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Tyrosine Phosphorylation and Structural Requirements Mediate Toll-Like Receptor 9 Function

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Abstract
Upon invasion of microbial pathogens, cells of the innate immune system respond through the activation of pattern recognition receptors (PRRs) that recognize pattern associated molecular patterns (PAMPs). Once these receptors bind ligand, they initiate a signaling cascade culminating in the expression of proinflammatory and antiviral cytokines, costimulatory molecules, and antimicrobial agents, all of which contribute to pathogen clearance and host defense. However, excessive signaling through these receptors can lead to inflammatory conditions resulting in damage to the host. Therefore, understanding the signaling events downstream of PRR activation is critical for gaining insight into targeting specific mediators for therapeutic intervention to combat infection and to limit host pathology. Canonically, Toll-like receptors (TLRs), one class of PRR, have been thought to signal through serine threonine kinases following ligand recognition. However, there is emerging evidence for the role of protein tyrosine kinases regulating TLR function, but their role is not entirely clear. We sought to understand how TLR9 function is affected by a conserved tyrosine residue in its cytoplasmic domain and by activation of the protein tyrosine kinase Syk. We initially hypothesized that Syk might be participating in tyrosine phosphorylation of TLR9 to induce downstream signaling following receptor activation with CpG DNA. Utilizing genetic deletion of Syk in dendritic cells in vivo and genetic knockdown in a macrophage cell line, here we demonstrate that Syk is important for the intracellular trafficking and exocytosis of the proinflammatory cytokine TNFa, but not IL-6, following CpG stimulation. This secretion event involved activation of calcium signaling and calcium calmodulin kinase II (CaMKII) downstream of Syk. Syk-deficient cells exhibited normal CpG-induced activation of the canonical TLR9 signaling machinery, suggesting that Syk mediates a signaling cascade to promote cytokine exocytosis independent of cytokine transcription and translation, a role unexpected based on its function downstream of ITAM-bearing receptors. These data implicate this signaling pathway in a novel role of cytokine sorting and may have broader implications for release of other cytokines downstream of various pattern recognition receptors.

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ABSTRACT

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Upon invasion of microbial pathogens, cells of the innate immune system respond through the activation of pattern recognition receptors (PRRs) that recognize pattern associated molecular patterns (PAMPs). Once these receptors bind ligand, they initiate a signaling cascade culminating in the expression of proinflammatory and antiviral cytokines, costimulatory molecules, and antimicrobial agents, all of which contribute to pathogen clearance and host defense. However, excessive signaling through these receptors can lead to inflammatory conditions resulting in damage to the host. Therefore, understanding the signaling events downstream of PRR activation is critical for gaining insight into targeting specific mediators for therapeutic intervention to combat infection and to limit host pathology. Canonically, Toll-like receptors (TLRs), one class of PRR, have been thought to signal through serine threonine kinases following ligand recognition. However, there is emerging evidence for the role of protein tyrosine kinases regulating TLR function, but their role is not entirely clear. We sought to understand how TLR9 function is affected by a conserved tyrosine residue in its cytoplasmic domain and by activation of the protein tyrosine kinase Syk. We initially hypothesized that Syk might be participating in tyrosine phosphorylation of TLR9 to induce downstream signaling following receptor activation with CpG DNA. Utilizing genetic deletion of *Syk* in dendritic cells in vivo and genetic knockdown in a macrophage

cell line, here we demonstrate that Syk is important for the intracellular trafficking and exocytosis of the proinflammatory cytokine TNFα, but not IL-6, following CpG stimulation. This secretion event involved activation of calcium signaling and calcium calmodulin kinase II (CaMKII) downstream of Syk. Syk-deficient cells exhibited normal CpG-induced activation of the canonical TLR9 signaling machinery, suggesting that Syk mediates a signaling cascade to promote cytokine exocytosis independent of cytokine transcription and translation, a role unexpected based on its function downstream of ITAM-bearing receptors. These data implicate this signaling pathway in a novel role of cytokine sorting and may have broader implications for release of other cytokines downstream of various pattern recognition receptors.

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Chapter 1

Introduction

Innate recognition of microbial products: A historical perspective

The late Charles Janeway, one of the leading immunologists of his generation, proposed a theory on pattern recognition which provided a conceptual framework for understanding the role of innate immunity and how it activated the adaptive immune response (Janeway, 1989). At the time, it was known that microbial stimuli could induce macrophage function (Steinman and Moberg, 1994), and that poly IC, a synthetic analog of pathogenic RNA, activated type I interferon genes (Nagata et al., 1980). However, integration between innate and adaptive immunity was not well appreciated, and studies on either system were occurring independently of others. In his publication, Janeway hypothesized that costimulatory signals activating lymphocytes were induced by conserved microbial products. These agents, termed pathogen-associated molecular patterns (PAMPs), were proposed to be recognized by germline-encoded receptors, distinct from the gene-rearranged receptors expressed by T and B lymphocytes. Furthermore, Janeway suggested that adjuvants "trick" the cells of the adaptive immune system into activation by first inducing costimulatory molecules via innate immune receptors. While his theory was overlooked at first, subsequent discoveries elucidating pattern recognition pathways validated Janeway's hypotheses.

It was postulated that cell surface receptors would recognize PAMPs and induce expression of costimulatory molecules via Rel-type transcription factors. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) was known to play a role in the innate immune response (Kopp and Ghosh, 1995), and one such receptor known to activate this molecule was the interleukin-1 (IL-1) receptor. Interestingly, the IL-1 receptor contained a cytoplasmic domain with structural homology to the Toll receptor

identified in *Drosophila* (Hashimoto et al., 1988). Proteins demonstrating homology to this TIR (Toll, IL-1R and resistance protein) domain were identified from a National Center for Biotechnology Information (NCBI) database in the mid-1990s (Nomura et al., 1994), and ultimately Toll like receptor (TLR) 4, homologous to the *Drosophila* Toll, was cloned and isolated. Consistent with a role in innate immune responses, this receptor demonstrated the ability to activate NF-κB. An exciting discovery was made soon after describing an antifungal role for the *Drosophila* Toll, suggesting that perhaps the mammalian Toll-like receptor would also play a role in immune function and activate costimulatory molecules (Lemaitre et al., 1996). Indeed, Medzhitov and Janeway demonstrated that the constitutively active human homologue of Toll could induce expression of the cytokines IL-1, IL-6, and IL-8 and the costimulatory molecule B7.1 (Medzhitov et al., 1997). Subsequently, other Toll-like receptors (Chuang and Ulevitch, 2000; Rock et al., 1998) and their ligands (Takeda et al., 2003) were identified.

