THE ROLE OF DOWNSTREAM OF KINASE (DOK)-3 IN TOLL-LIKE RECEPTOR SIGNALLING IN MACROPHAGES

SOO YEON KIM

(M.Res, Imperial College)

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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Soo Yeon Kim

08 August 2012

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Dum vita est spes est

This thesis represents the achievement of a long-time goal in my scientific journey from an undergraduate in microbiology, to a master's degree in analytical chemistry, to my present focus on the immunology of innate receptors. This journey included many detours, obstacles and doubts together with phases filled with heartache and sheer exhaustion. However looking back this was the time I felt inspired and that nothing is indomitable as long as I have a life filled with hope. I also have had the unequivocal support and encouragement from people around me so I have been able to find my feet. Therefore I remain optimistic for the future and leave with a stronger passion for research and abiding interest in my research field of innate immunity.

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SUMMARY

Toll-like receptors (TLR) are germ-line encoded pattern recognition receptors (PRR) that detect infectious agents specific signatures present on pathogens collectively referred to as pathogen associated molecular pattern (PAMP). LPS, an endotoxin and poly(I:C) which is a synthetic oligonucleotide that mimics double-stranded RNA (dsRNA) binds to TLR-4 and 3 respectively and activates the TIR-containing adaptor inducing interferon-β (TRIF)-dependent signal transduction cascade. Because Downstream of kinase (Dok)-1 and Dok2 were known to regulate TLR, we hypothesized that Dok3 may be involved in TLR regulation as well. We hence investigated if Dok3 is involved in TLR signalling in macrophages.

In examining the role of Dok3 in TLR3 signalling, we showed that Dok3 unexpectedly regulates TLR signalling positively. Dok3 is phosphorylated upon TLR3 and TLR4 stimulation. Dok3-deficient mice are more resistant to LPS- and poly(I:C)- induced septic shock and Dok3 is required for TNFα, IL-12, and IL-6 production by TLR3 stimulation *in-vitro*. In addition, IFNβ induction by TLR3 and TLR4, mediated through TRIF adaptor molecule, is significantly reduced by Dok3 deficiency, owing to a defect in interferon regulatory factor (IRF)-3 activity and nuclear translocation. In particular, Dok3 is required for phosphorylation of upstream IRF3 kinase, AKT and TBK-1 by TLR3 signalling, and is required for induction of IRF3-dependent CXCL10 gene expression. Mechanistically, this is mediated through Dok3 direct interaction with TBK1 and TRAF-3 as determined by overexpression and confocal imaging studies in HEK293T cells. These data suggest a possible role of Dok3 in mediating an anti-viral response.

Poly(I:C) is known to activate RIG (retinoic acid-inducible gene 1)-I-Like Receptors (RLR) signalling in the cytosolic nucleic acid sensing pathway. We therefore further explored if Dok3 is also required for the RIG-I/MDA5 pathway by using transfected poly(I:C) as a ligand. We report here that indeed Dok3 is also required for RLR signalling as activation of IRF3 as well as production of IFNβ was defective in Dok3-deficient cells upon RLR stimulation. Physiologically, we employed a viral infection assay and infected wildtype and Dok3-deficient macrophages with influenza virus and showed that IFNβ gene upregulation was impaired in Dok3-deficient cells and concomitantly, the virus replication was enhanced by Dok3 deletion.

To investigate novel protein interacting partners of Dok3 in TLR4 signalling, we undertook a proteomic analysis approach. Mass spectrometry analysis identified one novel target of Dok3 binding, ABIN1 (A20-binding inhibitor of NFκB) in RAW 264.7 cells upon stimulation with LPS. The interaction of Dok3 and ABIN1 was then confirmed by immunoprecipitating Dok3 and immunoblotting for ABIN1 upon LPS stimulation. Further investigation delineated the signal transduction pathways that are effected downstream of Dok3 and ABIN1 in TLR signalling leading to a deleterious reduction in TNFα production. These possible pathways include activation of A20, TPL-2 and p105 proteins. We showed here A20 protein expression is impaired in Dok3-deficient cells upon LPS stimulation whereas TPL-2 degradation is unaffected by loss of Dok3. These data suggested that Dok3 is required for A20 expression and may explain the defect in TNFα cytokine production in LPS-stimulated Dok3-deficient macrophages.

Taken together, these data demonstrated the importance of Dok3's role in anti-viral immunity. Immuno-therapeutic applications for anti-viral studies in future may hence be aimed at fine-tuning the activity of Dok3 *in vivo*.

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ABBREVIATIONS

APC	Antigen-presenting cells
ABIN1	A20-binding inhibitor of NFκB
BCR	B cell receptor
BLNK	B cell linker protein
BTK	Bruton's tyrosine kinase
CD	cluster of differentiation
DBD	DNA binding domain
Dok	Downstream of kinase
IKK	IκB kinase
IL	Interleukin
IRF	Interferon regulatory factor
IRAK	IL-1R-associated kinase
ISRE	IFN-stimulated response element
LPS	Lipopolysaccharide
LRR	Leucine-rich-repeat
MAL	MyD88 adaptor-like
MAPK	Mitogen-activated protein kinase
MyD88	Myeloid differentiation factor 88
NFκB	Nuclear factor κB
NLR	NOD-like receptors
NOD	Nucleotide Oligomerization Domain
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell

PH	Pleckstrin homology
PI3K	Phosphoinositide 3-kinase
PLCγ2	Phospholipase Cγ2
Poly(I:C)	Polyinosine-polycytidylic acid
PRR	Pattern recognition receptor
PTK	Protein tyrosine kinase
RHD	Rel homology domain
RIP	Receptor-interacting protein
RLR	RIG-I-Like Receptors
SH2	SRC-homology 2
SYK	Spleen tyrosine kinase
TAK-1	TGF-β-activated kinase 1
TANK	TRAF family member-associated NFκB activator
TBK1	TANK Binding Kinase-1
TIR	Toll/IL-1 receptor domain
TIRAP	Toll/IL-1R domain-containing adaptor Protein
TLR	Toll-Like Receptors
TNIP1	TNFAIP3 interacting protein 1
TNFα	Tumor necrosis factor-α
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-containing adaptor inducing interferon-β

LIST OF PUBLICATIONS

Kim S. -Y., Lee K.-G., Chin C.-S, Ng S. -K., Xu S and Lam K.-P. A novel role for DOK3 in IFN-β production by facilitating TRAF3/TBK1 complex formation and TBK1 activation. (Manuscript in submission).

CHAPTER I. INTRODUCTION

1.1 Human Immune System

The immune system comprises two distinct arms: innate immunity and adaptive immunity. The innate immune system is conserved evolutionary and is an ancient response for host organisms to rapidly mount an immune response to foreign pathogens. This response involves cells of myeloid lineage such as macrophages, dendritic cells and neutrophils. The adaptive immune system however requires 'pre-education' of B and T lymphocytes with specific pathogenic components prior to eliciting highly specific immune responses (Hoffmann *et al*., 1999; Janeway, Jr. and Medzhitov, 2002).

The recognition of pathogen associated molecular patterns (PAMP) by immune cells is mediated by various mechanisms. The host cell is equipped with an arsenal of receptors known as pattern recognition receptors (PRR) that can recognise PAMPs and evoke an immune response. These receptors include toll-like receptors (TLR), NOD-like receptors (NLR) and the retinoic-like receptors (RLR). More recently, newly discovered pathogen-sensing pathways include the intracellular nucleic-acid sensing pathways (Keating *et al*., 2011; Kumar *et al*., 2009).

The engagement of the pathogen by innate immune receptors leads to an activation of a wide array of signal transduction pathways that are essential in the production of a range of cytokines and cellular events to eradicate the infection. Immune homeostasis is achieved by the balanced activation and shutting down of the signalling events in an orderly and timely manner that otherwise could lead to deleterious effects and result in immune disorders such as inflammatory diseases and autoimmunity (Parkin and Cohen, 2001) (Figure I-1).

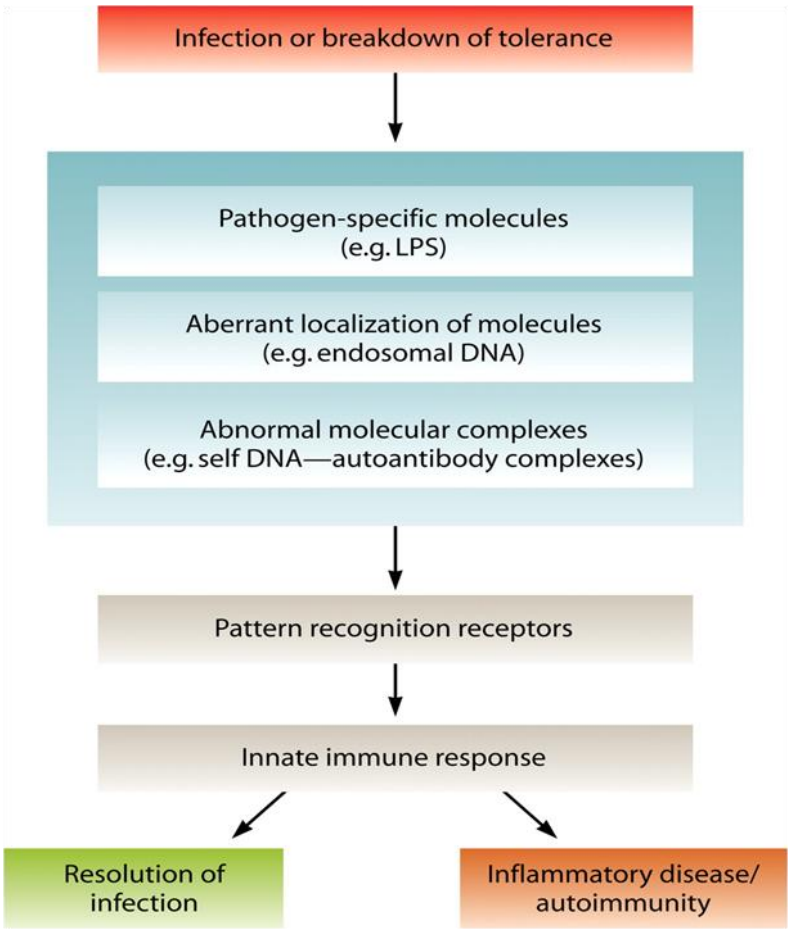

Figure I-1 Principles in innate immune recognition by PRRs

During microbial infection or breakdown of tolerance, accumulation of PAMPs, aberrant localization of foreign or self molecules or abnormal molecular complexes are recognized by PRRs. This event triggers PRR-mediated signalling and induction of an innate immune response, which ultimately results in clearance. However disregulation may cause inflammatory diseases or autoimmunity (Mogensen, 2009).

1.2 Pathogen recognition patterns: TLRs

One class of PRRs comprise the Toll-like receptors (TLR). To date, there are about 11 and 13 TLRs known in humans and mice, respectively. TLRs are type I integral transmembrane glycoproteins that consist of an extracellular domain, transmembrane domain and intracellular domain. The extracellular domain of different TLRs consists of tandem leucine-rich-repeat (LRR) motifs of varying length to mediate ligand

specificity. All TLRs share a common structural feature in their intracellular portions in the form of a Toll/IL-1 receptor (TIR) domain that is critical for signal transduction (Ferrao *et al*., 2012). Most TLRs (1, 2, 4, 5 and 6) are membrane-bound with the exception of TLRs 3, 7, 8 and 9 which are localised in the endosomes. Some TLRs function as a heterodimer, for example TLR1/TLR2 and TLR2/TLR6. All other TLRs function as homodimer (for example TLR3) or in conjunction with other receptors (for example TLR4 and CD14). Also, it was also demonstrated that CD14 which is expressed on the cell surface may enhance dsRNA-mediated TLR3 activation in myeloid DCs by binding to poly(I:C) directly and facilitating cellular uptake, internalization and trafficking to the endosome where TLR3 is localized or cooperates with TLR3 on the cell surface of human fibroblasts to internalize dsRNA (Lee et al., 2006). In addition, it was recently demonstrated that MHC class II functions as a novel adaptor protein with Btk and CD40 at the endosome to further prime the TLR response, notably mediated by TLR3, 4 and 9 (Liu et al., 2011). These findings thus help to explain how TLR3 in particular can recognise a wide variety of substrates. The TLRs recognise different ligands, for example TLR1/TLR2 and TLR2/TLR6 bind to triacylated and diacylated lipoproteins of bacterial and microbial origin respectively while TLR4 and TLR5 recognise LPS and flagellin of bacterial origin correspondingly. TLR3 detects double-stranded RNA from viral origins whilst TLR7/8 binds to viral component of single-stranded RNA. TLR9 on the other hand recognises double stranded DNA viruses and methylated CpG motifs of bacterial origin (Takeda and Akira, 2005; Uematsu and Akira, 2008)) (Figure I-2).

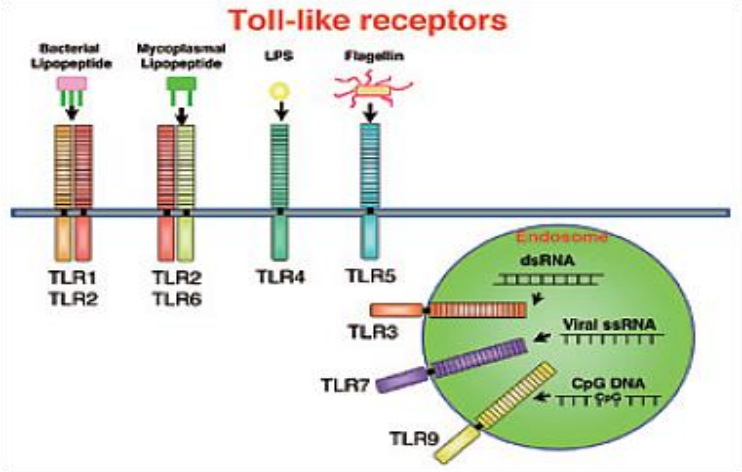

Figure I-2 Recognition of PAMPs from different classes of microbial pathogens.

Viruses, bacteria, fungi, and protozoa display several different PAMPs, some of which are shared between different classes of pathogens. Major PAMPs are nucleic acids, including DNA, dsRNA, ssRNA, and 5-triphosphate RNA, as well as surface glycoproteins (GP), lipoproteins (LP), and membrane components (peptidoglycans [PG], lipoteichoic acid [LTA], LPS, and GPI anchors). These PAMPs are recognized by different families of cell surface or endosomal PRRs such as TLRs (Mogensen, 2009)

The binding of ligands by various TLRs result in the activation of TLR-induced signal transduction pathways. This is mediated primarily through the usage of two different adaptor proteins downstream of the receptors. These adaptors include the TIR-receptor inducing to IFNβ (TRIF) and Myeloid-differentiation 88 (MyD88) which further recruits MyD88-like adaptors (MAL) and TRIF-related adaptors (TRAM) respectively (O'Neill and Bowie, 2007). All TLRs transduce signals via the MyD88 dependent pathway except for TLR3 which signals exclusively through TRIF and TLR4 and thus is able to utilize both adaptors (Yamamoto *et al*., 2003). The TLR signalling pathways subsequently culminate in the activation of various transcription factors that are responsible for specific gene expressions by binding to promoters of gene encoding inflammatory mediators. This activation of the various transcription factors subsequently results in a co-ordinated event in an on-going immune response including the transcription and translation of genes required to combat infection.

Some of these events such as cellular proliferation, migration and cytokine production are critical for overcoming infections (Sandor and Buc, 2005).

1.2.1 Lipopolysaccharide

Lipopolysaccharide (LPS) is commonly known to immunologists as an endotoxin (Raetz and Whitfield, 2002) and form a component of the outer membrane of Gram-negative bacteria and can potentially elicit strong immune responses in various immune cell types such as dendritic cells, macrophages and B cells (Netea *et al*., 2002). LPS is also commonly used by immunologists as a component in animal *in-vivo* model study to examine septic shock. This model mimics the causative factors for the development of sepsis in potentially fatal human disease (Stewart *et al*., 2006). The innate immune receptor for recognising LPS was identified as TLR4. These critical discoveries were fundamentally impactful on the scope of modern medicine (Poltorak *et al*., 1998).

1.2.2 Double-stranded RNA

Double-stranded RNA (dsRNA) comprises two complementary strands and is the genetic material of some RNA viruses. dsRNA is also known as an intermediate product of some viruses during their replication cycles. One example is the dengue virus (Tsai *et al*., 2009). dsRNA from viruses can trigger type I interferon (IFN) response in vertebrates. Poly(I:C) is a synthetic analogue of dsRNA, a molecular pattern associated with viral infection. Both natural and synthetic dsRNAs are known to induce type I IFN and other proinflammatory cytokines production. dsRNA is primarily recognized by the pattern recognition receptor, TLR3, in the endosome and RIG-I/MDA5 in the cytoplasm for signal transduction in host cell anti-viral immune response (Vercammen *et al*., 2008).

1.2.3 Toll-like receptor 4

TLR4, also known as cluster of differentiation (CD) 284, is one of the toll-like receptors that is most extensively studied to date (Reeves and Wang, 2002). The receptor, its functional role and mode of activation on dendritic cells was discovered and deciphered by various research studies in different laboratories, with these research findings deemed as groundbreaking discoveries (Lemaitre *et al*., 1996; Poltorak *et al*., 1998; Steinman and Witmer, 1978). TLR4 recognises LPS, which is a component of the cell wall of Gram negative bacteria (Poltorak *et al.*, 1998). Clinically, patients with severe toxins contamination from bacterial infection can develop sepsis that can be potentially fatal (Reading and Brecher, 2001). The LPS challenge is also commonly used as an *in-vivo* laboratory experimental protocol in mice to trigger endotoxin or septic shock (Copeland *et al*., 2005). As such, the identification of TLR4 as the main receptor for LPS presents answer for medical intervention.

TLR4 has been shown to require a co-receptor CD14 (Kim *et al*., 2007), for signalling. Upon activation of TLR4 by its ligand, several signalling pathways are propagated downstream for specific gene expression (Rallabhandi *et al*., 2006). Signalling by TLR4 is unique as it is the only TLR present on the plasma membrane that utilises four different adaptor proteins to orchestrate specific signal transduction pathways. These adaptors include MyD88/MAL and TRIF/TRAM (O'Neill *et al*., 2003). Following the assembly of adaptor proteins and kinases, the TLR4 pathway leads to the activation of two distinct responses. One of these pathways is required for the production of inflammatory cytokine production which is dependent on MyD88 signalling to NFκB activation. The other pathway is required for the production of

type 1 IFN which is mediated by TRIF-dependent signalling to TBK1 and IRF3 (Fitzgerald *et al*., 2003b).

1.2.4 Toll-like receptor 3

TLR3, otherwise also known as cluster of differentiation (CD) 283, was identified in 2001 as the receptor that binds dsRNA (Alexopoulou *et al*., 2001). There are multiple prerequisites as to how the receptor excludes its self-activation. Firstly, the structure of TLR3 showed that it resembles a solenoid horseshoe shape of which one side of it is masked by carbohydrate while the other side is glycosylation-free, presumably to prevent self-activation (Choe *et al*., 2005). Secondly, this receptor pre-exists as a dimer and activation is allowed only when its ligand, dsRNA cross-links TLR3 (de Bouteiller *et al*., 2005). Last but not least, the receptor prevents pre-and/or self-activation in order to mediate host self-to-foreign RNA recognition by its unique localisation in the endosome as opposed to other TLRs that are present on the plasma membrane (Schroder and Bowie, 2005). This is one way the TLR3 ensures that dsRNA will cross-link and activate a foreign invading source such as bacteria or viruses that have been subjected to cell-mediated phagocytosis or endocytosis and have trafficked to the endosome for TLR3-mediated antiviral immune response, similar to TLR9 activation (Latz *et al*., 2004). This finding was confirmed by studies whereby when chloroquine (a compound that disrupts endosome formation, hence disabling TLR3 proper assembly) was added to cells in culture, it resulted in a defective host innate antiviral response (de Bouteiller *et al*., 2005).

Poly(I:C), which mimics dsRNA and binds TLR3 and activates the TRIF pathway TLR3 is activated by tyrosine phosphorylation at two distinct tyrosine residues, 759 and 858 (Sarkar *et al*., 2007). The protein kinase responsible for this action was

recently identified to be BTK (Lee *et al*., 2012). In both TLR3 and TLR4 signalling, TRIF can also interact with the TNF receptor-associated factor 3 (TRAF3) that subsequently activates downstream IKK-related kinases, TANK-binding kinase 1 (TBK1) and inhibitor of kB kinase ε (IKKε). These IKK-related kinases then phosphorylates interferon response factor 3 (IRF3) and IRF7 which leads to IFNβ production, a cytokine important for an effective antiviral host immune response (Vercammen *et al*., 2008) (Figure I-3).

When TLR3 is phosphorylated at tyrosine 759 residue, it leads to the recruitment of PI3K which in turn activates its downstream substrate AKT. This is termed the detour pathway or TRIF-independent signalling branch downstream of TLR3. More recently, studies have also demonstrated that AKT can activate TBK1 (Joung *et al*., 2011). In addition, this TRIF-independent signalling branch of TLR3 is also important for other cellular aspects of the host cell, including cell migration, proliferation and adhesion (Yamashita *et al*., 2012b). It is now well-characterised that upon tyrosine phosphorylation of TLR3, PI3K is recruited to the tyrosine 759 residue leading to a full activation of IRF3 via AKT, in addition to TRIF-mediated IRF3 activation (Hiscott, 2004).

On the other hand, TRIF-dependent signalling pathway requires tyrosine phosphorylation of TLR3 at tyrosine 858 residue which recruits and mediates binding to BB loop of TRIF adaptor (Toshchakov *et al*., 2005). TRIF then allows the docking of RIP1 for subsequent NFκB activation and TBK1 for IRF3 activation, two distinct signalling arms that bifurcate at TRIF, leading to IFNβ production (Seya *et al*., 2005).

c-SRC, a SRC-family kinase is also implicated in TLR3 signalling in antiviral immune response (Johnsen *et al*., 2006). Recently SRC and EGFR have been demonstrated to co-localise with TLR3 in the endosome and phosphorylate the two activation residues in TLR3. Furthermore abrogation of EGFR impaired TLR3-mediated antiviral responses (Yamashita *et al*., 2012a). Thus it appears that TLR3 signalling is more complex than originally thought and there may be more novel proteins awaiting discovery, adding on to the diversity of pathways downstream of TLR3.

1.2.5 Critical signalling components of TLR pathway

Adaptor proteins play a critical role immediately downstream of TLR signalling by coupling the receptor crosslinking to signalling cascades leading to the activation of transcription factors. In TLR signalling, five of these adaptor proteins have been identified. These include MyD88, MAL, TRIF, TRAM and SARM (O'Neill and Bowie, 2007). The commonality these proteins lies in the presence of the hallmark TIR domain that also serves as a conduit between the TLRs and the adaptor proteins themselves (Hultmark, 1994). All the adaptor proteins are known to positively regulate TLR signalling with the exception of SARM being known to function instead as a negative regulator (Peng *et al*., 2010). In addition, the RLR pathway in the cytosolic signalling pathway for antiviral immune response uses another adaptor protein, IPS-1 to signal IFNβ production (Kawai *et al*., 2005).

We will discuss the adaptor protein TRIF in more detail as it is a crucial adaptor protein which transduces signal for IFNβ production. Whereas all other TLRs almost

ubiquitously use MyD88 for signal transduction, TRIF is exclusively used by TLR3 and TLR4 to mediate downstream signalling. TRIF is the largest protein among the other adaptor proteins in TLR signalling. It was identified by both database screening for TIR-domain containing proteins (Yamamoto *et al*., 2002) and in a yeast-2 hybrid screening with TLR3 as a bait (Oshiumi *et al*., 2003). The protein structure of TRIF is mainly divided into its N-terminal domain containing binding sites for TRAF6 and TBK1, an intermediary TIR domain for binding to TLR3 and a C-terminus containing a RIP1 binding site (Oshiumi *et al*., 2003). In TLR4 signalling, TRIF is known to mediate signalling with another adaptor protein, TRAM that functions as a scaffold (Rowe *et al*., 2006). TRIF-deficient mice were generated in 2003 and analyses of the mutant mice highlight its importance in activating IFNβ via the TBK1-IRF3 signalling axis (Oshiumi *et al*., 2003; Yamamoto *et al*., 2003). This finding also resolved the long-standing enigma of the existence of a MyD88-independent signalling arm in innate TLR signal transduction pathways. More recently, TRIF was also identified to participate with DDX1, DDX21 and DHX36 to form a complex that senses dsRNA in DCs, further adding complexity and specificity to innate immune signalling in host antiviral defense (Zhang *et al*., 2011).

1.2.2.1 TNF receptor-associated factor 3

TNF receptor-associated factors (TRAFs) proteins are known to be evolutionarily conserved from *Caenorhabditis elegans* to mammals and they function as adaptor or scaffold proteins to recruit and assemble multiple proteins together to amplify downstream signals (Chung *et al*., 2002). There are about 7 known family members to date (Arch *et al*., 1998). The protein structure unambiguously include the C-terminal TRAF domain, an N-terminal RING-finger domain and several zinc-finger motifs

(Chung *et al*., 2002). Historically, most studies performed with TRAF proteins have focused on their roles in the TNF receptor superfamily with the exception of TRAF6 (Bradley and Pober, 2001). TRAF6 was extensively studied in TLR4 signalling in disease conditions (Zhou *et al*., 2010). It is known to interact with IRAK, MyD88 and TRIF to signal to NFκB downstream of the receptor (Bradley and Pober, 2001; Zhou *et al*., 2010). However, knockout studies with TRAF6-deficient mice revealed that IRF3 activation mediated via TRIF was relatively unaffected downstream of TLR3 and suggested an alternative TRAF to be responsible for this signalling pathway instead (Hacker *et al*., 2006; Zhou *et al*., 2010). This promoted a surge in studies to identify the missing TRAFs in this pathway and led to the discovery of TRAF3 in antiviral responses. TRAF3 was identified previously as a protein that binds the CD40 cytoplasmic tail and studies were focused on its role in adaptive immunity (Cheng *et al*., 1995). The studies pertaining to TRAF3 in TLR signalling have been increasing in recent years. A yeast-2-hybrid screening using TRAF3 as a bait pulled out TANK whereas a separate screen using TANK as a bait pulled out TBK1 (Chariot *et al*., 2002; Pomerantz and Baltimore, 1999). Together, these findings suggest that TRAF3 and TBK1 may have some interacting role. Indeed, TRAF3 and TBK1 were found to interact with TRIF in overexpression experiments in HEK293T cell (Guo and Cheng, 2007; Pomerantz and Baltimore, 1999). However, genetic ablation of TRAF3 was found to confer embryonic lethality and suggests that TRAF3 has a broader and indispensable role in organism development (Xu *et al*., 1996).

Finally, studies with TRAF3$^{-/-}$ MEFs revealed that TRAF3 has a specific function in signalling to TBK1-IRF3 for IFNβ production in TLR3 activation and is required for IL-10 production downstream of the same receptor (Oganesyan *et al*., 2006). How

TRAF3 is activated remains unclear. We can at least hypothesise using TRAF6 activation as an analogy and design experiments to prove the theory. TRAF3 may function similarly to TRAF6 as an E3 ligase (Deng *et al*., 2000; Oganesyan *et al*., 2006). E3 ligase catalyses both K48 and K63-linked ubiquitination, a form of protein post-translational modification, of which either leads to degradation of target protein via the proteasome or mediates activation by ubiquitin-mediated scaffold assembly of target proteins respectively (Chen, 2005). TRAF3 may self-polyubiquitinate upon activation and assembles proteins to activate TBK1 to phosphorylate IRF3, in a manner similar to TRAF6 assembly of TAB1 and TAB2 that subsequently activate TAK1 for NFκB signalling (Kanayama *et al*., 2004). The mechanism of TRAF3 activation in antiviral immunity therefore awaits further clarification.

1.2.2.2 TANK-binding kinase 1

TANK-binding kinase (TBK)-1 also known as NFκB-activating kinase (NAK), shares a relatively high degree of homology with IKKε and belongs to the IκB kinase family that includes IKKα, IKKβ and IKKγ or NEMO (Chau *et al*., 2008). However, TBK1 and IKKε functions deviate from the other IκB kinases that were mainly implicated in activating NFκB and as such are also termed as non-canonical IκB kinase proteins. These kinases, especially TBK1 were predominantly required to activate the IRF3 transcription factor for signalling to IFNβ (BURNETT and KENNEDY, 1954). TBK1, a serine/threonine kinase forms an integral large network of signalling proteins that are involved in TLR activating pathways including IRAKs, the IKKs and TAK1 (MAPK) (Takeda and Akira, 2005).

TBK1 was originally identified by yeast-2 hybrid screening using TANK as a bait (Guo and Cheng, 2007). TBK1 knockout mice displayed embryonic lethality at E14.5 and suggests that this kinase is critically important for development (Hemmi *et al*., 2004). Also, studies with TBK1$^{-/-}$ MEFs underscore the importance of this kinase in directly activating IRF3 for IFNβ production in TLR3 and TLR4 signalling through the TRIF pathway (Hemmi *et al*., 2004). Last but not least, TBK1 is known to activate an antiviral immune state by forming several different complexes of which the constituting component depends on the type of cell and cellular stimuli. Some of these scaffolding molecules including FADD, TRADD, MAVS or SINTBAD have been identified to be recruited to the TBK1-containing-complexes (Chau *et al*., 2008). As such, it is possible that there are more novel proteins awaiting discovery, increasing the list of TBK1-containing protein complexes.

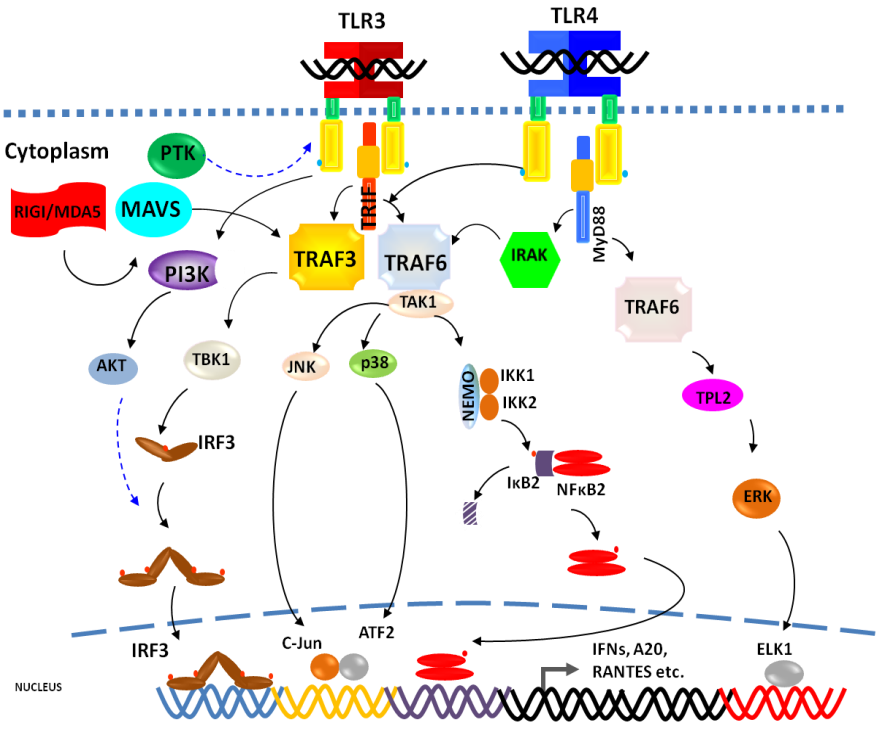

Figure I-3 Regulation of IRFs and NFκB via MyD88-dependent and MyD88-independent pathways by TLR pathways

TLR4 uses MyD88-dependent and MyD88-independent pathways to activate NFκB. NFκB transcription factors are initially inactive and retained in the cytoplasm of cells by the IκB subunits. Upon receptor activation, the IκB subunits are phosphorylated and degraded, thus allowing the translocation of NFκB p65/p50 subunits to the nucleus to effect proinflammatory gene transcription. In TLR4 signalling, the MyD88-independent pathway uses TRIF via adaptor TRAM to activate NFκB in either a TRAF6-dependent manner or a TRAF6-independent mechanism. TLR3 however, interacts directly with TRIF to trigger these MyD88-independent pathways. TRIF also associates with TBK1 and IKKε, which in turn phosphorylate IRF3 and IRF7 respectively, leading to their nuclear translocation and induction of type I IFN genes. Upon activation, TLR3 also interacts with PI3K and activates AKT, leading to further phosphorylation and maximal activation of IRF3 (Sandor and Buc, 2005).

1.2.6 Positive and negative regulators of TLR signalling

The study of TLR signalling has become increasingly complex with the surge in novel proteins being identified in recent years. In addition, proteins that are involved in TLR signalling usually display multifaceted roles and exhibit multi-tasking abilities. The main bulk of proteins that are associated with TLR signalling are generally classified into positive and negative regulators based on their functions that are usually delineated by gene ablation studies in mice or by siRNA application in cells. However, one added tier of complexity is the fact that some of these well-characterised proteins with defined positive or negative regulatory roles as demonstrated in previous studies are now challenged by more recent advanced technical studies to assume the opposite identity.

We will describe some of these examples. MyD88 was long identified as the main adaptor protein in most TLR signalling. In MyD88 knockout mice, the inflammatory cytokine and IFNβ production were compromised in multiple TLR signalling (Muraille *et al*., 2003; Scanga *et al*., 2004). MyD88 mice were also more susceptible to challenge with Leishmania *in vivo*, pointing to the fact that MyD88 functions as a

positive regulator (Muraille *et al*., 2003). More recently however, a separate study involving MyD88 appears to suggest that the adaptor protein functions as a negative regulator instead. The study showed that MyD88 negatively regulates TLR3-induced inflammation in human corneal epithelial cells (HCECs). This is possible by inhibiting the activation of JNK pathway (Johnson *et al*., 2008). Apart from this, another study also demonstrated that MyD88 negatively regulates the actions of TRIF adaptor, thereby inhibiting TLR3-induced IFNβ and CCL5 gene induction (Siednienko *et al*., 2011). One other study also clearly showed that MyD88 functions as a negative regulator to control hypergammaglobulinemia with the production of autoantibody in a bacterial infection (Woods *et al*., 2008).

The other major adaptor TRIF, was also largely characterised as being a positive regulator in TLR signalling (Yamamoto *et al*., 2002; Yamamoto *et al*., 2003). This paradigm was challenged by a recent study that surprisingly demonstrated a negative role of TRIF in TLR-activated DCs. The authors found that the IL-12 production and co-stimulatory molecule expression by r-EA-as well as TLR4 and TLR9-treated DCs were significantly higher in TRIF-deficient mice and cells (Seregin *et al*., 2011).

