investigator and author of this paper.

2-1A)



2-1B)

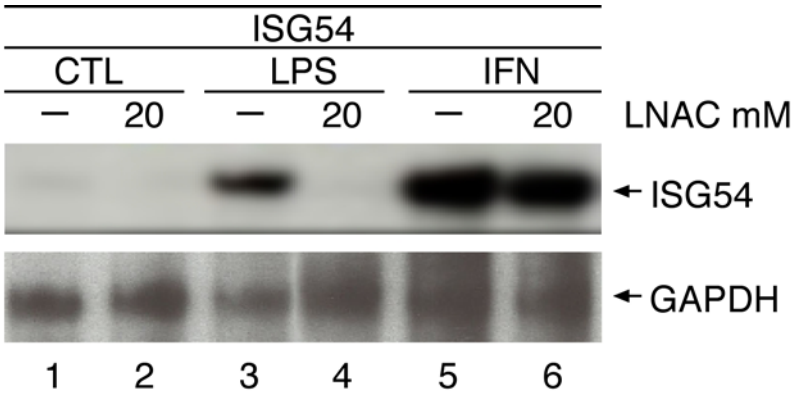
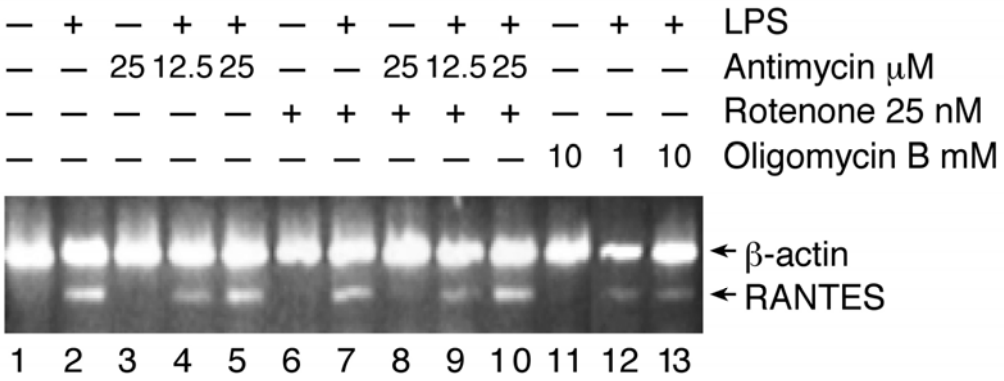


Figure 2-1: Delayed, ROS-dependent activation of ISG induction by LPS: A) U373 cells were treated with 1μg/mL LPS or 1000U/mL IFNβ for the indicated times in the presence of 50μg/mL cycloheximide, and ISG54 mRNA analyzed by RPA. B) Same as A), except cells were pretreated for 1h with 20mM L-NAC prior to stimulation with LPS or IFNβ.

2-2A)



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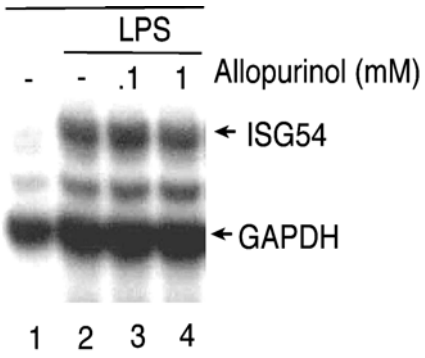


2-2C)

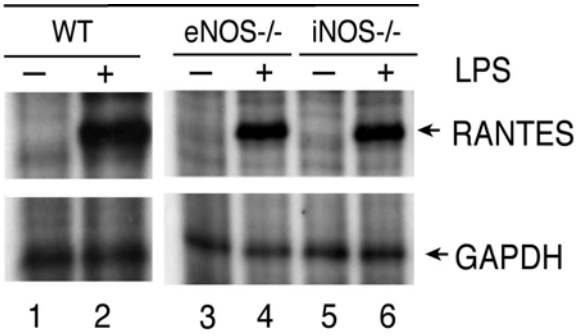
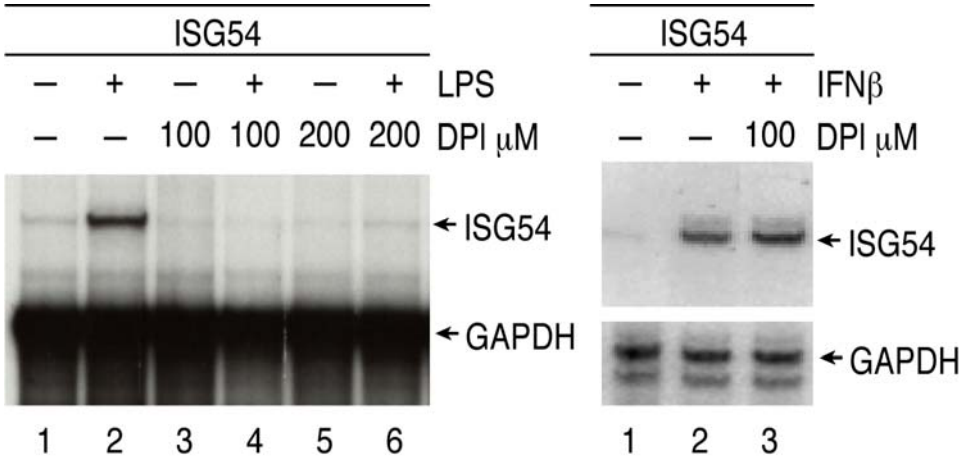


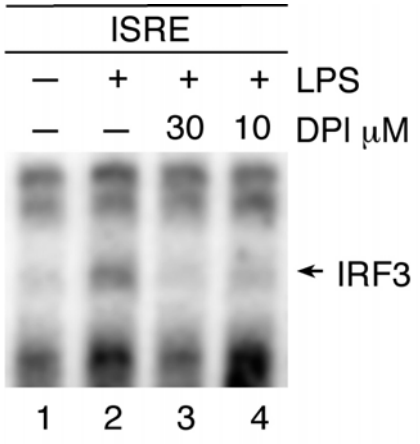
Figure 2-2: ROS-dependent activation of ISG induction by LPS: A) U373 cells were treated with the indicated inhibitors for 30min prior to stimulation with 1μg/mL LPS for 6h. hRANTES mRNA levels were analyzed by RT-PCR. B) Same as A), except ISG54 mRNA levels were analyzed by RPA. C) WT, eNOS or iNOS-deficient peritoneal macrophages were stimulated with 1μg/mL LPS for 6h. mRANTES mRNA levels were analyzed by RPA.

Figure 2-3: DPI inhibits LPS-mediated ISG induction and IRF-3 DNA-binding: A) U373 cells were treated with DPI for 1h in the presence of 50μg/mL cycloheximide, and stimulated with 1μg/mL LPS for 6h, or 1000U/ml IFNβ for 1h, and ISG54 mRNA levels were analyzed by RPA. B) Same as A) except extracts were analyzed for IRF-3 binding to the ISRE. C) U373 cells were stimulated with 1μg/mL LPS for 6h in the presence of 10μM DPI prior to immunostaining for IRF-3 (top panel). Nuclei were counterstained with DAPI (bottom panel) D) U373 cells transfected with TRIF and ISRE-luciferase were treated with DPI at the indicated concentrations for 12h prior to analysis of luciferase activity (a representative of 3 independent experiments is shown). E) RAW264.7 cells were transfected with siRNA against either LacZ or murine NOX4. After 48h, cells were stimulated with 100ng/ml of LPS for 6h, and ISG54 mRNA levels analyzed by Real-time PCR. ISG54-expression was normalized using β-actin as an internal control.