In addition to TLRs, other receptors engaging microbial products and their functions were discovered. These include NOD1 (Inohara et al., 1999) and NOD2, cytosolic sensors of bacterial peptidoglycans (Chamaillard et al., 2003; Girardin et al., 2003a; Girardin et al., 2003b; Inohara et al., 2003). The fungal component β-glucan was found to trigger the receptor Dectin-1 (Brown and Gordon, 2001). In 2004, the cytosolic sensors of double-stranded RNA, RIG-I and MDA-5, were identified (Yoneyama et al., 2004). Subsequent studies of these receptors demonstrated important roles in immune recognition and host defense.

Activation of these pattern recognition receptors triggers the adaptive immune system by the upregulation of costimulatory molecules and through the secretion of effector cytokines. These studies provided experimental evidence that Janeway's hypothesis was correct. Because of the essential role these receptors play in innate and adaptive immune responses, it is important to continue to study their signaling properties

and functions. It is likely that new receptors and new mechanisms of immune activation will be uncovered with further investigation.

The biology of Toll-like receptors

As Janeway predicted, pattern recognition receptors contribute to the activation of adaptive immunity by causing the upregulation of costimulatory molecules on antigen presenting cells, like dendritic cells (DCs). In addition to their ability to upregulate expression of CD80 and CD86, pattern recognition receptors also signal for the production of chemokines and proinflammatory cytokines, including IL-6, IL-12, and tumor necrosis factor alpha (TNFα). Upon secretion, these effectors contribute to the immune response by recruiting other cells of the innate immune system, like monocytes and neutrophils, facilitating differentiation of T lymphocytes, activating phagosomal lysis of pathogens, and promoting inflammation. While these responses are crucial for host defense, excessive signaling through these receptors can lead to inflammatory conditions. Discussed below is the role of one type of PRR, TLRs, in pathogen clearance and in inflammatory conditions.

TLRs are type I transmembrane proteins containing a leucine rich repeat domain for pathogen recognition and a cytoplasmic TIR domain mediating downstream signaling. These receptors are primarily expressed by cells of the hematopoetic system, including the innate immune cells macrophages and DCs. There are ten TLRs present in humans and twelve expressed in mice. Each TLR is triggered by a different PAMP. These PAMPs include nucleic acids, lipoproteins, lipids, and proteins derived from such pathogens as viruses, bacteria, and fungi. TLRs gain access to their ligands via their localization within the cell. For example, nucleic acids are recognized by the endosomal TLRs, TLRs 3, 7, 8, and 9, while other PAMPs activate the cell surface receptors, TLRs

1, 2, 4, 5, 6, 11, 12, and 13. Compartmentalization of TLRs offers a useful strategy for regulation of the initiation of appropriate immune responses (Barton and Kagan, 2009).

Toll-like receptors in host defense

Numerous studies utilizing mice deficient in various TLRs have uncovered a central role for these receptors in host cell recognition and defense against microbial pathogens, including bacteria, viruses, fungi, protozoa, and parasites. As noted above, their activation results in the production of proinflammatory cytokines and antiviral molecules that aid in pathogen clearance by recruiting cells of the innate immune system and by stimulating pathogen-specific adaptive immunity. Mice lacking these receptors or adaptor molecules associated with TLR signaling have severe defects in their ability to control certain pathogens, often resulting in death.

Bacteria contain a variety of PAMPs, including nucleic acids, LPS, lipopeptides, and flagellin, that trigger many different TLRs. TLR4-mutant mice are impaired in their ability to clear mycobacterium from the lungs, and expression of proinflammatory cytokines and chemokines is reduced in the lungs of these mice, demonstrating a role for TLR4 in the control of *Mycobacterium tuberculosis* (Abel et al., 2002). TLR4-deficient mice are also more susceptible to *Salmonella typhimurium* infection (Weiss et al., 2004). Furthermore, the Weiss study demonstrated that mice deficient in both TLR2 and TLR4 succumb to infection even earlier than TLR4 knockouts, implicating cooperation between the two receptors for maximal control of *S. typhimurium*. More recently, TLR11 was shown to be important in the control of this bacterium, as TLR11-/- mice demonstrated greater dissemination of *Salmonella* into the spleen and liver (Shi et al., 2012). In this model, TLR11 was proposed to prevent penetration of the bacterium into the Peyer's patches, thereby blocking systemic propagation into host organs. Mice lacking TLR1 had reduced survival following oral infection with *Yersinia enterocolitica* due to a defect in

their ability to induce differentiation of T helper 17 cells for pathogen clearance (DePaolo et al., 2012).

TLRs are also critical for their role in antiviral immunity. Strong inducers of antiviral molecules like type I interferons (IFN) include the nucleic acid sensing receptors, TLR3, TLR7, TLR8, and TLR9. TLR9 expression is necessary for cytokine responses to herpes simplex virus 1 (HSV1) in vitro (Lund et al., 2003), and TLR9 knockout mice inefficiently controlled replication of mouse cytomegalovirus (MCMV) in vivo (Krug et al., 2004; Tabeta et al., 2004). The Tabeta study also revealed a role for TLR3 in host defense against MCMV, suggesting redundancy in the ability of TLRs to mount an antiviral response against this pathogen. The ligand for TLR7 is single stranded RNA, and mice deficient in TLR7 demonstrated reduced serum IFNα levels in vivo after infection with either the single stranded RNA viruses vesicular stomatitis virus or influenza virus (Lund et al., 2004). These studies demonstrate the importance of TLRs in antiviral immunity for their induction of proinflammatory cytokines and their ability to activate cells of the adaptive immune system to assist in pathogen control.