Other examples of a TLR-signalling associated protein that displayed dual regulatory functions depending on the context of receptor signalling and cell type studied include the PI3K, BTK and Lyn which are classified as protein kinases (Page *et al*., 2009). The PI3K was generally demonstrated as positive players in TLR signalling in many studies (Lindmo and Stenmark, 2006). However, it was also demonstrated to play an inhibitory role in other TLR-related studies (Fukao and Koyasu, 2003; Keck *et al*., 2010). One particular landmark study showed that PI3K negatively regulates IL-12 production in TLR-activated DCs and concomitantly, the PI3K-deficient mice were

observed to have an enhanced T helper type 1 (Th1) in response to Leishmania major infection (Fukao and Koyasu, 2003). Last but not least, another interesting study demonstrated that Lyn, a SRC-family kinase, is a negative regulator of TLR4 signalling in macrophages and the stimulated cells overproduce inflammatory cytokines including IL-6, TNFα and anti-viral IFNα/β as a result of the deficiency (Keck *et al*., 2010) whereas in many other studies Lyn is clearly depicted as a positive regulator (Avila *et al*., 2012; Seo *et al*., 2001).

1.3 Other Pathogen Recognition Receptors

1.3.1 RIG-I-like Receptors

RIG-I-like receptors **(**RLRs) are cytoplasmic sensors of viral RNA in host cell. There are three RLRs discovered to date. They are RIG-I (also known as DDX58), MDA5 (also known as Helicard) and LGP2 (Martinon and Tschopp, 2005). The structure of RIG-I and MDA5 comprise a caspase recruitment domain (CARD) and a RNA helicase domain (RHD) (Yoneyama *et al*., 2005). The CARD is responsible for binding interacting partners in the signalling pathway to induce interferon production against viral infections and the RHD is important to bind viral nucleic acid (Saito *et al*., 2007). RIG-I can sense both viral RNA from virus infections and synthetic RNA that is transfected into the cytoplasm of the cell (Onoguchi *et al*., 2010).

Activation of RIG-I leads to the production of type I interferon. These viruses include ssRNA viruses such as influenza viruses, vesicular stomatitis viruses (VSV) and paramyxoviruses. Genetic ablation studies using RIG-I-deficient mice underscore the importance of RIG-I in protecting host animals against these virus infections as these

mutant mice display a compromised innate immunity when infected with the RNA virus and resulted in increased susceptibility (Kato *et al*., 2006) (Figure I-4).

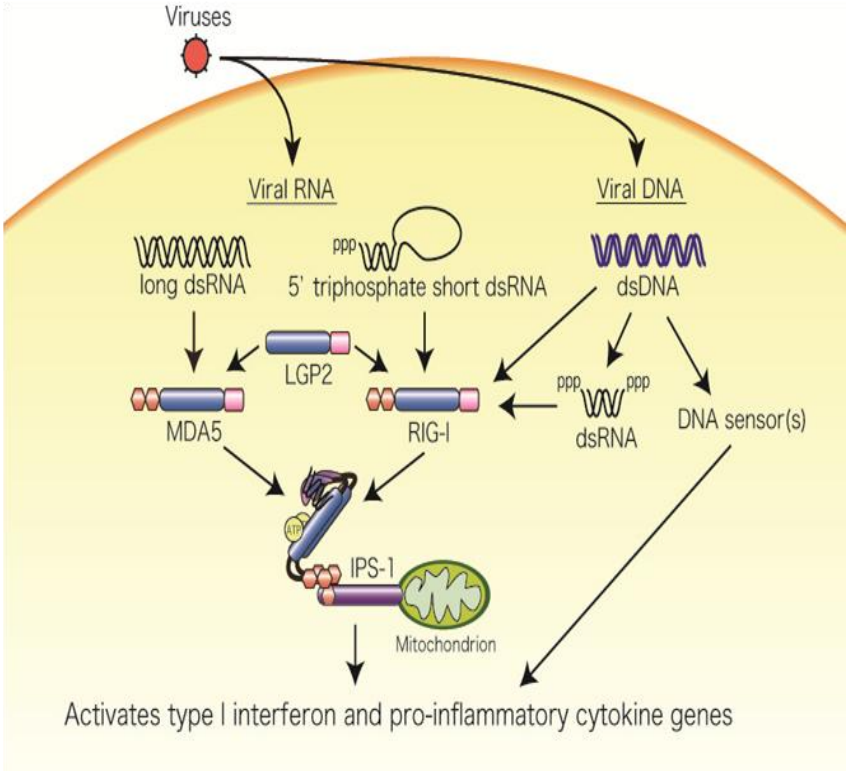

Figure I-4 Intracellular RNA recognition and signalling.
Cytosolic dsRNA or 5-triphosphate ssRNA is recognized primarily by the cytoplasmic RNA helicases RIG-I and MDA5, which mediate interaction with the adaptor IPS-1 is localized to mitochondria, and trigger signalling to NFκB and IRF3 via IKK and TBK/IKKε, respectively (Takeda and Akira, 2005)

There is an abundance of host-self RNA in the cell but this host-self RNA is different from foreign or viral RNA in that it does not activate RIG-I. This unique property of self versus foreign (non-self) RNA mediated activation of RIG-I is possible because of the presence of 5'triphosphate (5'PPP) cap or an attachment to the end of the RNA nucleic acid that allows discrimination from the host innate immune surveillance mechanism. During the maturation of nucleic acid in the host cell, the endogenous RNA will lose their 5'PPP attachment by default and therefore is able to escape RIG-I detection. In contrast RNA from viral source contains 5'PPP and is readily detected by RIG-I in the cytoplasm of the host cell which then immediately activates the anti-

viral immune response (Schmidt *et al*., 2009). In spite of this, there are also short dsRNA usually less than 1kb that can also activate RIG-I in a 5'PPP-independent manner (Kato *et al*., 2008). Some examples of these include short regions of reovirus, which is a dsRNA virus, and short poly(I:C) that have been found to activate RIG-I signalling (Kato *et al*., 2008). DNA virus infection can also activate RIG-I. This is because these viral DNA can be processed by RNA polymerase III in the cell to produce dsRNA and this is recognised by RIG-I (Ablasser *et al*., 2009).

Binding to viral RNA by RIG-I is direct and occurs via recognition in the C-terminal domain (CTD) of the helicase. The crystal structure of RIG-I revealed that the CTD forms a cleft with positively charged amino acids that possibly interact with the dsRNA (Schmidt *et al*., 2010). The mechanism of how RIG-I specifically recognises the 5'PPP moiety however remains elusive and is of intense interest to structural biologists. The binding of viral RNA to RIG-I subsequently results in a conformational change to RIG-I protein structure and expose the N-terminal CARD domain that allows protein interaction to occur. One immediate downstream interacting partner is the mitochondria-localised adaptor protein IFN-β promoter stimulator 1 (IPS-1) which possesses a CARD-like domain that can interact with the CARD domain of RIG-1. The assembly of RIG-I and IPS-1 complexes trigger major activation of downstream signalling pathways such as NFκB and IRFs for IFNβ production (Xu *et al*., 2005).

The protein structure of IPS-1 comprises a CARD-like domain at the N-terminal and a transmembrane region at the C-terminal for localisation to mitochondria (Kawai *et al*., 2005). Several studies have highlighted the role of IPS-1 in antiviral defense as IPS-1

deficiency in mice or cells impairs host ability to mount an antiviral immune response to RNA viruses with defects in inflammatory cytokines production and production of type I IFN (Kumar *et al*., 2008; Miyake *et al*., 2009). However the role of IPS-1 exhibits cell type specificity. In studies using pDCs, it was found that IPS-1 deficiency did not affect its production of type I IFN in response to RNA virus. Therefore, it was suggested that TLRs constitute a more important antiviral defense arsenal as compared to RLR in pDCs. In other cell types like macrophages, conventional DCs and fibroblasts, both TLRs and RLRs are equally important in mediating antiviral response and are not mutually exclusive (Sun *et al*., 2006).

Other proteins such as TRAF family proteins, more specifically TRAF3 and TRAF6, RIP-1 and caspases including caspase 8 and 10 and Fas-associated death domain (FADD) were demonstrated in various studies to be involved in RIG-I signalling for antiviral responses (Balachandran *et al*., 2007; Hacker *et al*., 2006; Takahashi *et al*., 2006). However the exact mechanistic involvement of these proteins in the RIG-I pathway remains to be elucidated.

Melanoma differentiation-associated protein 5 (MDA5) is structurally analogous to RIG-I as MDA5 also consists of RHD and CARD domains and is able to signal via IPS-1. However in contrast to RIG-I, MDA5 was demonstrated to recognise long forms of poly(I:C). More specifically, these long forms refer to length of more than 1kb as a determinant for nucleic acid ligand requirement (Kato *et al*., 2008). Under the taxonomy classification of virus, positive sense single-stranded RNA virus, and in particular the Picornaviridae family, including the Enceplomyocarditis virus and Mengo virus, are recognised by MDA5 (Kato *et al*., 2006).

Lastly, laboratory of genetics and physiology 2 (LGP2) is the third member of the RLR family proteins. Similar to RIG-I and MDA5, LGP2 contains RH domain but lacks an CARDs. Therefore LGP2 was initially referred to as a negative regulator of RLR signalling. However analysis of LGP2 deficient mice showed that these mice were susceptible to infection. Therefore LGP2 was reassigned as a positive regulator RLR signalling (Satoh *et al*., 2010).

1.3.2 NOD-like Receptors

The NOD-like receptors (NLR) belongs to another family of PRRs that are able to recognise PAMPs in the cytoplasm. These PAMPs are usually of microbial origins and include derivatives from Gram-positive and Gram-negative bacteria (Chen *et al*., 2009). The NLRs mainly include NOD1 and NOD2 and they bind to specific ligands. These ligands include dipeptide g-D-glutamyl-meso-diaminopimelic acis (iE-DAP) and muramyl dipetide (MDP) (Chamaillard *et al*., 2003).

The NODs proteins structure is mainly made up of three functional domains. First is a nucleotide-binding oligomerisation domain (NOD) that binds nucleoside triphosphate that forms critical component of pathogen nucleic acid that is required for nucleotide binding and self-oligomerisation. Next is a N-terminal effector binding region that consists of protein-protein interaction domains such as the caspase recruitment domain (CARD) domain, and finally a C-terminal leucine-rich repeats (LRR) to detect conserved microbial patterns and to modulate NLR activity (Inohara and Nunez, 2003).

At present, there are around 23 NLR genes in humans and 34 NLR genes in mice as predicted based on bioinformatics analysis (Harton *et al*., 2002). Structurally, the NLRs can be classified into three subfamilies also referred to as CARD-containing NODs, PYD-containing NALPs, or BIR-containing NAIPs based on the N-terminal domains whereas the LRRs in the C terminus of NLR proteins are thought to fold back onto the NOD domain, thereby inhibiting spontaneous oligomerisation and activation of the NLR protein (Duncan *et al*., 2007). It is thought that when NLRs engages ligands via the C-terminal LRR, it undergoes structural conformational changes such that it allows the oligomerisation via the NOD domain. As a result, the effector domains of the NLRs are now exposed and are able to induce the recruitment and subsequent activation of the CARD and PYD-containing signalling proteins due to close proximity and oligomerisation. These proteins include RIP2 that will trigger downstream signal transduction cascades (Kobayashi *et al*., 2002).

The signalling pathways that are evoked when NOD binds its ligands include NFκB and MAPKs that result in proinflammatory cytokine production (Franchi *et al*., 2009; Inohara and Nunez, 2003). A key serine-threonine enzyme that participates in NOD-mediated signalling is RIP2, a member of the RIP family protein that also includes RIP1. This protein mediates interaction with NOD via the CARD domain and is mainly responsible for directly binding and promoting the K63-type polyubiquitlation of the regulator IKKγ and activation of the kinase TAK1, a prerequisite for the activation of the IKK complex. These events result in the degradation of the NFκB inhibitor IκBα and the subsequent translocation of NFκB to the nucleus, where transcription of NFκB-dependent target genes occurs (Hall *et al*., 2008; Inohara and Nunez, 2003). The importance of NOD1 and NOD2 are underscored by their genetic

implications to human diseases mostly associated with inflammation. Polymorphisms in NOD1 are also associated with asthma and eczema (Hysi *et al*., 2005). On the other hand, there were three mutations (R702W, G908R and L1007insC) in amino acid residues found within the NOD2 LRR region that is responsible for the development of Crohn's disease (Hugot *et al*., 2001).

$NOD1^{-/-}$ and $NOD2^{-/-}$ mice were generated to study the physiological roles of these 2 proteins *in vivo* but the controversy generated in findings using NOD1 knockout mice further added more complexity rather than understanding. For example, in a study where $NOD1^{-/-}$ primary fibroblasts were infected with *Chlamydia trachomatis*, the mutant cells produced lower proinflammatory cytokines as compared to wildtype control indicating that NOD1 can function as an antagonist against Chlamydia. Interestingly, when *Chlamydia trachomatis* was introduced vaginally into mutant mice, they were relatively unaffected and were able to clear the infection, suggesting redundancy of NOD1 and compensation by other NOD proteins or TLRs (Welter-Stahl *et al*., 2006). Studies with $NOD2^{-/-}$ mice were however more straightforward and direct and revealed that these proteins were critical in sensing intracellular pathogens like *Listeria monocytogenes* (Kobayashi *et al*., 2005).

1.4 Transcription factor

There are several transcription factors that play a critical role in the immune responses. Transcription factors are also known as sequence-specific DNA-binding factors that are proteins that binds to specific DNA sequences, thereby controlling the flow (or transcription) of genetic information from DNA to mRNA (Latchman, 1997). They can either function alone or in a complex with other proteins to either activate or repress the recruitment of RNA polymerase to a specific gene. TLR signalling leads to

a controlled transcription of inflammatory-related genes mainly mediated by a niche subset of transcription factors including NFκB, AP-1 and IRFs (Sandor and Buc, 2005).

1.4.1 Nuclear Factor κB

The NFκB transcription factor is responsible for many gene expressions in an immune response. It is made up of various subunits, the most common being p65 and p50 subunit heterodimer. The p65/p50 heterodimer is usually held in an inactive state and prevented from activation by being bound to an inhibitor protein known as IκBα. Upon receptor engagement following a stimuli, it leads to the activation of the IKK complex containing NEMO (IKKγ), IKKα and IKKβ, of which IKKβ then phosphorylates IκBα. A phosphorylated IκBα is then targeted for degradation following Ly48-ubiquitination in the proteasome. A released p65/p50 is then free to translocate to the nucleus to bind DNA on the consensus region and activate specific gene expression (Gilmore, 2006).

1.4.2 Interferon Regulatory Factor

The IRF family of transcription factors is largely responsible for mediating the production of interferon in an immune response. There are about 9 members of these family proteins that exist in both human and mice. The most recently identified member is IRF10 but its expression is found only in chicken and not human or mice (Honda and Taniguchi, 2006). These proteins played diverse regulatory roles in innate immunity, to prime the adaptive immunity and also to prevent tumor development (Honda *et al*., 2005b; Honda *et al*., 2006). For example, IRF1, 3 and 7 was found to be involved in interferon production by various cell types in different toll-like

receptor signalling. IRF1 was activated downstream of TLR9 in myeloid DCs to produce interferon-β (Schmitz *et al*., 2007) whereas IRF3 and 7 was activated downstream of TLR3 and TLR4 in various cell types including B cells, macrophages and different subsets of DCs to induce interferon-β production via TRIF (Doyle *et al*., 2002; Honda *et al*., 2005a; Oganesyan *et al*., 2008). IRF5 was reported to be involved in macrophage polarization that subsequently determines a T cell immune response to Th1 or Th17 bias (Krausgruber *et al*., 2011).

As transcription factors, these proteins are required to bind to specific regions of DNA to induce target gene expression. IRFs possesses DNA-binding domain (DBD) in their protein structure and are known to bind to the consensus DNA region known as the ISRE characterised by the presence of DNA repeats (consensus sequence: $_{A/G}$NNGAAANNGAAACT) (Honda *et al*., 2006). Genetic studies using different IRF knockout-mice have revealed overlap and redundancy in the functions of the IRFs (Honda and Taniguchi, 2006). One example is studies with IRF3 and IRF7 knockout mice, which indicated that single knockout mice is still able to induce residual interferon-β production upon activation while a double IRF3 and 7 knockout in mice showed complete ablation of IFNβ (Daffis *et al*., 2009).

As thesis touches on IRF3, we will discuss this transcription factor in more detail. This particular transcription factor was identified by EST database search and is characterised because of its homology to IRF1 and IRF2 (Au *et al*., 1995). Its protein structure depicts a nuclear export signal, a DNA-binding domain and several regulatory serine phosphorylation sites in its C-terminus (Hiscott and Lin, 2005).

IRF3 signalling leads to the activation of IFNβ, a type I IFN. The anti-viral cytokine is important for the eradication of viral infections. The transcription factor is normally inactive and predominantly resides in the cytoplasm. Upon appropriate activation signals, IRF3 is phosphorylated by its upstream direct kinase, TBK1 on serine 396 residue and undergoes homodimerisation before translocating to the nucleus. Recent studies have also highlighted that IRF3 is not only activated by TLRs, but also by the intracellular RIG-1/MDA5 pathway that uses IPS-1 to induce IFNβ (Xu *et al*., 2005). In addition, new and novel DNA sensing pathways uncovered in recent innate immune studies have also demonstrated that the adaptor protein STING or MITA activates IFNβ production via TBK1 signalling to IRF3 (Ishikawa *et al*., 2009).

IFNβ protein expression is translated from the gene transcription of the IFNβ enhanceosome (Kim *et al*., 2000). The IFNβ enhanceosome is mediated by co-ordination of various transcription factors binding to different promoters and inducing the cytokine IFNβ. These 3 major transcription factors required for the activation of IFNβ are NFκB, IRF3 and AP-1. The upstream activators of these transcription factors include MAPKs, TBK1 and IKKs (Honda *et al*., 2005b).

1.5 Type I Interferon

Interferons are specific cytokines that are released by activated innate cells when they are subjected to viral or bacterial invasion. The term 'Interferon' was coined from "interfering with viral replication". The family of interferons include type I IFN, type II IFN and type III IFN. Type I IFN include IFNα, IFNβ and type II IFN include IFNγ whereas type III IFN include the IFNλ (Honda *et al*., 2005b). The members of type I and type II IFN mainly mediate anti-viral effects while IFNγ functions to prime the adaptive immune response (Singh *et al*., 2003). The biological effects of type I IFN

include the induction of myxovirus resistance gene Mx proteins and the 2'-5' oligoadenylate synthetases (OAS) (Leroy *et al*., 2005). There are about 13 genes encoding for IFNα but only one gene is responsible for encoding IFNβ (Honda *et al*., 2006).

IFNβ is induced via co-ordinated induction of specific transcription factors that lead to transcriptional activation of the IFNβ promoter. These transcription factors are NFκB, IRFs, and AP-1 (Kim *et al*., 2000). The IFNβ enhanceosome region is made up of 4 positive regulatory domains (PRD I-IV) (Nourbakhsh *et al*., 1993). The upstream activators of NFκB include the IKKα, IKKβ and NEMO (Gilmore, 2006). The upstream activators of IRF3 and IRF7 involve TBK1 and IKKε, respectively (Fitzgerald *et al*., 2003a) and the upstream activators of AP-1 involves the MAPKs (Ameyar *et al*., 2003).

Type I IFN can amplify its own production through an autocrine feed-forward mechanism by binding to IFNAR1 and IFNAR2 receptor (Ihle *et al*., 1997). Signalling via the IFN receptor triggers the JAK-STAT pathway to enable further gene transcription (Ihle *et al*., 1997).This is mediated through the phosphorylation of the STATs proteins which is dissociated from the receptors, dimerise and translocate across the nucleus and associates with IRF9 to bind to the ISRE DNA region and activate the transcription of IFN-inducible genes (Schafer *et al*., 1998).

Finally, there is emerging evidence to indicate the importance of type I IFN in modulating immune disease outcomes. Some of these diseases include viral and bacterial infection, autoimmunity and cancer (Hancock *et al*., 2012). For example,

protective effects of type I IFN have been described in infections with Listeria, Mycobacteria, Brucella and Chlamydia (Trinchieri, 2010) as well as many influenza virus infections (Pauli *et al*., 2008). The increase in the levels of Type I IFN was also described in SLE and was found to be pathogenic (Banchereau and Pascual, 2006). In cancer, type I IFN have been demonstrated to be a potential therapeutic application and there were several studies that used type I IFN as treatments for various cancers including chronic myeloid leukemia and B cell lymphoma (Verma and Platanias, 2002).

1.6 Tumor necrosis factor α

TNFα is a proinflammatory cytokine that is secreted by innate cell types such as macrophages and dendritic cells in an infection. It was first identified as a serum factor that was induced upon endotoxin challenge and was found to be able to lead some mouse specific tumors to necrosis in the *in vivo* model. This cytokine has now been demonstrated to play critical roles in chronic inflammation and infection and also to exhibit antitumor activities (Copeland *et al*., 2005). TNFα is expressed as a 26-kDa protein. It typically lacks a classic signal peptide. Newly translated TNFα is normally translated and expressed on the plasma membrane of cells. It can be subsequently cleaved in its extracellular domain by the compound, matrix metalloproteinases into a soluble 17-kDa protein.

TNFα can activate TNFα-mediated signal transduction pathway via binding to two receptors, namely type 1 TNFα receptor (TNFR1) and type 2 TNFα receptor (TNFR2). These two transmembrane receptors have many rich cysteine repeats in the extracellular N-terminal domains and share a high homology. In contrast however, their intracellular domains are relatively distinct and signalling via these two TNF

receptors exhibited overlapping and distinct downstream pathways. The knockout mice of these two receptors have been generated and their phenotypes outcomes are relatively different. For example, when these two TNFR knockout mice were subjected to LPS sepsis model, the mice deficient in TNFR1 are resistant to lethality, whereas mice deficient in TNFR2 remain sensitive (MacEwan, 2002).

1.7 Downstream of kinase

The Dok family of adapters comprises seven family members, Dok1-7 (Cong *et al*., 1999) (Figure I-5). Mutations in c-Abl was known to cause chronic myelogenous leukemia (CML), an aggressive form of lymphoid cancer or commonly known as cancer of the white blood cells (de Klein *et al*., 1982). This disease results from the uncontrolled expansion of myeloid lineage bone marrow cells including the macrophages, neutrophils and basophils. The diagnosis for this disease is usually performed by the detection of the Philadelphia chromosome (Ph) which is a chromosomal abnormality in patients. Ph was originally described in 1960s by Peter Nowell and David Hungerford at the University of Philadelphia (and hence the term Ph). This aberration in the chromosome results from a translocation where the 5' exons of the Bcr (breakpoint cluster) gene on chromosome 22 are fused to the c-Abl tyrosine kinase on chromosome 9 (de Klein *et al*., 1982). The subsequent by-product is a fusion or chimeric protein known as bcr-Abl and exists in either p185(Bcr-Abl) or p210(Bcr-Abl). In comparison to c-Abl which is inactive in its nascent state, Bcr-Abl is constitutively active by tyrosine phosphorylation and in turn continually turns on the cell-cycle related signalling cascade (Hantschel and Superti-Furga, 2004). On the other hand, the detrimental effects of Bcr-Abl also include its ability to suppress DNA repair thus contributing to genomic instability in host cells and chromosomal abnormalities arises as a result. This is evident in a number of studies where infecting

cells with viral-transducing methods and therefore a stable incorporation of Bcr-Abl results in malignant transformation of a variety of host cell lines including fibroblastic cell lines and hematopoietic cell lines (Konopka *et al*., 1984).

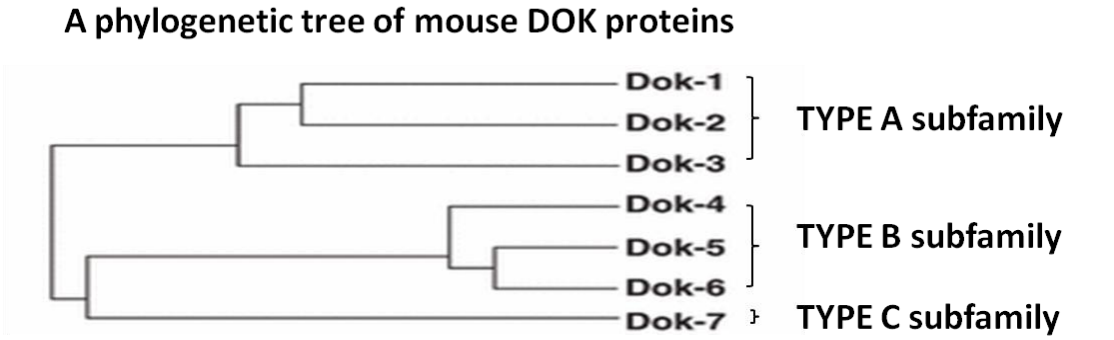

Figure I-5 A phylogenetic tree demonstrating Dok family members.
The phylogenetic tree was calculated using full-length sequences. Dok family members ranging from 1 to 7 were classified accordingly into Type A, B and C subfamily (Favre *et al*., 2003).

Dok1 was first identified as a novel protein with a molecular size of 62 kDa that may be associated with Bcr-Abl or c-Abl. p62 protein is found to be constitutively phosphorylated at the tyrosine residues and can be co-immunoprecipitated with p120 rasGTPase-activating protein (GAP) from hematopoietic cells are isolated from CML patients (Carpino *et al*., 1997). The tyrosine phosphorylation of p62 can also be detected when cell lines were stimulated with various ligands under different conditions including platelet-derived growth factor (PDGF), vascular-endothelial growth factor (VEGF), colony-stimulatory factor-1 (CSF-1) and insulin growth factor (IGF) (Hosomi *et al*., 1994; Sanchez-Margalet *et al*., 1995). When B lymphocytes were cross-linked via BCR or via Fcγ receptors, a tyrosine phosphorylated p62 can also be detected (Yamanashi *et al*., 2000). Taken together, p62 was suggested to be activated by a wide range of stimuli and then undergoes rapid tyrosine

phosphorylation and is a likely protein substrate for many of the tyrosine kinase in signal transduction.

It was not until 1997 that this p62 protein was cloned by 2 different laboratories and identified and named as $p62^{dok}$ or p62 Dok1 (Carpino *et al*., 1997; Yamanashi and Baltimore, 1997). Follow-up experiments revealed that Dok1 was phosphorylated by several tyrosine kinases including Tec, Lyn and SYK via HEK293T cells using overexpression systems (Yoshida *et al*., 2000).

Further data analysis done in NCBI GenBank identified Dok1 to be highly conserved in many animal species, ranging from humans and primates to lower order species like zebra fish. Dok1 mRNA is estimated to be approximately 2kb by northern blot analysis and encodes for 481 amino acid residues (Carpino *et al*., 1997). Human and mouse Dok1 have a high overall sequence homology of approximately 83% (Yamanashi and Baltimore, 1997).

The protein structure of Dok1 comprises an N-terminal Pleckstrin homology (PH) domain, intermediary phospho-tyrosine binding (PTB) domains and a C-terminal SH2 target motif. The PH domain is responsible for membrane localization and the PTB domain is responsible for binding to phospho-tyrosine containing proteins. In addition to these unique protein domains, Dok1 also has as many as fifteen tyrosine residues of which most are located within the C-terminal SH2-target motif domain. It is notable that six of these residues contain the unique pYXXP sequence which is the preferential recognition sites of kinases including Abl and Crk (Songyang *et al*., 1993). There are also many proline-rich motifs in the Dok1 protein structure

comprising of PXXP motifs. This conserved motif is expressed commonly in SH3 domain-containing proteins and hence suggests that Dok1 may function as an adaptor or scaffold protein to mediate protein-protein interaction via this conserved region. Indeed, an example that best describes this property of Dok1 is via an extrapolation of known interacting partners of insulin receptor substrate-1 (IRS-1) which has a protein structure highly homologous to Dok1 (Shi *et al*., 2004). IRS-1 binds to its receptor via its PH and PTB domain and subsequently undergoes tyrosine phosphorylation. Upon activation, its C-terminal region functions as docking sites for SH2-containing proteins. For example, these proteins include PI3K and SHP2 (Myers, Jr. *et al*., 1998; Yenush *et al*., 1998). This suggests Dok1 may function similarly to IRS-1 as an adaptor protein to orchestrate and propagate downstream signal transduction events. Dok1 was also the first of the Dok family proteins to be identified and subsequently more of the Dok family members were uncovered (Figure I-6).

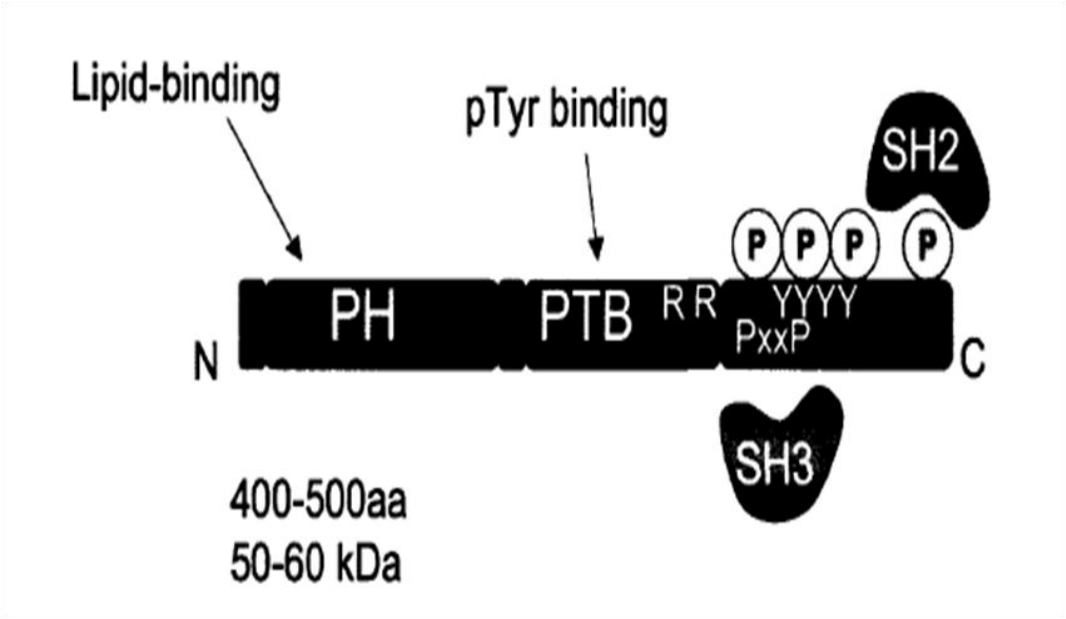

Figure I-6 Domain structure of Dok family protein shown with tyrosine phosphorylation sites indicated.

Y denotes a tyrosine residue in the C-terminal region. Many of these tyrosines act as core residues in their respective SH2-binding motifs. PH, Pleckstrin homology domain; PTB, Phospho/tyrosine Binding, SH3, SRC-homology 3 domain; SH2, SRC-homology 2 domain (Favre *et al*., 2003)

Dok2 was the second member of the Dok-family proteins to be discovered. It was cloned by three laboratories in 1998 (Di Cristofano *et al*., 1998; Jones and Dumont, 1998; Nelms *et al*., 1998). It exists as a 56 kDa RasGAP binding phospho-binding protein isolated from a cell line that expresses $p210^{bcr\text{-}Abl}$. In addition to uncovering Dok2 or $p56^{dok2}$, an analysis of expressed sequence tag (EST) database helped uncover more of the Dok family proteins ranging from Dok1 to Dok6. Dok2 was also separately identified in two different yeast-two-hybrid screenings. Nelms *et al* used the interleukin-4 (IL-4) receptor motif as a bait and pulled down Dok2 which they named IL-4 receptor interacting protein whereas Dumont *et al* used the intracellular domain of the murine Tek receptor as a bait and pulled down Dok2 which they named Dok-related (Dok-R) (Jones and Dumont, 1998; Nelms *et al*., 1998).

Dok2 mRNA is translated into a 412 amino acid Dok2 protein and there is no disparity between the *homo sapiens* and murine mRNA. The protein structure of Dok2 is similar to Dok1 and comprise the general PH and PTB and SH2 target motifs. It has however thirteen possible tyrosine residues of which six of them are of PXXP motifs. These two proteins exhibited an overall 34.8% similarity and expression of these two Dok family proteins is restricted to tissues of hematopoietic origins such as thymus, spleen and the lymph nodes.

Dok1 and Dok2 have been speculated to mediate critical signal transduction events functioning as adaptor proteins. This is based on the fact that they are known to undergo tyrosine phosphorylation upon specific receptors stimulation. Dok1 and Dok2 are highly expressed in lymphoid cells including B and T cells, mast cells and macrophages (Mashima *et al*., 2009). As such, their particular roles in receptors such

as BCR, Fc receptor and TLRs are of immense interest. The generation of Dok1 and Dok2-single deficient mice or Dok1 and Dok2 double-deficient mice provided a model to critically study these proteins functions in a physiological setting.

Single Dok1 and Dok2-knockout (KO) mice were generated in 2000 and 2004 (Niki *et al*., 2004; Yamanashi *et al*., 2000). The single deletion of Dok1 or Dok2 however did not affect the overall development of the organism and these mice were born at a normal Mendelian ratio. As Dok1 is highly expressed in B cells, studies were performed to elucidate its role in BCR signalling. Upon ligation of BCR with anti-IgM, Dok1 was found to be phosphorylated in B cells. The phosphorylation of Dok1 was much higher when B cells were stimulated with whole anti-IgG antibody, which co-ligate both the BCR and FcγRIIB. Interestingly, proliferation of B cells upon BCR ligation proceeded normally in the absence of Dok1 deficiency compared with wildtype controls. Dok1 is however required for dampening ERK activation in BCR-induced signal transduction and Dok1-deficient lymphocytes exhibited hyper-phosphorylation of ERK2 in BCR signalling as compared to its wildtype counterparts (Yamanashi and Baltimore, 1997). This finding suggests a negative regulatory role of DOK1 in BCR signalling.

Dok1 and Dok2 inhibitory roles were also evident in TLR signalling. Dok1 or Dok2 single knockout mice were found to exhibit enhanced susceptibility to LPS induced sepsis (Shinohara *et al*., 2005). Combined deficiency in Dok1 and Dok2 however exacerbated the disease progression of the mice and they succumbed to death in less than a year due to the development of myelomonocytic leukemia (Yasuda *et al*., 2004). Anatomical analysis of these mice revealed that they have splenomegaly and

enlarged kidney. This occurred as a result of excessive expansion and infiltration of granulocytes and lymphocytes into the central periphery blood circulation. The mechanism behind the development of leukemia in Dok1 and Dok2 double-knockout mice was explained by the overly excessive responsiveness of the myeloid cells upon treatment with cytokines such as stem cell factor (SCF). In addition, these cells could not undergo normal apoptosis, further aiding cancer progression (Yasuda *et al*., 2004). As such, Dok1 and Dok2 exhibited a negative regulatory role in overall immune homeostasis and is required to suppress the development of leukemia.