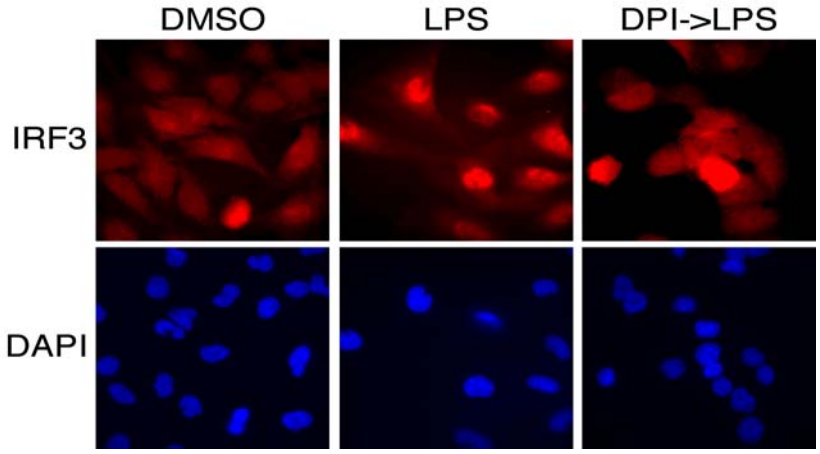
2-3A)


ISG54
ISG54
– + – + – + LPS
– + + IFNβ
– – 100 100 200 200 DPI μM
– – 100 DPI μM
ISG54
GAPDH
ISG54
GAPDH
1 2 3 4 5 6
1 2 3

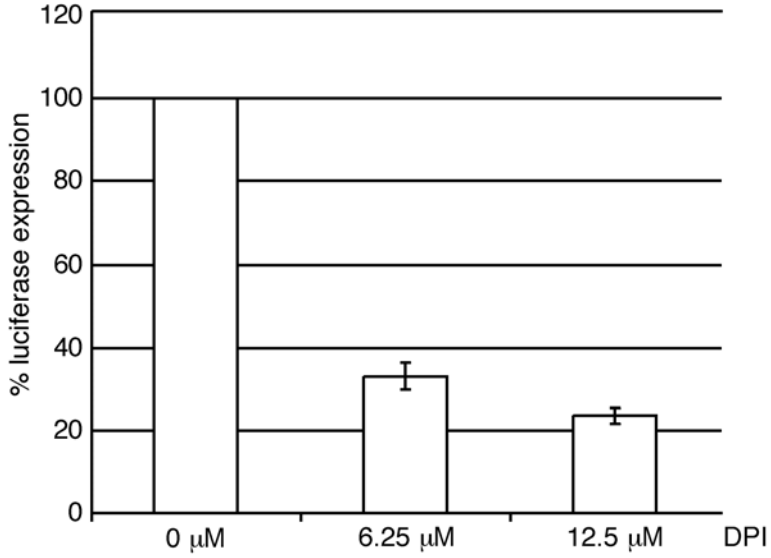
2-3B)


ISRE
– + + + LPS
– – 30 10 DPI μM
IRF3
1 2 3 4

2-3C)


DMSO
LPS
DPI->LPS
IRF3
DAPI

2-3D)



2-3E)

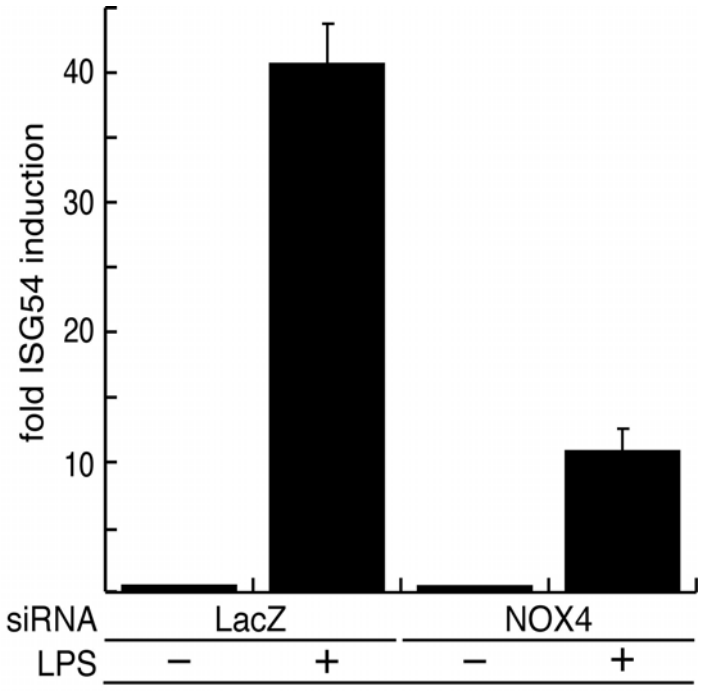
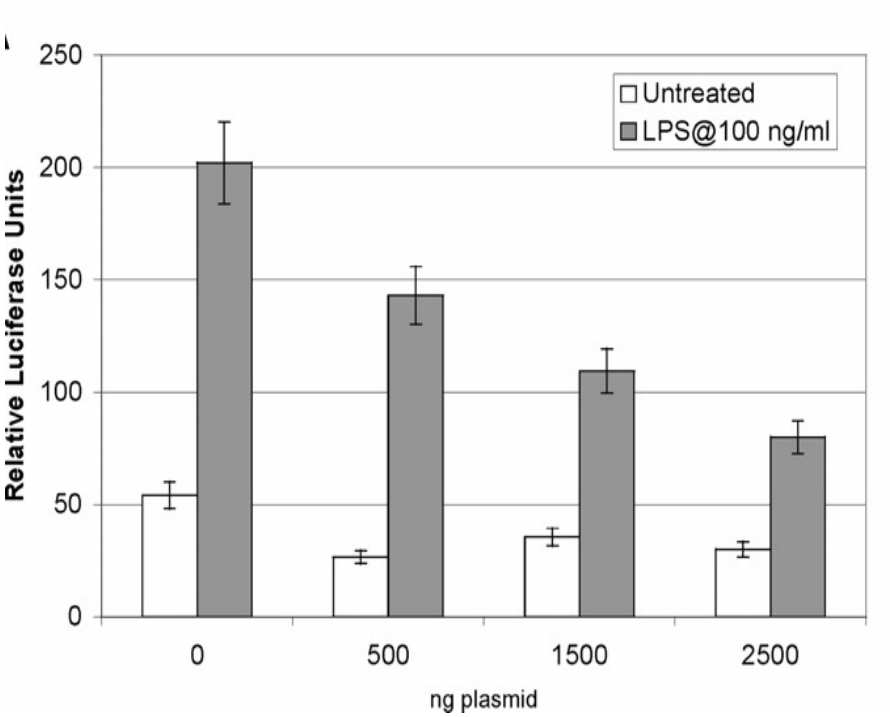


Figure 2-3 continued

Figure 2-4: Kinase activity of ASK1 is required for ISRE-mediated transcription by LPS: A) U373 cells were transfected with ISRE-Luciferase and the indicated amount of TRX plasmid. After 16h cells were stimulated with 1μg/mL LPS for 24h and luciferase production analyzed. B) Cells were treated as in A), however, WT and kinase-inactive ASK1 plasmid was used instead of TRX.