Infection with protozoa including *Leishmania*, *Trypanosoma cruzi*, *Trypanosoma brucei*, *Plasmodium*, and *Toxoplasma gondii* accounts for immeasurable human suffering and is a major health issue world wide. Parasitic PAMPs, including glycosylphosphatidylinositol (GPI) anchors, genomic DNA, and profilin, can activate host defense via TLR recognition (Gazzinelli and Denkers, 2006). Mice deficient in TLR12, shown to recognize profilin, are highly susceptible to infection with *Toxoplasma gondii* (Koblansky et al., 2013). TLR12-induced production of IL-12 was necessary to trigger NK cell secretion of IFNγ to combat the pathogen. TLR11 also recognizes *T. gondii* profilin, but TLR11 knockout mice survived infection with this pathogen (Yarovinsky et al., 2005). This can be explained by the ability of TLR12 to heterodimerize with TLR11 or to homodimerize for recognition of parasitic profilin (Koblansky et al., 2013).

DNA from *T. cruzi* stimulates bone marrow derived macrophages in a TLR9-dependent manner, while TLR2 is necessary for recognition of GPI anchors (Bafica et al., 2006). Consistent with these in vitro data, mice deficient in both TLR2 and TLR9 succumb to *T. cruzi* infection, suggesting cooperation between these two receptors in parasite control. TLR9 also plays a role in control of parasitemia in vivo in mice infected with *T. brucei* (Drennan et al., 2005). Mice deficient in myeloid differentiation primary response protein 88 (MyD88), an adaptor molecule utilized by all TLRs except TLR3, demonstrate impaired cytokine responses and increased susceptibility to *L. major* (de Veer et al., 2003; Muraille et al., 2003), *T. brucei* (Drennan et al., 2005), *T. cruzi* (Campos et al., 2004), and *T. gondii* (Scanga et al., 2002), suggesting the importance of TLR signaling for the control of parasitic infection.

As reported above, it is evident that TLRs play a central role in host defense against a variety of invading pathogens. However, excessive signaling through TLRs can result in inflammatory conditions and host tissue damage. Therefore, understanding the signaling molecules that regulate TLR signaling is crucial for identifying targets for therapeutic intervention to ensure appropriate responses. Below, we discuss canonical and noncanonical signaling intermediates that participate in the control of cytokine responses downstream of TLR ligand recognition.

Toll-like receptor signaling

Upon ligand recognition, TLRs signal through two adaptor molecules, TIR domain-containing adaptor inducing IFNβ (TRIF) and MyD88. TLR4 is the only TLR that signals through both adaptors. All of the other TLRs signal through MyD88, except for TLR3, which utilizes only the TRIF signaling pathway. The receptors can bind TIR domain-containing adaptor protein (TIRAP)/Mal and MyD88 via TIR-TIR interactions resulting in activation of downstream signaling (Jiang et al., 2006). This interaction

recruits interleukin-1 receptor-associated kinase (IRAK) (Nunez Miguel et al., 2007). The IRAK family members become serine/threonine phosphorylated and subsequently dissociate from MyD88 in order to activate tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 exerts its function by ubiquitinating transforming growth factor-β-activated protein kinase 1 (TAK1). Ubiquitination is yet another post-translational event that can mediate signal transduction (Chen, 2005). Upon forming a complex with other proteins, ubiquitinated TAK1 activates the IκB kinase (IKK) complex, which results in phosphorylation and degradation of IκBα leading to nuclear translocation of the transcription factor NF-κB. TAK1 can also serine/threonine phosphorylate members of the mitogen activated protein kinase (MAPK) family, leading to phosphorylation and activation of c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase (Erk). These molecules, along with NF-κB, contribute to transcriptional activation of proinflammatory cytokines, like IL-6, IL-12, and TNFα. Throughout this thesis, we will refer to the canonical TLR9 signaling pathway as depicted in Figure 1.1.

The other TLR adaptor molecule, TRIF, signals downstream of TLR3 and TLR4 with the help of TRIF-related adaptor molecule (TRAM) (Yamamoto et al., 2003b). Like MyD88, TRIF can also activate NF-κB through its recruitment of TRAF6. Through activation of noncanonical IKKs, interferon regulatory factors (IRFs) are phosphorylated, resulting in transcription of type I IFNs. Indeed, TRIF-deficient mice showed defective type I IFN production in response to LPS, while MyD88 signaling remained intact (Hoebe et al., 2003; Yamamoto et al., 2003a).

Canonically, the kinases identified downstream of TLRs participate in serine/threonine phosphorylation events. However, there is accumulating evidence for a role for tyrosine kinases in the regulation of TLR signaling. Receptor tyrosine phosphorylation induces signaling downstream of TLR2 (Arbibe et al., 2000; Xiong et al.,

2012), TLR3 (Sarkar et al., 2004), TLR4 (Medvedev et al., 2007), TLR5 (Ivison et al., 2007), and TLR9 (Sanjuan et al., 2006). These reports suggest that tyrosine phosphorylation of the receptor itself mediates downstream signaling upon ligand recognition. Furthermore, the mutation identified in the C3H/HeJ mice, rendering the animals non-responsive to LPS (Poltorak et al., 1998), prevents TLR4 phosphorylation and results in abrogated downstream signaling in vitro (Medvedev et al., 2007). Additionally, the adaptor Mal is tyrosine phosphorylated in response to LPS, and mutations of tyrosine residues in its TIR domain prevented LPS-induced activation of p38 and NF-κB (Gray et al., 2006; Piao et al., 2008). Because of these reports implicating phosphorylation of the receptor as an event initiating downstream signaling, some groups focused on identifying the potential kinase(s) involved. It was reported that treatment of a human monocytic cell line (THP-1) with a pharmacological inhibitor of spleen tyrosine kinase (Syk) prevented LPS-induced phosphorylation of TLR4 (Chaudhary et al., 2007). However, it is known that Syk inhibitors can lead to off-target effects and prevent activity of other kinases (Geahlen and McLaughlin, 1989; Lin et al., 2010). Therefore, it is possible that a kinase other than Syk was responsible for TLR4 tyrosine phosphorylation in that system. Vogel's group demonstrated that in a heterologous expression system the Src family kinase Lyn associated with TLR4, and Src inhibition resulted in reduced TLR4 phosphorylation in response to LPS (Medvedev et al., 2007). Similarly, treatment of THP-1 cells with Src family kinase or Syk inhibitors results in abrogated CpG-induced TLR9 phosphorylation (Sanjuan et al., 2006). Sen's group reported that the tyrosine kinase phosphoinositide kinase-3 (PI3K) associated with TLR3 in transfected HEK 293 T cells, suggesting that PI3K participates in the phosphorylation of TLR3 (Sarkar et al., 2004). It will be important to validate these results with endogenous proteins and genetic deletion to prevent potential nonspecific interactions seen in heterologous expression systems and to avoid off-target effects of