Dok1 and Dok2 were also involved in T cell functions. This is based on a study where Dok1 and Dok2 double-deficient mice were analyzed for their role in thymocytes development (Yasuda *et al*., 2007). Consistent with the negative regulatory roles of Dok1 and Dok2 in other receptor signalling, these proteins also demonstrated inhibitory functions in T cells. When the mice were immunized with antigens and examined for T-dependent (TD) responses, they exhibited a negative regulatory role. For example *in vitro* assays using $CD4^+$ T cells from the Dok1 and Dok2 single knockout mice showed that these cells have increased proliferative response in culture and a higher production of IL-2 cytokine and these effects were synergistically further enhanced in the double knockout (DKO) mice and cells. In the context of TCR-mediated signalling, Dok1 and Dok2 were also demonstrated to be essential players. Dok1 and Dok2 are required to negatively regulate signal transduction pathways downstream of the TCR. In the absence of Dok1 and Dok2, T cells activated by TCR crosslinking displayed increased phosphorylation of T lymphocytes adaptor protein including ZAP70 and LAT as well as MAPKs such as ERK. Mechanistically, Dok1 and Dok2 compete with ZAP70 for binding to the ITAMs of TCR and CD3 in TCR

signalling through their PTB protein domain and so prevented a continual positive signalling in TCR activation. More interestingly, Dok1 and Dok2 double knockout mice developed autoimmune disease over time, characterized by the presence of high levels of autoantibodies to dsDNA and presence of immune complexes deposits in kidneys (Yasuda *et al*., 2007). These research findings suggested that Dok proteins may be attractive targets for therapeutic intervention in autoimmunity.

Several other studies have highlighted the roles of Dok1 and Dok2 in more diversified receptors signalling, indicating such signalling is not limited to only receptors from cells of hematopoietic origins. There is increasing literature covering these areas and some of the conclusions suggest controversy to the current known knowledge of the Dok1 and Dok2 established roles. For example, Dok1 and Dok2 were demonstrated in one study to be important for osteoclasts formation and in its absence, osteopenia formed as a result (Kawamata *et al*., 2011). In a separate study, Dok1 and Dok2 were showed to be differentially regulated in platelets activation in response to thrombin and the authors raised an interesting possibility that Dok2 may play an important role in integrin outside-in signalling through a physical and functional interaction with integrin $\alpha_{IIb}\beta_3$. It is interesting to note that in this context scenario, Dok1 and Dok2 do not play a cooperative role but differential roles instead. Another study also demonstrated that Dok1 and Dok2 play opposing roles. In this study, the authors investigated the role of Dok1 and Dok2 in CD200 receptors which negatively regulate myeloid cells upon binding to its ligand CD200. They showed that when CD200R is activated, Dok2 phosphorylation preceded that of Dok1 and recruited individual specific subsets of signalling proteins downstream of the receptor and exhibited different binding affinities to rasGAP (Mihrshahi and Brown, 2010). They concluded

that Dok1 and Dok2, despite having high homology in protein structure, could display opposing effects in certain receptor systems. The conclusions redefined some of the current paradigms that suggest that the two proteins act in a cooperative manner most of the time.

1.7.1 Downstream of kinase-3

Dok3 was identified by two independent groups in 1999 and 2000 and was named Dok-L (Cong *et al*., 1999; Lemay *et al*., 2000). It is the third member of the Dok family of adaptor proteins. Dok3 expression is restricted to B cells and cells of the myeloid lineage including macrophages and dendritic cells. Both research groups utilised yeast-two-hybrid to fish out Dok3. The first group made use of the knowledge that Dok proteins have preferential binding sites for Abl and hence by using Abl as a bait, Dok3 was pulled down and sequenced (Cong *et al*., 1999). The other group used Csk instead as a bait together with SRC kinase and pulled down a novel adaptor that when sequenced, was found to be Dok3 (Lemay *et al*., 2000). Dok3, like the earlier discovered Dok1 and Dok2, comprises of a common protein domain structure including the amino terminal PH and PTB structures and the C terminal region of SH2-target motif with around four potential tyrosine phosphorylation residue sites. Interestingly Dok1, Dok2 and Dok3 share a high degree of similarity in their PH and PTB domains but not in the carboxyl terminal region. The most notable difference of Dok3 from Dok1 and Dok2 was the absence of the repeated YXXP motifs that enable Dok1 and Dok2 to interact with rasGAP.

Similar to Dok1 and Dok2, Dok3 expression is limited to immune organs. It is expressed most abundantly in lymphoid organs such as the spleen and bone marrow but absent in the thymus (Lemay *et al*., 2000). This suggests that Dok3 may not play a

role in T lymphocytes development and function. In comparative expression studies of the overlapping roles of Dok1, Dok2 and Dok3 using sorted primary cells, Dok1 and Dok3 were discovered to be exclusively expressed during B cell developmental stages and in peripheral circulating B cells whereas Dok1 and Dok2 messenger levels were found to be upregulated in developing thymocytes. Therefore it is currently accepted that Dok1 and Dok2 function mainly in T cells whereas Dok1 and Dok3 are more important for B cells (Yasuda *et al*., 2007).

In yeast-two hybrid studies performed by Cong *et al*, it was found that Dok1 and Dok3 interact with Abl via their PTB domains and this interaction is dependent on Abl kinase activity (Cong *et al*., 1999). Upon cross-linking of the BCR, Dok3 readily undergoes tyrosine phosphorylation and interacts with SHIP1 and Csk (Lemay *et al*., 2000). Biochemical assays to decipher the effect of Dok3 in B cell activation were performed by overexpressing Dok3 in A20 B cell line and it was shown that Dok3 played a negative role as BCR-induced IL-2 production was inhibited when there was increased levels of Dok3 in A20 (Robson *et al*., 2004). Site-directed mutagenesis studies were performed on Dok3 to investigate if tyrosine phosphorylation is required for interaction with its known partner, SHIP1. Indeed, when the tyrosine residues of Dok3 were mutated to phenylalanine, Dok3 binding to SHIP1 was abrogated (Robson *et al*., 2004). In the same study, Dok3 directly inhibited JNK activation via SHIP1 however the other known substrates of SHIP1 such as AKT activation appeared unaffected. Therefore this suggests other novel negative signaling pathways in BCR involving Dok3/SHIP1 (Robson *et al*., 2004).

Dok3 was also demonstrated to interact with Grb2 (Honma *et al*., 2006). This novel interacting partner was sequenced from immunoprecipitated lysates that were co-expressed with Dok3 and SRC in HEK293T cells. Further biochemical studies including site-directed mutagenesis identified the tyrosine phosphorylation sites of Dok3 responsible for binding to Grb2. The tyrosine 398 and tyrosine 432 sites on Dok3 has to be phosphorylated by SRC to allow Dok3 binding to the SH2 domain of Grb2. The authors envisaged that as Grb2 can form a stable complex with son-of-sevenless (SOS), therefore Dok3 may be required to sequester the Grb2-SOS complex from Shc to inhibit downstream RAS-ERK pathway (Honma *et al*., 2006).

Grb2 was previously identified to be a negative regulator of BCR-induced $Ca2^{+}$ flux. $Ca2^{+}$ signalling was found to be enhanced in Grb2-deficient DT40 cells upon BCR crosslinking (Stork *et al*., 2007). The authors hypothesized that since Dok3 binds to Grb2, they may function in the same regulatory manner. Indeed, when Dok3-deficient DT40 cells were generated and analyzed for $Ca2^{+}$ activation upon BCR crosslinking, they were found to be enhanced as compared with wildtype DT40 cells treated similarly (Stork *et al*., 2007). This phenotype was consistent with that of Grb2-deficient DT40 cells. Furthermore the mechanistic role of Dok3 in $Ca2^{+}$ signaling has been investigated in detail. When tyrosine 331 residue was mutated in Dok3, it abolished binding to Grb2 which led to a defective $Ca2^{+}$ flux. Also, this amino acid residue is indispensible for Dok3 interaction to SHIP1 (Stork *et al*., 2007). As SHIP1 was already known to be a negative regulator of BCR-induced $Ca2^{+}$ flux, it was tempting to assume that Dok3 exerts its negative effects of BCR-induced $Ca2^{+}$ flux via SHIP1 and Grb2. However subsequent experiments revealed that in Dok3-deficient DT40 cells, SHIP1 phosphorylation was unaffected by BCR ligation but PLCγ2 activation was significantly enhanced in these cells. The authors therefore

concluded that Dok3 and SHIP1 interaction and the signalling event which leads to $Ca2^{+}$ flux upon BCR ligation were mutually exclusive in DT40 cells. Dok3 together with Grb2 negatively regulated PLCγ2-mediated $Ca2^{+}$ flux in BCR signalling in DT40 cells (Stork *et al*., 2007).

Finally, Dok3-deficient mice were generated in our laboratory and upon analysis with respect to B cell development and signalling via BCR, we found that Dok3 is non-redundant in B cell receptor signalling, as the phenotype of $Dok3^{-/-}$ mice is distinguishable from $Dok1^{-/-}$ mice. The data from the analysis of $Dok3^{-/-}$ mice in B cell development and activation showed that although the mice exhibited normal B cell development, normal T-dependent immune response and germinal centre formation had enhanced basal serum IgM and were hyper-responsive toward T-independent Type I and type II antigen. In the *in vitro* studies of Dok3 in BCR signalling, the mutant B cells were hyperproliferative and induced a higher level of $Ca2^{+}$ flux compared to wildtype controls upon BCR crosslinking. The major signal transduction pathways were examined and Dok3 was shown to inhibit the activation of NFκB and MAPK including JNK and p38 as well as the activation of SHIP1 but not its translocation to lipid raft (Ng *et al*., 2007). In conclusion Dok3 played a negative regulatory role in some aspects in BCR signalling and suggested that there could be compensatory functions mediated by Dok1. This hypothesis may be tested by the analysis of BCR induced activation and the function of B lymphocytes in $Dok1^{-/-}$ $Dok3^{-/-}$ (DKO) mice.

1.7.2 Tyrosine kinases that can potentially phosphorylate Dok3

Tyrosine kinases are catalytic enzymes that are able to transfer a phosphate group from ATP to a protein in the cell. The accepting protein is defined as the substrate. The mode of action allows the tyrosine kinase to function perpetually as a molecular "switch" that can turn on or off many cellular functions as a result of the signal transduction events that follow when the enzyme is active (Hanks *et al*., 1988).

When tyrosine kinase functions abnormally such as in a mutated form where it is constitutively switched "on", it is possible to result in cancerous events where cells are dividing in an unrestricted and proliferative manner owing to disregulated signal transduction pathways responsible for cell division (Gunby *et al*., 2007). Parts of effective cancer treatments that are currently under clinical trials include those of tyrosine kinase inhibitors. An example of one such drug is PCI-32765 which is a BTK inhibitor for the treatment of B cell lymphoma (de Rooij *et al*., 2012). Other kinase inhibitors that are already approved drug therapies in the pharmaceutical market include Imatinib for the treatment of CML (Kantarjian *et al*., 2011)

1.7.2.1 c-Abelson tyrosine kinase

While Abelson tyrosine kinase (c-Abl; Abl1) is a member of the tyrosine kinase family, the Abl family of non-receptor tyrosine kinase beside c-Abl, also includes its paralogue, the Abl-related gene (Abl2) (Hanks *et al*., 1988). The structure of c-Abl comprises a tyrosine kinase domain, a SH2 and a SH3 domain as well as a 'CAP' region present in the amino terminus of the protein (Superti-Furga and Courtneidge, 1995). c-Abl is activated similarly to SRC-family kinases in that it is kept in an inactive state by autophosphorylation on a tyrosine kinase residue that restricts its

access to its substrate (Superti-Furga and Courtneidge, 1995). c-Abl is activated when its interacting protein partners bind to its regulatory domains which release c-Abl from the inhibitory conformation. A free c-Abl is then able to carry out its enzymatic activity and begins to add phosphate groups to its signalling substrate (Smith and Van Etten, 2001). c-Abl functions have to be tightly regulated as it is implicated in many cellular processes including cell growth and differentiation, oxidative stress and DNA-damage, actin filaments reorganization and cell migration (Hernandez *et al*., 2004). One chromosomal aberration that is well characterised in disregulation of c-Abl function is bcr-Abl (Ren, 2005). This is formed by a chromosomal translocation between two chromosomes. For example, the fusion of c-Abl with the Bcr gene leads to the formation of the BCr-Able oncogene.

Bcr-Abl is devastating in two ways. Firstly, Bcr-Abl results in a new protein folding structure and owing to some perturbations, the regulatory domain of the original c-Abl protein does not function any more to keep itself in an inhibited state and the Bcr-Abl protein becomes uncontrollable (Ren, 2005). Secondly, because of the fact that four of the Bcr-Abl chains are now formed in close proximity, it became effortless for the kinase to carry out catalytic activity on its substrates. Bcr-Abl perpetually self-activates other Bcr-Abl proteins and this result in hyperactivity and constitutive positive signalling. Unregulated growth of blood cells happens as a result and leads to CML (Nowell, 2007). Fortunately it was the intensive research that was carried out on drug screening that Imatinib was discovered to be able to block the kinase site of Bcr-Abl and thus dampening the response that was otherwise on-going in CML patients (Sawyers, 2003).

1.7.2.2 Bruton's tyrosine kinase

Bruton's tyrosine kinase (BTK) is a member of the Tec family of kinases that plays a critical role in B cell maturation and activation, activation via Toll-like receptors in macrophages and dendritic cells as well as mast cell activation through the high-affinity immunoglobulin E receptor (IgE) receptor (Mohamed *et al*., 1999). Mutations in the *BTK* gene resulted in the development of primary immunodeficiencies X-linked agammaglobulinemia (XLA) in humans (Lee *et al*., 2010) and X-linked immunodeficiency (Xid) in mice (Lindvall *et al*., 2004). Human XLA patients have normal pre-B cell populations in their bone marrow but severely compromised mature B cell numbers in secondary lymphoid organs and circulating blood. They are incapable of producing antibodies and are consequently presented with recurrent bacterial and enteroviral infections, as over 800 such cases have been reported (Fruman *et al*., 2000). But the effects of many of these on the normal physiology of relevant immune cells and the specific contribution to the XLA or Xid phenotype are still unclear. This is in part due to the hitherto limited knowledge of which signalling molecules interact with BTK when different immune receptors are engaged, and also for assembling what is called the BTK interactome or signalosome, although several signalling partners such as spleen tyrosine kinase (SYK), the adapter protein B-cell linker protein (BLNK) and phospholipase Cγ2 (PLCγ2) are well established (Satterthwaite and Witte, 2000). Besides its extensively characterised role in B cells, BTK has been shown to be involved in Fcε-receptor I-mediated mast cell degranulation and cytokine production (Hata *et al*., 1998). However, the role of BTK in lymphomagenesis is more controversial as different studies described the presence or absence of inactivating mutations of BTK in lymphoid tumours. The most compelling evidence of such a role for BTK comes from mice with inactivated BLNK

in which combining this null mutation with BTK deficiency potentiates tumor development (Kersseboom *et al*., 2006). Taken together, it is therefore not only of immense biological but also clinical interest to identify the signalling molecules which constitute the BTK interactome in different immune cell types activated via different receptors. It was demonstrated that Dok proteins could interact with BTK and Grb2 in BCR signalling and hence make BTK a likely tyrosine kinase candidate that possibly phosphorylates Dok family proteins in certain receptor signalling systems (Stork *et al*., 2007). Structurally, BTK contains multiple protein-protein interaction domains. It has a N-terminal Pleckstrin homology (PH) domain for membrane localization following its activation, and also a Tec homology (TH) domain as well as SRC-homology 2 (SH2), and a C-terminal SH3 domain binding proline-rich sequences and a SH1 kinase domain. In addition, BTK possesses multiple tyrosine phosphorylation sites (Lindvall *et al*., 2005).

1.7.2.3 Lyn

Among the tyrosine kinase proteins, Lyn is one in the SRC-family kinase. The function of Lyn has been a subject of controversy in the B cell immunology for a long time. It is generally accepted that Lyn is the tyrosine kinase that first initiates BCR signalling by phosphorylating the Igα and Igβ ITAM motifs upon activation. Interestingly, Lyn is kept in an inhibitory state by autophosphorylation in tyrosine 507 residue and phosphorylation of tyrosine 416 residue instead of relieving its inhibition and allowing activation to occur where it can carry out its intrinsic enzymatic activity (Hibbs and Dunn, 1997). The majority who have studied Lyn accept its identity as a positive regulator. However, the studies with Lyn-deficient mice have challenged this paradigm. $Lyn^{-/-}$ mice, although born viable without gross morphological defects and

in the normal Mendelian ratio, however developed autoimmunity at a later age. This strongly suggests a negative regulatory role for Lyn, as this phenotype closely mimics known negative regulatory proteins (including SHP1, SHP2 and SHIP1) in knockout mice. (Nishizumi *et al*., 1998).

In these mutant mice, the B lymphocytes exhibited increased $Ca2^{+}$ flux and proliferative responses upon BCR ligation as well as enhanced levels of autoantibodies as compared to its wildtype counterparts (Tsantikos *et al*., 2010). It is however notable that Lyn-deficiency does not affect B cell developmental stages in the bone marrow suggesting that other SRC-family kinase (SFK) may compensate for Lyn absence in this specific developmental regulatory role of B lymphocytes (Meade *et al*., 2002). One other possibility is Lyn's redundancy in this aspect of B cell maturation as studies have showed that SYK (a critical kinase downstream of BCR signalling) is known to also phosphorylate the Igα and Igβ of the BCR (Cornall *et al*., 2000). These findings therefore indicate a multi-faceted role for Lyn and suggest that although Lyn's positive regulatory function may be redundant and could be replaced by other SFKs, Lyn's negative regulatory function is nonetheless indisputable and indispensable for some of its downstream signal transduction effects of BCR leading to cellular functions of B lymphocytes. Lyn was demonstrated to phosphorylate a negative co-receptor CD22 on the cell surface, which in turn can recruit SHP1. SHP1 belongs to the family of tyrosine phosphatase, which include SHP-2, and SHP-1 functions to inhibit on-going signalling events in BCR activation by dephosphorylating the positive effector proteins. These effector proteins include SYK, PLCγ and BLNK (Kurosaki, 2002). More recently, it was demonstrated that Lyn could phosphorylate even some known negative regulators of BCR signalling. One of

these proteins is the Dok family proteins. When Dok family proteins are phosphorylated by Lyn, they in turn interact with the SH2 domain of SHIP1 and rasGAP to exert its inhibitory effects including the activation of ERK MAPK (Yamanashi *et al*., 2000).

1.8 Aim of research

Most investigations on TLRs have focused on cells of the innate system. These include professional antigen-presenting cells (APCs) such as dendritic cells and macrophages. Our previous study demonstrated that Dok3 played a critical role in BCR signalling in B cells by functioning as a negative regulator (Ng *et al*., 2007). However, Dok3 is also highly expressed in macrophages (Mashima *et al*., 2009) and it is not known if Dok3 has a role in TLR signalling. Since TLR signalling is complex, it may be possible that more molecules could be involved in their signal transduction pathways. We therefore hypothesise that Dok3 might play a novel role in TLR signalling in macrophages. This hypothesis is further supported by findings that Dok1 and Dok2 have been demonstrated as negative regulators in LPS signalling. Thus Dok3 may also participate in some as yet undefined way in TLR signalling in macrophages.

The main aim of the research is to determine if Dok3 is involved in TLR signalling. This was accomplished by dissecting the signal transduction pathways of Dok3 in TLR3-mediated activation pathways leading to the production of inflammatory cytokine and other cellular aspects of host innate immune response.

The results will hopefully provide new insights into the complexities of TLR signalling and identify Dok3 as a central molecular target for therapeutic intervention against microbial and viral infection, sepsis and autoimmunity.

CHAPTER II. MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents, Buffers and Cell culture media

Gene	Forward Primers 5'-3'	Reverse Primers 5'-3'
Actin	AGATGACCCAGATCATGTTTGAGA	CACAGCCTGGATGGCTACGTA
iNOS	AACGGAGAACGTTGGATTTG	CAGCACAAGGGGTTTTCTTC
TNF	TCCCAGGTTCTCTTCAAGGGA	GGTGAGGAGCACGTAGTCGG
IFNβ	CAGCTCCAAGAAAGGACGAAC	GGCAGTGTAACTCTTCTGCAT
RANTES	GCTGCTTTGCCTACCTCTCC	TCGAGTGACAAACACGACTGC

Table II.1 Sequences of primers used in RT-PCR.

Gene	Primer Sequence 5'-3'	Vectors
DOK3F	CGGGATCCATGGAGTCTGTGGAGCC	PXJ40
DOK3R	CCCAAGCTTTCAGGGCCGGTCAC	PXJ40
D1F	ATAGGATCCGAGTCTGTGGAGCCCCCG	PXJ40
D1R	ATAAAGCTTTCACGGGAAGGCCAGCTGACA	PXJ40
D2F	ATAGGATCCGAGTCTGTGGAGCCCCCG	PXJ40
D2R	ATAAAGCTTTCACCGCTGGCGGGCAATGGC	PXJ40
D3F	ATAGGATCCGCCACCGAGCGTCTTCCAGAGCTG	PXJ40
D3R	ATAAAGCTTTCACCGCTGGCGGGCAATGGC	PXJ40

D4F	ATAGGATCCGCCACCATGGGTACCGGAGAATGTTCG	PXJ40
D4R	ATAAAGCTTTCATCAGGGCCGGTCACAGGG	PXJ40
D5F	ATAGGATCCGCCACCATGGAGTCTGTGGAGCCC	PXJ40
D5R	ATAGGATCCGCCACCATGGGTACCGGAGAATGTTCG	PXJ40
TBK1F	ATAGGATCCCAGAGCACCTCCAACCAT	PXJ40
TBK1R	ATACCCGGGCTAAAGACAGTCCACATTGCG	PXJ40
TRAF3F	ATAGGATCCGAGTCAAGCAAAAAGATG	PXJ40
TRAF3R	ATACCCGGGTCAGGGGTCAGGCAGATCCGA	PXJ40
Luc-IFNbF	ATACCCGGGGGCTTTTCAGTGGACACT	pGL3-Basic
Luc-IFNbR	ATAAAGCTTGCAGTGAGAATGATCTTC	pGL3-Basic

Table II.2 Gene-specific primers and plasmid constructs used in this thesis.

Cytokine	**Company**	**Application**
IL6	BD Pharmingen	ELISA
IL12p40	BD Pharmingen	ELISA
TNFα	eBioscience	ELISA

Table II.3 Recombinant cytokines used for ELISA.

Cell Culture Media	**Component**	**Concentration**
General Culture	DMEM (4500 mg/ml glucose)	NA
	Fetal calf serum	10 %
	L-glutamine	2 mM
	penicillin/streptomycin	100 units/m
	non-essential amino acids	100 mM
	sodium pyruvate	1 mM

	β-mercaptoethanol	5 mM
BMMϕ Culture	M-CSF L929 supernatant	30 %
	Fetal calf serum	20 %
	L-glutamine	2 mM
	penicillin/streptomycin	100 units/m
	non-essential amino acids	100 mM
	sodium pyruvate	1 mM
	β-mercaptoethanol	5 mM

Table II.4 Component specification for cell culture media used in this thesis.

All solutions are filter-sterilized and stored at 4°C.

Cell Culture Buffers	**Component**	**Concentration**
Macrophage Detach Buffer	Glucose	10 mM
	EDTA, pH 8.0	3 mM
	PBS	NA
Red Blood Cell Lysis Buffer	NH_4Cl	0.15 M
	$KHCO_3$	1 mM
	EDTA, pH 8.0	0.1 mM
	H_2O	NA

Table II.5 Component specification for cell culture buffers used in this thesis.

All solutions are filter-sterilized and stored at 4°C.

Reagents	**Component**	**Concentration**
10 X DNA loading dye	Bromophenol blue	0.1 %
	Xylene cyanol	0.1 %

	Glycerol	40%
5X Protein Loading dye	Tris-HCl, pH 6.8	0.25M
	SDS	15%
	Glycerol	50%
	β-mercaptoethanol	25%
	Bromophenol blue	0.01%
Phospho-lysis buffer	Tris-Cl, pH 8.0	10 mM
	EDTA, pH 8.0	1 mM
	NaCl	150 mM
	NP-40	1 %
Triton-X buffer	Tris-Cl, pH 7.5	15 mM
	NaCl	150 mM
	EDTA	5 mM
	Triton-X	0.1 %

Table II.6 Reagents required for gel electrophoresis.

All reagents are stored at room temperature.

Name	Company	Species	Working dilution
A20	Santa Cruz	Goat	1:1000
ABIN1	Cell Signalling	Rabbit	1:1000
AKT	Cell Signalling	Rabbit	1:1000
BTK	Santa Cruz	Goat	1:1000
COX2	Cayman chemical	Mouse	1:1000
Dok3	Santa Cruz	Goat	1:500
Dok3	Santa Cruz	Rabbit	1:1000

ERK2	Santa Cruz	Rabbit	1:1000
FLAG	Sigma	Rabbit	1:1000
HA	Sigma	Mouse	1:1000
HDAC-1	Santa Cruz	Rabbit	1:1000
IκBα	Santa Cruz	Rabbit	1:1000
IRF3	Santa Cruz	Rabbit	1:1000
JNK1	Santa Cruz	Rabbit	1:1000
p105	Santa Cruz	Goat	1:1000
p38	Santa Cruz	Rabbit	1:1000
Phospho-AKT (Thr308)	Cell Signalling	Rabbit	1:1000
Phospho-AKT (S473)	Cell Signalling	Mouse	1:1000
Phospho-ERK	Santa Cruz	Mouse	1:1000
Phosphotyrosine HRP-conjugated (4G10)	Upstate	Mouse	1:5000
Phospho-IRF3 (S396)	Cell Signalling	Rabbit	1:1000
Phospho-SAPK/JNK (Thr183/Tyr 185)	Cell Signalling	Rabbit	1:1000
Phospho-p38	Cell Signalling	Rabbit	1:1000
Phospho-TBK1 (S172)	BD Pharmingen	Mouse	1:1000
TBK1	Cell Signalling	Rabbit	1:1000
TNFα	Santa Cruz	Goat	1:1000
TPL-2	Santa Cruz	Goat	1:1000
TRAF3	Cell Signalling	Rabbit	1:1000
TRIF	IMGENEX	Rabbit	1:1000

Table II.7 List of antibodies used in this thesis.

Ligand	Type	Company
LPS	*E. coli* O111:B4	Sigma
poly(I:C)	Synthetic (HMW)	InvivoGen
Poly(I:C)/LyoVec™	Synthetic (HMW)	InvivoGen

Table II.8 Ligands used to stimulate cells.

Cell lines	Company
Raw264.7	ATCC
HEK 293T	ATCC
L929	Sigma

Table II.9 List of cell lines used in this thesis

2.1.2 Viruses

Influenza A virus, IVR-116 (A/New Caledonia/20/99 (H1N1) x IVR-6 (H3N2), NIBSC code 06/108) was obtained from the National Institute for Biological Standards and Control, United Kingdom.

2.1.3 Mouse Strains

Wildtype C57BL/6 and Dok3$^{-/-}$ mice were obtained from Biological Resources Centre (BRC) (A-STAR). TLR3$^{-/-}$ and TRIF$^{-/-}$ mice were bred and housed in the BRC facilities. Experiments with mice were performed according to guidelines issued by the National Advisory Committee on Laboratory Animal Research (NACLAR). TLR3$^{-/-}$ and TRIF$^{-/-}$ mice were kindly provided by Dr. Shizuo Akira (Osaka University, Japan).

2.2 Methods

2.2.1 Characterisation of the effect of Dok3 deficiency on antiviral response *in vivo*

For the measurement of poly(I:C)/LPS-induced septic shock, wildtype and Dok3$^{-/-}$ mice (6-8 weeks old) were injected intraperitoneally with 12.5 μg of poly(I:C) or 10 ng LPS with 20 mg of D-GalN (Sigma). The dose of poly(I:C) and LPS was calculated per 20 g of body mass. Survival was monitored for more than 32 hours.

2.2.2 Characterisation of Dok3 deficiency *in vitro*

2.2.2.1 Bone marrow-derived macrophage culture

Bone marrow cells were obtained by injecting culture media into the femur and tibia. All cells were spun down by centrifuging at 1000 rpm for 5 mins at 4 ^{o}C. To eliminate erythrocytes, cells were treated with 1 ml of red blood cells lysis buffer for 5 mins by incubating on ice. The cells were washed in 10 ml culture media and collected by centrifuging at 1000 rpm for 5 mins at 4 ^{o}C. Bone marrow cells were counted using haemocytometer and 10^{6}cells were plated on 10cm culture plate containing 10ml of BMMϕ culture media.

2.2.2.2 TLR ligand Stimulation

After 6 days of culture BMMϕ were harvested using macrophage detach buffer and plated according to the assay performed. BMMϕ were incubated for 24hrs and stimulated with poly(I:C) [50ug/ml] or LPS [10-100ng/ml].

2.2.2.3 Virus infection

Influenza A virus was propagated 3 times in Vero cells (ATCC CCL-81) in OptiPro SFM (Invitrogen, Grand Island, NY, Cat. No. 12309-019) supplemented with 5 μg/ml porcine trypsin (Sigma-Aldrich, St. Louis, MO, Cat. No. T5266, 1500 BAEE unit/mg) and subsequently stored at -80 ^{o}C. The virus was then thawed at room temperature and used to infect WT and Dok3$^{-/-}$ primary macrophage cells in OptiMEM (Invitrogen) at a multiplicity of infection (MOI) of 10 TCID50/cell for 3, 6, 12 or 24 hours. The experiment was performed in triplicate. Total RNA was extracted from virus-infected cells using TRIzol (Invitrogen).

2.2.2.4 Enzyme-Linked Immuno-Sorbent Assay (ELISA)

Prior to incubation with biotinylated detection antibodies (BD Pharmingen) samples were washed in PBS containing 0.05% Tween-20, followed by streptavidin-coupled horseradish peroxidase secondary antibody. Finally BD OptEIA detection reagent (Becton Dickinson) was applied and colorimetric changes were quantified by measuring absorbance at 450 nm with a TECAN Genios multi-plate reader. The concentrations of IL-6, TNFα, IL-12p40 were determined using commercial ELISA kits.

2.2.2.5 Quantitative RT-PCR

Cells were lysed and homogenized by passing through 26G needles in 1ml of Trizol (Invitrogen Cat. No. 15596-026). The homogenized mixture was incubated at room temperature for 5 min to ensure complete dissociation of nucleoprotein complexes. 200 μl of chloroform was then added to the mixture and tubes were vortexed before incubation at room temperature for another 2 min. The tubes were centrifuged at

12,000 rpm at 4°C for 15 min. After centrifugation, the upper aqueous layer was collected in a clean tube and 500 µl isopropyl alcohol was added, mixed and incubated at room temperature for 10 min. The tubes were centrifuged again at 12,000 rpm at 4°C for 10 min. The RNA pellet was washed with 70% ethanol and allowed to air dry before dissolving in RNAase-free water. The first strand cDNA synthesis was done using Superscript^{TM} III First-Strand Synthesis System for RT-PCR (Invitrogen, Cat. No. 18080-051) according to the manufacturer's protocol. Briefly, the following mixture was prepared in sterile PCR tubes for each reaction of 5 µg of RNA: Total RNA was extracted from wildtype and Dok3$^{-/-}$ cells using an RNeasy Mini kit (Qiagen) and cDNA was synthesized with Superscript II reverse transcriptase as per manufacturer's protocol (Invitrogen). Quantitative PCR was performed on Applied Biosystems 7500 real-time PCR system using specific primers and SYBER green (Applied Biosystems).

2.2.2.6 Western blotting

Cells were lysed on ice for 30 mins in a phospho-lysis buffer containing 1% NP40, 10 mM Tris-HCl pH8.0, 150 mM NaCl, 1 mM EDTA, 0.2 mM Na_3VO_4 and a cocktail of protease inhibitors (Roche) and sonicated. Cell homogenates were centrifuged at 13,000 rpm for 15 min at 4^0C and supernatants were recovered for protein quantification by BCA protein assay kit (Pierce). 30 µg of whole cell lysates or 5 µg of nuclear extracts were electrophoresed in 10% SDS-polyacrylamide gels and transferred onto immunoblot polyvinylidene difluoride membranes (Millipore, MA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 hr at room temperature and incubated separately with the various antibodies that recognized the different molecules being studied. Protein

bands were visualized using horseradish peroxidase-coupled secondary antibodies and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Densitometric analyses of protein bands was carried out using Bio-Rad Imaging Densitometer and Multi-Analysis Software (Bio-Rad Laboratories), and pixel intensity (absorbance unit/mm^2) was normalized to the corresponding total protein and expressed as -fold increase in intensity over the control non-stimulated samples.

2.2.2.7 Immunoprecipitation

For immunoprecipitation studies, antibodies were first coupled to Protein A/G Plus-Agarose (SC-2003, Santa Cruz) at 4 °C overnight. The beads were washed twice in lysis buffer and incubated with pre-cleared cell lysates for 1 h at 4 °C. Subsequently, beads were boiled in a loading buffer for 5 min to release the proteins. The released proteins were resolved in SDS-polyacrylamide gels as described in Western blotting.

2.2.2.8 Nuclear translocation Assays

For analysing translocation of proteins to the nucleus, 5ug of nuclear extracts from cells that were stimulated with LPS and Poly(I:C) for an hour were isolated using Nuclear and cytoplasmic Extraction Reagents kit (Pierce Biotechnology, USA) prior to immunoblotting. Tubulin and HDAC1 were used as markers for cytosolic and nuclear fractions separately and respectively.

2.2.3 Mass spectrometric analysis

BMMϕ cells were stimulated with LPS or poly(I:C) and lysate and then subjected to endogenous immunoprecipitation using Dok3 antibody. After the SDS-PAGE proteins were visualised by Coomassie Brilliant Blue staining the band of interest

were excised. Proteins in gel were washed, trypsin digested and MS analysis were performed in a similar manner according to (Senis et al., 2009).

2.2.4 Molecular Cloning

2.2.4.1 Polymerase chain reaction

To clone DNA fragments PCR reactions were carried out using gene specific primers and DNA polymerase (Finnzyme) according manufacturer's protocol. Thermal cycling was performed according to thermal profile below:

Step	Temperature	Time	Cycles
Initial denaturation	94 ^{o}C	1 mins	1
Denaturation	94 ^{o}C	30 s	
Annealing	50 to 60 ^{o}C	30 s	30-35
Extension	72 ^{o}C	30 s	
Final extension	72 ^{o}C	10 mins	1
Cooling	4 ^{o}C	Infinite time	

2.2.4.2 Agarose gel electrophoresis

To cast a 1 % (w/v) agarose gel, 1 g of agarose was added to 100 ml 1 X TAE and microwaved till the agarose melted. Then 0.5 μg/ml ethidium bromide was added to the melted agarose, poured into a gel casting apparatus and allowed to solidify. 10 X DNA loading dye was added to DNA samples to a final 1 X working concentration, loaded into gel submerged in 1 X TAE buffer and electrophoreses at 120 volts for 30 mins. The separated DNA bands were visualized using UV trans-illuminator and photographed for the record.