2-4A)


Relative Luciferase Units
250
200
150
100
50
0
Untreated
LPS@100 ng/ml
0
500
1500
2500
ng plasmid

2-4B)

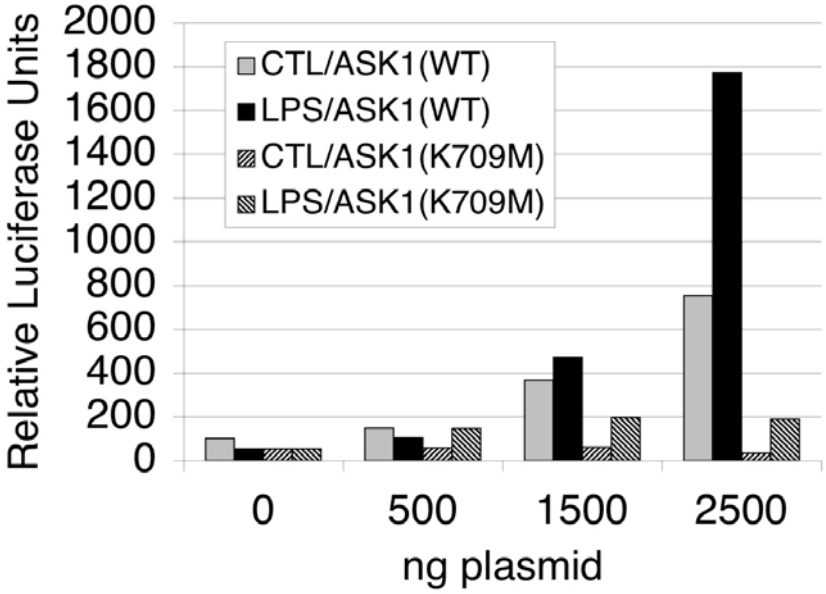
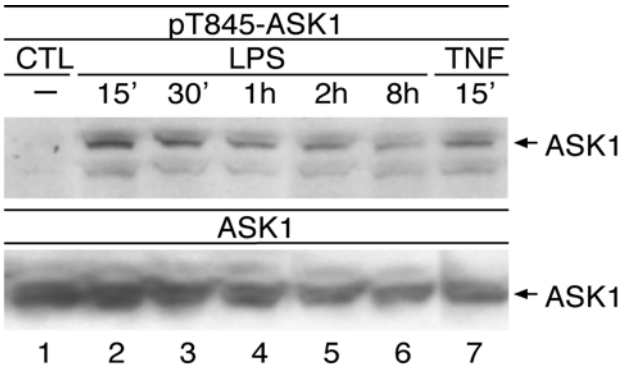

Relative Luciferase Units
2000
1800
1600
1400
1200
1000
800
600
400
200
0
CTL/ASK1(WT)
LPS/ASK1(WT)
CTL/ASK1(K709M)
LPS/ASK1(K709M)
0
500
1500
2500
ng plasmid

Figure 2-5: Activation and requirement of ASK1 in LPS signaling: A) U373 cells were stimulated with 1μg/mL LPS or TNFα for the indicated times, and blots were probed with anti-phosphoT845-ASK1 or ASK1 antibodies. B) Peritoneal macrophages from WT and ASK1-/- mice were stimulated with 1μg/mL LPS for the indicated times, and ISG54 and GAPDH mRNA levels were analyzed by RT-PCR. C) WT and ASK1-/- MEFs were transfected with murine TRIF and/or IRF-3, and ISRE-Luciferase. After 24h, luciferase activity analyzed. A representative of three independent experiments is shown.

2-5A)



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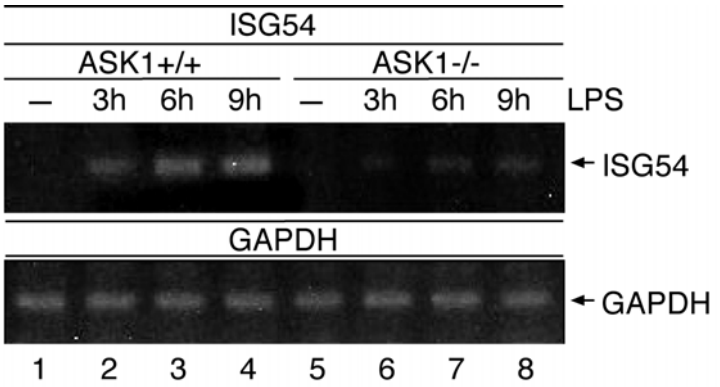
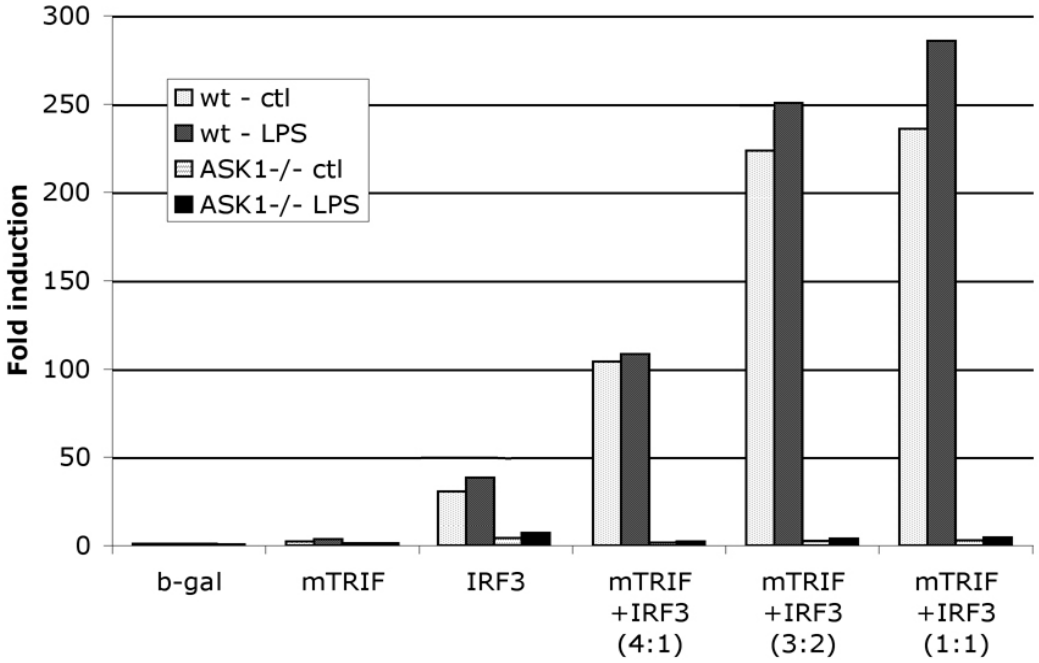


Figure 5

2-5C)



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CHAPTER 3:

Calcium Is Required for the Activation of IFN Regulatory Factor 3 by Lipopolysaccharide

ABSTRACT:

Previously, we have characterized interferon regulatory factor 3 (IRF-3) activation requires the reactive oxygen specie (ROS) that's produced from NADPH oxidasae 4 (NOX4). ROS activates ASK1/MKK6/p38 MAPK pathway and leads to IRF-3 activation(1-3). Production of ROS occurs frequently concomitant with an increase in cytosolic Ca^{2+} (4, 5). In this study, we reported calcium is required for IRF-3 activation. Inositol 1,4,5-triphosphate receptors (IP_3Rs) mediates the release of calcium from endoplasmic reticulum into the cytosol. Antagonist of IP_3Rs, 2-APB, impaired IRF-3 nuclear translocation and IRF-3 mediated ISG54 expression in response to LPS. Furthermore, phospholipase C gamma 2 (PLCγ2) mediates the cleavage of phosphatidylinositol (4,5)-bisphosphate (PIP2) into Inositol 1,4,5-triphosphate (IP_3) which regulates IP_3R activity in response to LPS. Calcium specifically acts on IRF-3 activation whereas the regulation of NF-κB mediated genes remains intact. The results suggest a novel role for calcium in the innate immune response as signaling intermediates in the IRF-3 activation pathway.

INTRODUCTION:

Toll receptors were initially characterized as a critical player in the development of embryonic dorsoventral polarity in *Drosophila* (6, 7). Furthermore, Toll-deficient *Drosophila* showed reduced resistance to fungal infection (8). Peptide sequence analysis demonstrated the Toll receptor is evolutionarily conversed in mammals (9). Mammalian Toll like receptors (TLRs) are germ line encoded pattern recognizing proteins (PPRs)

which detect the invasion of foreign pathogens. PRRs recognize microbial components, known as pathogen associated molecular patterns (PAMPs), that are essential for the survival of the microorganism and are therefore difficult for the microorganism to alter (10). To date, 12 members of the TLR family have been identified in mammals. Different TLRs react with specific PAMPs, show distinct expression patterns, activate specific signaling pathways, and lead to distinct antipathogen responses (11).