pharmacological inhibition. Nonetheless, these studies provide evidence to implicate the involvement of tyrosine kinases in the initiation of TLR signaling.

Tyrosine kinase function downstream of TLRs

In addition to tyrosine phosphorylation events of the receptor mediating TLR signaling, there are also reports implicating tyrosine kinases in the regulation of downstream signaling. For example, PI3K, whose activation depends on tyrosine phosphorylation, was implicated in TLR2-mediated activation of NF-κB by way of Rac1 GTPase independent of IκBα (Arbibe et al., 2000). Pharmacologic inhibition of PI3K resulted in decreased secretion of TLR4-induced monocyte chemoattractant protein-1 (MCP-1) in human neutrophils (Arndt et al., 2004). Moreover, genetic deletion or knockdown of PI3K prevented LPS-induced secretion of TNFα in macrophages (Low et al., 2010).

Bruton's tyrosine kinase (BTK) was tyrosine phosphorylated following LPS stimulation and interacted with TLR4, MyD88, and IRAK-1. Introduction of a dominant negative BTK abrogated LPS-induced NF-κB activation (Jefferies et al., 2003). Its association with TLR4 suggests that BTK might also participate in phosphorylation of the receptor following ligand recognition. BTK also interacted with TLR2, and its inhibition resulted in reduced mRNA expression of some cytokines in a mouse macrophage cell line (Liljeroos et al., 2007). In B cells, CpG stimulation of TLR9 caused BTK phosphorylation, and BTK-deficient B cells demonstrated increased levels of secreted IL-12, suggesting specific negative regulation of this cytokine (Lee et al., 2008). It is important to note that the Liljeroos study utilized pharmacologic inhibition of BTK, the potential off-targets of which could confound the interpretation of the results. It will be

important to extend genetic deletions of this molecule to studies investigating BTK function in innate immune cells.

LPS stimulation of bone marrow derived DCs (BMDCs) caused tyrosine phosphorylation of the Src family kinases Lyn, Hck, and Fyn, and CD45-deficient cells demonstrated dysregulation of the activation of these proteins (Cross et al., 2008). Furthermore, this study found dysregulated cytokine secretion in response to various TLR ligands, but normal activation of the signaling molecules MAPKs and Akt. However, the link between Src family kinase phosphorylation and cytokine secretion remains to be determined. Expression of Src family kinases was abolished in Lyn-deficient bone marrow derived DCs, and these cells demonstrated reduced maturation, based on CD86 and CD40 surface expression, in response to LPS, CpG, and poly IC (Chu and Lowell, 2005). Secretion of IL-12 was abrogated in the absence of Lyn, while TNFα and IL-6 levels were normal. The mechanism by which Lyn exerts its effects on IL-12, however, was not investigated in this study. More recently, it was reported that myeloid differentiation factor-2 (MD-2), a component of the LPS receptor complex, was tyrosine phosphorylated and interacted with Lyn kinase in the endosome (Gray et al., 2011). The implication is that Lyn phosphorylated MD-2 prior to its trafficking to the plasma membrane. Mutation of MD-2 tyrosine residues resulted in reduced LPS-induced IL-8 secretion with only a modest defect in NF-κB activity. It is possible that MD-2 tyrosine phosphorylation initiates a signaling cascade to export IL-8 out of the cell after its synthesis. It will be interesting to investigate how MD-2 tyrosine phosphorylation or the absence of Lyn affects protein translation of cytokines in addition to their secretion.

The protein tyrosine kinase Syk has also been implicated in signaling downstream of TLRs. Activation of TLR4 by minimally oxidized low-density lipoprotein (mmLDL) in macrophages resulted in phosphorylation of Syk and its downstream target phospholipase C gamma (PLCγ) independent of MyD88, resulting in generation of

reactive oxygen species (ROS) (Bae et al., 2009). Knockdown of Nox2, downstream of Syk and PLCγ activation, caused a defect in ROS generation and a reduction in production of only some cytokines, including IL-6 and IL-1β, while production of TNFα, MCP-1, and macrophage inflammatory protein 1 alpha (MIP1α) was unaffected. These data suggest that Syk-dependent ROS generation differentially regulates cytokine production in response to mmLDL in macrophages. Cliff Lowell's group reported that neutrophil-specific deletion of Syk resulted in impaired TNFα secretion, while mRNA production was intact, in response to opsonized bacteria, suggesting a role for Syk in TNFα exocytosis (Van Ziffle and Lowell, 2009). Furthermore, studies with Syk inhibitors resulted in reduced TLR-induced cytokine secretion (Chaudhary et al., 2007).