2.2.4.3 Restriction digestion of DNA

To clone DNA fragments into a mammalian expression vector, both vector and insert DNA were cut with the appropriate restriction enzymes. The restriction digestion was set up as follows:

Components	**Stock concentration**	**1X Reaction**
DNA template	1 μg/μl	1 μl
Reaction Buffer	10X	2 μl
Restriction enzyme(s)	10 u/μl	1μl
BSA	10 mg/ml	0.3
Water	NA	15.6μl
	Total Volume	20 μl

Reaction mix was incubated in 37 ^{o}C water bath for at least 1 hour. The genomic DNA was kept overnight for complete digestion.

2.2.4.4 Ligation of DNA

Ligation of the vector for DNA insertion was carried out as follows:

Components	**Stock concentration**	**1X Reaction**
vector DNA	1 μg/μl	0.2 μl
insert DNA	1 μg/μl	0.8 μl
Reaction buffer	10 X	2 μl
T4 DNA Ligase	400 units/μl	1
Water	NA	16 μl
	Total Volume	20 μl

Reaction mix was incubated at 16 ^{o}C overnight or 37 ^{o}C water bath for at least 1 hour.

2.2.4.5 Transformation of DH5α by heat shock method

DH5α competent cells (50μl) was added into the ligation mixture and incubated on ice for 30 mins. Next, the DNA-DH5α mix was immersed into a 42 ^{o}C water bath for 90 seconds and immediately chilled on ice for a further 1 min. The transformed cells were allowed to recover from heat shock by the addition of 1 ml of antibiotic-free LB broth and incubated in a 37 ^{o}C water bath for at least 30 mins. Finally, the transformants were centrifuged at 3000 rpm, resuspended in 200 μl of antibiotic free LB and plated onto selection agar and incubated overnight at 37 ^{o}C.

2.2.4.6 Bacterial DNA mini-/maxi-prep by alkaline lysis

Antibiotic-resistance transformants were inoculated into 2 ml selection LB broth and grown overnight by shaking at at 37 ^{o}C. 1.5 ml of overnight culture was collected by centrifugation at 14000 rpm in an eppendorf tube. Mini scale plasmid DNA was prepared using Qiagen Plasmid mini-prep Kit according to the manufacturer's protocol. Large scale plasmid DNA was also prepared using Qiagen Plasmid Maxi-prep Kit according to the manufacturer's protocol.

2.2.4.7 DNA sequencing

DNA sequencing was carried out using Bigdye Version 3 according to the manufacturer's protocol. After sequencing PCR cycle reaction mixture was purified using capillary electrophoresis and sequenced using Applied Biosystems 3730xl instrumentation and BigDye terminator chemistry.

2.2.5 Protein interaction studies using overexpression system

2.2.5.1 Transfection of HEK293T and RAW264.7 cell lines

For transient transfection of HEK293T or RAW264.7 cells, Lipofectamine 2000 (Invitrogen) or FuGENE was used and the transfection was done as according to the manufacturer's protocol. Briefly, approximately 2 x 10^6 cells were added onto each well of a 6-well plate 24 hours prior to transfection. Approximately 1 μg of DNA was added into a sterile microcentrifuge tube with 250 μl of OptiMEM. The solution was mixed and then followed by quick spin to collect the material and incubated at room temperature for 5 min. Subsequently 10 μl of Lipofectamine in OptiMEM was added to the mixture followed by gentle mixing before drawing the sample which was then incubated at room temperature for 20 min to allow the formation of the transfection

complex. Culture media was removed from the cells and 1.5 ml of fresh media was added. To each of the transfection mixture, 400 µl of media was added and the mixture was aggregated by gently pipetting up and down before adding to the cells in a drop-wise manner.

2.2.5.2 Confocal microscopy

Cells were washed twice with cold PBS with 1% BSA (PBS/1%BSA) and fixed 20 min on ice with 4% paraformaldehyde in PBS. After permeabilization in 0.2% saponin-0.03M sucrose in PBS/1%BSA at room temperature for 10 min, the cells were washed twice with cold PBS. The cells were blocked with 5% normal goat serum in PBS/1%BSA room temperature for 1 hr before incubation with primary antibodies overnight at 4°C. The slides were washed three times with PBS/1% BSA, incubated for 1 hr at room temperature with Alexa 546 goat anti-mouse and Alexa 488 chicken anti-anti-rabbit to reveal the respective primary antibodies. The slides were washed three times with PBS/1%BSA, mounted and viewed under a confocal laser scanning microscopy (Olympus).

2.2.5.3 Luciferase Assay

HEK293T cells seeded on 48-well plates (10^5cells/well) were transfected with 100ng of luciferase reporter vector controlled by the IFNβ promoter together with a total of 500ng of different expression vectors or control vector as well as 2ng of Renilla-luciferase reporter gene for internal control. Approximately twenty-four hours later cell were harvested and luciferase activity in the total cell lysate was analysed using Luciferase Reporter Assay System kit (Promega).

2.2.6 Statistics

Statistical analysis was performed using an unpaired student *t* test (Prism; Graph Pad Software, San Diego, CA). All *p* values less than 0.05 were considered significant and marked with an asterisk *. Survival curves (Kaplan-Meyer plots) were compared using a log-rank test. Final mortality rates (deaths per total) were compared with Chi-square test.

CHAPTER III. ROLE OF DOK3 IN TLR3 SIGNALLING IN MACROPHAGES

3.1 Introduction

Toll-like receptors (TLR) are innate immune receptors that recognise PAMPs found on microbes and activate signalling pathways that induce the expression of immune and inflammatory genes in the host (Takeda and Akira, 2005). There are altogether 11 TLRs in humans and 13 in mice (Takeda and Akira, 2005). Of these, TLR3 is involved in the anti-viral response by recognizing dsRNA, a replication intermediate of numerous viruses (Vercammen *et al*., 2008). In particular, TLR3 has been implicated in the host response to influenza, dengue, SARS coronavirus and other respiratory viruses (Tsai *et al*., 2009; Vercammen *et al*., 2008). TLR3 engagement by dsRNA or poly(I:C) triggers the secretion of inflammatory cytokines such as IL-6 and TNFα and more importantly, IFNβ (Alexopoulou *et al*., 2001). TLR3 is also involved in inflammatory responses by detecting host-derived nucleic acids (Bernard et al., 2012; Cavassani et al., 2008). TLR3 signals via the adaptor TRIF, and induces the formation of the TRAF3/TBK1 complex that subsequently activates the transcription factor IRF3, which is necessary for interferon gene expression (Hiscott and Lin, 2005). Despite this, much remains unknown in terms of TLR3 signalling. It is postulated that there could be other as yet to be discovered signalling molecules involved in TLR3 signal transduction. A detailed understanding of the TLR3 signal transduction pathways could yield novel molecular targets that are amenable for the development of therapeutic drugs such as those that could enhance the antiviral response or those that could dampen the induction of any potential "cytokine storm" that would be detrimental to the host. Since TLR signalling is complex, we hypothesized that more molecules are therefore involved.

In contrast to other Dok family members, there are no studies investigating the role of Dok3 in TLR3 signalling. Previous studies have revealed negative regulatory roles of Dok1 and Dok2 in TLR4 signalling (Shinohara *et al*., 2005). Using Dok3-knockout mice our laboratory generated, the characterisation of Dok3 indicated that it played an inhibitory role in BCR signalling (Ng *et al*., 2007). DOK3 was shown to be tyrosine phosphorylated upon B-cell antigen receptor engagement (Ng et al., 2007). Because Dok1 and Dok2 were known to to be also tyrosine phosphorylated and regulate TLR in macrophages, we hypothesized that Dok3 may be involved in TLR regulation as well. We hence investigate if Dok3 may play a role in TLR signalling in macrophages. To confirm this we assessed wildtype and Dok3-knockout macrophages abilities to produce inflammatory cytokines in response to poly(I:C) challenges.

We also examined the possible signalling pathways that might be affected by Dok3 deficiency in TLR3 signaling in macrophages in many of our subsequent experiments. Lastly we examined the mechanistic role of Dok3 in TLR3 signal transduction.

3.2 Dok3 is phosphorylated upon poly(I:C) stimulation of macrophages

Tyrosine phosphorylation of Dok family members is known to precede its effector role as adaptor an protein (Lemay *et al*., 2000). To determine if Dok3 is involved in TLR3 signalling in macrophages, we investigated if Dok3 undergoes tyrosine phosphorylation upon poly(I:C) treatment. We treated wildtype macrophages with poly(I:C) that was recognised by TLR3. As shown in Figure III-1, Dok3 undergoes tyrosine phosphorylation upon poly(I:C) stimulation. The tyrosine phosphorylation of Dok3 was not evident in the untreated cells. Next we examined if the TRIF adaptor protein is required for Dok3 tyrosine phosphorylation. This is because TRIF is known

to be the only adaptor protein that is solely used by TLR3 in contrast to the other TLRs which also uses MyD88 (Yamamoto *et al*., 2003). We show here in Figure III-2, that Dok3 phosphorylation was impaired as a result of TRIF deficiency. The data taken together suggest that Dok3 undergoes tyrosine phosphorylation upon TLR3 signalling and may possibly be involved in the signal transduction pathways activated downstream of TRIF.

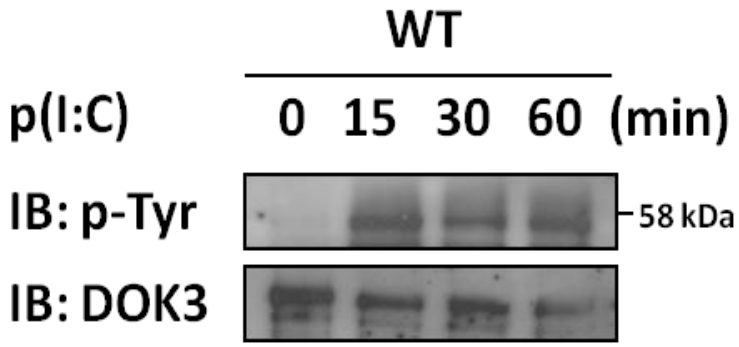

Figure III-1 Dok3 is phosphorylated in macrophages upon poly(I:C) stimulation.
Bone marrow-derived macrophages (BMMϕ) were stimulated for various times as indicated with 50 μg/ml poly(I:C) and subsequently lysed. Dok3 was immunoprecipitated and probed with anti-phosphotyrosine (pY) or anti-Dok3 antibodies. Figure shown is representative of two independent experiments.

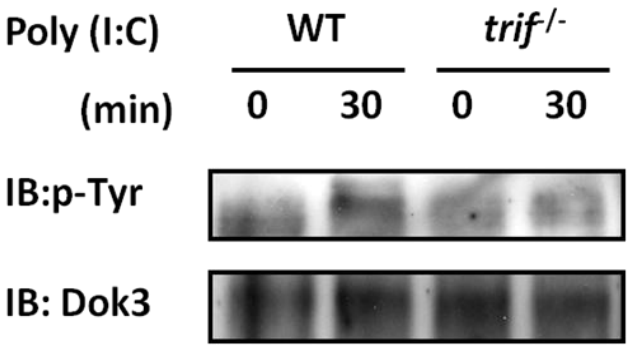

Figure III-2 Dok3 phosphorylation in macrophages upon poly(I:C) stimulation is dependent on TRIF.
Wildtype and TRIF$^{-/-}$ BMMϕ were stimulated with 50 μg of poly(I:C) for 30 mins and subsequently lysed. Lysates were immunoprecipitated with Dok3 antibodies and subsequently immunoblotted probed with anti-phospho-tyrosine (pY) or anti-Dok3 antibodies. Figure shown is representative of two independent experiments.

3.3 Dok3 deficiency affects cellular response to poly(I:C) *in vivo* and *in vitro*

It has been demonstrated that TLR3 is the receptor involved in the inflammatory response of mice challenged with poly(I:C) (Alexopoulou *et al*., 2001). To determine if Dok3 plays a role in mediating the inflammatory effects of poly(I:C)-induced activation of TLR3, we employed a septic shock model by challenging wildtype and Dok3-deficient mice with poly(I:C) together with D-galactosamine (D-GalN). D-GalN sensitises the mice to the lethal effects of poly(I:C) injection via the overproduction of proinflammatory cytokine TNFα in the liver (Dejager and Libert, 2008). Interestingly, as shown in Figure III-3, Dok3-deficient mice were more resistant to poly(I:C)-induced sepsis as opposed to its wildtype control mice that readily succumbed to death after 8 hours from injection. Since this *in vivo* model is a result of excessive TNFα and other inflammatory cytokine production the data suggests that Dok3 unexpectedly mediate a positive regulatory role in TLR3 signalling. This is in contrast to its other family member proteins including Dok1 and Dok2 that played an inhibitory role in LPS signalling (Shinohara *et al*., 2005). As Dok3 mice were more resistant to sepsis in our *in vivo* animal model, it may indicate that the inflammatory cytokines that were produced upon TLR3 signalling were perturbed by Dok3 deficiency.

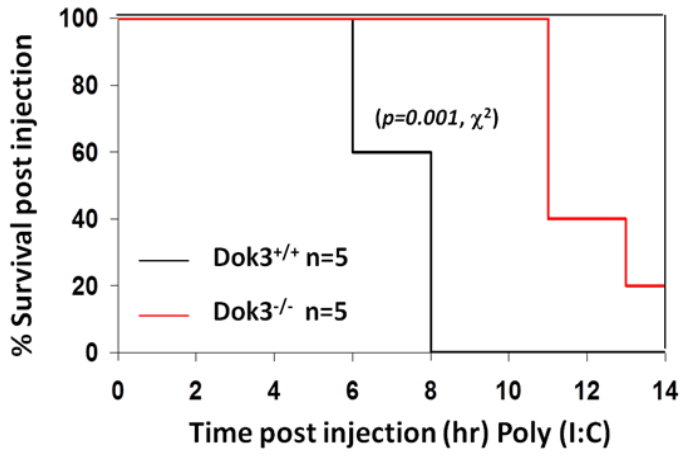

Figure III-3 Dok3-deficient mice are more resistant to poly(I:C)-induced septic shock.
Wildtype (Black line) and Dok3$^{-/-}$ mice (Red line) were injected with 12.5 μg poly(I:C) and 20 mg of D-GalN. The dose of poly(I:C) was calculated per 20 g of body mass. Survival of mice was monitored for over 24h. Statistical significance was established by paired student's t test, *p<0.05.

To test this hypothesis, we sought to measure the proinflammatory cytokines that are known to be produced upon poly(I:C) stimulation of wildtype and Dok3-deficient macrophages. We performed both ELISA and intracellular cytokine staining (ICS) using flow cytometry (data not shown). As shown in Figure III-4, the production of proinflammatory cytokines including IL-6, IL-12 and TNFα in TLR3-stimulated macrophages were significantly reduced in the absence of Dok3. As controls, these analyses were performed alongside with TRIF and TLR3-deficient macrophages and the data indicated that Dok3-deficency exhibited the same phenotype as TRIF and TLR3-deficiency and suggest that Dok3 participates in a linear signal transduction pathway initiating from the activated receptor.

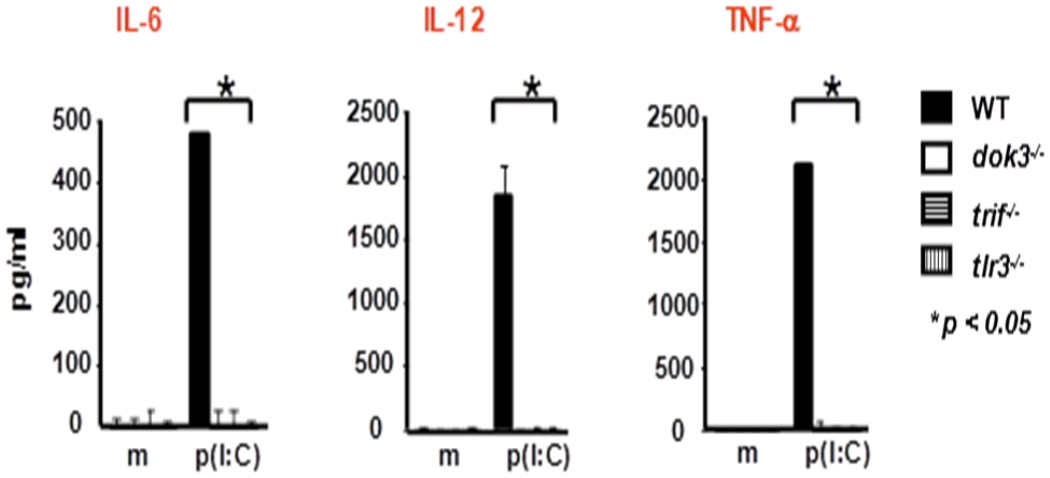

Figure III-4 Dok3 positively regulate cytokine production in poly(I:C)-stimulated macrophages.
Wildtype (black columns) and Dok3$^{-/-}$ (white columns), TLR3$^{-/-}$ (horizontal shaded columns) and TRIF$^{-/-}$ (vertical shaded columns) macrophages were untreated or treated with 50 μg/ml poly(I:C) for 6h and their secretion of IL-6, IL-12p40 and TNFα quantified via ELISA using known standards. Statistical significance was established by paired student's *t* test, *p<0.05. Figures shown are representative of at least 3 independent experiments.

3.4 Dok3 is involved in TLR3-dependent IFNβ gene induction and IFN-dependent gene response

TLR3 is known for its antiviral role in innate immunity. It primarily achieves its anti-viral effects via the production of IFNβ, a type 1 IFN. Engagement of dsRNA by TLR3 in the endosome allows signalling to IFNβ. As such, we asked if Dok3-deficiency would affect TLR3 induced IFNβ production. To this end, we stimulated wildtype and Dok3-deficient macrophages with poly(I:C) and assayed for IFNβ gene induction using quantitative real-time RT-PCR. We showed here in Figure III-5 that while we were able to readily detect IFNβ gene upregulation in wildtype macrophages stimulated with poly(I:C) for 2 hours, the IFNβ gene expression was largely defective in poly(I:C)-stimulated Dok3-deficient macrophages, similar to poly(I:C)-stimulated TRIF-deficient macrophages. The production of IFNβ, a hallmark cytokine of antiviral immune response, is amplified by its autocrine feed-forward loop of binding to its own receptor (Ihle *et al*., 1997). The signal transduction pathways emanating from either the TLR or IFNα/β receptor lead to the activation of the IRF3 transcription factor that in turn induces a specific set of genes expression. These genes are termed the IRF3-dependent gene response. One of the genes induced is RANTES (Regulated on Activation Normal T cell Expressed and Secreted). RANTES is known to be a chemokine important in the recruitment of neutrophils at the site of inflammation in an on-going viral infection (Rojas-Ramos *et al*., 2003). Given the fact that IFNβ gene induction is defective arising from Dok3 deficiency in TLR3 signalling, it is possible that other gene inductions may be abrogated. We then investigated if Dok3 is required for RANTES gene induction following TLR3 stimulation. As shown in Figure III-6, we were able to detect RANTES gene induction in TLR3-stimulated wildtype macrophages but RANTES induction was

defective in TLR3-stimulated Dok3$^{-/-}$ macrophages. These results suggest that Dok3 plays a role in signalling to IFNβ and RANTES production via TLR3.

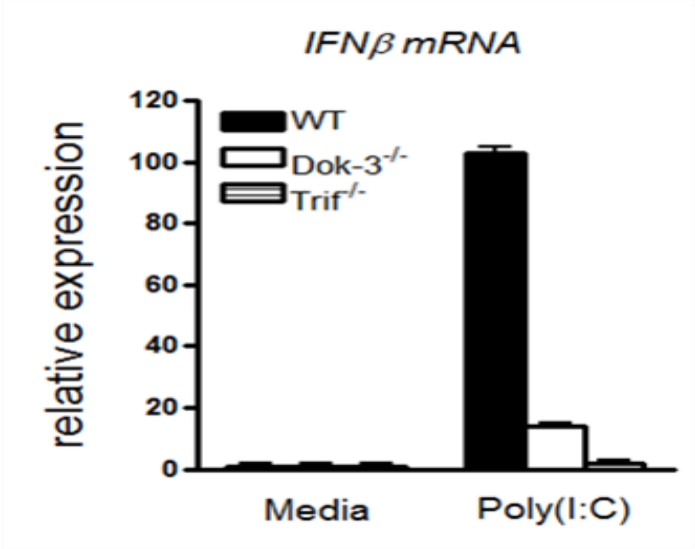

Figure III-5 Dok3 regulates IFNβ gene induction in poly(I:C)-stimulated macrophages.
Real-time qPCR analysis of IFNβ mRNA expression in wildtype (black columns), Dok3$^{-/-}$ (white columns) and TRIF$^{-/-}$ (shaded columns) macrophages stimulated with 50 μg/ml poly(I:C) for 2h. The amount of IFNβ mRNA expression was normalized to that of actin mRNA. Figures shown are representative of at least 3 independent experiments.

3.5 Dok3 is required for TLR3-mediated activation of PI3K but not MAPK and NFκB

Multiple signalling pathways are known to be activated downstream of TLR3 engagement. These include the MAPKs, PI3K and NFκB (Schroder and Bowie, 2005). These signal transduction pathways culminate in the activation of various transcription factors which lead to specific gene expression and translation of required proteins that are critical in co-ordinating the magnitude of host innate immune response. For example, the MAPKs will activate the AP-1 transcription factors that are known for their roles in the production of proinflammatory cytokines (Ameyar *et al.*, 2003). The NFκB signalling pathway on the other hand activates the p65/p50 heterodimer transcription factor that is also known to induce inflammatory

components (such as TNFα and COX2) which acts as a co-factor on the IFN enhanceosome to induce IFNβ.

The PI3K pathway activated downstream of TLR3 was recently defined as a detour signalling pathway downstream of TLR3 that does not involve TRIF but nonetheless also culminates in the activation of TBK1 that is a direct kinase of IRF3 activation (Fitzgerald *et al*., 2003a; Hiscott, 2004). IRF3 is predominantly activated via the TRIF-TBK1 signalling axis to induce IFNβ production (Doyle *et al*., 2002). We have shown earlier that the production of cytokines such as IL-6, IL-12, TNFα and IFNβ was defective due to Dok3 deficiency upon TLR3 stimulation in macrophages. As such, we asked if the major signal transduction pathways activated downstream of TLR3 would be affected in the absence of Dok3. We show here in Figure III-7 that when wildtype macrophages were stimulated with poly(I:C), it readily leads to the phosphorylation of MAPKs including ERK, JNK and p38. However, Dok3 deficiency did not lead to any drastic effect on MAPK activation as the phosphorylation of ERK and p38 were comparable and JNK was only slightly reduced in Dok3-deficient macrophages compared to wildtype control upon poly(I:C) stimulation. We next measured the activation of NFκB in TLR3 signalling by examining IκBα degradation. As shown in Figure III-8, the kinetics of IκBα degradation upon poly(I:C) stimulation were also comparable between wildtype and Dok3-deficient macrophages, suggesting that Dok3 does not play a role in TLR3-mediated NFκB signalling.

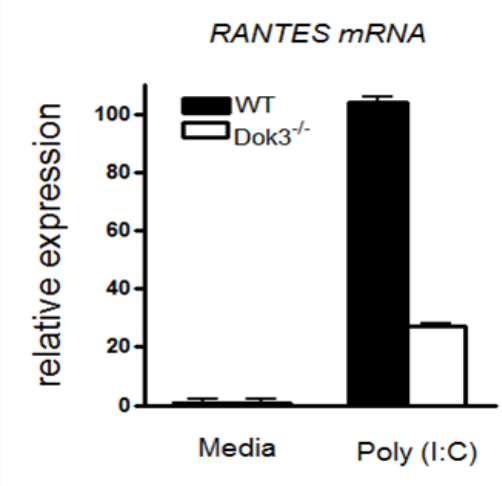

Figure III-6 Dok3 regulates RANTES gene induction in poly(I:C)-stimulated macrophages.
Real-time qPCR analysis of RANTES mRNA expression in wildtype (black columns) and Dok3$^{-/-}$ (white columns) macrophages stimulated with 50 μg/ml poly(I:C) for 2h. The amount of RANTES mRNA expression was normalized to that of actin mRNA. Figures shown are representative of at least 3 independent experiments.

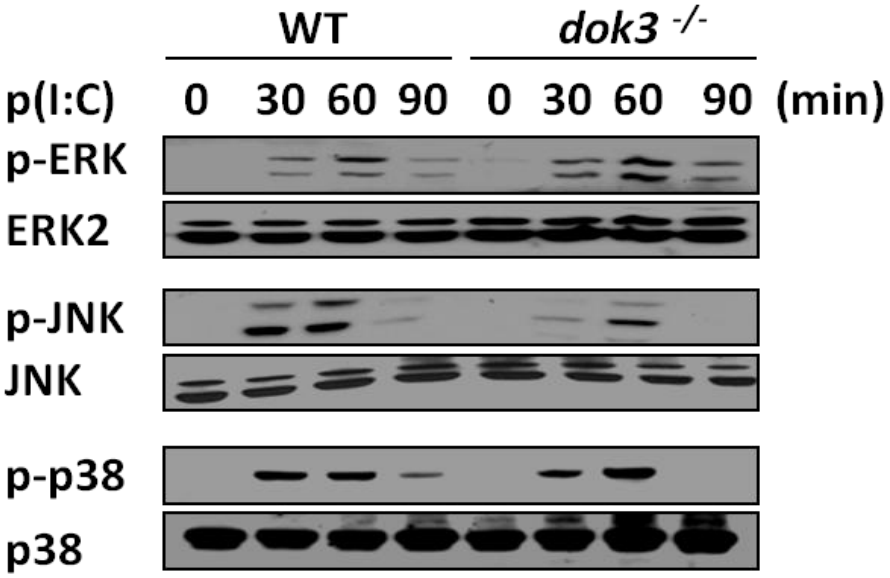

Figure III-7 Normal MAPK activation in poly(I:C)-stimulated Dok3$^{-/-}$ macrophages.
Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 50 μg/ml poly(I:C) for the indicated timepoints. Cells were lysed and analysed by immunoblotting with phospho-antibodies to (A) ERK, (B) JNK and (C) p38. Loading of equal amount of lysates were verified by immunoblotting with antibodies to total ERK, JNK and p38. Data shown are representative of three independent experiments.

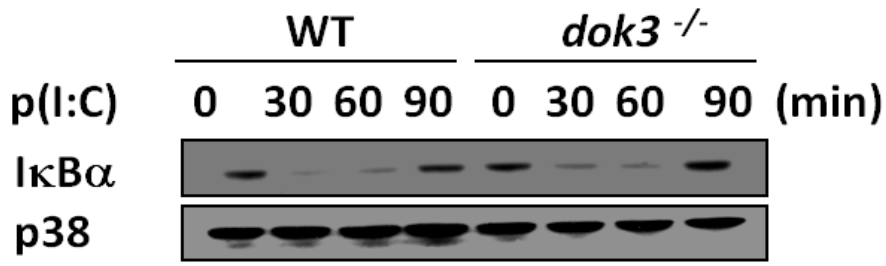

Figure III-8 Normal NFκB activation in poly(I:C)-stimulated Dok3$^{-/-}$ macrophages.

Normal IκBα degradation in poly(I:C)-stimulated Dok3$^{-/-}$ macrophages. Wildtype and Dok3$^{-/-}$ macrophages were simulated for various times with 50 μg/ml poly(I:C) and examined by Western blot analysis for the degradation of IκBα. Anti-p38 blot was included as control for equal loading of cell lysates.

As we have shown that Dok3 is redundant in TLR3- mediated MAPK and NFκB pathway, we next investigated if Dok3 would play a role in PI3K signalling following TLR3 activation. We stimulated wildtype and Dok3-deficient macrophages with poly(I:C) and assayed for the phosphorylation of AKT at two activation residues, threonine 308 and serine 473 residue. The activation of AKT at these two acceptor sites was indicative of substrate activity for PI3K kinase activity following recruitment to TLR3 when the receptor is phosphorylated at tyrosine 759 residues (Sarkar *et al*., 2004). We show here in Figure III-9 that Dok3 deficiency impairs the phosphorylation of AKT and this suggests that Dok3 is required for PI3K signalling to activate IFNβ production in TLR3 signalling.

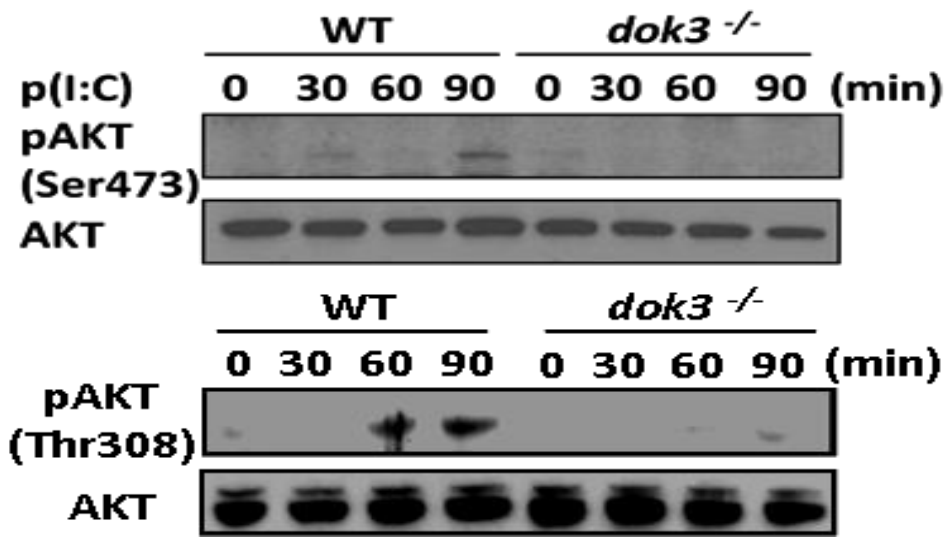

Figure III-9 Impaired AKT pathway in poly(I:C)-stimulated Dok3$^{-/-}$ macrophages.

Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 50 μg/ml poly(I:C) for the indicated timepoints. Cells were lysed and analysed by immunoblotting with phospho-antibodies to AKT (Serine 473) (top panel) and AKT (Threonine 308) (bottom panel). Loading of equal amounts of lysates were verified by immunoblotting with antibodies to total AKT. Data shown are representative of two independent experiments.

3.6 Impaired nuclear translocation of IRF3 transcription factor in LPS and poly(I:C) stimulated Dok3$^{-/-}$ macrophages

The IRF3 transcription factor belongs to a family of transcription factors that are involved in type 1 IFN production in an innate host immune response (Honda and Taniguchi, 2006). The notable anti-viral cytokine IFNβ can be activated by IRF binding to its promoter (Kim *et al*., 2000). These factors include IRF3 and IRF1 (Honda and Taniguchi, 2006) and its activation is dependent on various upstream kinases. In TLR3 and TLR4 signalling, production of IFNβ is mainly triggered via TRIF which signals to TBK1 and subsequently activates IRF3. IRF3 normally exist as a monomer in the cytoplasm of the cells in a quiescent state and dimerises when it is active. This event coupled with phosphorylation of the transcription factor on serine 396 residue allows the translocation of IRF3 to the nucleus to bind genomic DNA (Hiscott and Lin, 2005). Since IFNβ production is defective in TLR3-stimulated

Dok3$^{-/-}$ macrophages we investigated if Dok3 is required for the activation of IRF3 transcription factor. We first performed fractionation studies to divide the protein pool of macrophages from wildtype and Dok3-deficient mice into cytosolic and nuclear fractions after stimulation with LPS and poly(I:C). As shown in Figure III-10, we were not able to detect significant amounts of nuclear IRF3 protein in Dok3-deficient macrophages as compared to wildtype macrophages upon TLR3 and TLR4 stimulation. This suggests that Dok3 deficiency impairs the translocation of IRF3 into the nucleus by TLR3 and TLR4 signalling which uses the TRIF adaptor protein for this signalling event. We further confirmed these findings by applying confocal microscopy to analyse the effect of IRF3 translocation following poly(I:C) stimulation. As depicted in Figure III-11, we detected the co-localization of IRF3 with DAPI, a nuclear marker in wildtype macrophages upon poly(I:C) stimulation. In contrast, the IRF3 was mainly localised in the cytoplasm upon stimulation with poly(I:C) in Dok3$^{-/-}$, TRIF$^{-/-}$ and TLR3$^{-/-}$ macrophages, similar to macrophages that were untreated, suggesting an impairment of IRF3 nuclear translocation as a result of Dok3 deficiency in TLR3 signalling.

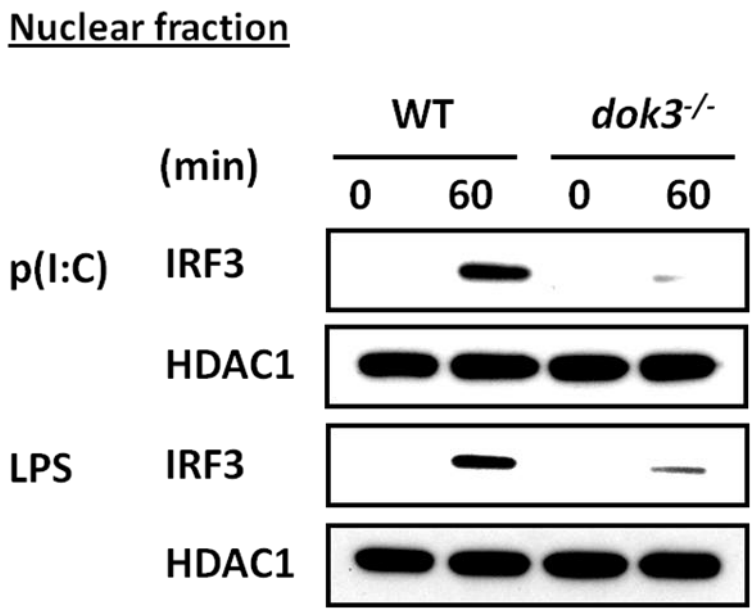

Figure III-10 Impaired nuclear translocation of IRF3 in poly(I:C) and LPS-stimulated Dok3$^{-/-}$ macrophages.

Nuclear extracts were prepared from wildtype and Dok3$^{-/-}$ macrophages stimulated with poly(I:C) (upper panel) and LPS (lower panel) for 1 hr and examined for the presence of IRF3 by western blot analyses. The anti-HDAC1 blot was included as control for equal loading of nuclear extracts. The results shown were representative of 2 independent experiments.