After ligand binding, TLRs recruit TIR-domain-containing adaptor molecules to the TIR domain of the TLR. Toll like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS) that is expressed on the cell wall of gram negative bacteria (12, 13). TLR4 is unique in utilizing four adaptor proteins in specifying the downstream transcription profiles. The MyD88 dependent pathway utilizes both MyD88 and TIRAP (TIR-associated protein) which lead to NF-κB activation (14-16). This pathway is critical in mediating the induction of pro-inflammatory cytokines. The MyD88 independent pathway signals though TRIF (TIR-doimain-containing adaptor protein-inducing IFN-β) and TRAM (TRIF-realted adaptor molecule) which activate IKKi/TBK1 and this leads to IRF-3 activation (17-19). Interferon regulatory factor 3 (IRF-3) plays a pivotal role in up-regulating interferon stimulated genes (ISGs) in response to bacterial and viral infection (20-23). IRF-3 is also important in the induction of co-stimulatory molecules in the dentritic cells (24). The interaction of CD28 with the co-stimulatory molecules, CD80 and CD86, is required for the subsequent T cell activation.

In the previous study, we show that ISG induction by LPS, which occurs in an IRF-3-dependent manner, requires the generation of ROS by the NADPH-dependent oxidase NOX4. Subsequent activation of ASK1 links LPS-induced ROS production to

the activation of MKK6 and p38, two kinases that we had previously identified as components of the LPS-induced IRF-3 activation cascade. Production of ROS occurs frequently concomitant with an increase in cytosolic calcium (4, 5). Calcium signals are required to initiate several types of transcriptional events and growth responses such as proliferation and apoptosis (25, 26). In most eukaryotic cells, calcium signals are triggered by the secondary messenger inositol-1,4,5-triphosphate (IP_3), the cleavage product of phosphatidylinositol-4,5-bisphosphate (PIP_2) by the enzymatic activity of phspholipase C (PLC). IP_3 binds to IP_3Rs which are located in the endoplasmic reticulum, thereby stimulating the release of calcium from internal stores (27). Here we report that calcium mediates IRF-3 activation in response to LPS. Phospholipase C gamma 2 (PLCγ2) specifically mediates the cleavage of PIP_2 into IP_3 which regulates the release of calcium from ER into cytosol through the IP_3Rs.

MATERIAL AND METHODS:

Cells Lines – RAW264.7 cell line was purchased from American Type Culture Collection (ATCC). 293T cells expressing TLR2-CD14-MD2 was generously gifted by Dr. Katherine Fizgerald (the University of Massachusetts). Both RAW26.4 and 293T cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100ug/ml streptomycin and 2mM L-gluctamine.

Reagents – 2-aminoethoxydiphenyl borate (2APB), Thapsigargin (Tg), were obtained from Calbiochem. Murine IFN-β was a generous gift from Biogen. Anti murine IRF-3 antibody was purchased from Invitrogen Life Technologies**.** Anti Histone H3,

PLCγ1 and PLCγ2 antibodies were purchased from Cell Signaling Technology. siRNA against PLCγ1 and PLCγ2 were purchased from Dharmacon with the catalogue number 040978 and 040979 respectively.

Transfection – Transfection was done using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturere's instructions. 1 x 10^5 RAW264.7 cells were seeded in a 24-well plate and transected with 20 μM of siRNA.

RNase protection assay (RPA) – Total RNA was isolated using TRIzol Reagent (Invitrogen Life Technologies). Ten micrograms of RNA and 32P-labeled riboprobes for ISG54, RANTES, and GAPDH were incubated in buffer (4:1 formamide and 5x stock (200 mM PIPES (pH 6.4), 2 M NaCl, and 5 mM EDTA)) overnight at 56°C before digestion with T1 RNase (Invitrogen Life Technologies). Protected fragments were solubilized in 98% formamide plus 10 mM EDTA and subjected to electrophoresis on a 4.5% polyacrylamide/urea gel.

Western Blotting – Cells were lysed in RIPA buffer (50mM Tris-HCl pH8.0, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM sodium vanadate, and 1 mM PMSF). Cell lysates were resolved by SDS-PAGE, and blots were immunoblotted with the indicated Abs. All blots were developed with HRP-conjugated secondary Abs and ECL (Amersham Biosciences).

Nuclear extract – Nuclear extraction was performed using CelLytic™ NuCLEAR™ Extraction Kit (Sigma-Aldrich) according to the manufacturer's instruction.

Luciferase Assay – 4 x 10^5 293T cells were transfected in suspension in a 24 well plate by LF-2000. Cells were lyzed by passive lysis buffer (Promega) 48 hours post transfection. The ISRE driven luciferase and SV-40 driven Renilla activities were

quantified by the Dual Luciferase System (Promega) according to the manufacturer's protocol. The ISRE luciferase activity was normalized by the SV-40 driven Renilla activity to account the differences in transfection efficiency between samples. Furthermore, the fold of expression was normalized against ISRE-Luciferase activity in GFP transfection cells.

Real Time PCR – Total RNA was extracted by using RNeasy Mini Kit according to the manufacture's instruction (Qiagen). 100ng of RNA sample was reverse transcribed using the Omniscript kit (Qiagen). PCR products were detected using the fluorescent dye SYBR green (Applied Biosystems). The primers used for the analysis are shown in (Table 1). Formation of a unique DNA product was confirmed by verifying that products had a single melting temperature. Fluorescence-monitored PCR values were normalized to β-actin values to account for any differences in cDNA recovery between samples.

Table 3-3: Oligonucleotides used for SYBR green quantification of gene expression:

Murine β-actin:
Forward Primer: ACGGCCAGGTCATCACTATTG
Reverse Primer: CAAGAAGGAAGGCTGGAAAAGAG

Murine IκBα:
Forward Primer: GCGGGATGGCCTCAAGA
Reverse Primer: ATTTGCTCGTACTCCTCGTCCTT

Murine ISG54:
Forward Primer: TCTGATTCTGAGGCCTTGCA
Reverse Primer: CTTGCTGACCTCCTCCATTCTC

Murine RANTES:
Forward Primer: GCAGTCGTGTTTGTCACTCGAA
Reverse Primer: GATGTATTCTTGAACCCACTTCTTCTC

Table 3-3 continued

Murine TNFα:
Forward Primer: TGGCCTCCCTCTCATCAGTT
Reverse Primer: GCTTGTCACTCGAATTTTGAGAAG

Murine TRAIL:
Forward Primer: AGACCTTAGGCCAGAAGATTGAATC
Reverse Primer: ATTCCTAAAGAGCACGTGGTTGAG

RESULTS AND DISCUSSION:

Calcium released from intracellular source is essential for IRF-3 mediated LPS response:

The ISG54 gene is under the sole control of an ISRE in its promoter region; therefore, the induction of ISG54 by interferon α/β (IFN α/β) occurs strictly in an ISGF3-dependent manner whereas its activation as a consequence of viral infection or LPS stimulation requires the activation of IRF-3. In earlier studies, we characterized ISG54 up-regulation in response to LPS requires reactive oxygen species (ROS). ROS production by NADPH oxidase 4 (NOX4) activates Apoptosis-regulating signal kinase 1 (ASK-1), a mitogen activated kinase kinase kinase (MAPKKK). The association of ROS to the ASK-1 inhibitor, thioredoxin, results in its conformational change and therefore can no longer exert its inhibitory effect toward ASK-1 activation (28). Activated ASK-1 signals through the MKK6/p38 axis and this leads to IRF-3 activation. Production of ROS occurs frequently concomitant with an increase in cytosolic calcium (4, 5). We therefore decided to explore the potential role of calcium signaling in the LPS activation of IRF-3. To address this possibility, we ectopically supplied RAW264.7 cells with calcium ionophore, A23187. At the presence of A23187, ISG54 expression increased in

a dosage dependent manner in response to LPS (Figure 3-1). To exclude the possibility that A23187 was acting in a nonspecific manner, we also examined its effect on ISG54 mRNA levels upon IFNβ treatment. As anticipated, A23187 failed to exert stimulatory effect upon the IFN-induced, Jak/STAT-mediated expression of ISG54. This suggests that calcium mediates IRF-3 activation in response to LPS.