However, Syk has also been implicated in the negative regulation of TLR responses. Cao's group reported that TLR-induced integrin activation resulted in Syk phosphorylation that mediated the degradation of TLR adaptor molecules to dampen the TLR response (Han et al., 2010). Additionally, Syk deficient bone marrow derived DCs demonstrated increased cytokine secretion in response to TLR ligands (Hamerman et al., 2005). The interpretation of this study was that TLR signals activate the immunoreceptor tyrosine-based activation motif (ITAM)-bearing adaptor molecule DAP12. This leads to Syk recruitment and phosophorylation resulting in its suppressive effect on TLR signaling. However, the mechanism by which Syk acts on the TLR pathway was not identified in this study, and the use of Syk KO cells could have resulted in developmental defects in DCs, possibly confounding the interpretation of the results. It will be important to utilize cell-specific deletions or conditional knockouts of Syk to ensure normal development of these cells to investigate Syk function.

Historically, the canonical TLR pathway leading to cytokine responses involves signaling through serine/threonine kinase events, and secretion of these cytokines has

been linked to the signaling machinery itself. Most investigators have focused on cytokine transcription and translation as the critical events driving cytokine secretion. Of course, after cytokine translation, there necessarily must be some mechanism by which the cytokine moves to the outside of the cell to be secreted. As noted above, there is emerging evidence for a role of tyrosine kinases in the regulation of TLR responses, and many studies point to these non-canonical signaling molecules in the regulation of cytokine vesicle transport (Kamen et al., 2011; Low et al., 2010; Nanamori et al., 2007). Therefore, it is possible that PAMP recognition elicits a distinct pathway necessary for cytokine exocytosis that relies on tyrosine kinases in addition to the canonical serine/threonine kinase pathway resulting in cytokine transcription. Discussed below is a review on cytokine exocytosis.

Regulation of cytokine responses

As mentioned previously, TLRs signal for the production of cytokines, which can facilitate the immune response by recruiting other immune cells to the site of infection or by contributing to the differentiation of T helper cells, among numerous other functions. However, aberrant TLR signaling can lead to inappropriate cytokine responses and subsequent tissue damage and inflammatory conditions. Therefore, gaining an understanding of the signaling events and mechanisms by which cells control the export of cytokines is critical for identifying possible therapeutic targets to shape the immune response. Discussed below is a review of cytokine secretion pathways.

Most cytokines are secreted via the classic secretory pathway, in which proteins traffic through the endoplasmic reticulum (ER) and are packaged in the Golgi apparatus into vesicles for their transport out of the cell (Stanley and Lacy, 2010). Classical secretion can be divided into two categories: regulated exocytosis and constitutive exocytosis. Constitutive exocytosis of cytokines is defined by de novo transcription of the

cytokine following receptor stimulation, like that from TLRs, cytokine receptors, Fc receptors, and other PRRs, among others. Once the cytokine is transcribed and translated, a coordinated series of events occurs allowing for appropriate packaging and trafficking of the cytokine to ensure proper exocytosis. Regulated exocytosis of cytokines is defined as signal-mediated release and secretion of vesicles containing preformed cytokine in the absence of de novo cytokine transcription. In this mode of exocytosis, cytokines are already packaged into vesicles in a resting cell and are released within minutes of a specific receptor engagement. Orchestrating the movement of many different cargo molecules to achieve the desired cytokine response involves an array of trafficking machinery, including soluble *N*-ethylmaledimide-sensitive factor attachment protein receptors (SNAREs), cytoskeleton motor proteins, golgins, and the Rab and Rho proteins (Stanley and Lacy, 2010). SNAREs mediate the fusion of membranes on the donor cargo-containing vesicle with a target membrane, either from another endosomal vesicle or the plasma membrane, to release the contents. As discussed below, these proteins can participate in the differential sorting and transport of cytokines and chemokines throughout the cell. It is important, therefore, to elucidate the trafficking machinery and pathways required to transport individual cytokines from the cell to adequately understand how immune responses are generated.

Mast cells, eosinophils, and neutrophils are examples of cell types that participate in regulated exocytosis of cytokines. For example, mast cells can release secretory granules containing preformed cytokine in response to Fc receptor ligation, a process known as degranulation, in the absence of de novo transcription (Gordon and Galli, 1990; Tiwari et al., 2008). Fusion of these granules, depending on the cargo, can be differentially regulated. For example, deletion in a specific SNARE molecule, vesicle associated membrane protein (VAMP)-8, resulted in impaired Fc-epsilon-induced release of serotonin and cathepsin D, but not of TNFα (Puri and Roche, 2008). Instead,

TNFα localized with VAMP3 at the cell surface in mast cells (Tiwari et al., 2008). Indeed, several molecules have been described in differential vesicular trafficking in mast cells (Lorentz et al., 2012). Interestingly, inhibition of PI3K, whose activation depends on phosphorylation of its tyrosine residues, resulted in diminished phosphorylation of trafficking proteins in mast cells, suggesting a role for tyrosine kinase substrates in vesicular transport (Nanamori et al., 2007). Additionally, tyrosine phosphorylated proteins were identified in degranulating neutrophils and thought to participate in exocytosis (Luerman et al., 2011), and neutrophils deficient in proline rich kinase 2 (Pyk2) were unable to degranulate downstream of integrin activation (Kamen et al., 2011). These studies suggest that individual cytokines depend on a specific set of trafficking molecules for their degranulation and exocytosis in mast cells and neutrophils and that tyrosine kinases can facilitate this process.

Constitutive release of cytokines has been well described in macrophages. Similar to what has been observed in mast cells during regulated exocytosis, macrophages also utilize a wide array of trafficking molecules to export various cytokines and chemokines after receptor-induced transcription. Endosomal vesicles carrying distinct cargo molecules rely on expression of various components of the trafficking machinery, including SNAREs, Rab GTPases, and golgins. Upregulation of several different SNARE molecules, including syntaxin 7, VAMP3, VAMP8, syntaxin 6, Vti1b, syntaxin 4, syntaxin 3, and SNAP 23, among others, has been reported in macrophages after TLR stimulation (Achuthan et al., 2008; Han et al., 2009; Murray et al., 2005a; Murray et al., 2005b; Pagan et al., 2003). Rab GTPase proteins have also been identified for their role in constitutive exocytosis in macrophages and participate in formation of vesicle membranes and the fusion of vesicles to target membranes for cargo release (Hutagalung and Novick, 2011). The golgin family of molecules is expressed at the Golgi complex and function by forming vesicular carriers and by

tethering vesicles to the Golgi membrane. The golgins p230 and golgin-97 participate in macrophage constitutive exocytosis (Lieu et al., 2008; Stanley et al., 2012). These molecules are induced during TLR stimulation and assist with cargo loading and endosomal trafficking to ensure appropriate packaging and release of cytokines. Because cytokines can exert profound effects on both host defense and damage to host tissues, it is likely that secretion of these molecules must be exquisitely regulated by numerous trafficking intermediates to achieve the desired response.