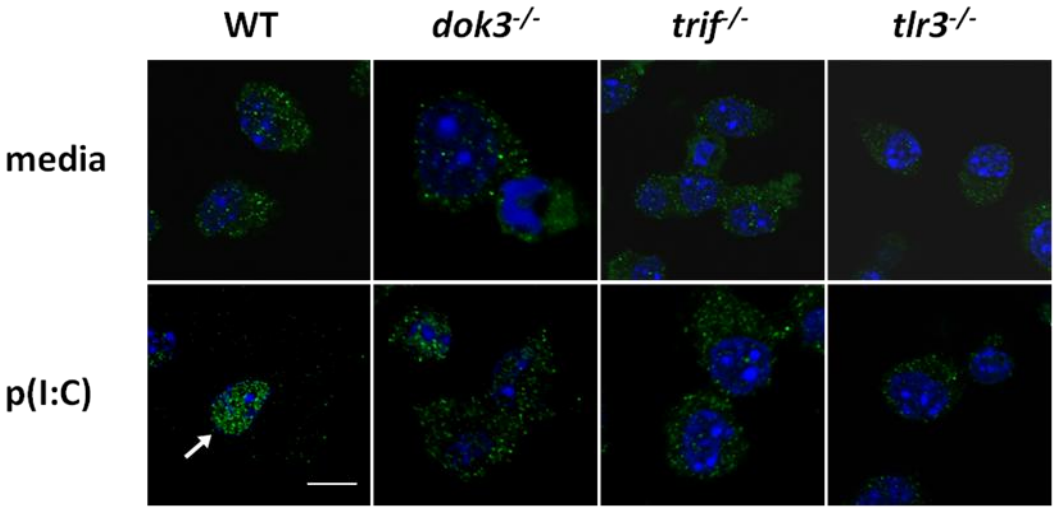

Figure III-11 Confocal images of impaired nuclear translocation of IRF3 in poly(I:C)-stimulated Dok3$^{-/-}$, TRIF$^{-/-}$ and TLR3$^{-/-}$ macrophages.

Macrophages from wildtype, Dok3$^{-/-}$, TRIF$^{-/-}$ and TLR3$^{-/-}$ mice were treated with poly(I:C) for 1 hr and stained with IRF3 antibody and DAPI and imaged with confocal microscope for IRF3 translocation. The results shown were representative of 2 independent experiments.

3.7 Dok3 is critical for TRIF-dependent TBK1 and IRF3 phosphorylation

Another prerequisite that is indicative of IRF3 activity is the phosphorylation of IRF3 at serine 396 residue (Fitzgerald *et al*., 2003a). This event happens in the cytosol (Hiscott and Lin, 2005). The phosphorylation of IRF3 on serine 396 residue however depends on the presence of an active TBK1 as a study previously demonstrated that a defective or kinase-dead TBK1 is unable to phosphorylate IRF3 (McWhirter *et al*., 2004). To determine whether phosphorylation of the TBK1-IRF3 signalling axis via TRIF to induce IFNβ production requires Dok3 in TLR3 and TLR4 signalling, we examined the activation of TBK1 on serine 172 residue as well as activation of IRF3 on serine 396 residue. As shown in Figure III-12, when wildtype macrophages were treated with poly(I:C) and LPS in a time-dependent manner, it led to the robust phosphorylation of IRF3 on serine 396 residue. In contrast, we did not detect IRF3 phosphorylation in Dok3-deficient macrophages treated in the same manner. We then investigated if the phosphorylation of TBK1 is affected downstream of TLR3 by the lack of Dok3. To this end, we immunoprecipitated TBK1 from TLR3-stimulated wildtype and Dok3$^{-/-}$ macrophages and examined via Western Blotting, its phosphorylation at the serine 172 residue. We show here in

Figure **III-13**, that although TBK1 activation at Serine 172 was detectable in TLR3-stimulated wildtype macrophages, they were however impaired by Dok3 deficiency. Altogether, the current data indicates that Dok3 plays a non-redundant role in TLR3 signalling to induce IFNβ production. The presence of Dok3 is also required for the activation of TBK1 which is necessary for IRF3 activity, including phosphorylation on its Serine 396 residue and subsequent nuclear translocation from the cytoplasm of the cell.

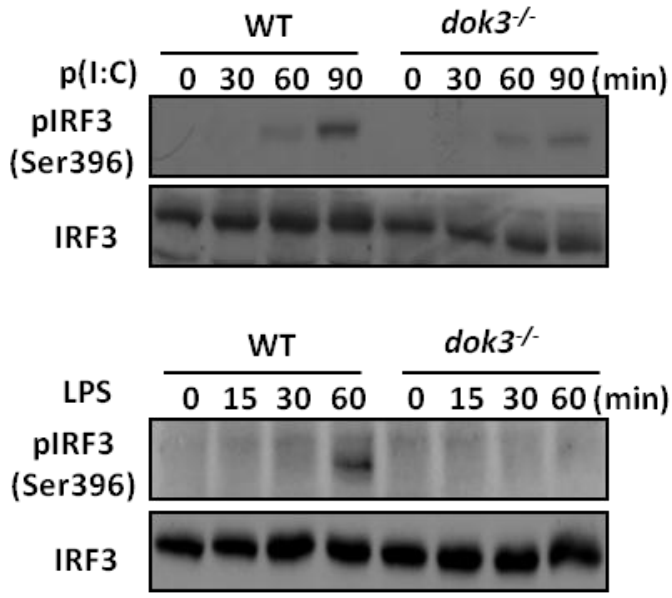

Figure III-12 Impaired IRF3 phosphorylation in poly(I:C)-stimulated Dok3$^{-/-}$ macrophages.
Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 50 μg/ml poly(I:C) (upper panel) and 100 ng/ml LPS (lower panel) for the indicated timepoints. Cells were lysed and analysed by immunoblotting with phospho-antibodies to IRF3 (Serine 396). Loading of equal amounts of lysates was verified by immunoblotting with antibodies to total IRF3. Data shown are representative of 2 independent experiments.

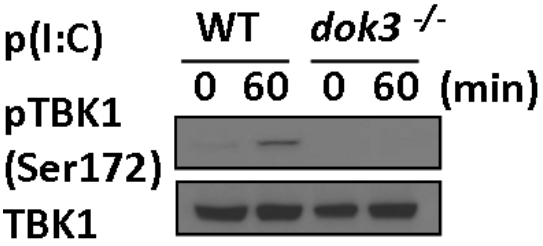

Figure III-13 Impaired TBK1 phosphorylation in poly(I:C)-stimulated Dok3$^{-/-}$ macrophages.
Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 50 μg/ml poly(I:C) for the indicated timepoints. TLR3-stimulated wildtype and Dok3$^{-/-}$ lysates were immunoprecipitated with TBK1 and immunoblotted with phospho-antibodies to TBK1 Serine 172. Loading of equal amounts of lysates was verified by immunoblotting with antibodies to total TBK1. Data shown are representative of 2 independent experiments.

3.8 Dok3 interacts with TRAF3 and TBK1 and is required for TBK1 binding to TRAF3 in TLR3 signalling

A previous study demonstrated that the proper assembly of TRAF3 and TBK1 complex formation is essential in LPS signalling to induce IFNβ (Gatot *et al*., 2007). It was also shown in several other studies that the binding of TBK1 to the TRIF adaptor protein upon activation requires TRAF3 and this binding enables downstream signalling to occur (Guo and Cheng, 2007; Hacker *et al*., 2006). As such, TRAF3 and TBK1 complex formation is deemed important prerequisites for signalling to IFNβ. Dok3 intrinsically functions as a adaptor protein in several receptor signalling studies, namely BCR and FcγRIIb (Lemay *et al*., 2000). Hence we asked if Dok3 could also function as an adaptor to stabilize the association between TRAF3 and TBK1 in TLR3 signalling. To answer this question, we immunoprecipitated TRAF3 from poly(I:C)-stimulated macrophages and examined TBK1 association by western blotting. We can see here in Figure III-14, that whereas stable TBK1 and TRAF3 complex formation was detected in TLR3-stimulated wildtype cells, the lack of Dok3 protein appears to abolish this complex formation as they are undetectable in TLR3-stimulated Dok3-deficent macrophages. This data from endogenous immunoprecipitation (IP) suggest that in primary macrophages Dok3 is required for the formation of the TBK1 and TRAF3 complex in TLR3 signalling. However we were unable to distinguish if the associations to TBK1 and TRAF3 occurred via direct interaction. Dok3 possesses an SH2-target motif domain containing tyrosine motif which is commonly known to mediate protein and protein interaction (Pawson and Gish, 1992). The above results prompted us to ask if Dok3 could interact with TRAF3 and/or TBK1 directly. To address this we constructed Dok3, TBK1 and various TRAF family member plasmids tagged with HA or FLAG and tested the interaction of these

proteins in IP assays using a mammalian cell overexpression system, HEK293T. As shown in

Figure **III-15**, Dok3 interacts directly with TRAF3. As a control, it did not bind specifically to TRAF2 or 6. We also showed here that Dok3 interacts directly with TBK1 in HEK293T cells (

Figure **III-16**). The findings that Dok3 interact with TBK1 (fig upper panel) and TRAF3 (fig lower panel) directly were also confirmed via confocal microscopy as the proteins exhibited co-localisation when overexpressed together in HEK293T cells (Figure III-17B).

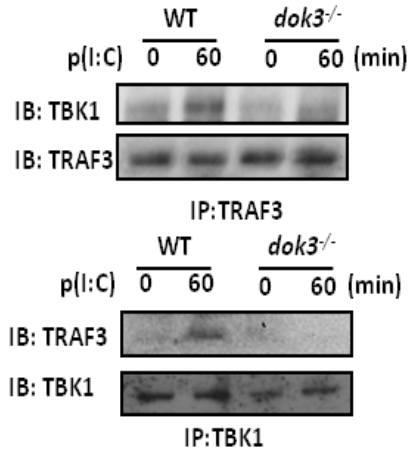

Figure III-14 Dok3 is required for TRAF3 association with TBK1 upon poly(I:C) stimulation.
Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 50 μg/ml poly(I:C) for 30 mins. Cells were immunoprecipitated with antibodies to TRAF3 and analysed for TBK1 binding or vice versa by western blot analysis. Loading of equal amount of lysates was verified by immunoblotting with antibodies to total TRAF3 and/or TBK1. Data shown are representative of at least 2 independent experiments.

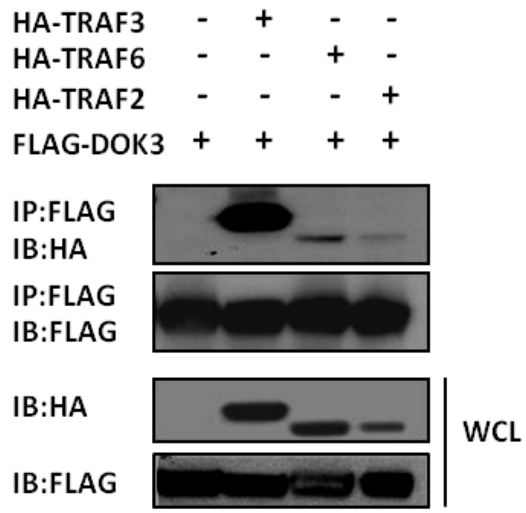

Figure III-15 Dok3 interacts with TRAF3.

Immunoblot analysis of HEK293T cells 24 hrs after co-transfection of haemagglutinin (HA)-tagged TRAF3 with FLAG-tagged Dok3, HA-tagged TRAF6 with FLAG-tagged Dok3 and HA-tagged TRAF2 with FLAG-tagged Dok3, followed by immunoprecipitation with anti-FLAG antibodies and immunoblot with anti-HA antibodies. Whole cell lysates (WCL), immunoblot analysis of total cell lysates with anti-HA and anti-FLAG. Data are from one experiment representative of 3 independent experiments.

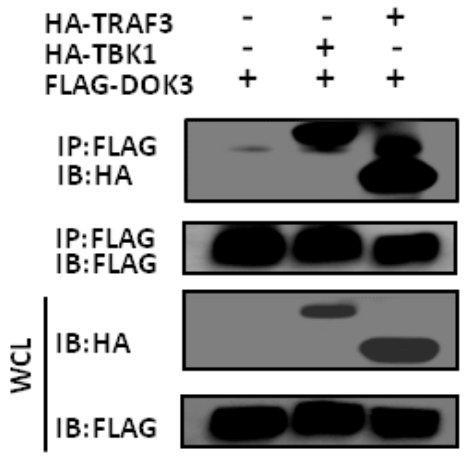

Figure III-16 Dok3 interacts with TBK1.

Immunoblot analysis of HEK293T cells 24 hrs after co-transfection of haemagglutinin (HA)-tagged TRAF3 with FLAG-tagged Dok3 and HA-tagged TBK1 with FLAG-tagged Dok3 followed by immunoprecipitation with anti-FLAG antibodies and immunoblot with anti-HA antibodies.. WCL, immunoblot analysis of total cell lysates with anti-HA and anti-FLAG. Data are from one experiment representative of 3 independent experiments.

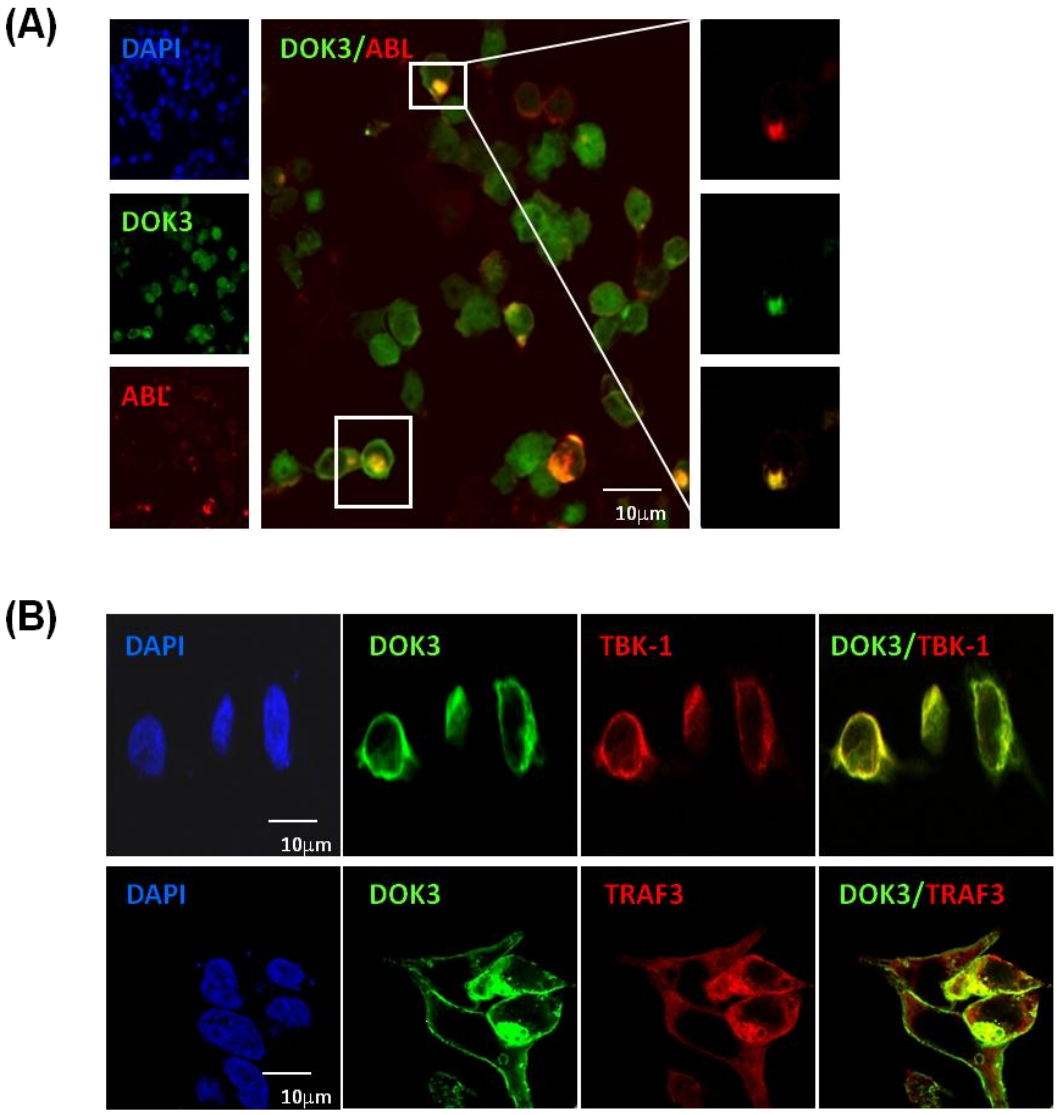

Figure III-17 Confocal images of Dok3 co-localisation with TBK1 and TRAF3.
(A) HEK293T cells were transfected with Dok3-GFP and HA-c-Abl and immunofluorescence staining was performed using antibodies against HA-Alexa 546 goat anti-mouse as a positive control to demonstrate co-localisation. (B) HEK293T cells were transfected with Dok3-GFP and HA-TBK1 or HA-TRAF3 and immunofluorescence staining was performed using antibodies against HA-Alexa 546 goat anti-mouse. Confocal images were taken using and Olympus confocal microscope to determine co-localisation. Images shown are representative of two independent experiments.

3.9 Dok3 binds TRAF3 and TBK1 via SH2 target motif

Dok3 protein structure is divided into mainly the N-terminal PH domain, an intermediary PTB domain and the C-terminal SH2 target motif containing tyrosine motifs (Lemay *et al*., 2000). As we showed Dok3 interacted with TBK1 and TRAF3

directly, we were interested in elucidating the domains of Dok3 that bind to TBK1 and TRAF3, thus allowing an in-depth understanding of the molecular mechanisms that mediate the signal transduction events leading to IFNβ production in TLR3 signalling. To map the binding site of Dok3 to TBK1 and TRAF3, we prepared truncated versions of Dok3 and conducted pull-down assays with TBK1 and TRAF3 (Figure III-18). As showed in Figure III-19, we observed that Dok3 interacts with TRAF3 via the SH2 target motif. The interaction between Dok3 and TBK1 also requires the SH2 target motif (Figure III-20).

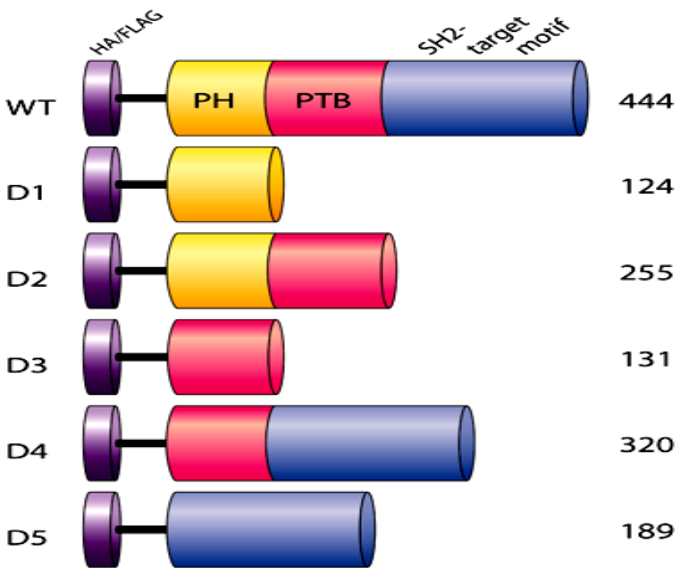

Figure III-18 Pictogram depicting HA or FLAG-tagged Dok3 wildtype (WT) and truncated mutant Dok3.

Dok3 full length protein (WT) or mutants harbouring various combinations of the PH (yellow), PTB (red) or tyrosine-rich carboxyl-terminal (blue) domains (D1 to D5). Numbers on the right indicate total number of Dok3 amino acid residues.

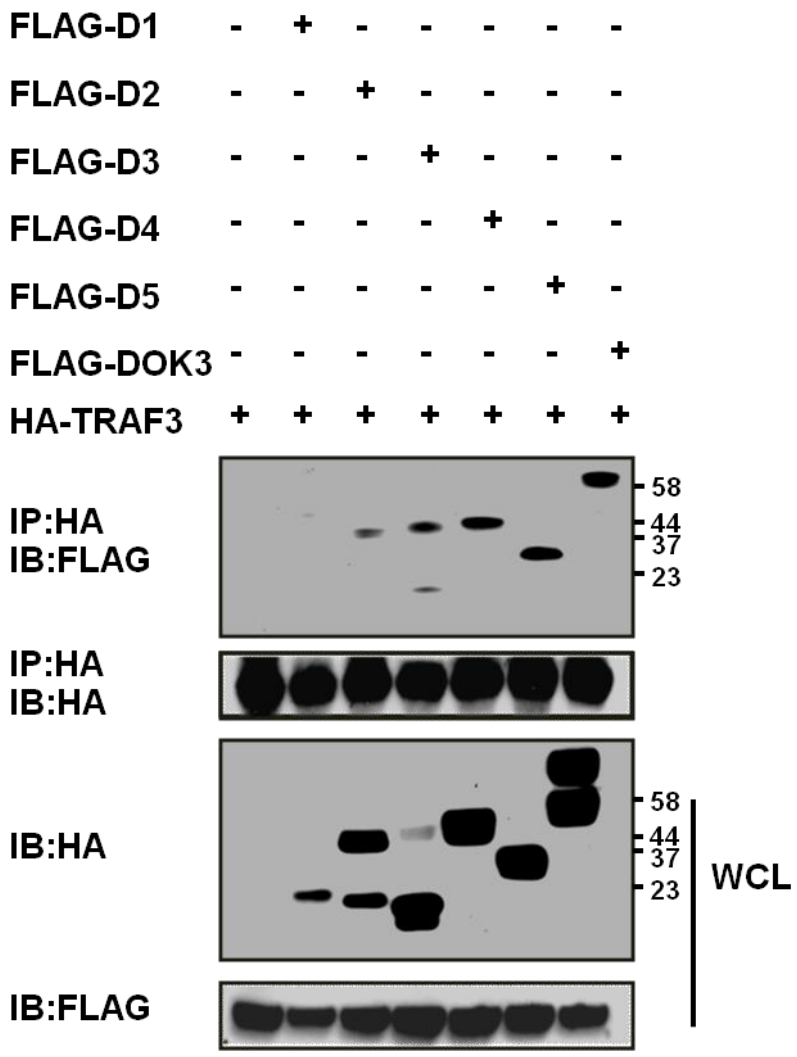

Figure III-19 Dok 3 binds TRAF3 via SH2-target motif domain.

HEK293T cells were transfected with HA-tagged TRAF3 together with FLAG-tagged Dok3 full length or various mutants and cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG antibodies. WCL were also included to ensure expression of the various transfected genes constructs. Data shown are representative of more than 2 independent experiments.

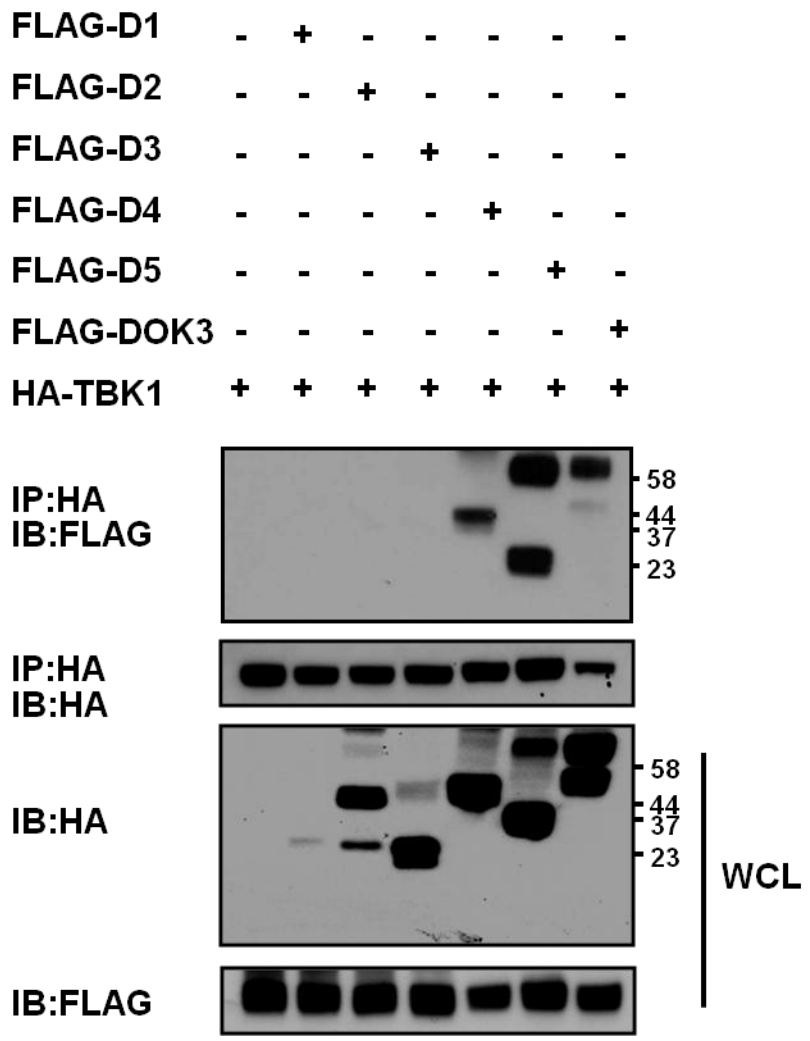

Figure III-20 Dok3 binds TBK1 via its SH2-target motif domain.

HEK293T cells were transfected with HA-tagged TBK1 together with FLAG-tagged Dok3 full length or various mutants and cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG antibodies. WCL were also included to ensure expression of the various transfected genes constructs. Data shown are representative of more than 2 independent experiments.

3.10 Dok3 does not bind to TRIF adaptor protein

Since Dok3 binds to TBK1 and TRAF3 directly, we next investigated if Dok3 can interact with the TRIF adaptor protein, which is the immediate signalling protein downstream of TLR3 (Oshiumi *et al*., 2003). We performed immunoprecipitation experiments with HA/FLAG-tagged Dok3, TRIF and TRAF3 in overexpressed HEK293T cell lysates and as shown here in Figure III-21, in the positive control

lanes, TRAF3 can bind to TRIF directly. However, Dok3 could not be immunoprecipitated with TRIF and this suggests that Dok3 does not interact with TRIF and perhaps acts at the level of TBK1 and TRAF3 in TLR3 signal transduction pathways.

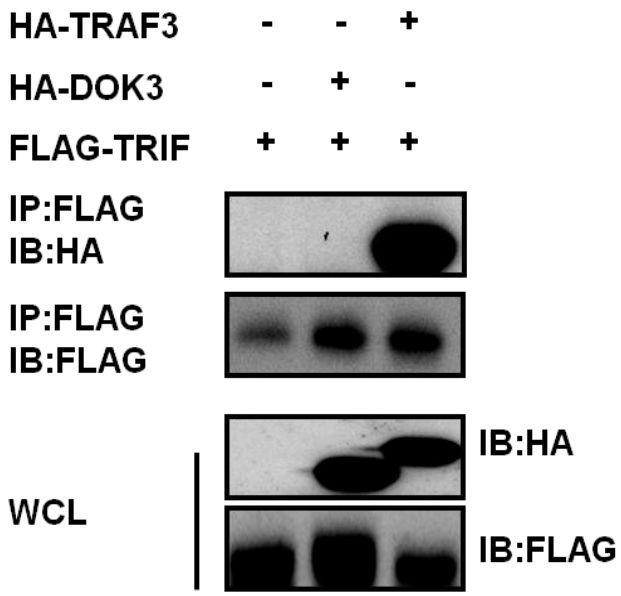

Figure III-21 Dok3 does not bind to TRIF adaptor protein.

HEK293T cells were transfected with HA-tagged TRAF3 and HA-tagged Dok3 together with FLAG-tagged TRIF and cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-HA antibodies. WCL were also included to ensure expression of the various transfected genes constructs. Data shown are representative of more than 2 independent experiments.

3.11 BTK phosphorylate Dok3 for optimal IFNβ production

We demonstrated earlier in this chapter that Dok3 undergoes tyrosine phosphorylation upon poly(I:C) stimulation (Figure III-1). Tyrosine phosphorylation of Dok3 is not only indicative of Dok3 activation status but is also known for its adaptor function (Lemay *et al.*, 2000). The identity of the tyrosine kinase that could possibly phosphorylate Dok3 in TLR3 signalling is unknown and is of interest. One such tyrosine kinase recently shown to be involved in TLR3 signalling is BTK but not Lyn and it was shown that BTK directly phosphorylated the receptor to initiate TLR3

activation (Lee *et al*., 2012). We then asked if BTK could be the tyrosine kinase that phosphorylates Dok3 in TLR3 signalling. We first checked if Dok3 can interact with BTK as these two proteins were known to be associated with each other in BCR signalling (Stork *et al*., 2007). To this end, we immunoprecipitated HA and/or FLAG-tagged Dok3 and BTK in overexpressed HEK293T lysates and showed here in Figure III-22 that Dok3 interacts with BTK directly. Next, we asked if BTK can phosphorylate Dok3. We transfected HA and/or FLAG-tagged BTK and Dok3 into RAW 264.7 macrophage cell lines that endogenously expresses TLR3 and stimulate these cells with poly(I:C). Further examination of Dok3 tyrosine phosphorylation from immunoprecipitated RAW cell lysates by phospho-tyrosine antibodies (4G10) showed (Figure III-23) that co-expression of both BTK and Dok3 resulted in detectable Dok3 tyrosine phosphorylation upon TLR3 stimulation. These results verify that BTK is the tyrosine kinase that phosphorylates Dok3 in TLR3 signalling. We next investigated if Dok3 may be a substrate for BTK's enzymatic activity. We transfected FLAG-tagged Dok3 together with HA-tagged BTK or BTK mutants plasmids including point mutations that lead to constitutive (E41K) kinase activity or point mutations that abolished (K430R) BTK kinase activity into HEK293T cells and examined the phosphorylation of ectopically expressed Dok3. We showed here in Figure III-24 (A) that Dok3 phosphorylation as indicated by 4G10 staining was visibly detectable by co-expression with BTK or its constitutive form but not by the kinase-dead BTK. In addition, Dok3's phosphorylation in TLR3-stimulated macrophages was defective by Btk's deficiency (Figure III-21 B). These results indicate that Dok3 is a substrate of BTK in TLR3 signalling leading to the activation of IRF3 and production of IFNβ.

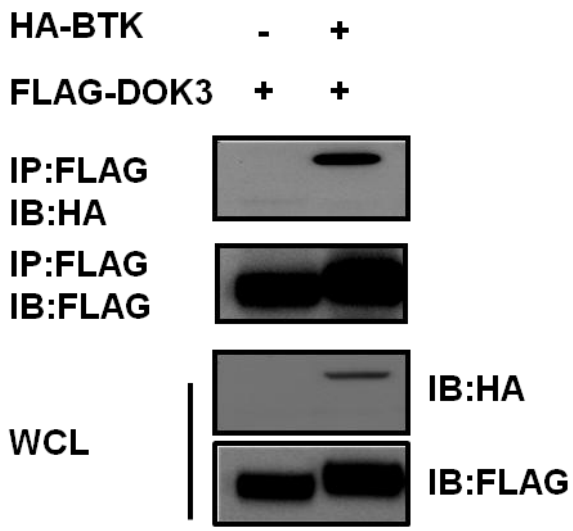

Figure III-22 Dok3 interacts with BTK.

HEK293T cells were transfected with HA-tagged BTK together with FLAG-tagged Dok3 and cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-HA antibodies. WCL were also included to ensure expression of the various transfected genes constructs. Data shown are representative of more than 2 independent experiments

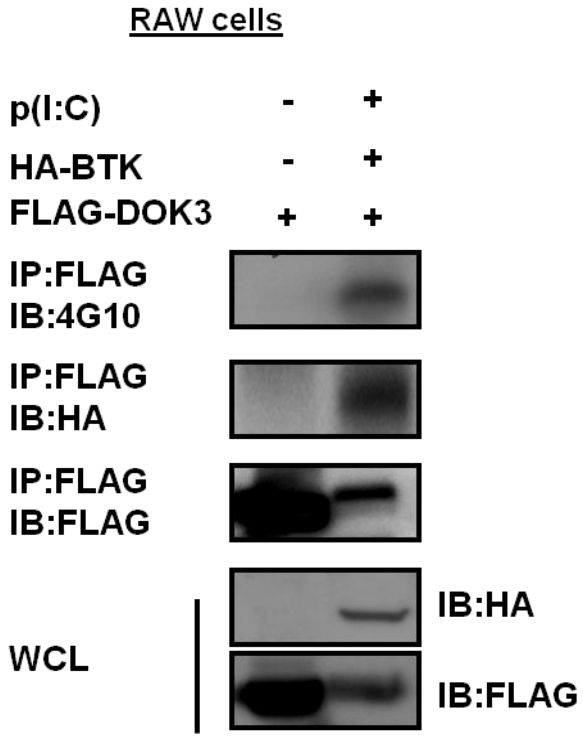

Figure III-23 BTK phosphorylates Dok3 in RAW264.7 cells.

RAW264.7 macrophage cell lines were transfected with HA-tagged BTK together with FLAG-tagged Dok3 and cell were stimulated with or without poly(I:C) and immunoprecipitated with anti-FLAG antibody and immunoblotted with 4G10 antibodies. WCL were also included to ensure expression of the various transfected gene constructs. Data shown are representative of more than 2 independent experiments.

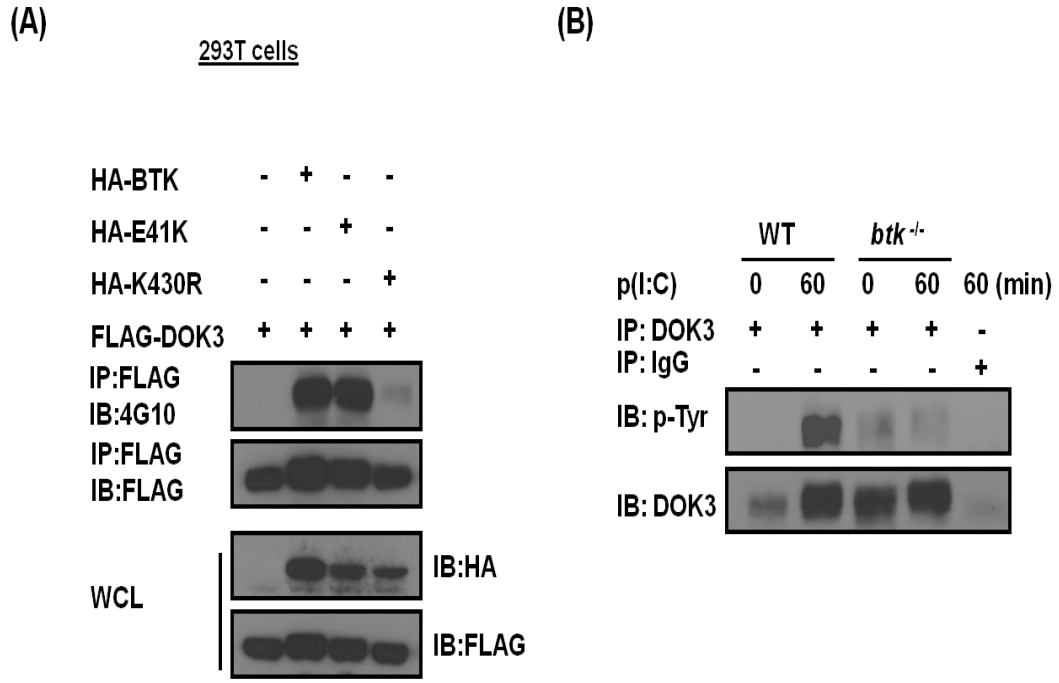

Figure III-24 BTK kinase activity is required for Dok3 phosphorylation.