The calcium that's mobilized in response to LPS can be originated from either intracellular or extracellular sources. To determine the source of calcium, cell permeable intracellular calcium chelator, BAPTA, was loaded onto RAW 264.7 cells prior to LPS stimulation. LPS-induced ISG54 activation was completely abrogated in the absence of cytosolic free calcium (Figure 3-2). In contrast, depletion of extracellular calcium due to the presence of EGTA in the culture media had no effect on IRF-3 mediated ISG54-induction (data not shown). This suggests that calcium released from the intracellular store is required for IRF-3 activation in response to LPS.

Calcium is released from endoplastic reticulum through Inositol 1,4,5-triphosphate receptors:

The ER and its muscle equivalent, the sarcoplasmic reticulum (SR), are major sites of intracellular calcium storage. IP_3Rs and ryanodine receptors (RyRs) are two types of receptors expressed on the ER/SR which mediate the release of calcium form ER into the cytosol. IP_3Rs are virtually universal, whereas RyRs are most evident in excitable cells such as skeletal and cardiac muscles. To determine which one of these two receptors mediated the release of calcium from the ER into the cytosol in response to LPS, we used cell permeable 2-APB (2-aminoethoxydiphenyl borate) (29, 30) and Dantrolene which antagonized IP_3Rs and RyRs, respetively. Treating RAW264.7 cells

with 2-APB prior to LPS stimulation impaired ISGS54 gene expression in a dosage dependent manner whereas its expression in response to IFN-β remained intact. In contrast, the ISG54 expression at the presence of dantrolene was intact (Figure 3-3). The expression profile of two other IRF-3 dependent genes, TRAIL and RANTES (31, 32), were parallel to the ISG54 expression (Figure 3-4). This suggests the calcium released from IP_3Rs mediates IRF-3 activation in response to LPS. However, no such inhibition was seen when the constitutively active IRF-3-5D mutant was used to activate the ISRE-luciferase reporter construct (32) (Figure 3-5). This confirms the effect of 2-APB specifically acting on the IRF-3 pathway.

One of the early events in IRF-3 activation is its nuclear translocation following appropriate phosphorylation on serine and threonine residues. We therefore decided to test whether antagonizing IP_3R by 2-APB would trigger a change in the subcellular distribution of IRF-3. IRF-3 subcellular distribution was determined by western blotting of the purified nuclear extracts. As shown Figure 3-6, IRF-3 translocation in response to LPS was abrogated at the presence of 2-APB.

PLC gamma 2 mediates the IRF-3 activation:

The activity of IP_3Rs are under the regulation of a secondary messenger, IP_3. Eleven PLC isozymes have been classified into five families based on their structure and sequence relationships. Namely, the eleven PLCs are PLCβ (1–4), PLCγ (1 and 2), PLCδ (1, 3 and 4), PLCε (1) and PLCζ (1). PLCβ is activated by G protein mediated mechanisms involving members of the Gq, Gi or Go families. PLCγ is unique in possessing one SH3 domain and two SH2 domains, inserted between the X and Y

sequences of the catalytic domain. PLCγ is therefore able to interact with either receptor protein tyrosine kinase or non-receptor protein tyrosine kinase to convey the signal cascade. PLC ζ is characterized to be the physiological stimulus for egg activation and development at mammalian fertilization (33, 34). In contract to PLCβ and PLCγ, the mechanisms for the activation of PLC-δ and PLC-ε require substantial further study (35). Therefore, the possibility of PLCβ, PLCδ, PLC-ε and PLCζ in the IRF-3 mediated TLR4 signaling is excluded. Both PLCγ1 and PLCγ2, however, were shown to be tyrosine phosphorylated in response to LPS in RAW264.7 cell (36). To determine the specific PLCγ isoform that mediates IRF-3 activation in the LPS response, RAW264.7 cells were transfected with siRNAs that target either PLCγ1 or PLCγ2. Both PLCγ1 and PLCγ2 protein expression level was reduced by 60% after transfecting with the cognate siRNAs (data not shown). Knocking down of PLCγ2 but not PLCγ1 reduced ISG54 mRNA level by 60% in response to LPS (Figure 3-7A). Furthermore, the expression of TRAIL and RANTES, were impaired by 75% and 50%, respectively, at the presence of PLCγ2 but not PLCγ1 siRNA (Figure 3-7B).

Calcium specifically mediates IRF-3 but not NF-κB activation:

TLR4 signaling is unique in mediating the activation of both MyD88-dependent and MyD88 independent pathways. We have established that IRF-3 activation requires calcium that's released from ER. To determine the possibility of calcium may be acting upstream of the TLR4 pathway and therefore affecting both the MyD88-dependent and TRIF-dependent pathway, we assayed the effect of 2APB and the PLCγ2 siRNA on the transcriptional regulation of TNF-α and IκBα, whose expressions are NF-κB dependent.

Upregulation of both TNF-α and IκBα remained intact at the presence of 2-APB and PLCγ2 siRNA in response to LPS (Figure 3-8). This suggests calcium specifically mediates IRF-3 but not NF-κB activation.

The SH2 domains of PLCγ2 allow their recruitment to activated receptor complexes through specific sites of tyrosine phosphorylation on receptor chains. The PH domain is speculated to be important in docking the enzyme to inner membrane by binding PIP_3. PLCγ2 is critical in the B cell development. In B lymphocytes, signaling complex that contributes to cell fate decisions is the B-cell antigen receptor (BCR). Upon BCR ligation, the non-receptor tyrosine kinase, Syk, is recruited to the Igα-Igβ chain of the B cell receptor complex. Syk in turn tyrosine phosphorylates the adaptor protein, BLNK. Tyrosine phosphorylated BLNK provide a docking site for both Burton tyrosine kinase (Btk) and PLCγ2 (37). How PLCγ2 link to TLR-4 receptor signaling is unknown. However, given PLCγ2 is tyrosine phosphorylated by Btk upon BCR activation, it is not surprising that Btk may also mediate PLCγ2 activation in responsive to LPS stimulation. In fact, it was reported that Btk is involved in TLR4 signaling by its direct interaction with TLR4, MyD88 and TIRAP (38, 39).

Previously, we reported that ISG induction by LPS, which occurs in an IRF-3-dependent manner requires the generation of ROS by the NADPH-dependent oxidase NOX4. How ROS and calcium cooperate in regulating IRF-3 mediate TLR-4 signaling requires further characterization. However, the identification of the interdependent relationship of calcium and ROS in regulating B cell receptor (BCR) signaling provides insight to this question. Singh *et al.* reported ROS directly involves in the transient inhibition of BCR-associated protein tyrosine phsophatase (PTP) activity.

Inhibition of PTP activity shifts the equilibrium in favor of kinase activation. However, the BCR dependent ROS production is significantly inhibited in the presence of either EGTA or TMB-8 (inhibits the release of calcium from the endoplasmic reticulum). Intriguingly, suppression of BCR-initiated ROS generation also results in an attenuated calcium response. The evidence that inhibition of one leads to a concomitant inhibition of the other suggests a positive cooperativity between the BCR-dependent calcium and BCR-dependent ROS pathways. While both the BCR-dependent calcium and ROS pathways influence each other, it was the ROS that ultimately affected BCR signaling. This was determined from experiments indicting that, whereas the inhibitory effect of calcium could be overcome by independent elevation in ROS levels, the reverse was not true.