TNFα, a potent proinflammatory cytokine, has many post-translational regulators that facilitate in its secretion from the cell. The mechanism of TNFα transport has been most closely investigated in macrophages participating in constitutive exocytosis. Once TNFα is synthesized as a membrane-bound protein in the ER in response to a stimulus, it is packaged in the Golgi complex into tubulovesicular structures (Shurety et al., 2000). Loading of the cytokine into vesicles and its subsequent entry into the endosomal compartment are regulated processes involving many members of the trafficking machinery. TNFα colocalized with the golgin p230 at the Golgi, and knockdown of p230 resulted in diminished trafficking of LPS-induced TNFα in macrophages (Shurety et al., 2000). Following packaging, TNFα-containing vesicles must be released from the Golgi membranes to enter the trafficking pathway to the cell surface. Deletion of the δ isoform of PI3K or the GTPase dynamin 2 caused these vesicles to remain tethered to the Golgi membrane, thereby preventing secretion of TNFα in response to LPS (Low et al., 2010). Interestingly, IL-6 secretion was unaffected by the absence of PI3K, suggesting that this kinase is involved in the regulation specifically of TNFα transport in macrophages. Indeed, because such a diverse array of molecules traffics from this organelle, regulation must be tightly coordinated to ensure the appropriate response is being generated. Thus

it is not surprising that generation of a coordinated immune response relies on regulation of so many different trafficking intermediates (De Matteis and Luini, 2008).

Once TNFα vesicles detach from the Golgi membrane, they must fuse with membranes of trafficking endosomes, another highly regulated process. Fractionation studies in LPS-stimulated macrophages demonstrated that budded vesicles (shown to be recycling endosomes) contained TNFα, syntaxin 6, and vt11b (Murray et al., 2005b). Additionally, VAMP3 knockdown resulted in diminished TNFα secretion, and TNFα colocalized with Rab11 in recycling endosomes (Murray et al., 2005a). TLR-induced upregulation of secretory carrier membrane protein-5 (SCAMP5) promoted TNFα trafficking from the Golgi to the cell surface (Han et al., 2009). SCAMP5 interacted with many different SNARE molecules, and its knockdown prevented LPS-induced TNFα secretion. Rab27 and Munc13-1 were also reported to interact in recycling endosomes and to promote TNFα secretion (Mori et al., 2011). Interestingly, TNFα and IL-6 were loaded into different vesicles at the Golgi complex (Manderson et al., 2007). These investigations suggest that a complex network of trafficking proteins is important for cytokine exocytosis in response to TLR stimulation and that these molecules can differentially sort cytokines to regulate their transport.

Prior to its secretion, TNFα is delivered to the plasma membrane as an integral membrane protein and must be cleaved at the cell surface such that soluble cytokine is released into the extracellular space. This is achieved by TNFα-converting enzyme (TACE) / ADAM17 (Black et al., 1997). Even TACE trafficking is regulated to ensure appropriate release of TNFα (Adrain et al., 2012; McIlwain et al., 2012). The fusion of TNFα-containing endosomes with the plasma membrane also relies on coordinated activity of the SNARE machinery. Syntaxin 4 associated with cholesterol-rich lipid rafts at the plasma membrane to form a phagocytic cup structure to which VAMP3 endosomes

containing TNFα were delivered following LPS stimulation (Kay et al., 2006). TACE also colocalized with these molecules at phagocytic cups to guarantee cleavage and release of TNFα (Murray et al., 2005a).

Trafficking pathways have also been described for the exocytosis of other cytokines. IL-6, a soluble cytokine, was reported to rely on similar SNARE molecules as TNFα, but these cytokines could be sorted into different vesicles at the Golgi complex (Manderson et al., 2007). Furthermore, in contrast to TNFα, IL-6 vesicles were not directed to the phagocytic cup at the cell surface. These data suggest that undefined molecules might participate in the differential sorting of these two cytokines at the Golgi complex. While several molecules have been described for cytokine exocytosis in macrophages, additional work remains to be done regarding the differential regulation of these intermediates to achieve a specific cytokine response. Because these cytokines impose disparate effects on the immune response, understanding how they are sorted and trafficked will uncover potential targets for therapeutic intervention.

Structure of thesis

This thesis examines how tyrosine phosphorylation events affect signaling downstream of TLR9. In particular, we are interested in the protein tyrosine kinase Syk and TLR9 itself as a potential substrate for tyrosine phosphorylation. Syk phosphorylation has been reported in response to TLR3, TLR4, and TLR9 stimulation (Han et al., 2010), but the exact role of this kinase in TLR signaling is unresolved. Conflicting reports in the literature portray Syk as a negative regulator of TLR signaling or as a positive regulator of TLR signaling. Many of these studies have utilized pharmacological inhibition of Syk, which may impose off-target effects and affect kinases other than Syk. Moreover, groups utilizing Syk knockout (KO) cells might be imposing