(A) HEK293T cells were non-transfected or transfected with FLAG-tagged Dok3 together with HA-tagged BTK, constitutively-active BTK (E41K) or kinase-dead BTK (K430R). Cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-phospho-tyrosine (4G10) or anti-FLAG antibodies. WCL were also included to ensure expression of the various transfected genes constructs. (B) Wildtype and btk $^{-/-}$ macrophages were stimulated with 50 μg/ml poly(I:C) for 60 mins. Cells were immunoprecipitated with antibodies to Dok3 and control IgG antibodies and analysed for Dok3 phosphorylation by pTyr antibodies. Loading of equal amount of lysates was verified by immunoblotting with antibodies to total Dok3. Data shown are representative of more than 3 independent experiments.

3.12 Dok3 acts in concert with BTK and TBK1 to induce IFNβ promoter

Using biochemical approaches, we have shown that Dok3 interacts with TBK1, TRAF3 and BTK but not TRIF in TLR3 signalling. To independently confirm that Dok3 positively regulates IFNβ production we investigated the effect of Dok3 interacting with BTK and TBK1 in cellular responses induced by TLR3. To achieve this we overexpressed these proteins and measured the physiological outcomes using IFNβ-luciferase promoter assays. As demonstrated in Figure III-25, Dok3 protein expression alone does not drive IFNβ promoter activity whereas TBK1 protein

expression can induce some levels of basal IFNβ promoter activity. Indeed, when Dok3 was co-expressed with TBK1, there was a higher induction of IFNβ promoter activity as compared to the expression of single proteins. We found from this data that Dok3 and TBK1 could cooperatively promote IFNβ activity in the luciferase promoter assay. Previously, we have demonstrated that the interaction of TBK1 and Dok3 is mediated via the Dok3 SH2-target motif. Hence we checked if this protein domain responsible for Dok3 interaction with TBK1 is required to promote IFNβ activity.

To this end, we expressed TBK1 and Dok3 singly as well as co-expressed TBK1 with wildtype Dok3 or its truncated mutants (lacking PH domain (D4), lacking in SH2 target motif (D2) and containing only SH2 target motif (D5)) and examined their ability to promote IFNβ activity. As shown in Figure III-26, we found that Dok3 and TBK1 together induce higher levels of IFNβ promoter activity but this process requires the Dok3 SH2-target motif protein domain. Expression of the SH2 target motif protein domain of Dok3 (D5) is enough to synergise with TBK1 to induce IFNβ luciferase activity. Last but not least, we were interested to elucidate if there is an additive effect of promoting IFNβ activity under a triple expression of Dok3 together with TBK1 and BTK. As shown in Figure III-27, the triple expression of three proteins drive IFNβ promoter activity much further than when compared to expressing TBK1 or TBK1 together with Dok3. These findings together affirm the role of Dok3 adaptor protein in the positive regulation of IFNβ production.

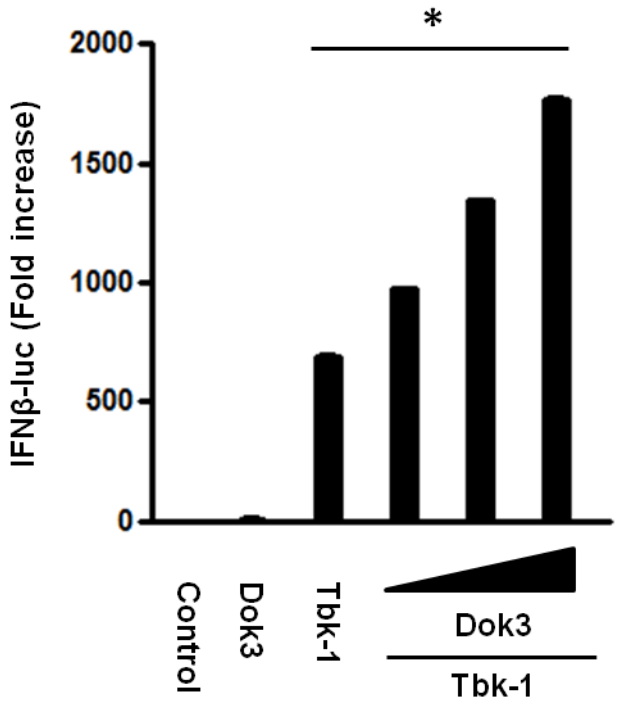

Figure III-25 Dok3 synergizes with TBK1 to induce IFNβ promoter activity.

HEK293T cells were transfected with Dok3 (20 to 100ng) and/or TBK1 (100ng)-encoding plasmids together with reporter (IFNβ luciferase) and control plasmids (Renilla luciferase) into 48-well plates. After 24 hrs the activation of reporter was measured. Data shown is representative of 5 independent experiments.

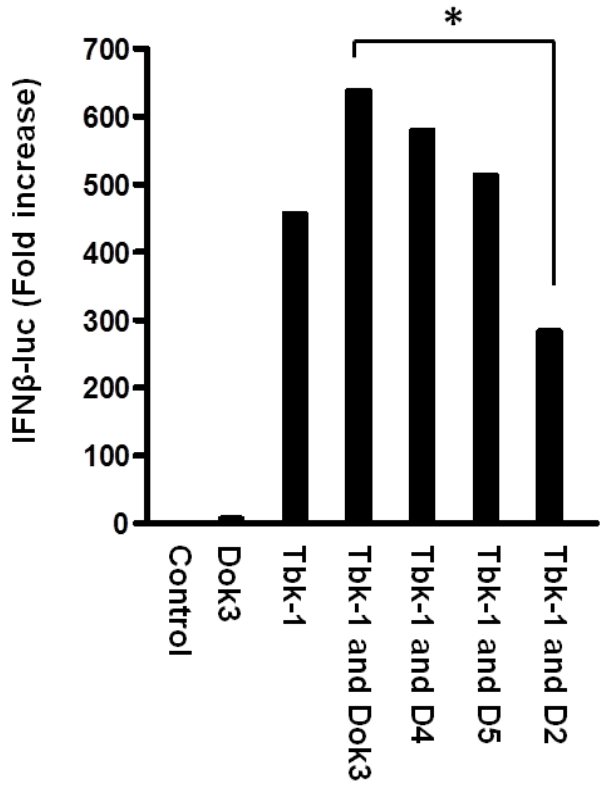

Figure III-26 The SH2-target motif of Dok3 is required for binding to TBK1 to induce IFNβ promoter activity.

HEK293T cells were transfected with either Dok3 (100ng) or various Dok3 mutants lacking either PH (D4), SH2-target motif (D2) or containing only SH2- target motif (D5) (all 100ng) and/or TBK1 (100ng)-encoding plasmids together with reporter (IFNβ luciferase) and control plasmids (Renilla luciferase) into 48-well plates. After 24 hrs, the activation of reporter was measured. Data shown is representative of 3 independent experiments.

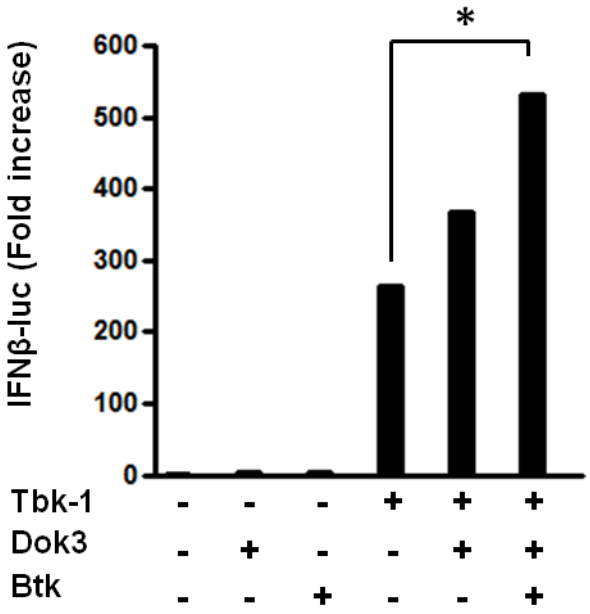

Figure III-27 BTK acts in concert with Dok3 and TBK1 to drive IFNβ gene expression.

HEK293T cells were transfected with Dok3 (100ng), TBK1 (100ng) and/or BTK (100ng)-encoding plasmids together with reporter (IFNβ luciferase) and control plasmids (Renilla luciferase) into 48-well plates. After 24 hrs, the activation of reporter was measured. Data shown is representative of 3 independent experiments.

3.13 Dok3 played a role in intracellular RIG-1 pathway and is required for clearance of influenza virus

Various studies have demonstrated that poly(I:C) stimulation of cells can be recognized not only by endosomal bound TLRs such as TLR3 but also by RLRs that can activate the antiviral response via the RIG-I/MDAS pathway (Kato *et al*., 2006; Kato *et al*., 2008). We have demonstrated definitively that poly(I:C) stimulation of Dok3-deficient macrophages led to defective production of IFNβ as compared to

wildtype control cells (Figure III-5). Experimentally, the defect was observed with the addition of poly(I:C) into the culture media to stimulate the cells. It is established in a number of publication that this method of addition of the poly(I:C) ligand stimulates the cell via the TLR3 pathway as the ligand is taken up via endocytosis with the poly(I:C) or eventually engaging TLR3 once being trafficked to the endosome (Vercammen *et al*., 2008). Therefore, in order for poly(I:C) to activate the RIG-I/MDA5 pathway of antiviral response optimally, the poly(I:C) ligand has to be transfected or introduced directly into the cytosol of host cells by various transfection reagents including lipofectamine or LyoVec (Kawai and Akira, 2007) or by electroporation (Busillo *et al*., 2011). Since TRAF3/TBK1 complex is also involved in RIG-I pathways and we showed that Dok3 is important for TRAF3/TBK1 complex formation, we hypothesize that Dok3 might also participate in RLR signalling. To address this hypothesis, we examined IFNβ gene expression in wildtype and Dok3-deficient macrophages that were stimulated with poly(I:C) packaged in the transfectant reagent LyoVec that allowed the introduction of the dsRNA mimetic directly into the cytoplasm of host cells to activate the RLR-dependent RIG-I/MDA5 signalling pathway. We showed here in Figure III-28 that whereas IFNβ gene expression was detectable in wildtype cells upon transfecting poly(I:C), it was however defective in transfected Dok3 cells$^{-/-}$. This data indicated that Dok3 is not only required for TLR3 signalling but also suggest that Dok3 may play a role in RLR signalling. We next asked if Dok3 is phosphorylated by transfected poly(I:C) as indicated by its involvement by RLR signalling. As seen in Figure III-29, tyrosine phosphorylation of Dok3 was clearly detectable 3 hours after stimulation by transfected poly(I:C) in wildtype macrophages. The RLR pathway activates antiviral responses via the RIG-I/MDA5 pathway that signals through IPS-1 adaptor protein.

This subsequently leads to the activation of TBK1 that phosphorylates IRF3 for inducing IFNβ cytokine production (Kawai *et al*., 2005). We next examined if phosphorylation of IRF3 is affected by Dok3 deficiency in macrophages transfected with poly(I:C). We show here in Figure III-30 that when wildtype macrophages are stimulated with transfected poly(I:C), it leads to the phosphorylation of IRF3 after 6 hours of post-stimulation. However phosphorylation of IRF3 was absent when Dok3-deficient macrophages were treated in a similar manner. Taken together our data here also suggest that Dok3 could participate in RLR signalling in addition to our demonstration that Dok3 is involved in TLR3 signalling.

It is also known that TLR3 and RLRs are key antiviral immune pathways against infectious viral diseases such as influenza and dengue (Kawai and Akira, 2007). Intracellular replication of virus within the host cells generate dsRNA intermediates that are known to be recognised by TLR3 and RLRs (Pauli *et al*., 2008; Tsai *et al*., 2009). The question then is whether there is a physiological role for Dok3 in combating virus infections. To this end, we employed an influenza virus infection model to decipher if Dok3 played a role in limiting virus infection and replication with the notion that Dok3 is required for optimal antiviral IFNβ production. To test whether reduced production of IFNβ by TLR3 and RLR-stimulated $Dok3^{-/-}$ macrophages would compromise antiviral responses, we infected wildtype and Dok3-deficient macrophages with influenza viruses. As shown in Figure III-31 A, quantitative real-time PCR analyses indicated that $Dok3^{-/-}$ macrophages were defective in the production of IFNβ upon influenza virus infection and concomitant with this defect, the replication of the influenza virus as measured by the presence of viral RNA, was much more pronounced in the mutant compared to wildtype

macrophages (Figure III-31 B). The data altogether demonstrated the importance of Dok3 in antiviral TLR3 and RLR signalling.

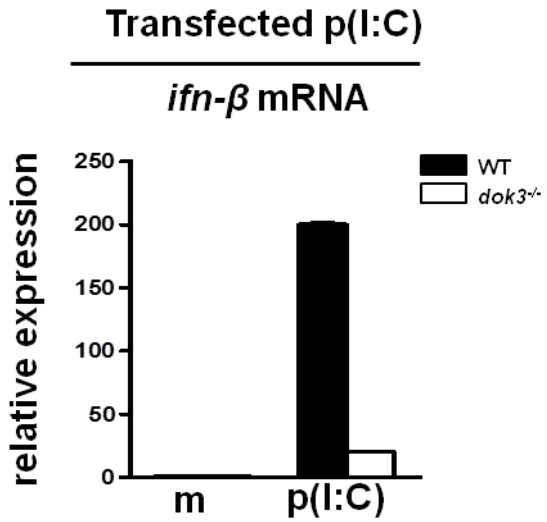

Figure III-28 Dok3 regulates IFNβ gene induction in transfected poly(I:C)-stimulated macrophages.

Real-time qPCR analysis of IFNβ mRNA expression in wildtype (black columns), Dok3$^{-/-}$ (white columns) macrophages stimulated with 10 μg/ml poly(I:C)/LyoVec for 6 hr. The amount of IFNβ mRNA expression was normalized to that of actin mRNA. Figures shown are representative of at least 2 independent experiments.

Figure III-29 Dok3 is phosphorylated in macrophages upon transfected poly(I:C) stimulation.

BMMϕ were stimulated for 3 hrs as indicated with 10 μg/ml poly(I:C)/LyoVec and subsequently lysed. Dok3 was immunoprecipitated and probed with anti-phospho-tyrosine (pY) or anti-Dok3 antibodies. Figure shown is representative of two independent experiments.

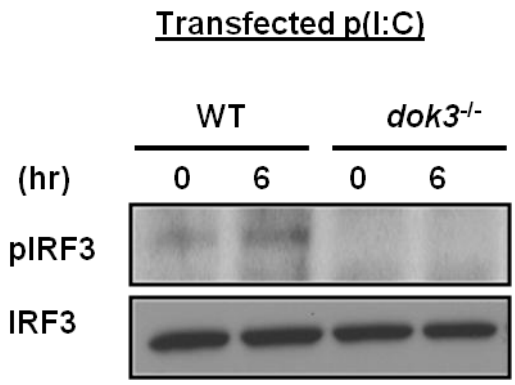

Figure III-30 Impaired IRF3 phosphorylation in transfected poly(I:C)-stimulated Dok3$^{-/-}$ macrophages.

Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 10 μg/ml poly(I:C)/LyoVec for 6 hr. Cells were lysed and analysed by immunoblotting with phospho-antibodies to IRF3 (Serine 396). Loading of equal amounts of lysates was verified by immunoblotting with antibodies to total IRF3. Data shown are representative of 2 independent experiments.

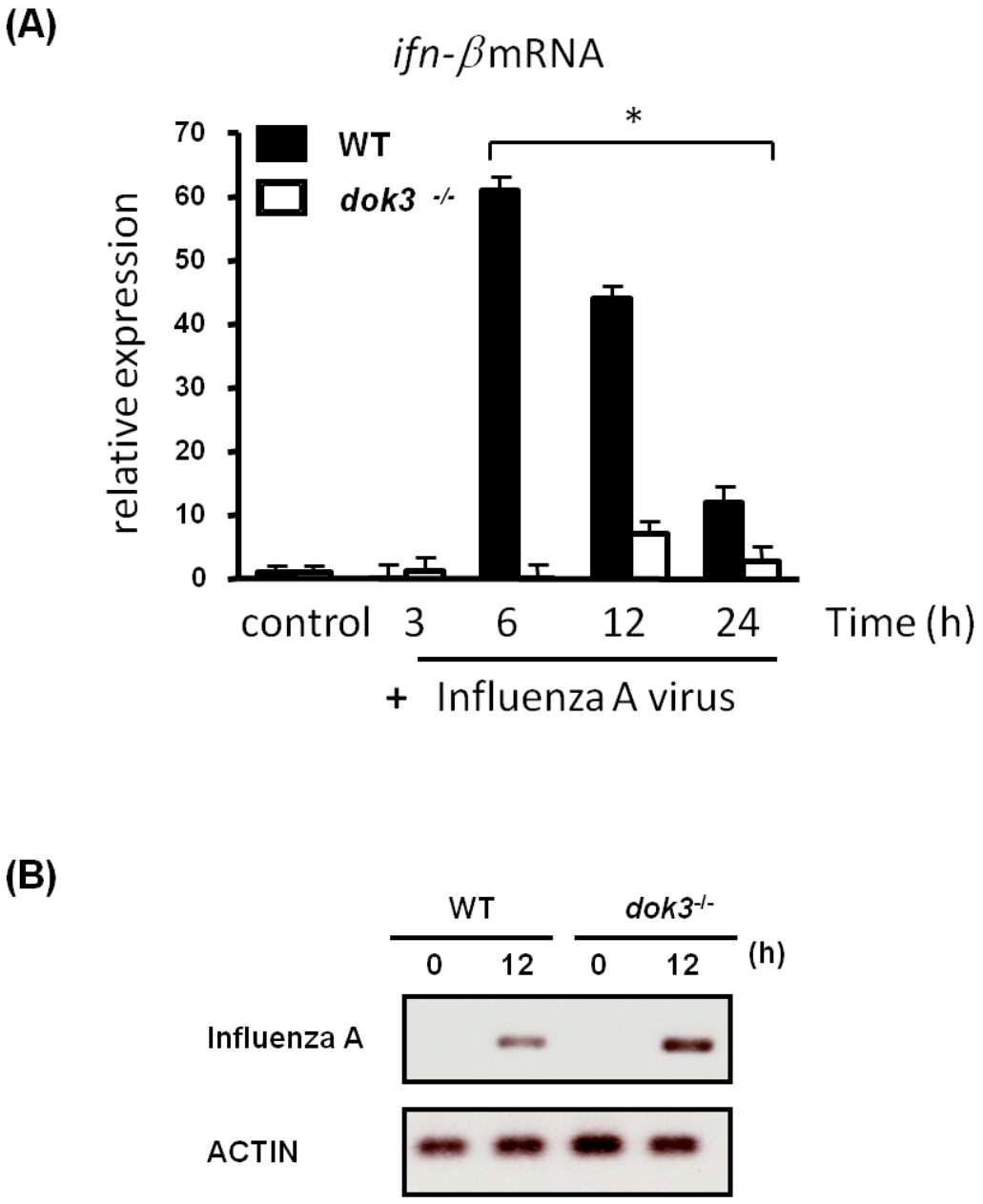

Figure III-31 Dok3 is required for inhibition of influenza A virus replication.

WT and Dok3$^{-/-}$ macrophages were infected with dengue virus and at 72 hrs post-infection assayed for *IFN-β* mRNA (A) and presence of influenza A virus RNA (B) via semi-quantitative RT-PCR. The actin RT-PCR served as control for loading of templates. . Statistical significance was established by paired student's *t* test, *p<0.05. Data shown are representative of 2 independent experiments.

3.14 Discussion

Several studies have implicated the role of Dok family members in TLR signalling. More recently, Dok3 was shown to be important in lung carcinoma development (Berger *et al*., 2010). The role of Dok3 in TLR pathway however is elusive. In this chapter study, we utilised Dok3$^{-/-}$ mice to discern the physiological role of Dok3 in TLR3 signalling. In our initial screening experiments of assaying inflammatory cytokine production via ELISA using a panel of TLR ligands against wildtype and Dok3-deficient macrophages, we found no observable differences for cells that were stimulated with the TLR ligand that signals through MyD88, for example CpG, ligand for TLR9 and R837, ligand for TLR7 and TLR8 (data not shown), with the exception of poly(I:C), ligand for TLR3 and LPS, ligand for TLR4 (Figure III-4 and Figure IV-2). The commonality between TLR3 and TLR4 lies in the usage of TRIF adaptor protein. It has been shown interestingly that in LPS signalling via TLR4, the cytokine production predominantly occurs via TRIF and not MyD88 (Yamamoto *et al*., 2003). This enabled us to exclude the possibility that Dok3 has a functional relevance in signalling through MyD88 and prompted us to examine in greater detail its role in TRIF-dependent signalling mediated via TLR3 and TLR4. We primarily used naked poly(I:C) as a TLR3 ligand in our assays. The poly(I:C) is a synthetic analogue and mimics viral dsRNA that is exposed to host immune cells and trigger TLR3 specifically in the endosome.

We report here that Dok3 undergoes tyrosine phosphorylation upon poly(I:C) stimulation in macrophages. This indicates that Dok3 is “activated” during TLR3 signalling. More importantly, we also showed that the induction of Dok3 phosphorylation requires the presence of TRIF. We went on to delineate the

physiological aspects of Dok3's role in TLR3 signalling. Many studies have suggested an inhibitory role of Dok family members (for example Dok1 and Dok2) in TLR signalling (Shinohara *et al*., 2005). In this current study however, we report an unexpected and surprising discovery that Dok3 mediates a positive regulatory role in TLR3 signalling. The poly(I:C)-induced sepsis model showed that mutant mice were more resistant to poly(I:C)-induced sepsis compared with wildtype control mice. This *in-vivo* result is consistent with our *in-vitro* ELISA data that indicate that the proinflammatory cytokines production upon TLR3 signalling was defective in $Dok3^{-/-}$ cells and mice.

We have examined the different major signalling pathways that are known to be activated downstream of TLR3 activation. These include PI3K, MAPK, NFκB and IRF3 (Schroder and Bowie, 2005). IFNβ, a type 1 IFN, is an important anti-viral cytokine against viral invasion. The optimal production of this cytokine is known to require the co-ordination of various transcription factors including AP-1, NFκB and IRF3 binding to the IFNβ promoter region (PRDI-IV) known as the IFNβ enhanceosome (Kim *et al*., 2000). We found that Dok3 is dispensable for MAPK and NFκB signalling downstream of TLR3 and this suggests the possibility of other proteins or related family members compensating for this specific role. Dok3 however is required for signalling to IRF3, the key transcription factor for inducing IFNβ as evident by several of our findings. We also discovered that Dok3 is important for activating the PI3K-AKT pathway to TBK1 and TRIF-mediated TBK1 signalling. Signalling via these two pathways from TLR3 eventually converges upon TBK1 which is an essential kinase that phosphorylates and activates IRF3 to allow nuclear translocation and DNA binding to allow antiviral gene expression.

In elucidating the molecular mechanism of Dok3's action in TLR3 signalling we found that Dok3 acts at the level of stabilizing TBK1 and TRAF3 complex formation to signal to IFNβ by directly interacting with these proteins. This is consistent with the intrinsic role of Dok3 behaviour as an adaptor protein. We further delineate and dissect the domain of Dok3 that mediate the interaction and specifically identify the SH2-target motif and/or PTB domain for binding to TBK1 and TRAF3, respectively. We also report that signalling via PI3K-AKT is defective due to the lack of Dok3. This signalling pathway downstream of the receptor, TLR3, was identified as a detour as opposed to the mainstream signalling pathway that is dependent on TRIF (Hiscott, 2004). The phosphorylation of TLR3 on tyrosine 759 was found to be important in the recruitment of PI3K in mutagenesis studies (Sarkar *et al*., 2004). More recently, this pathway, otherwise termed TRIF-independent branch of TLR3 signalling, was reported to be essential in other cellular activities including cell migration and adhesion (Yamashita *et al*., 2012b). Attempts made to identity the tyrosine kinase critical for the phosphorylation of TLR3 at tyrosine 759 residue led to the identification of BTK and c-SRC as important mediators of TLR3 signalling (Johnsen *et al*., 2006; Lee *et al*., 2012). As BTK, an SRC-family member, Lyn and Dok3 were largely well-characterised in BCR signalling and exhibiting mutually non-exclusive roles in B cell development, it is useful to extrapolate their roles for TLR3 signalling. One possible scenario is that upon TLR3 engagement by dsRNA, BTK undergoes SRC-dependent activation and phosphorylates TLR3 to initiate signalling events. It has been demonstrated that BTK binds TRIF (Liu *et al*., 2011) and Dok3 interacts with BTK (Figure III-32). Our work separately indicated that Dok3 undergoes tyrosine phosphorylation upon TLR3 activation and we thought that BTK may be the

kinase responsible. Indeed we proved that this is the case. This then points to Dok3 possibly exerting an adaptor function to bridge TBK1, TRAF3 to amplify the signalling events to IRF3, which is critical for the induction of IFNβ (Figure III-32). Moreover the absence of Dok3, i.e. loss of adaptor function results in impairment in the PI3K-AKT pathway as observed in TLR3-stimulated Dok3$^{-/-}$ macrophages as this pathway may be heavily dependent on BTK-Dok3 activity. In conclusion, these results highlight the importance of adaptor function in the context of Dok3 bridging and or stabilizing other proteins (i.e., BTK and/or TBK1 and TRAF3) in a signalosome formation in TLR3 signalling.

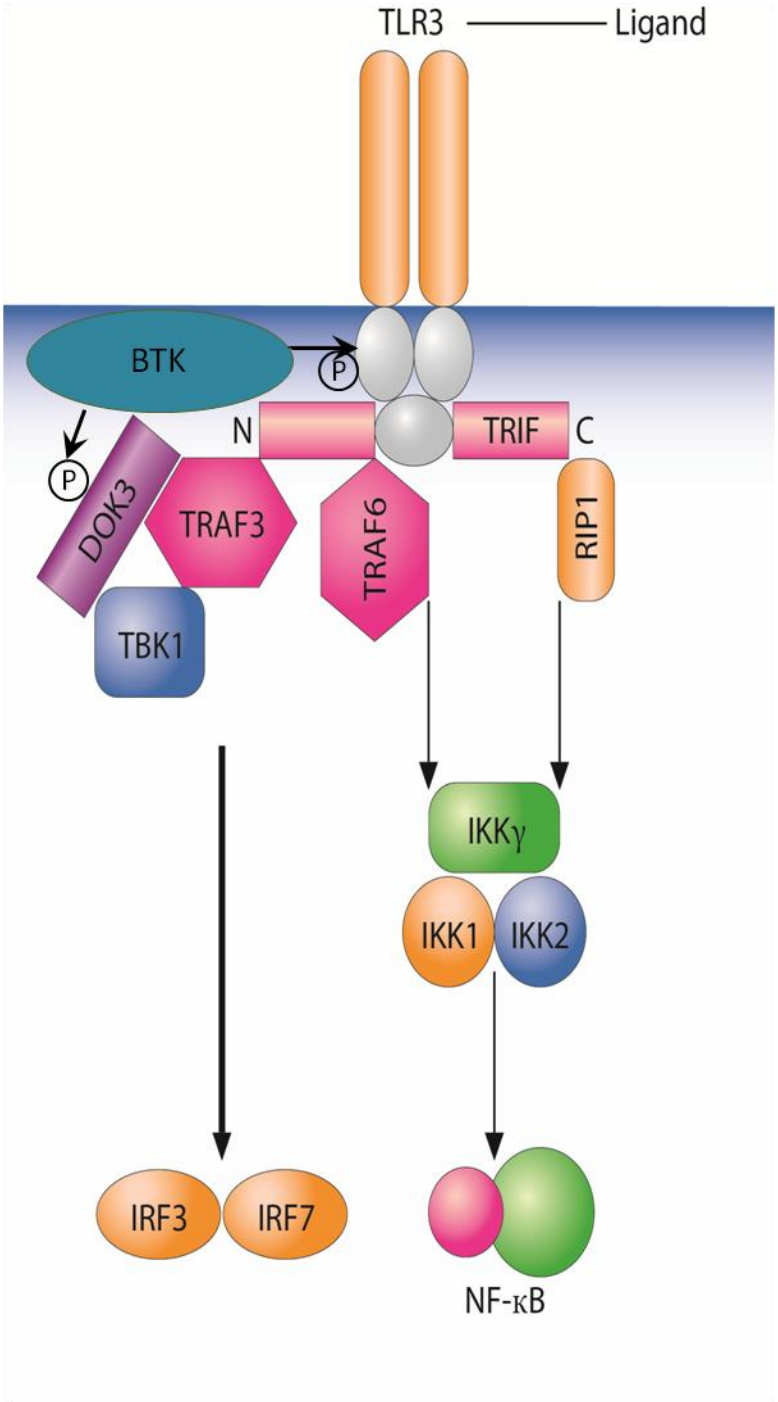

Figure III-32 A working model for Dok3's role in TLR3 signalling.

Upon activation by dsRNA, it leads to the dimerization and activation of TLR3. This likely result in the recruitment of TRIF and BTK of which BTK is required to fully phosphorylate TLR3 at tyrosine 759 residue. BTK then interacts with and phosphorylate Dok3. Dok3 is then required to interact with TBK1 and TRAF3 and mediates the interaction of TBK1/TRAF3 to TRIF for TRIF-dependent signalling pathways downstream of TLR3 including TBK1-mediated IRF3 activation of which is required for an effective assembly of a functional β-interferon enhanceosome to secrete IFNβ, a critical type I IFN for an anti-viral immune response.

CHAPTER IV. ROLE OF DOK3 IN TLR4 SIGNALLING IN MACROPHAGES

4.1 Introduction

Toll-like receptors are germ-line encoded pattern recognition receptors (PRR) that detect infectious agents collectively referred to as pathogen associated molecular pattern (PAMP). For example, lipopolysaccharide (LPS) is a Gram-negative bacteria cell wall component that upon contact with TLR4 can induce the intracellular signalling cascade known as TLR4 signalling. TLR dimerization upon ligand presentation allows the binding of cytoplasmic adaptor molecules such as MyD88, MyD88 adaptor-like MAL, TIRAP and TRIF. Almost all TLRs signal via the universal adaptor MyD88 with the exception of TLR3 and TLR4. In the case of TLR4 it utilizes MyD88 and TRIF to trigger the respective pathways. Activation of MyD88 recruits and phosphorylates two kinases (IRAK1 and 4). This is followed by the activation of the cytoplasmic adaptor TRAF6. Once TRAF6 forms a complex with Ubc13/Uev1 E2, it then functions as an ubiquitin ligase to polyubiquitinate TAK1. Activated TAK1 then upregulates the transcription factor NFκB through the IKK complex and the MAPK family. TRIF-dependent pathway also activates both the NFκB and the MAPK family but the IKK complex is activated via recruitment of RIP1 (Meylan *et al*., 2004).

The quintessential function of the abovementioned signalling cascades are to activate the necessary gene transcription to produce proinflammatory cytokines such as TNFα which are necessary to respond to pathogenic invasion. Whilst TLR activation is necessary to control dissemination of the pathogen it is also portrayed as a double-edged sword as sustained release of potent cytokines such as TNFα can result in pathogenesis of autoimmune, chronic inflammation and septic shock. Therefore

various negative regulators exist to maintain homeostasis between appropriate and inappropriate immune responses. However not all is fully understood especially regarding the mechanistic function of known negative regulators.

The family of proteins downstream of kinase (Dok) has been thought to play inhibitory functions in signalling transduction. They do not have enzymatic activities alone but are able to bring the necessary molecules to activate signal transduction. Therefore Dok is also referred to as the adaptor/docking proteins. The first member of the Dok family p62, was identified in the hematopoietic progenitors isolated from chronic myelogenous leukemia patients and was found to be highly phosphorylated by numerous cytoplasmic tyrosine kinases such as Lyn, SYK and BTK (Niki *et al*., 2004). To date there are seven known proteins that represent the Dok family. However only Dok1 (p62), Dok2 (p56/Dok-R/FRIP) and Dok3 (Dok-L) are highly expressed in hematopoietic cells. The basic overall structure of Dok consists of homologues PH-domain at the N terminus followed by a PTB region and a variant carboxyl terminal containing a few potential tyrosine kinase sites (Mashima *et al*., 2009).

Dok1 and Dok2 are involved in the negative regulation of numerous immune cell signalling transductions such as BCR, CD2 receptor, Fc receptor and TLR4 signalling. In 2005 a single knockout study carried out by Shinohara *et al* demonstrated that while Dok1 or Dok2 was not essential for organism growth, upon LPS challenge, mice underwent fatal septic shock. Peritoneal resident and bone marrow derived macrophages from single knockout mice showed elevated levels of

TNFα and NO production when stimulated with LPS. However, the responses to stimulation with other TLR ligands were normal (Shinohara *et al*., 2005).

Dok3 is also expressed in hematopoietic cells such as B lymphocyte, myeloid cells and macrophages but not T cells. Structurally, unlike Dok1 and Dok2, Dok3 does not contain the repeated YXXP motifs in the carboxyl tail containing the binding site to p120rasGAP. Instead Dok3 is phosphorylated at tyrosine 398 and 432 by SRC kinase, which allows the binding of Grb2. Physiological studies of Dok3 knockout mice revealed that Dok3 is not detrimental to the organism and B cell development. However these mice have elevated basal levels of the serum IgM and heightened humoral responses against T-cell independent antigens. Moreover upon BCR engagement, B cells hyperproliferate with enhanced upregulation of MAP Kinase, NFκB pathway and raised level of calcium signalling. Ng *et al* suggested that Dok3 negatively regulate BCR signalling via activation of SHIP-1 although the exact mechanistic role of Dok3 still remains unclear (Ng *et al*., 2007). Together with the evidence that Dok1 and Dok2 play negative roles in TLR4 signalling, it is likely that Dok3 may also be involved in the regulation of signalling downstream of Toll-like receptors.

In chapter 3, we examined the role of Dok3 in TLR3 signalling and showed that it played a positive regulatory role in inducing IFNβ production through the TRIF-dependent pathway. It is known that TLR4 also used TRIF adaptor proteins to signal to IFNβ (Fitzgerald *et al*., 2003b). In addition to TRIF, TLR4 also uses another adaptor protein known as TRAM (Fitzgerald *et al*., 2003b) but TRAM is not utilised by TLR3 for signalling. Since Dok3 is required for TLR3-mediated TRIF dependent

activation of IFNβ, it may be possible that Dok3 is also required by TLR4 in its signalling to IFNβ. To determine if there is a specific role for Dok3 in TLR4 signalling in macrophages, we examined the effect of the absence of Dok3 in TLR4 engagement, which is an innate anti-bacterial PPR that recognizes endotoxins. (Poltorak *et al*., 1998)

4.2 Dok3 is phosphorylated upon TLR4 stimulation

To determine if Dok3 is involved in TLR4 signalling in macrophages, we investigated if Dok3 undergoes tyrosine phosphorylation upon LPS stimulation. We treated wildtype macrophages with LPS that engages TLR4. As shown in Figure IV-1, Dok3 undergoes tyrosine phosphorylation upon LPS stimulation. The tyrosine phosphorylation of Dok3 was however not observed in the untreated cells or at 0 timepoint. This data indicated that Dok3 may be required for a regulatory role in TLR4 signalling.