In summary, we reported calcium as a novel component of TLR4 mediated IRF3 activation. Sequestering calcium by BAPTA inhibits TLR4 stimulated ISG54 induction, while increasing the calcium concentration A23187 enhances ISG54 induction in response to LPS. This calcium dependence is specific to LPS mediated ISG54 induction, and is not common to ISG54 induction by IFNβ. Furthermore, inhibiting either PLCγ2 or IP_3Rs abrogates LPS stimulated ISG54 induction and therefore must be responsible for calcium release from the ER/SR. This gene abrogation was also demonstrated in other IRF3 dependent genes, TRAIL and RANTES, but was not seen in NFκB dependent genes, TNFα and IκB. Calcium released from the ER/SR via the IP_3Rs is required for the nuclear translocation of IRF3 in response to LPS.

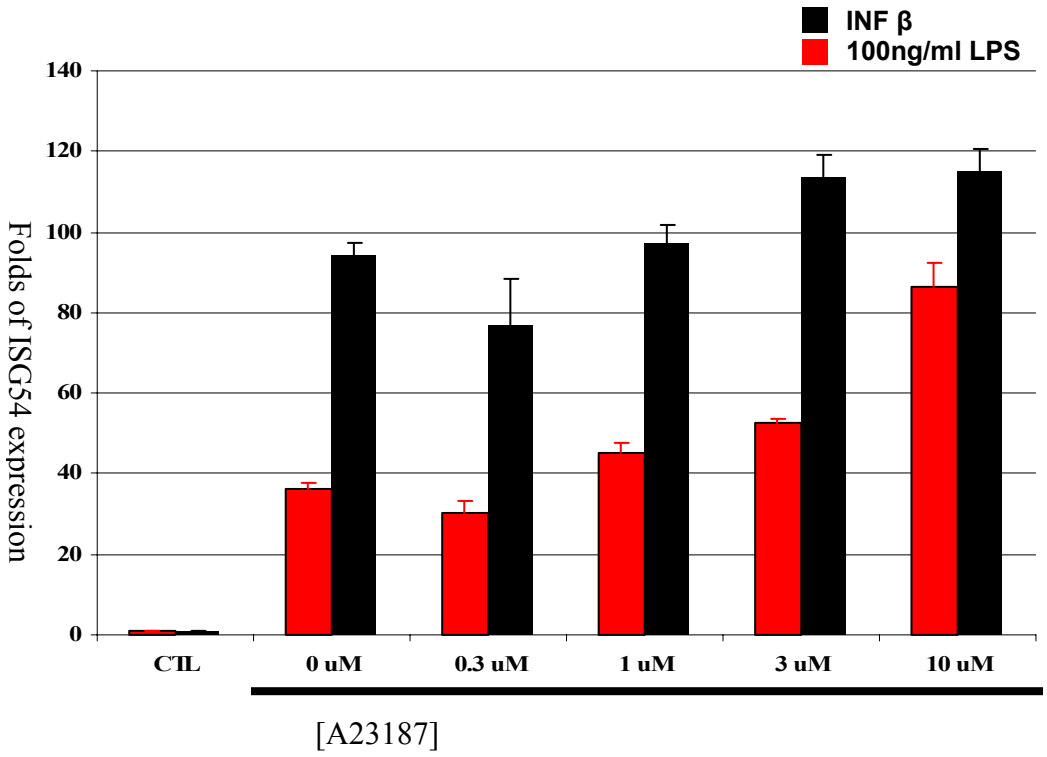

Figure 3-1: A23187 induces ISG54 expression in response to LPS. Total RNA was isolated from LPS or IFNβ stimulated RAW264.7 cells with or without preincubation with the calcium ionophore, A23187. ISG54 mRNA level was than quantified by real-time PCR. β-actin was used as an internal standard.

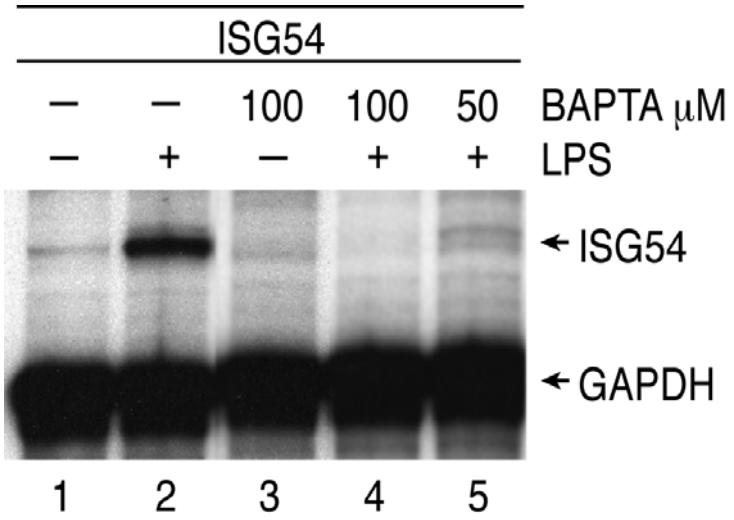

Figure 3-2: BAPTA abrogates ISG54 expression in response to LPS. Total RNA was isolated from LPS stimulated U373 cells with or without preincubation with the intracellular calcium chelator, BAPTA. RNase protectionh assays were performed using a probe corresponding to the human ISG54 gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard.

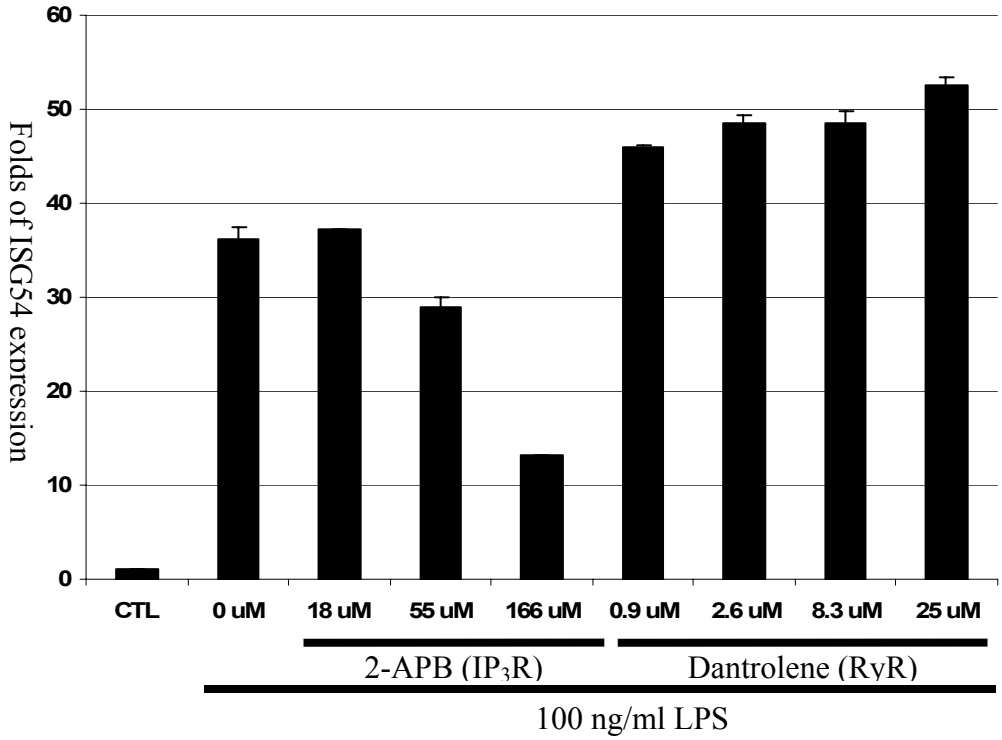

Figure 3-3: LPS induced ISG54 expression is impared at the presence of 2-APB but not dantrolene . Total RNA was isolated from LPS stimulated RAW264.7 cells with or without preincubation with the IP_3R and RyR antagonists, 2-APB and dantrolene, respectively. ISG54 mRNA level was than quantified by real time PCR. β-actin was used as an internal standard.