developmental defects in cells due to the absence of Syk. Therefore, in order to more rigorously investigate the role of this kinase in TLR signaling, we have emplyed genetic knockdown in a macrophage cell line and utilizied genetic deletion of *Syk* in dendritic cells in vivo. These approaches avoid potential off-target effects of inhibitors and allow cells to develop in the presence of Syk, thus preventing developmental defects. In Chapter II, we investigate the role of Syk downstream of CpG-induced activation of TLR9 using these genetic approaches. The data presented in this Chapter suggest that Syk positively mediates CpG-induced signaling by promoting the trafficking of TNFα, but not IL-6, to the cell surface. These experiments suggest that Syk participates in the differential secretion of cytokines and that TLR ligands must promote a signal for transport to the plasma membrane distinct from the signal leading to cytokine transcription in order to activate cytokine exocytosis completely. This was an unexpected finding because in other signaling systems, for example downstream of the B cell receptor, tyrosine kinases including Syk play a critical role in regulating proximal signaling events ultimately affecting gene transcription. However, here, we find that Syk is participating outside the canonical TLR9 signaling pathway to promote TNFα exocytosis. In Chapter III of this thesis, we address the biochemical mechanism by which Syk regulates TNFα exocytosis in response to CpG. Because calcium mobilization is a known downstream effector of Syk in other signaling systems (Mocsai et al., 2010), we ask if Syk-dependent calcium signals can promote TNFα secretion in response to CpG. Indeed, the data presented in this Chapter demonstrate that calcium calmodulin kinase II (CaMKII) is the target of Syk-dependent calcium signaling that facilitates TNFα exocytosis.

During the course of our studies investigating Syk function in TLR9 signaling, we became interested in the receptor itself as a potential modulator of downstream

signaling. Posttranslational modification of TLR9, including receptor proteolysis and phosphorylation, to promote downstream signaling has been previously reported. Though preliminary, we show data in Chapter IV to suggest that a conserved tyrosine residue in the TIR domain of TLR9 is important for receptor activation. When mutated to phenylalanine, the receptor cannot mature as efficiently, suggesting that tyrosine phosphorylation might be a mechanism by which TLR9 becomes fully activated to exert its effects. Further experimentation will be needed to confirm the effects of TLR9 phosphorylation in downstream signaling.

In the final Chapter of the thesis, we discuss the significance of our findings for our understanding in tyrosine kinase-induced regulation of cytokine responses. Additionally, we propose future investigations to explore based on our results.

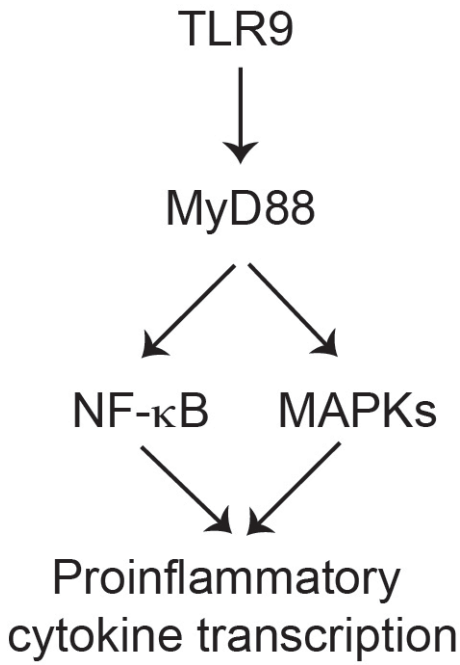

Figure 1.1 The canonical TLR9 signaling pathway. Canonically, upon CpG stimulation, TLR9 signals through the adaptor molecule MyD88. MyD88 recruitment leads to the activation of NF-κB and the MAPKs, Erk and p38. This leads to the transcription of proinflammatory cytokines including IL-6 and TNFα.

Chapter II

Results

Syk mediates trafficking of TNFα downstream of CpG signaling in innate immune cells

Introduction

Pattern recognition receptors, including Toll-like receptors, triggered by PAMPs activate a variety of signaling events leading to initiation of the immune response (Takeda et al., 2003). This response includes upregulation of costimulatory molecules on innate immune cells, production of antiviral molecules and proinflammatory cytokines, and activation of antigen-specific immunity. While TLR activation is crucial for host defense, excessive TLR signaling can lead to inflammatory and autoimmune disease (Kawai and Akira, 2010). TNFα is a potent proinflammatory cytokine produced following PAMP recognition by PRRs that is crucial for the clearance of certain pathogens including *L. monocytogenes* and *M. tuberculosis* but whose activity has also been linked to pathogenesis of inflammatory disease (Flynn et al., 1995; Pfeffer et al., 1993). How this balance between beneficial and detrimental effects of TNFα is determined represents a significant gap in our understanding of PRR signaling. Gaining an understanding of the signaling events that contribute to TNFα production and release is critical for identifying possible targets for directing the immune response downstream of PAMP recognition.

Syk is a protein tyrosine kinase that regulates many cellular processes, including B cell antigen receptor (immunoglobulin) and integrin signaling, inflammasome activation, phagocytosis, and reactive oxygen species production (Mocsai et al., 2010). Syk signaling is also important for cytokine release downstream of PAMP recognition;

however, the mechanism by which Syk regulates this process is unresolved. Conflicting studies suggest that Syk acts downstream of receptors either to promote cytokine production (Chaudhary et al., 2007; Rogers et al., 2005; Sanjuan et al., 2006; Van Ziffle and Lowell, 2009) or to limit cytokine production (Hamerman et al., 2005; Han et al., 2010; Zhang et al., 2009). The various approaches employed to study Syk may be contributing to the contradictory functions ascribed to this enzyme. Embryonic deletion of *Syk* results in perinatal lethality due to severe vascular abnormalities, hence mice with germline deletion of the gene cannot be used for analysis (Abtahian et al., 2003; Cheng et al., 1995; Turner et al., 1995). While use of radiation chimeras circumvents perinatal lethality, this produces developmental abnormalities including blocks in B cell maturation that may confound the interpretation of the effects of Syk deletion in innate immune cells. Other studies have relied on pharmacological inhibition of Syk which are likely complicated by off-target effects of these drugs (Geahlen and McLaughlin, 1989; Lin et al., 2010).

Recognizing the limitations of these approaches, we have used complementary methodologies in primary cells and in a model cell line to resolve the role of Syk in signaling downstream of one critical PRR, TLR9, which responds to CpG DNA. Utilizing genetic deletion selectively in DCs and genetic knockdown in a macrophage cell line, we observed that Syk deficiency resulted in impaired CpG-induced exocytosis of TNFα, but not IL-6. Syk-deficient DCs and macrophages exhibited normal activation of canonical TLR9 signaling molecules and normal production of TNFα. Our data suggest a novel role for Syk downstream of PAMP signaling and provide insight into how particular cytokine responses are generated post-translationally.