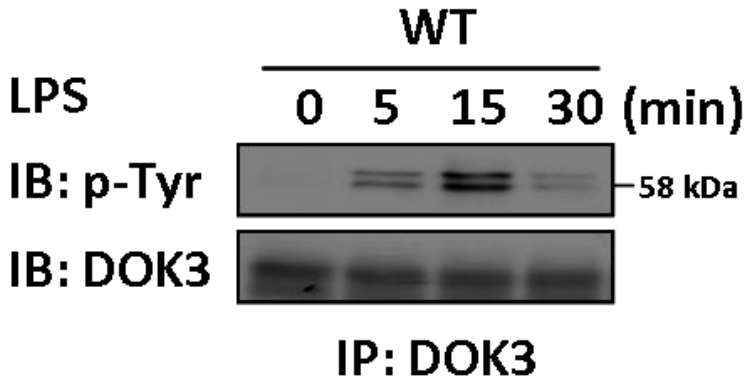

Figure IV-1 Dok3 is phosphorylated in macrophages upon LPS stimulation.

BMMϕ were stimulated for various times as indicated with 1 μg/ml LPS and subsequently lysed. Dok3 was immunoprecipitated and probed with anti-phospho-tyrosine (pY) or anti-Dok3 antibodies. Figure shown is representative of two independent experiments.

4.3 Dok3-deficient macrophages exhibit reduced IL-6 secretion but normal production of IL-12 and TNFα upon TLR4 stimulation

LPS stimulation is known to lead to the production of proinflammatory cytokines that are mediated via the usage of four adaptor systems (O'Neill *et al*., 2003). A previous study demonstrated that upon TLR4 signalling, proinflammatory cytokine production was dependent on the cooperation of TRIF and MyD88 (Yamamoto *et al*., 2003). As Dok3 is phosphorylated in macrophages upon LPS stimulation, we asked if Dok3 is required for the proinflammatory cytokine productions downstream of TLR4. We measured with ELISA and by ICS flow cytometry the production of IL-6, IL-12 and TNFα in TLR4-stimulated wildtype and Dok3-deficient macrophages (data not shown). As shown in Figure IV-2, Dok3-deficient macrophages exhibited reduced IL-6 secretion but normal production of IL-12 and TNFα upon TLR4 stimulation. As controls, these analyses were performed alongside with TRIF and TLR3-deficient macrophages. Cytokine production was defective in TLR4-stimulated $TRIF^{-/-}$ macrophages but however was normal in TLR4-stimulated $TLR3^{-/-}$ macrophages, consistent with previous reports (Yamamoto *et al*., 2003). The data indicated that Dok3 may play only a marginal role in TLR4 signalling where its absence resulted only in a defective IL-6 cytokine production upon LPS signalling.

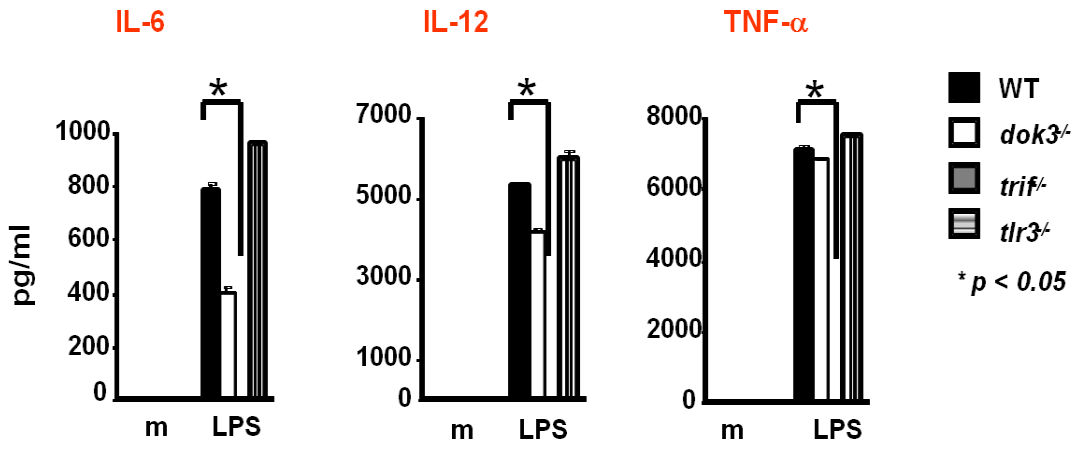

Figure IV-2 Dok3 positively regulate cytokine production in LPS-stimulated macrophages.
Wildtype (black columns) and Dok3$^{-/-}$ (white columns), TLR3$^{-/-}$ (grey columns) and TRIF$^{-/-}$ (shaded columns) macrophages were untreated or treated with 100 ng/ml LPS for 6 hrs and their secretion of IL-6, IL-12p40 and TNFα quantified via ELISA using known standards. Statistical significance was established by paired student's *t* test, *p<0.05. Figures shown are representative of at least 3 independent experiments.

4.4 TLR4 stimulated Dok3-deficient macrophages result in reduced *IFNβ*

Signaling through TLR4 is known to trigger IFNβ production via the TRIF-dependent signaling axis. We have demonstrated in chapter 3 that Dok3 is important for IFNβ production in the TRIF pathway downstream of TLR3. We next investigated if Dok3 is also required to operate in the TRIF-dependent signaling axis downstream of TLR4, analogous to TLR3, in inducing IFNβ. We show here in Figure IV-3, that in contrast to TLR4-stimulated wildtype macrophages that can readily induce IFNβ production, this was impaired in TLR4-stimulated Dok3 and TRIF-deficient macrophages. These results indicated that Dok3 is required in both TLR3 and TLR4-mediated signaling to IFNβ.

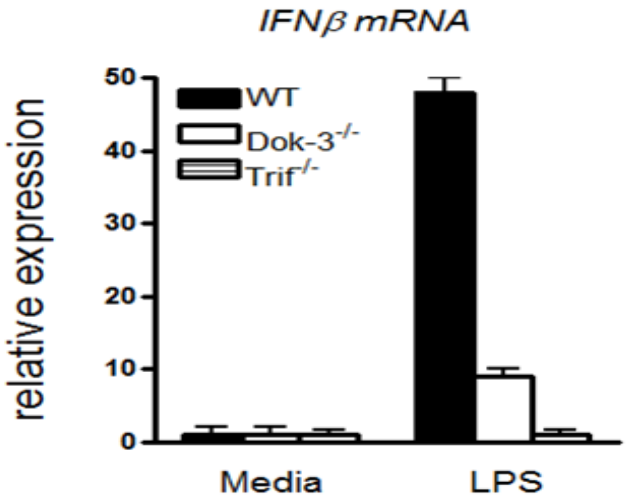

Figure IV-3 Dok3 regulates IFNβ gene induction in LPS-stimulated macrophages.

Real-time qPCR analysis of IFNβ mRNA expression in wildtype (black columns), Dok3$^{-/-}$ (white columns) and TRIF$^{-/-}$ (shaded columns) macrophages stimulated with 100 ng/ml LPS for 2 hr. The amount of IFNβ mRNA expression was normalized to that of actin mRNA. Figures shown are representative of at least 3 independent experiments.

4.5 TLR4-stimulated Dok3-deficient macrophages exhibit normal MAPK and IκBα activation

TLR4 signaling pathways are known to lead to MAPK and NFκB activation, mediated largely by signaling through MyD88 (Yamamoto *et al*., 2003). Thus we investigated if Dok3 is required for these signal transduction pathways downstream of TLR4. We stimulated wildtype and Dok3-deficient macrophages with 100 ng/ml of LPS and assayed for MAPK activation by western blotting. These MAPKs include phosphorylation of ERK, JNK and p38 (Takeda and Akira, 2005). We show in Figure IV-4 that the phosphorylation of ERK, JNK and p38 were however similarly induced between TLR4-stimulated wildtype and Dok3-deficient macrophages. We next assayed for NFκB activation by looking at IκBα degradation via western blot (Figure IV-5). However, IκBα degradation rate was comparable between TLR4-stimulated

wildtype and Dok3-deficient macrophages. These results suggest that there appears to be no requirement for Dok3 in the major signaling pathways downstream of TLR4 (including MAPKs and NFκB) with the dosage of LPS (100 ng/ml) that was utilized in the assays. A previous study identified that sublevels of LPS can be associated with the severity of pathogenesis in various human inflammatory diseases. Thus the LPS dosage utilized in experiments can be categorized into high and low dose and may yield different experimental outcomes. So far, we have been using 100 ng/ml LPS in our experiments. This is considered a high dosage of LPS stimulation in various literatures (Maitra *et al*., 2011; Stelter *et al*., 1998; Xu *et al*., 2007). As such, it may be possible that there could be some dosage-dependent effects for TLR4 signaling by Dok3.

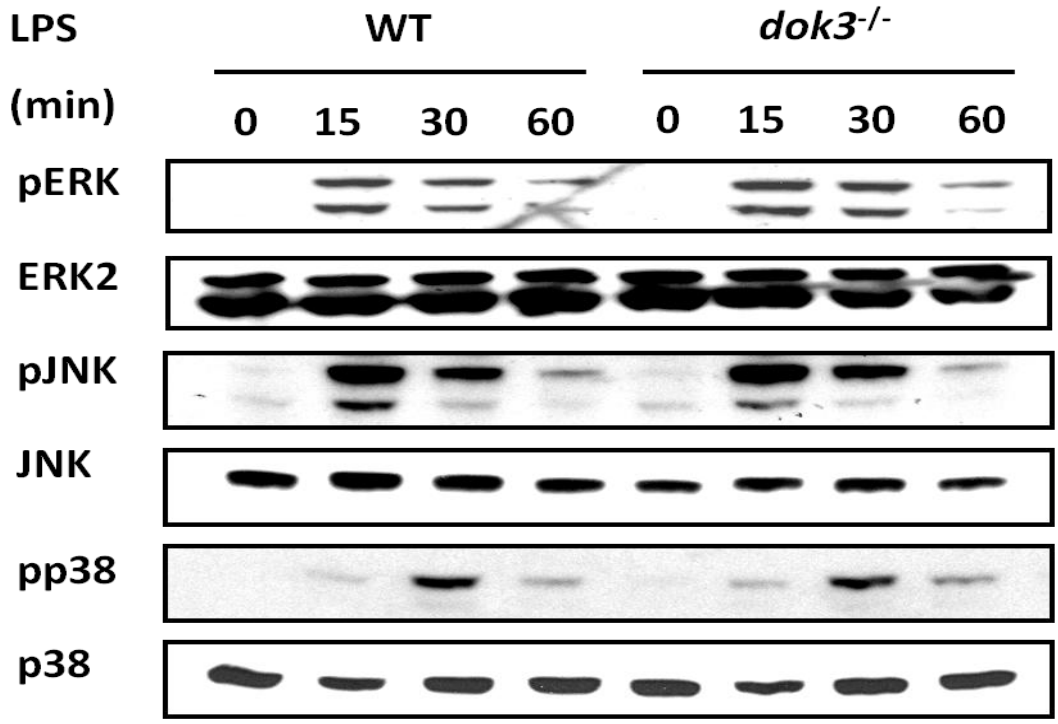

Figure IV-4 Normal MAPK activation in LPS-stimulated Dok3$^{-/-}$ macrophages.
Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 100 ng/ml LPS for the indicated timepoints. Cells were lysed and analyzed by immunoblotting with phospho-antibodies to (A) ERK, (B) JNK and (C) p38. Loading of equal amount of lysates were verified by immunoblotting with antibodies to total ERK, JNK and p38. Data shown are representative of three independent experiments.

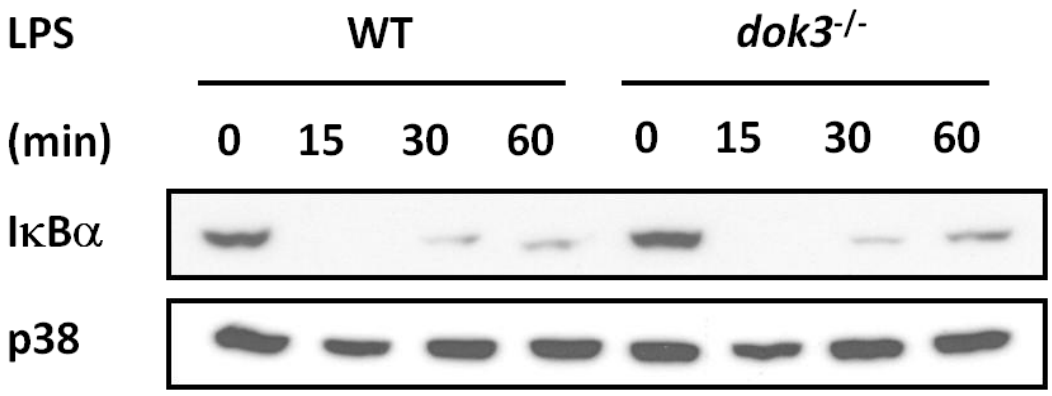

Figure IV-5 Normal NFκB activation in LPS-stimulated Dok3$^{-/-}$ macrophages.
Wildtype and Dok3$^{-/-}$ macrophages were simulated for various times with 100 ng/ml LPS and examined by Western blot analysis for the degradation of IκBα. Anti-p38 blot was included as control for equal loading of cell lysates.

4.6 Low Dose of LPS stimulated Dok3-deficient macrophages exhibit defective ERK activation

To test out this hypothesis, we stimulated wildtype and Dok3-deficient macrophages with a low dose of LPS (10 ng/ml) in a time-dependent manner and assayed for phosphorylation of ERK by western blotting. We show here in Figure IV-6 that the activation of ERK was defective in Dok3-deficient macrophages as compared to wildtype by this low LPS dose stimulation. The data indicated that there is LPS dosage dependent TLR4 signaling during which Dok3 shows its effect.

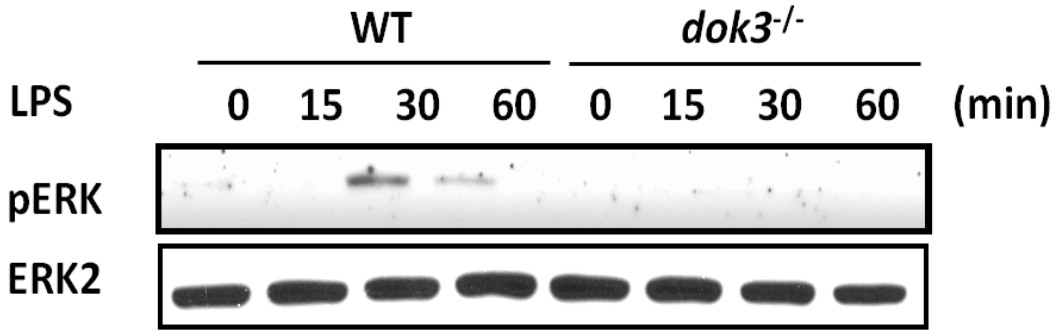

Figure IV-6 Defective ERK activation in low dose LPS-stimulated Dok3$^{-/-}$ macrophages.
Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 10 ng/ml LPS for the indicated timepoints. Cells were lysed and analysed by immunoblotting with phospho-antibodies to ERK. Loading of equal amount of lysates were verified by immunoblotting with antibodies to total ERK. Data shown are representative of two independent experiments.

4.7 Dok3 $^{-/-}$ Mice are Resistant to Septic Shock upon Immunization with low dose LPS

The septic shock model is established laboratory protocols that assess the ability of D-GalN-sensitized mice to mortality. Other factors that contribute to septic shock include prostaglandins, COX2 and proinflammatory mediators such as iNOS as well as excessive production of type I IFN (Ketteler *et al*., 1998; Rabuel *et al*., 2004). As a lower threshold of TLR4 signaling appears to require Dok3, we investigated if these mutant mice were viable with a low dose of LPS challenge in a septic shock model. We show here in Figure IV-7 that Dok3-deficient mice are resistant to LPS-induced septic shock whereas all the wildtype mice succumbed after 40 hours from immunization. The data suggest that Dok3 is required as a positive regulator in a suboptimal dose of TLR4 signaling.

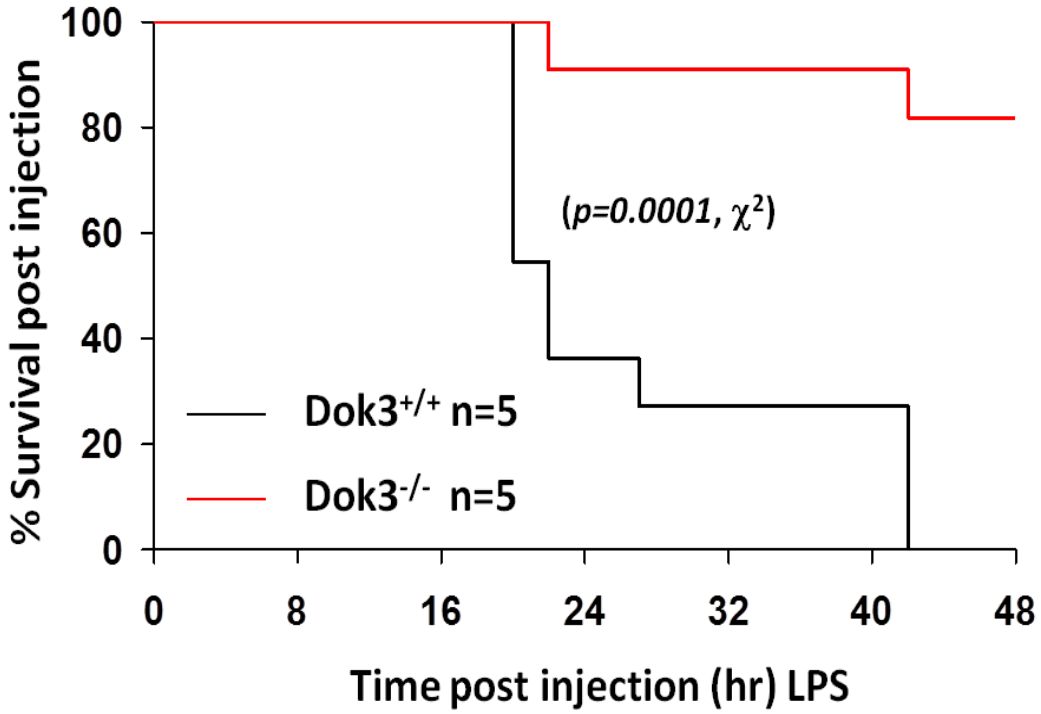

Figure IV-7 Dok3-deficient mice are more resistant to low dose LPS-induced septic shock.
Wildtype (black line) and Dok3$^{-/-}$ mice (red line) were injected with 10 ng LPS and 20 mg of D-GalN. The dose of LPS was calculated per 20 g of body mass. Survival of mice was monitored for over 24 hr. Statistical significance was established by paired student's *t* test, *p<0.05.

4.8 Impaired Production of TNFα by Dok3 Deficiency in low dose TLR4 Signalling

The TNFα-mediated liver damage is one of the key contributing factors to the mortality of mice in D-GalN-dependent LPS septic shock (Dejager and Libert, 2008). As the above results indicate that Dok3 deficiency confers resistance to death in a suboptimal dose of LPS-induced sepsis, it is possible that the TNFα proinflammatory cytokine production may be disregulated. To investigate this possibility, we stimulated *in vitro* wildtype and Dok3-deficient macrophages with varying dose of LPS including 10 ng/ml and 100 ng/ml and assayed for TNFα production by its protein secretion and protein expression. As shown in Figure IV-8 whereas we were able to detect by ELISA TNFα production in the suboptimal dose LPS-stimulated

wildtype macrophages, this was defective in Dok3-deficient macrophages treated in a similar fashion. To confirm these findings, we also stimulated wildtype and Dok3-deficient macrophages with low and high doses of LPS and assayed for TNFα protein expression by western blotting. As shown in Figure IV-9, TNFα protein expression was not detectable in untreated wildtype macrophages; however this TNFα protein expression can be detected when treated with both low and high dose of LPS. In contrast, TNFα protein expression was only defective in Dok3-deficient macrophages when treated with a low dose of LPS but not with a high dose of LPS.

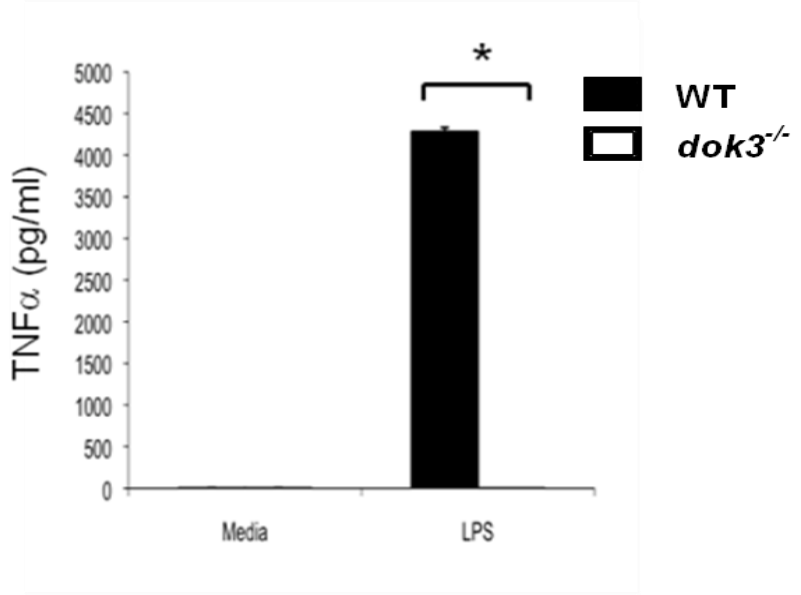

Figure IV-8 Dok3 regulates TNFα gene induction in low dose LPS-stimulated macrophages.
Real-time qPCR analysis of TNFα mRNA expression in wildtype (black columns) and Dok3$^{-/-}$ (white columns) macrophages stimulated with 10 ng/ml LPS for 2 hr. The amount of TNFα mRNA expression was normalized to that of actin mRNA. Figures shown are representative of at least two independent experiments.

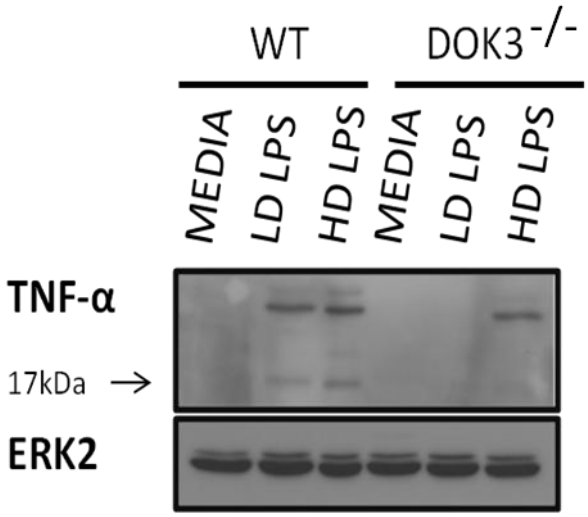

Figure IV-9 Defective TNFα production in low dose LPS-stimulated Dok3$^{-/-}$ macrophages.
Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 10 ng/ml (low dose) and 100 ng/ml (high dose) LPS for 2 hr. Cells were lysed and analyzed by immunoblotting with TNFα antibodies. Loading of equal amount of lysates were verified by immunoblotting with antibodies to total ERK. Data shown are representative of two independent experiments.

4.9 Impaired Production of COX2 and induction of iNOS mRNA Expression by Dok3 Deficiency in TLR4 Signalling

In addition to an excessive production of TNFα that can contribute to sepsis in mice, other known components of proinflammatory mediators of LPS-induced sepsis include prostaglandins such as COX2 and the reactive oxygen species such as iNOS. We have shown in *in vitro* experiments that TNFα production in a suboptimal dose of LPS stimulation requires Dok3 and *in vivo*, this defect in TNFα production by Dok3 deficiency confers resistance in the mice upon being challenged with low dose LPS. We investigated further if COX2 and iNOS induction may be perturbed by Dok3 deficiency. As shown in Figure IV-10, when wildtype macrophages are stimulated for 6 hours with LPS, COX2 protein expression was readily detected by western blotting. The COX2 protein expression however was defective by Dok3-deficiency when

stimulated in the same manner. We also stimulated wildtype and Dok3-deficient macrophages with LPS and measured iNOS gene expression via real-time qPCR and show here in Figure IV-11 that the gene expression of iNOS was defective in TLR4-stimulated Dok3-deficient macrophages but not in TLR4-stimulated wildtype macrophages. Taken together, these data indicated that Dok3 is required to induce TNFα, COX2 and iNOS production in a suboptimal threshold of TLR4 signaling.

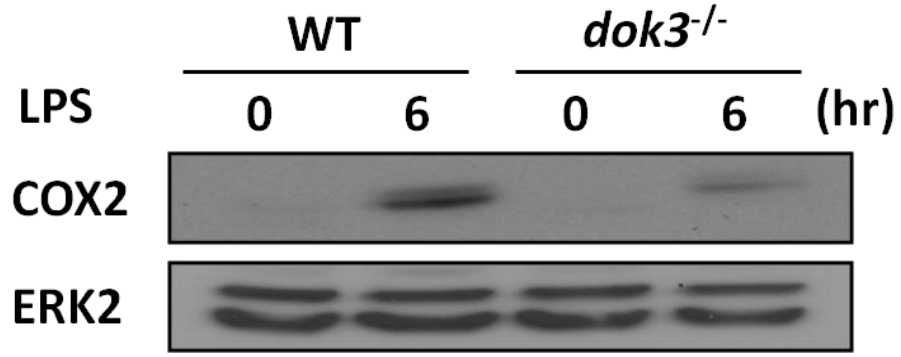

Figure IV-10 Defective COX2 production in LPS-stimulated Dok3$^{-/-}$ macrophages.
Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 10 ng/ml (low dose) LPS for 6 hours. Cells were lysed and analyzed by immunoblotting with COX2 antibodies. Loading of equal amount of lysates were verified by immunoblotting with antibodies to total ERK. Data shown are representative of three independent experiments.

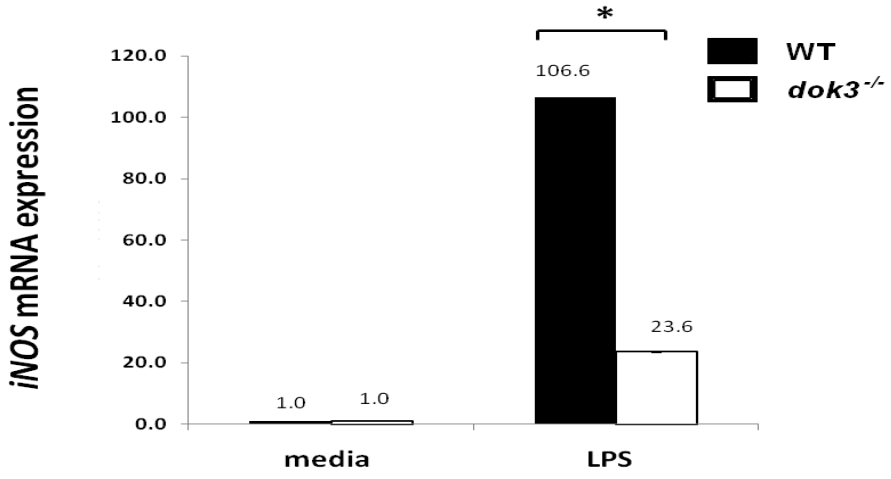

Figure IV-11 Dok3 regulates iNOS gene induction in low dose LPS-stimulated macrophages.
Real-time qPCR analysis of iNOS mRNA expression in wildtype (black columns) and Dok3$^{-/-}$ (white columns) macrophages stimulated with 10 ng/ml LPS for 2 hr. The amount of iNOS mRNA expression was normalized to that of actin mRNA. Figures shown are representative of at least 2 independent experiments.

4.10 Proteomic Analysis of Novel Dok3 Interacting Partner upon BMDM Stimulation with TLR4 Agonist

The results obtained so far with examining the role of Dok3 in TLR4 signaling reveal that the Dok3 regulatory role in TLR4 signaling is dependent on TLR4 signaling strength and dosage. In addition, we note that there appears to be no requirement of Dok3 in the MyD88 signaling axis in TLR4 activation in terms of inflammatory cytokine production but Dok3$^{-/-}$ mice are yet susceptible to low dose LPS septic shock. This makes Dok3 an interesting but complex target in TLR4 signaling and prompted us to ask if there could be other, novel interacting partners of Dok3 in TLR4 signaling. Thus we undertook a proteomic approach to examine possible Dok3 interacting partners in TLR signaling via mass spectrometry (MS). We show here in Figure IV-12, a list of different protein targets interacting with Dok3. One of these candidate targets that was pulled down by MS and is of interest is ABIN1.

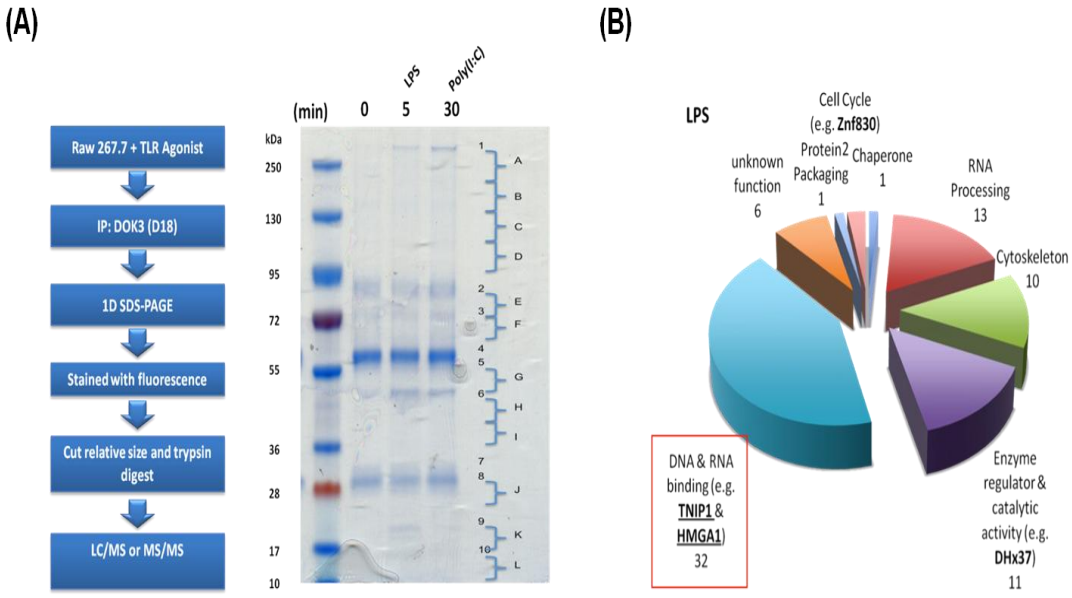

Figure IV-12 Mass spectrometry analysis of RAW264.7 stimulated with LPS.

(A) Protocol for mass spectrometry analysis and Coomassie Blue gel staining of immunoprecipitated RAW264.7 cell lysates stimulated with LPS and poly(I:C). (B) Pie chart demonstrating the novel proteins identified via mass spectrometric analysis of interacting partners of Dok3.

4.11 Dok3 interacts with ABIN1 *in vitro*

The A20-binding inhibitor of NFκB activation (ABIN1), also named as Naf1 (Nef-associated factor-1) or TNIP1 (TNFAIP3-interacting protein 1) was identified by yeast-two hybrid screening as an interacting partner to A20 (Heyninck *et al*., 1999). A20 was a known negative regulator of NFκB signaling and ABIN1 was found to modulate its inhibitory effect (Vereecke *et al*., 2009). NFκB played diverse roles in cellular gene expressions and processes (Gilmore, 2006). ABIN1 was demonstrated in one study where it can bind the MAP kinase ERK2 and down-regulate ERK2 signaling (Fang *et al*., 2011). ERK2 activation was known to be critical for cell growth and differentiation as well as apoptosis (Wada and Penninger, 2004). ABIN1-deficient mice were generated in 2009 but it was embryonically lethal with death resulting from fetal liver apoptosis, anemia and hypoplasia (Oshima *et al*., 2009).

To examine and validate our MS results on whether the candidate protein ABIN1 does indeed interact with Dok3 in TLR signaling, we stimulated wildtype macrophages in a time dependent manner with LPS and immunoprecipitated Dok3 and examined for ABIN1 interaction by western blotting. As shown in Figure IV-13, we could detect ABIN1 binding to Dok3 upon stimulation with LPS. This data confirms the results obtained with MS analysis and suggested that ABIN1 is a novel binding partner of Dok3 in TLR4 signaling in a physiological setting.

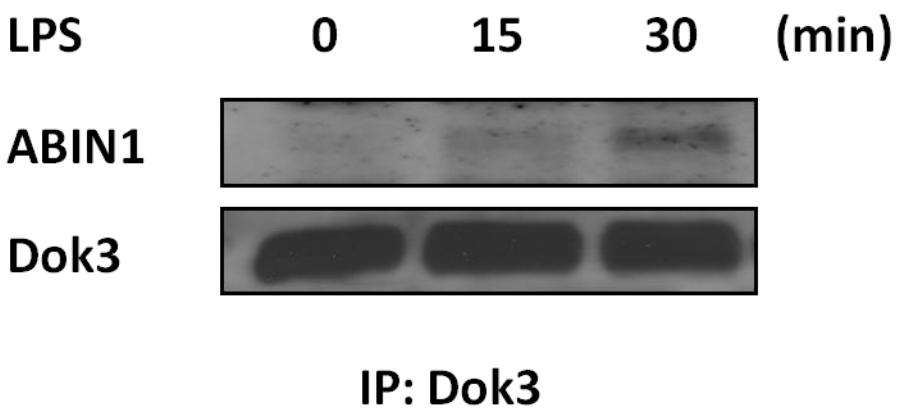

Figure IV-13 Dok3 interacts with ABIN1 upon LPS stimulation.

BMMϕ were stimulated for various times as indicated with 1 μg/ml LPS and subsequently lysed. Dok3 was immunoprecipitated and probed with antibodies to ABIN1. Figure shown is representative of two independent experiments.

4.12 A20 expression is impaired by Dok3 Deficiency in TLR4 signalling

ABIN1 was originally identified as an A20 interacting partner. A20 was demonstrated in a study to form a signaling complex with TPL-2 and p105 (Lang *et al*., 2004) and TPL-2 was known to be degraded upon LPS signaling (Beinke *et al*., 2004). Next we investigated if TPL-2, A20 and p105 protein expression could be perturbed in the absence of Dok3. To this end, we stimulated wildtype and Dok3-deficient macrophages with LPS and examined the protein expression of TPL-2, A20 and p105. We show here in Figure IV-14 that whereas TPL-2 degradation and p105 protein levels by LPS stimulation were comparable between TLR4-stimulated wildtype and Dok3$^{-/-}$ macrophages, A20 protein expression was defective from Dok3 deficiency in TLR4 signaling. This data suggest that Dok3 protein may be required for some regulatory role in A20/ABIN1 dependency in TLR4 signaling.