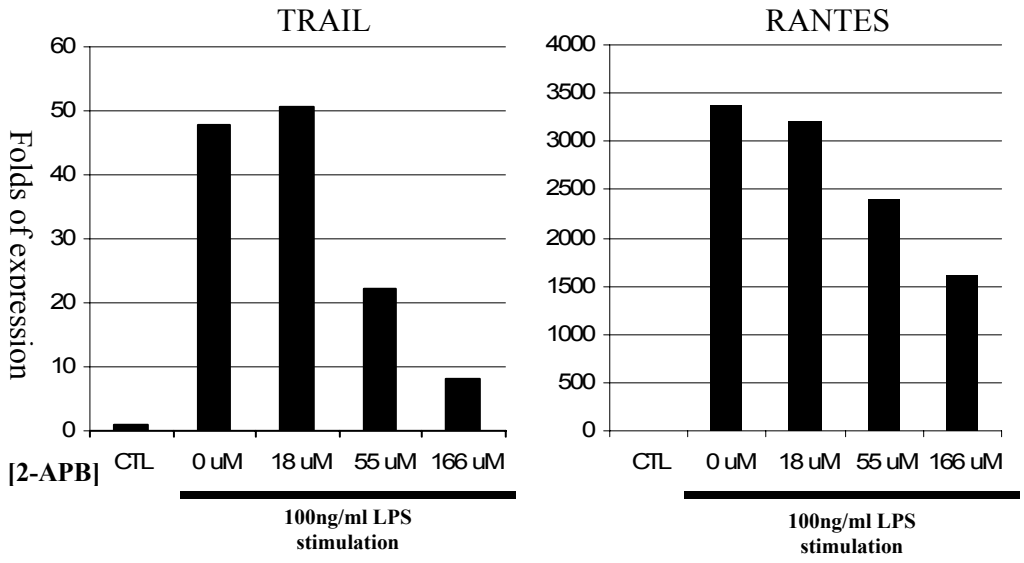

Figure 3-4: 2-APB impairs IRF3 dependent gene upregulation in response to LPS. Total RNA was isolated from LPS stimulated RAW264.7 cells with or without preincubation with the IP_3R antagonist, 2-APB. TRAIL and RANTES mRNA level was than quantified by real time PCR. β-actin was used as an internal standard.

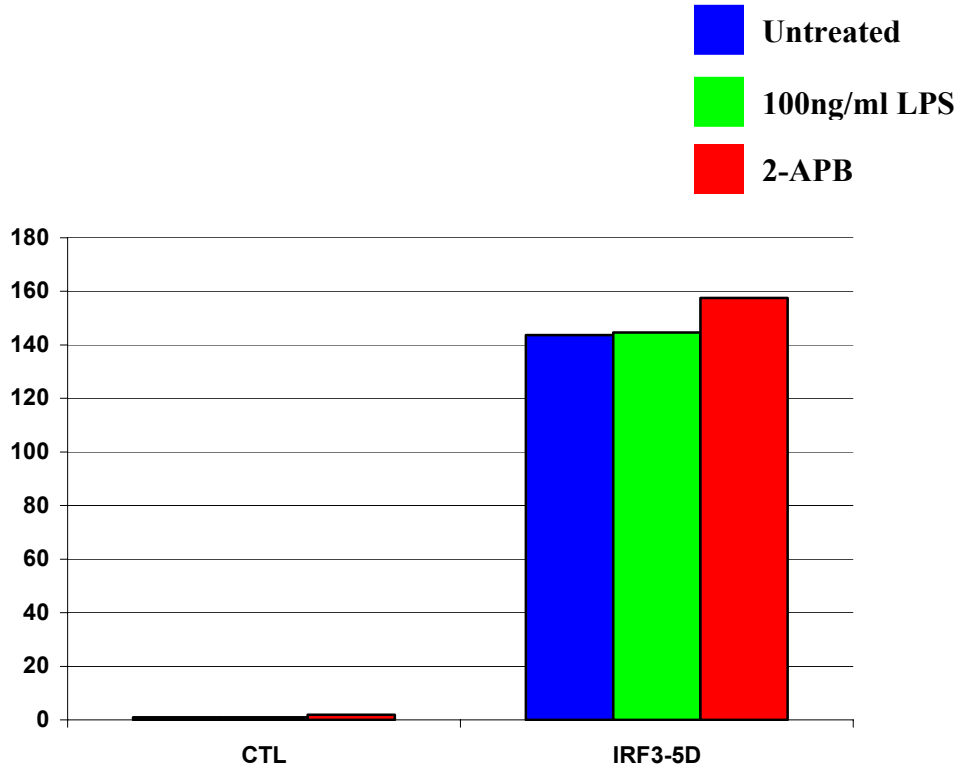

Figure 3-5: Overexpression of constitutive IRF-3 overcomes the inhibitory effect of 2-APB. 293T cells were transfected with constitutive IRF-3, IRF3-5D, ISRE-luciferase. Transfected cells were incubated with 2-APB for 6 hours. The luciferase activity was normalized by SV-40 Renilla activity, which serves as a transfection control.

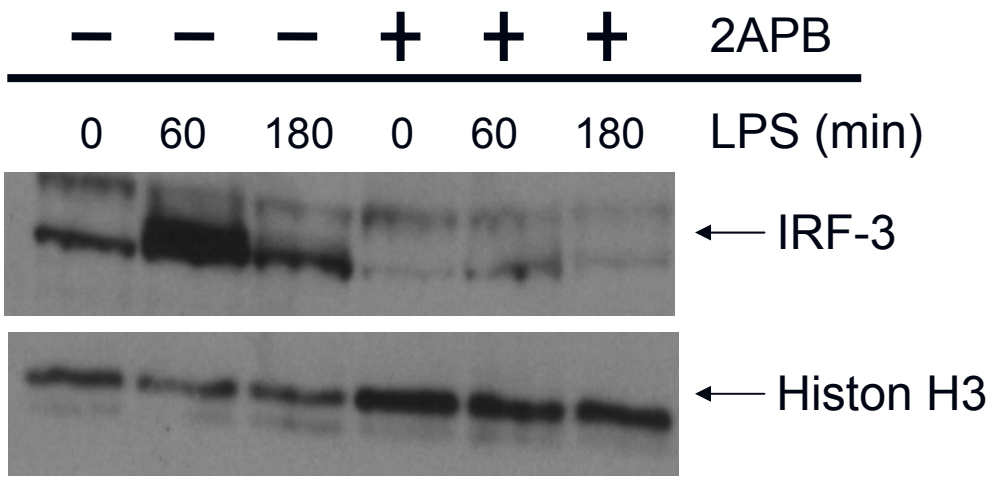

Figure 3-6: 2-APB impairs IRF-3 subcellular localization in response to LPS stimulation. Nuclear extract was purified from untreated or LPS stimulated RAW264.7 cells with or without preincubation with the IP3R antagonist, 2-APB. The nuclear extracts were then subjected to SDS-PAGE and western blotted by IRF-3 the specific antibody. Histon H3 was used as a loading control.