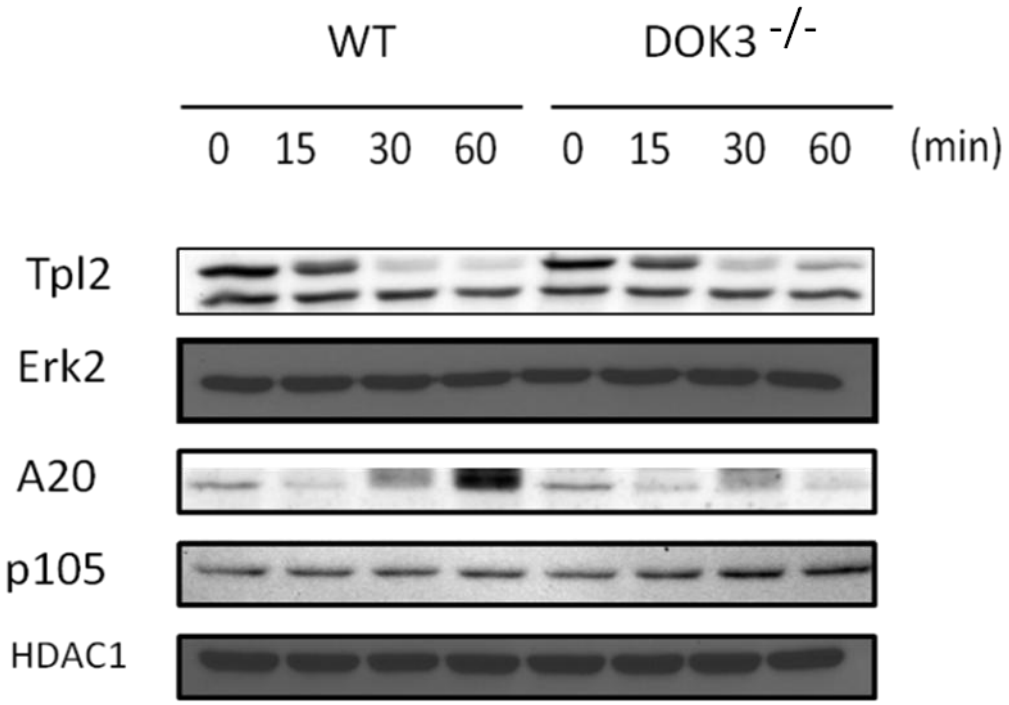

Figure IV-14 A20 protein expression is defective in LPS-stimulated Dok3$^{-/-}$ macrophages.
Wildtype and Dok3$^{-/-}$ macrophages were stimulated with 100 ng/ml LPS for the indicated timepoints. Cells were lysed and analyzed by immunoblotting with antibodies to (A) TPL-2, (B) A20 and (C) p105. Loading of equal amount of lysates were verified by immunoblotting with antibodies to total HDAC1. Data shown are representative of two independent experiments.

4.13 Discussion

Dok1 and Dok2 have been demonstrated to be negative regulators of TLR4 signalling (Shinohara *et al*., 2005). Negative regulators of signal transduction pathways may function in three different modes of actions. First, they can function to keep effector proteins from being activated in the quiescent state in the cells. Second, they can function to control the strength and threshold of activated signalling proteins at an appropriate level. Third, they can function to dampen or downregulate an on-going activated response (Sanada *et al*., 2008).

Dok3 differs from Dok1 and Dok2 in that it does not bind rasGAP (Mashima *et al*., 2009). It is unknown if this unique differential property of Dok3 from its other protein

family members will confer it a different identity in TLR signal transduction. The role of Dok3 in TLR4 signalling has not been demonstrated to date. Therefore, we utilised Dok3-deficient mice generated in our laboratory to examine the role of Dok3 in TLR4 signalling as part of this study. TLR4 signalling is unique as it utilises four adaptor proteins in its signal transduction pathways (Sandor and Buc, 2005). Unexpectedly and interestingly, we found that Dok3 positively regulates TLR4 signalling in a dose-specific manner, in contrast to its other family members Dok1 and Dok2. We demonstrated that although Dok3 is dispensable for high dose TLR4 stimulation in signalling to major signal transduction pathways including NFκB and MAPKs, Dok3 is however required for low dose TLR4 stimulation in signalling to activate ERK. Moreover, the requirement of Dok3 effect in low dose TLR4 signalling was exemplified in low-dose LPS sepsis model and this was attributable to the combined defects observed in proinflammatory TNFα secretion, activation of prostaglandins including COX2 and iNOS as well as in IFNβ production that resulted in the Dok3-deficient mice being more resistant to mortality as compared to wildtype mice.

The MyD88 signalling axis downstream of TLR4 is known to mediate proinflammatory cytokine production whereas the signalling mediate by TRIF downstream of TLR4 signals to produce IFNβ (Yamamoto *et al*., 2003). In this context, IL-6, TNFα (MyD88-dependent) and IFNβ (TRIF-dependent) production in TLR4 signalling requires Dok3 and suggest that Dok3 is required in both MyD88-dependent as well as TRIF-dependent pathway downstream of TLR4 for signalling to IFNβ. However, the signalling defect with Dok3-deficient cells was not easily observable when TLR4 signalling strength was increased. This perhaps suggests that Dok3 activation in TLR4 signalling is delicate and functions more as a modulator in

fine-tuning TLR4 activation. This may also explain why Dok3 loss-of-function phenotype could only be detectable when TLR4 signalling strength (i.e. LPS dose) is reduced in our studies.

Our hypothesis is that Dok3 may not only participate in TLR4 signalling via the current known signalling proteins such as MyD88 and TRIF, but that more proteins could be involved. To address this hypothesis, a MS analysis was performed to uncover novel proteins interacting with Dok3 in TLR signalling. A most interesting target that was immunoprecipitated in our TLR4 Dok3-interactome studies was ABIN1. ABIN1 is known as a negative regulator for TNFα production. ABIN1-deficient mice were generated previously and reported to exhibit embryonic lethality. However this developmental defect can be reversed by crossing the mice with TNFα-deficient homozygous background mice (Oshima *et al*., 2009). There are a number of studies on ABIN1 and its role in TLR signalling. For example, ABIN1 functions as a negative regulator of antiviral signalling by cooperating with TAXBP1 and A20 (Gao *et al*., 2011). In a separate study, ABIN1 was found to negatively regulate TLR-induced C/EBPβ activation and help protect the host from inflammatory disease (Zhou *et al*., 2011). It is intriguing as to why Dok3 and ABIN1 interact. There are several biological questions surrounding this interesting phenomena that warrant further investigation. First, what is the relationship between Dok3 and ABIN1? Dok3 appears to be a positive regulator in TLR signalling in our study whereas ABIN1 was documented as a negative regulator in other related studies (Fang *et al*., 2011; Oshima *et al*., 2009; Zhou *et al*., 2011). We speculate that ABIN1 may function to switch off TNFα production by Dok3 in TLR signalling upon activation to limit the excessive effects of inflammation that may be deleterious to the host cell. This observation is

consistent by our studies where we observed that the ABIN1 protein increasingly interacts with Dok3 in TLR4 stimulation in our immunoprecipitation experiments in macrophages when we pulled down Dok3. Second, how does Dok3 and ABIN1 effector functions affect each other in TLR-mediated signal transduction pathways? Dok3 contains a PH domain, a PTB domain and a SH2-target motif domain containing tyrosine residues whereas ABIN1 contains the ABIN homology domain (AHD), Ubiquitin Binding domain in ABIN proteins and NEMO (UBAN) and the Nucleotide binding domain (NBD). There appears to be no homologous or specific known domains of these two proteins that may mediate interaction. However, it is noteworthy that one particular post-translational modification (PTM), ubiquitination, is gaining increasing importance in signal transduction pathways as it is one critical way whereby immune homeostasis and balance is achieved. It may be possible that Dok3 may be ubiquitinated for either K48-mediated degradation or ubiquitin K63-linked activation and ABIN1 may act or inhibit PTM for subsequent biological activity. Last but not least, it is useful to understand if the phenotype of ABIN1 in TLR signalling is similar to Dok3 deficiency and if a combinatorial deletion in both molecules could yield a more striking phenotype as compared to the single knockout mice. However, ABIN1 is known to have a broad and indispensible role in organism development and its deletion in mice leads to embryonic lethality. Given all the implications, it is possibly necessary to investigate the defect of ABIN1 loss of function in TLR signalling in mice via a conditional knockout model. Another way to study the combined loss of function effect of Dok3 and ABIN1 would be to introduce siRNA to ABIN1 in Dok3$^{-/-}$ cells to remove ABIN1 expression and subject the double-deficient cells to *in vitro* immunological assays in TLR signalling to study combined loss-of-function biological effects.

Another family member of ABIN, ABIN2 has been demonstrated to form a protein complex with TPL-2 and p105, keeping them in their inhibitory state in TLR4 signalling. Upon TLR4 crosslinking, ABIN2 gets sequestered, leading to the degradation of p105 in the proteasome and the release of p50 homodimer to mediate NFκB activation. On the other hand, TPL-2 can activate the MAPK signal transduction pathway for TNFα and COX2 production via ERK signalling (Gantke *et al*., 2011). We postulate here that ABIN1 may function in the same manner as ABIN2 in TLR4 signalling and the sequestration of ABIN1 from the signalosome complex requires Dok3 (Figure IV-15). Future experiments will be aimed to fathoming out this possibility.

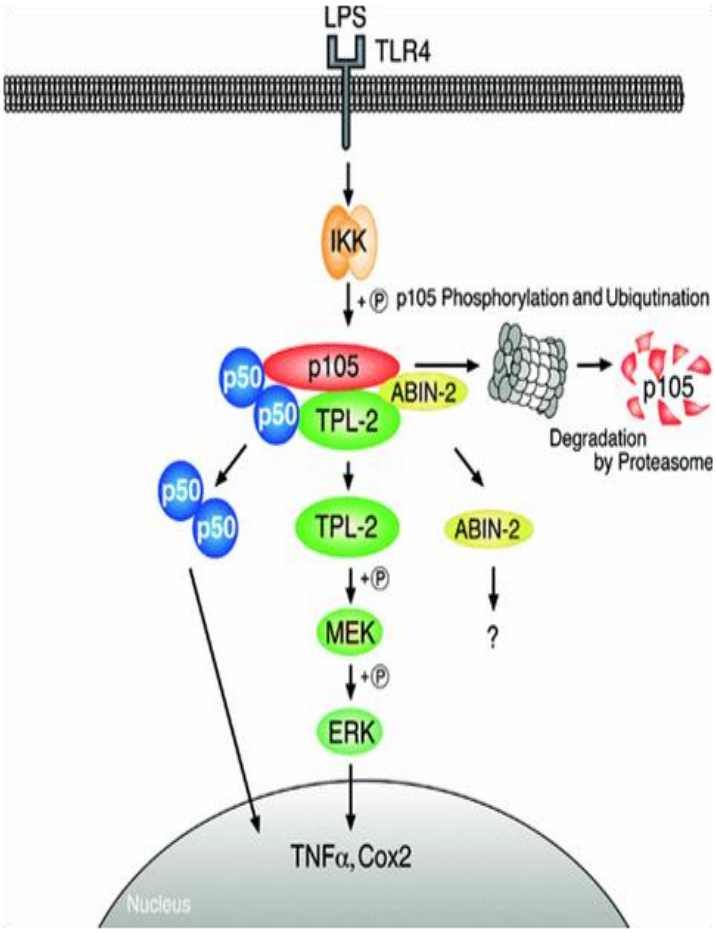

Figure IV-15 A model for ABIN2 role in TLR4 signalling.

Regulation of TPL-2 through IKK-induced p105 proteolysis. TPL-2 is confined to a cytoplasmic complex with the NFκB1 precursor protein p105, and the ubiquitin-binding protein ABIN2. NFκB1 p105 has multiple functions. First, it serves as a precursor molecule for the NFκB1 p50 subunit, which is generated by limited proteolysis (processing) of p105 by the proteasome, and removes p105's C-terminal. Second, p105 functions as a classical IκB, retaining associated NFκB subunits in the cytoplasm. Cellular stimulation with TLR ligands, for example LPS, induces the formation of receptor proximal complexes that trigger activation of the MAP3K, TAK1. Activation loop phosphorylation of IKK2 by TAK1, in turn, activates IKK2 to phosphorylate the target residues S927 and S932 in p105, creating a binding site for the SCFβTrCP ubiquitin E3 ligase complex. K48-linked ubiquitination of p105 by SCFβTrCP triggers its complete degradation by the 26S proteasome. IKK-induced proteolysis of p105 releases associated NFκB dimers, which then translocate to the nucleus and modulate expression of target genes, similar to the activation of NFκB dimers in the classical pathway. Stimulus-induced p105 proteolysis also couples activation of NFκB pathways to MAP kinase signalling by releasing TPL-2 from p105 inhibition. After liberation from p105, TPL-2 directly phosphorylates MEK and thereby activates downstream ERK MAP kinase signalling. Proteasomal degradation of p105 also releases ABIN2 from association with p105 and TPL-2. The function and downstream targets of ABIN2 are not known (Gantke *et al*., 2011).

CHAPTER V. GENERAL DISCUSSION

5.1 General Discussion

To gain more insight into the physiological role of adaptor protein Dok3 in the innate signalling pathway, we analysed the effects of Dok3 deficiency in various PRRs including membrane TLR4, endosomal TLR3 and cytosolic sensor RIG-I. We show in this study that Dok3 plays a critical role in antiviral PRRs signalling including TLR3 and RLR signalling. Dok3 is phosphorylated upon TLR3 and RLR engagement and its deficiency impaired the secretion of proinflammatory cytokines and IFNβ in naked poly(I:C)-treated macrophages as well as IFNβ induction in transfected poly(I:C)-treated macrophages. As a result of reduced cytokine production, $Dok3^{-/-}$ mice were more resistant to D-GalN-sensitized poly(I:C)-induced septic shock. In these aspects, the responses of $Dok3^{-/-}$ macrophages and mice to poly(I:C) stimulation resemble those of $TLR3^{-/-}$ and $BTK^{-/-}$ cells and mice. Both TLR3 and BTK-deficient cells also had reduced production of cytokines in response to poly(I:C)-stimulation and $TLR3^{-/-}$ and $BTK^{-/-}$ mice were also resistant to D-GalN/poly(I:C)-induced septic shock (Alexopoulou *et al*., 2001; Lee *et al*., 2012). These observations suggest that Dok3 could be part of the TLR3 signalling pathway.

Engagement of TLR3 triggers the induction of multiple signalling pathways that culminate in the activation of NFκB, AP-1 and IRF3 that are critical for the production of cytokines and interferons (Vercammen *et al*., 2008). At the biochemical level, our data indicated that Dok3-deficiency specifically affects TLR3-induced IRF3 activation and this led us to hypothesize that Dok3 could act somewhere along the TRIF/TBK1/TRAF3 signalling axis to IRF3 for IFNβ production. The *de novo* identity of Dok3 as an adaptor protein prompted us to examine if Dok3 is required to

function as a scaffold protein in TLR3 signalling to mediate the production of IFNβ. We ectopically expressed HA and/or FLAG-tagged plasmids of Dok3 independently with the various known signalling proteins involved in TLR3 pathway including TRIF, TBK1, TRAF3 and BTK and found that Dok3 interacts directly with BTK, TBK1 and TRAF3 but not with TRIF. As such, Dok3 may function as an adaptor protein at the level of TBK1 and TRAF3 to enable TBK1 and TRAF3 to form a complex that is known to be necessary for IRF3 activation. We further identified the SH2-target motif of Dok3 as important for its interaction with TBK1 and the PTB plus SH2-target motif for TRAF3. Consistent with this finding, the co-expression of Dok3 with TBK1 plasmid synergistically promoted IFNβ production in an IFNβ luciferase assay and a mutant Dok3 plasmid lacking the SH2-target motif but not the PH domain could ablate this enhanced IFNβ promoter effect. The net effect of a resultant loss of the Dok3 effect of interacting with TBK1 downstream of TLR3 is a compromised TBK1 activity, and the subsequent IRF3 phosphorylation and its nuclear translocation to bind the ISRE consensus region of the IFNβ enhanceosome and optimally induce IFNβ. It was recently demonstrated that BTK is the tyrosine kinase that phosphorylate critical tyrosine residues on TLR3 in order to initiate antiviral responses (Lee *et al*., 2012). We also showed in our studies that Dok3 undergoes tyrosine phosphorylation in its carboxyl tail. Our work also examined if BTK could be the tyrosine kinase that phosphorylate Dok3 and this was indeed the case. In addition, BTK and Dok3 could interact directly as demonstrated from our overexpression studies using HEK293T cells.

One of the most intriguing questions in this project lies in where Dok3 localizes upon TLR3 engagement. TLR3 is an endosomal bound receptor (de Bouteiller *et al*., 2005)

and Dok3 is known to be translocated to the plasma membrane via its PH domain in BCR signalling (Stork *et al*., 2007). However, the TRIF multimer complex that forms upon TLR3 signalling was shown to translocate from the receptor to form speckles in the cytosol (Funami *et al*., 2007). If Dok3 was part of this complex, it may suggest that Dok3 activity following TLR3 stimulation is independent of its PH domain. This would contrast with the current knowledge indicating the importance of Dok3 PH domain for BCR signalling (Stork *et al*., 2007) adding further complexity into the biology of Dok3's regulation. Further experiments such as confocal analysis of Dok3 localization in TLR3 stimulation will distinguish these possibilities.

In addition to the above findings, our studies indicated that Dok3 may participate in signalling pathways leading to IFNβ production in multiple receptors systems including TLR and RLR. This view comes from the fact that Dok3 absence leads to defective IFNβ signalling not only upon TLR3 and TLR4 activation (which signals through TRIF) but also via stimulation with transfected poly(I:C) and upon influenza virus infection where these stimulants were known to engage RLRs and signals through IPS-1 for IFNβ production. The commonality between signalling via the TLRs including TLR3 and TLR4 and the RLRs pathways is that despite the differential usage of adaptor proteins, they converge on TBK1 and TRAF3 to activate IRF3 transcription factor for IFNβ production. We have shown that in Dok3, it does not bind to TRIF, but instead bind to TBK1 via its SH2-target motif for signalling to IFNβ. We hypothesize that Dok3 might not interact with IPS-1 in this situation. Future experiments will be aimed at investigating this possibility. However, based on our current understanding of the Dok3 role in TLR3 signalling, Dok3 may not be required to bind IPS-1 (analogous to the fact that it does not bind TRIF) to signal

downstream of RLRs engagement for IFNβ production and the binding to TBK1 in RLR-mediated signalling may already be sufficient for Dok3 to exert its effect and induce IFNβ in RLR signalling.

The other question is whether BTK is required to phosphorylate Dok3 in RLR-dependent signalling instead. This is because although BTK is required to phosphorylate Dok3 in TLR3 signalling, it is dispensable for the RLR-mediated induction of IFNβ however (Lee *et al*., 2012). Dok3 may have a much broader and more significant role than BTK in the signal transduction pathway leading to an antiviral response since we showed that Dok3 is required for both TLR3 and RLR signalling pathway whereas BTK's role is only restricted to TLR3 only. It is also important to note that the multifaceted role of Dok3 as a modulator of antiviral interferon makes it a much more attractive therapeutic target or approach than those drugs that simply inhibit or activate kinases or phosphatases as shutting down or turning on a whole signal transduction pathway may yield a more detrimental outcomes in disease control as compared to fine tuning the effects to produce a better option.

We demonstrated that Dok3 is also involved also in TLR4-mediated TRIF dependent pathway of signalling to IFNβ. However, TLR4 is known to be the receptor for endotoxin responses that is usually activated by microbial infections. Thus, the role of Dok3 in TLR4 may be much broader than thought of currently as being required for the activation of IFNβ downstream of TLR4. We examined the effects of Dok3 in TLR4 signalling by LPS stimulation and showed that there is a dose-dependent requirement of Dok3 by the signalling strength of TLR4. For example, when we

investigated the requirement of Dok3 in the TLR4-induced MAPK using a higher dose of stimulant, there was no observable differences between wildtype controls and Dok3-deficient macrophages. More strikingly however, there was a defect in activating ERK MAPK by Dok3-deficiency when the cells were stimulated with a 10-fold dosage decrease of the LPS stimulant. The resultant effects of a lower signalling strength of LPS yielded more accompanying phenotypes in Dok3-deficient mice and cells including the mutant mice being resistant to LPS-induced sepsis and an impaired TNFα proinflammatory cytokine production. From such observations and findings, we speculate that Dok3 may be involved in endotoxin tolerance and future studies will investigate this possibility. Endotoxin tolerance is characterised by refractive responses in immune cells subsequent to LPS challenge prior to its initial stimulation in terms of inflammatory reaction (Chae, 2002). Our understanding of Dok3's requirement in only suboptimal TLR4 signalling may drive us to further investigate if the phenotype of Dok3-deficiency may perhaps only be unveiled in a subsequent LPS challenge after prior low dose LPS exposure in an endotoxin tolerance model.

The lack of effects of Dok3 on TLR4 signalling in impacting some of the major signal transduction pathways that were examined including NFκB and MAPKs have also prompted us to ask if there could be other novel molecules involved in TLR4 signalling that are associated with Dok3. In answer, we undertook a MS proteomics approach to identify novel proteins that could interact with Dok3 in this pathway. We immunoprecipitated Dok3 from TLR3- and TLR4- stimulated RAW cell (or BM derived macrophages), lysates and subjected it to MS to identify the possible candidates. Some of the candidates that were identified were of significant interest. One of these interesting candidates that are related to our research focus is ABIN1.

We decided to focus our understanding on the role of Dok3 interaction with ABIN1 as this interaction may be involved in the regulation of the TNFα contributing role in sepsis. The pro-inflammatory response in LPS signalling needs to be a tightly regulated process and this positive regulation was balanced by the up-regulation of several negative regulators. The NFκB inhibitor, IκBα was up-regulated along with TNFAIP3, also known as A20, and its interacting partner TNIP1 (ABIN1). A20 and ABIN1 inhibit the activation of NFκB through the de-ubiquitination of IKKγ (NEMO) (Song *et al*., 1996). We found that Dok3-deficiency results in defective TNFα production and this translates to better protection against sepsis in the LPS challenge *in vivo*. This suggests that Dok3 functions as a positive regulator in LPS-induced TNFα production and in its absence, the defective production of this deleterious cytokine helped protect the mice from the lethal effects of sepsis. In contrast, ABIN1 was demonstrated to be a negative regulator of NFκB and TNFα production and conditional ABIN1 KO mice showed that they were more susceptible to mortality by LPS-induced sepsis compared to wildtype mice (Zhou *et al*., 2011). It is therefore intriguing that we found these two proteins in our MS studies and immunoprecipitation studies from LPS-stimulated macrophages. We hypothesized the following scenarios.

First, Dok3 acts as a positive regulator and at the same time binds to a negative regulatory protein to control the duration and magnitude of the inflammation process to avoid overt inflammation. This is a scenario whereby cross-talk between signalling pathways happen and the cell evolve a delicate balance by having both positive and negative proteins acting together to maintain homeostasis.

Second, it was found in BCR signalling that Dok3 sequesters Grb2 away from the GTP exchange factor (GEF) son-of-sevenless (SOS), thereby preventing efficient activation of the Ras/ERK cascade (Stork *et al*., 2007). A similar scenario may happen whereby in a physiological setting upon LPS signalling, ABIN1 has to be turned on to downregulate the positive signalling to TNFα, and over time Dok3 functions then begin to sequester away ABIN1. This may explain why in Dok3$^{-/-}$ macrophages, where there is a lack of Dok3 protein expression to sequester the activating function of ABIN1, ABIN1 functions as a constitutive negative regulator with A20 and prevents TNFα production by inhibiting NFκB activation (Figure V-1).

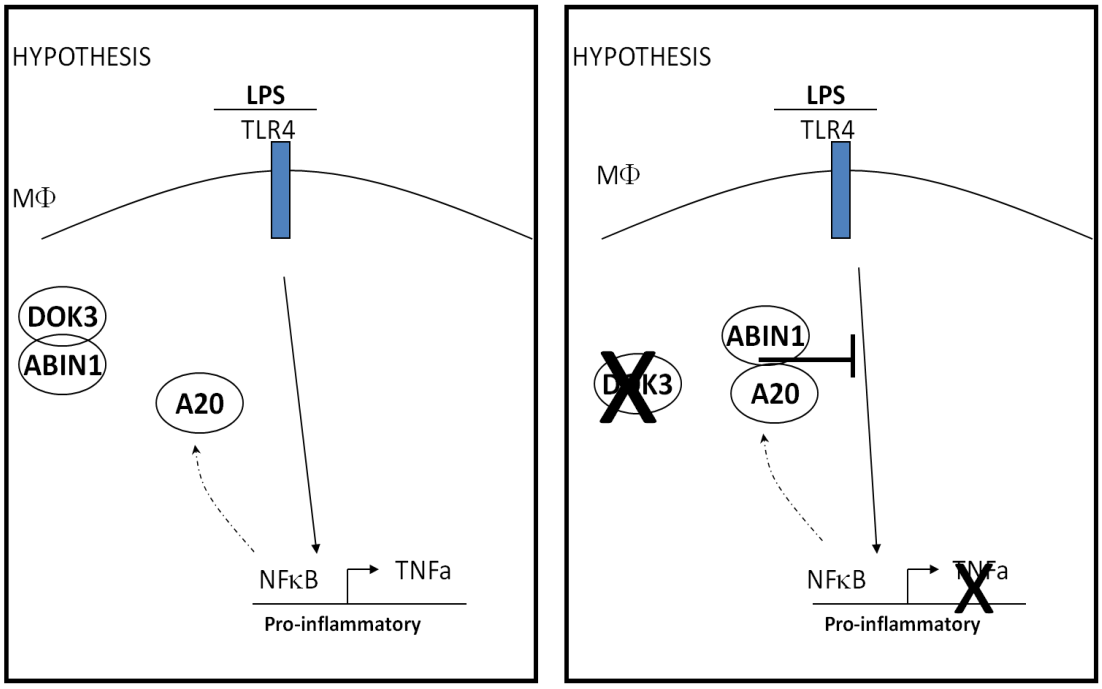

Figure V-1 Hypothetical model of Dok3 and ABIN1 roles in TLR4 regulation of TNFα.

Upon activation of TLR4 by LPS, ABIN1 has to be turned on to downregulate the positive signalling to TNFα, and Dok3 functions to sequester ABIN1 over a regulated timeframe to allow TNFα production to occur in order to maintain immune homeostasis. However in the absence of Dok3 there is no sequestration of ABIN1 from A20 and therefore these two proteins are constitutive active and continually inhibit the NFκB pathway from activating TNFα production.

This would explain the lack of TNFα being detected by Dok3$^{-/-}$ macrophages upon LPS signalling. However, the kinetics of Dok3 and ABIN1 regulation and activation has to be carefully considered as critical parameters in this TLR-induced setting to definitively place the hierarchy of these proteins in the signalling pathways evoked by LPS.

Third, ABIN1 was demonstrated to be able to bind the MAP kinase ERK2 and down-regulate ERK2 signaling. We have also observed that with a suboptimal dose of LPS signaling, Dok3-deficient macrophages exhibited defective activation of ERK. To take these two observations together, it appears that Dok3 and ABIN1 may have some opposing effects in regulating ERK activation. This would also be consistent with their regulatory functions in that Dok3 is a positive while ABIN1 is a negative regulator from ours and other studies (Oshima *et al*., 2009; Zhou *et al*., 2011). It is interesting to check if Dok3 may perhaps regulate the protein expression of ABIN1. One likely scenario is that if this is the case, it may explain that in the absence of Dok3 in macrophages, ABIN1 mRNA and protein expression may be abrogated as a result or even be increased, if Dok3 negatively regulate ABIN1. In this postulation, there will be more ABIN1 protein which in turn binds to ERK2 and inhibit its activation. This possibility may also be extended to explain how Dok3 deficiency leads to an increase in ABIN1 expression which in turn leads to a constitutive inhibition of TNFα production. More experiments would have to be performed to confirm these hypotheses.

In summary, we demonstrated that Dok3 is involved in TLR signalling, namely with TLR3 and TLR4, both of which use a common TRIF adaptor protein to induce type 1

interferon, IFNβ. Here, we showed that in contrast to other Dok family members that were demonstrated to be negative regulators of TLR signalling (Shinohara *et al*., 2005; Zhou *et al*., 2011), Dok3, to our surprise exists as a positive regulator in inducing IFNβ. We defined in detail the mechanism of the Dok3 positive regulatory role in TLR3 and TLR4 signalling to IFNβ and found BTK to be the kinase involved in phosphorylating Dok3 for its regulatory function. Dok3$^{-/-}$ mice are more resistant to poly(I:C)-induced endotoxin shock, owing to a defective IFNβ signalling.

We see a scenario for the role of Dok3 in TLR3 signalling with our current data. Upon activation by dsRNA, it leads to the dimerisation and activation of TLR3. This possibly results in the recruitment of TRIF and BTK of which BTK is required to fully phosphorylate TLR3 at tyrosine 759 residue (Lee *et al*., 2012). Previously, c-SRC was shown to be induced by dsRNA and leads to IRF-3 activation. However Lyn, a SRC family member could not directly phosphorylate TLR3 (Johnsen *et al*., 2006). Indeed, c-SRC was shown to associate with TRAF3 and this occurred further downstream in TLR3 signalling. PI3K is then recruited to phosphorylate TLR3 on tyrosine 759 residue to mediate IRF3 activation via the AKT pathway. On the other hand, BTK activates the TRIF-dependent major signalling pathways downstream of TLR3 including MAPKs, NFκB and TBK1-mediated IRF3 activation. All of these cumulate in an effective assembly of a functional β-interferon enhanceosome to secrete IFNβ, a critical type I IFN for an anti-viral immune response. Additionally we found that Dok3 played a critical adaptor function to mediate the TBK1 signalling pathway to induce IFNβ. Furthermore upon TLR3 stimulation, BTK is activated and interacts with TRIF. Dok3 may also shuttle or bridge various signalling components to signalosome by interacting with BTK. Dok3 then binds to TBK1 via its SH2-target

motif and TRAF3 via its PTB and SH2-target motif. This adaptor function of Dok3 further allows TBK1 and TRAF3 to tether to TRIF for TBK1's activation. An activated TBK1 on serine 172 residue is now able to function as a serine threonine kinase and phosphorylate its substrate IRF3 on serine 396 which allows its dimerisation and nuclear translocation and binding to the ISRE region of the DNA to induce optimal IFNβ production. The AKT pathway activated by PI3K binding to the tyrosine phosphorylated residues of an activated TLR3 was also found to be defective by Dok3 deficiency. More recently, this pathway of AKT activation was found to converge downstream in the TLR3 pathway to activate TBK1 leading to an optimal activation of IRF3. Since lack of Dok3 affects AKT activation, it may be possible that Dok3 perhaps interact with AKT or interferes with TLR3 signalling by AKT in some way.

Finally, since dsRNA is a key replication intermediate of several viruses and is recognized by TLR3, our work suggests that drugs that activate Dok3 could be relevant for the treatment of various viral infections including dengue and influenza. However, there is to date no known chemical compound that could activate Dok3. Any chemical activator of Dok3 would have to activate the enzyme at an appropriate level and only transiently in order to prevent excessive inflammation in the host.

We found BTK to be the kinase that activates Dok3 in our studies. There are known chemical inhibitors of BTK undergoing clinical trials for the treatment of B-cell driven autoimmune diseases and B-cell malignancies such as non-Hodgkin lymphoma, mantle cell leukemia and chronic lymphocytic leukemia. BTK-inhibitors could also be of use in the treatment of certain viral infections where there is a need to

limit excessive inflammatory responses that could have a deleterious effect on patients. Some examples include the pandemic flu and SARS coronavirus infections where excessive host response results in a “cytokine storm” that ultimately kills the host. BTK-inhibitors could theorectically be used to dampen the “cytokine storm” and therefore appears to be a potential attractive target.

Although the current trend in therapeutic design appears to be directed towards kinases or phosphatases that either switch “on” or “off” signalling pathways, it may not be ideal as shutting down or activating bulk pathways may lead to detrimental or undesirable outcomes or side effects. We propose that new screening methodologies for drug targets be aimed at activating or deactivating proteins involved in moderation or “fine-tuning” specific responses, for example Dok3 in this case for antiviral studies. This is so as our research findings show, Dok3 is specific in activating the TBK1-IRF3 signalling axis to IFNβ and therapeutics based on targeting specificity in particular pathways may be a much more desirable prophylactic measure in clinical requirements for viral disease control.

In addition to Dok3’s role in TLR3 signalling, we have also examined its role in TLR4 signalling. TLR4 signalling is also required to signal IFNβ production via the TRIM-TRAM pathway. Our postulation is that Dok3 also plays a role in TLR4 signalling to IFNβ. Indeed we found that Dok3 impacts TBK1 and IRF3 activation and subsequently IFNβ production. TLR4 activation also evokes major signalling events including NFκB and MAPKs for the production of inflammatory cytokines required in an anti-microbial immune response. This explicit inflammation process has to be tightly regulated otherwise it will lead to sepsis (Raetz and Whitfield, 2002).

We found that Dok3 is required for TNFα production in a suboptimal dose of TLR4 activation and its absence confers resistance to mice in an LPS-induced sepsis model. In addition, many other key mediators of sepsis in mice including COX2 and iNOS activation were found to be defective with Dok3 deficiency. In contrast, there appears to be no major requirement for Dok3 when TLR4 signalling strength was increased. This phenomenon suggested that Dok3 may probably be required for a condition known as endotoxin tolerance where prior exposure of cells to suboptimal dose of endotoxin will confer a refractory response upon re-stimulation with a lethal dose later on. Further investigations in this area will be performed to prove the hypothesis. While this report writing was in progress, a paper was published describing Dok3 as a negative regulator of TLR4 signalling in the same manner as Dok1 and Dok2 by inhibiting ERK, contrary to our findings (Peng *et al*., 2012). However, the doses of LPS used by Peng *et al* were high doses ranging from 100 ng/ml to 1 μg/ml. Their claim that ERK was enhanced in the absence of Dok3 may be premised differently as their results clearly showed that the differences were not as significantly different as compared to wildtype controls upon LPS stimulation. In addition, the mice that were utilised in their studies were of 129S1/SvImJ strain, whereas our mice were of the C57BL6 strain. The different strains of mice used may yield different experimental outcomes under different stimuli as discussed in past literature (Kamath *et al*., 2003; Ripoll *et al*., 2010).

Last but not least, the lack of major signalling defects by Dok3 deficiency in higher doses of TLR4 stimulation prompted us to undertake a MS proteomic approach to identify novel interacting partners of Dok3 that would coherently participate in TLR4 signalling. We have identified one such candidate, ABIN1 to be the novel target.

Given that much research was performed to study the role of ABIN1 in endotoxin tolerance, it is intriguing to see how Dok3 and ABIN1 participate in activating TLR4 contribution to sepsis. Further experiments in establishing the overlapping roles of Dok3 and ABIN1 in TLR4 signalling will explain the oddity. Finally the discovery of Dok3's role in TLR4 signalling could mean that Dok3 represents a novel target for chemo-therapeutic development against sepsis.

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