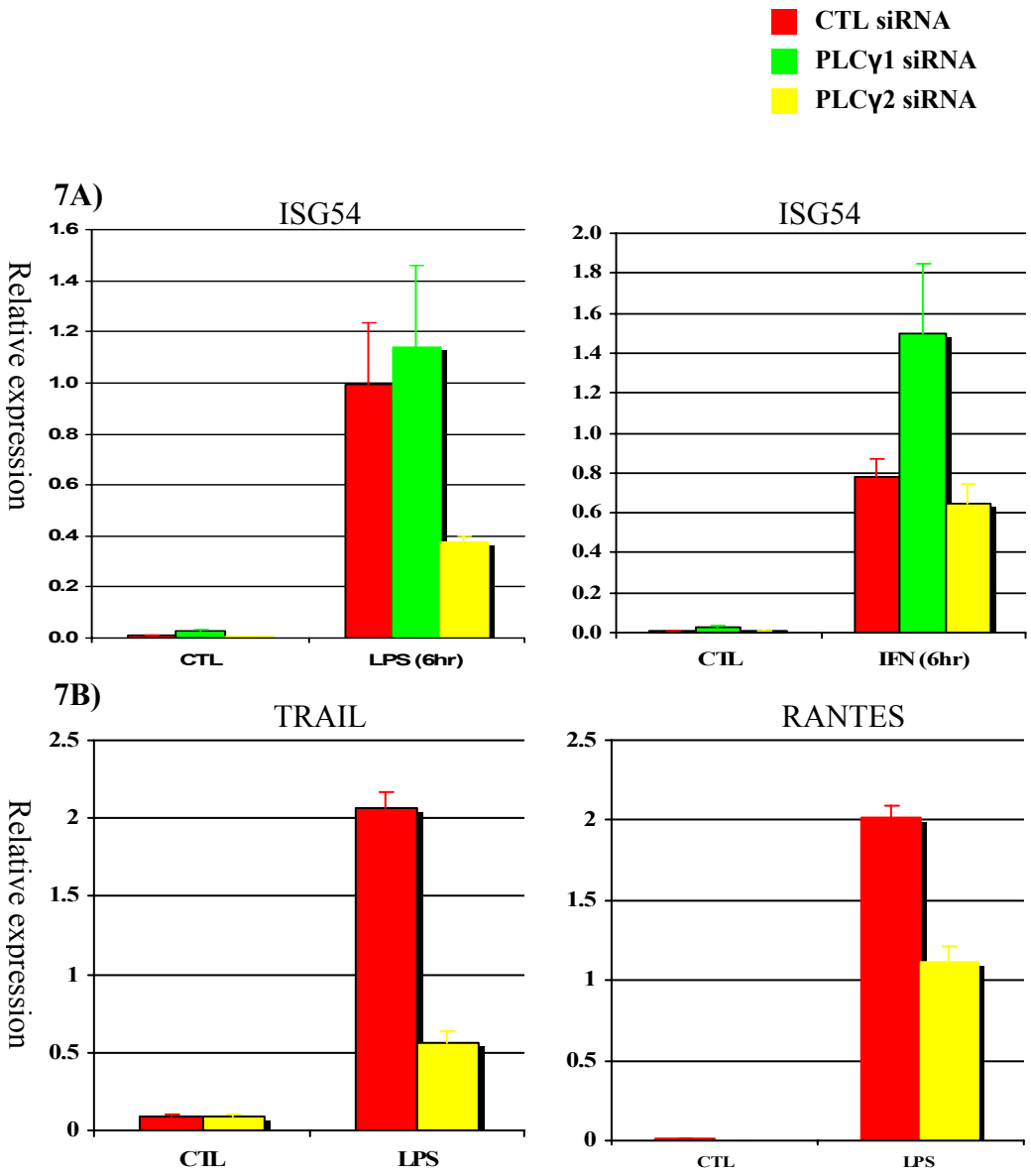

Figure 3-7: Knocking down of PLCγ2 but not PLCγ1 impairs IRF-3 dependent gene expression in response to LPS. Total RNA was isolated from LPS or IFN-β stimulated RAW264.7 cells which were transfected with control, PLCγ1 or PLCγ2 siRNA. A) ISG54 or B) TRAIL and RANTES mRNA levels were quantified by real time PCR.

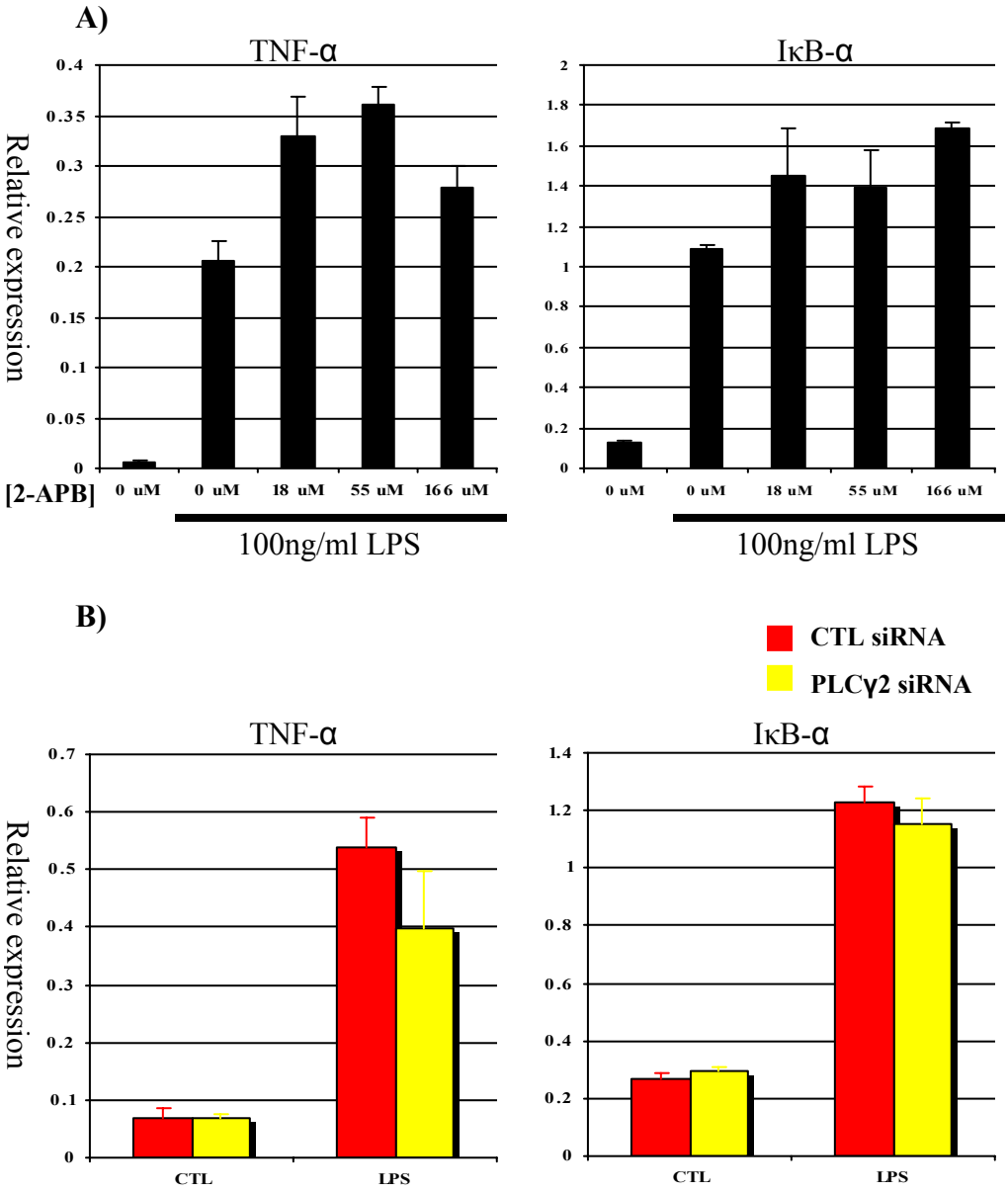

Figure 3-8: Upregulation of NF-κB depedent genes in response to LPS remains intact at the presence of 2-APB and PLCγ2 siRNA: A) Total RNA was isolated from LPS stimulated RAW264.7 cells with or without the preincubation of 2-APB. TNFα and IκBα mRNA level was than quantified by real time PCR. B) Total RNA was isolated from LPS stimulated RAW264.7 cells which were transfected with either control or PLCγ2 siRNA. TNFα and IκBα mRNA level was than quantified by real time PCR.

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