Results

Syk deficiency results in impaired secretion of CpG-induced TNFα.

To determine how Syk affects cytokine response downstream of CpG signaling, we first treated RAW cells with the Syk inhibitor piceatannol. Syk-inhibited cells showed a near complete absence of secreted TNFα in response to CpG (Figure 2.1). Because of the potential off-target effects of pharmacological inhibition (Geahlen and McLaughlin, 1989; Lin et al., 2010), we decided to use a genetic approach to study Syk function. To study the role of Syk in DCs in vivo, we crossed mice expressing Cre recombinase under the control of the *CD11c* promoter to mice with loxP sites flanking *Syk* (Saijo et al., 2003), resulting in the conditional deletion of Syk in CD11c+ DCs (Syk flox). Western analysis of splenic DCs and bone marrow derived DCs (BMDCs) from Syk flox mice showed near complete deletion of Syk protein (Figure 2.2). To assess the role of Syk in cytokine response, Syk flox and control (Syk flox, Cre negative) mice were injected with CpG. Serum from CpG-injected Syk flox mice contained markedly less TNFα compared to that of control mice (Figure 2.3A), demonstrating an in vivo role for Syk in the regulation of CpG-induced TNFα.

We next sought to investigate the contribution of CD11c+ cells to TNFα production and secretion. Culture supernatants from splenic DCs and BMDCs isolated from Syk flox mice contained a two-fold reduction in CpG-induced TNFα compared to supernatants from cells isolated from control mice (Figure 2.3B-C). We confirmed this phenotype using shRNA knockdown of *Syk* in the RAW macrophage cell line. Syk-deficient RAW cells also showed a decreased level of TNFα in the supernatant following CpG stimulation compared to cells transduced with empty vector (control) (Figure 2.3D

and 2.4) or to RAW cells transduced with an irrelevant shRNA sequence that did not result in effective knockdown of Syk (Figure 2.4). Moreover, we found that Syk-deficient RAW cells and splenic DCs were impaired in their ability to secrete TNFα following stimulation with the TLR4 ligand LPS (Figure 2.5), demonstrating that Syk-dependent regulation of TNFα is not specific to TLR9. In contrast to the reduction in TNFα, Syk-deficient splenic DCs and BMDCs secreted IL-6 and produced IFNβ normally in response to CpG (Figure 2.6A-C), indicating that Syk deficiency does not result in global dysfunction of the CpG signaling pathway. However, Syk knockdown resulted in decreased CpG-induced secretion of the chemokine monocyte chemotactic protein-1 (MCP-1) (Figure 2.6D). Together, these data suggest that Syk selectively regulates TNFα and MCP-1, but not IL-6 or IFNβ, levels.

We speculated that Syk regulates CpG-induced TNFα levels by promoting its production or secretion, however it is possible that Syk regulates TNFα consumption or biological activity as has been seen in other cellular systems (Tartaglia and Goeddel, 1992). To test the possibility that Syk deficiency might alter consumption of secreted TNFα, CpG-stimulated or unstimulated control and Syk-deficient RAW cells were exposed to a fixed amount of exogenous TNFα in the presence of a TNFα converting enzyme (TACE) inhibitor, which prevents cleavage and secretion of synthesized, endogenous TNFα into the supernatant. We measured no difference in TNFα levels in culture supernatants from cells replete or deficient in Syk (Figure 2.7), suggesting that the defect observed from Syk-deficient cells is not due to increased cellular consumption of secreted TNFα.

Soluble TNF receptor cleaved from the cell surface can bind to soluble TNFα, thereby acting as a TNFα antagonist (Van Zee et al., 1992). To rule out the possibility

that Syk-deficiency promotes the production of soluble proteins binding secreted TNFα and, thus, preventing its detection by ELISA, we examined TNFα levels in supernatants that were boiled and denatured to disrupt protein-protein interactions, thus allowing for detection of all secreted TNFα. Western analysis of denatured supernatants from CpG-treated RAW cells revealed a decrease in secreted TNFα in the absence of Syk, arguing against selective production of an interfering TNFα binding protein in the context of Syk deficiency (Figure 2.7).

Canonical signaling downstream of TLR9 is intact in the absence of Syk.

Syk is known to be activated downstream of CpG stimulation (Lin et al., 2010; Sanjuan et al., 2006). To determine the role of canonical TLR9 signaling in Syk-dependent regulation of TNFα, we investigated signaling events downstream of TLR9 in control and Syk-deficient RAW cells and BMDCs stimulated with CpG. Control and Syk-deficient cells expressed similar levels of TLR9 (Figure 2.8A), and CpG-induced phosphorylation of the MAP kinases ERK and p38 occurred similarly in the presence and absence of Syk (Figure 2.8B). IκBα, a cytoplasmic protein whose interaction with NF-κB blocks its nuclear translocation, was similarly degraded after 30 minutes of CpG stimulation in control and Syk-deficient cells (Figure 2.8C), thus allowing nuclear translocation of NF-κB (Figure 2.8D). These data demonstrate that canonical signaling downstream of TLR9 is intact in the absence of Syk.

MAP kinase and NF-κB activity can regulate TNFα transcription (Collart et al., 1990; Dumitru et al., 2000; El Gazzar et al., 2007). Consistent with the observation that activation of these signaling intermediates was intact, TNFα mRNA levels were also similar in control and Syk-deficient cells following CpG stimulation (Figure 2.9A).

Moreover, Syk-deficient RAW cells, splenic DCs, and BMDCs produced TNFα protein similar to control cells following CpG stimulation in the presence of Brefeldin-A, which prevents exocytosis (Figure 2.9B). Taken together, these data demonstrate that in response to CpG, TNFα mRNA and protein generation occurs independently of Syk.

TNFα trafficking to the cell surface is impaired in the absence of Syk.

We next considered whether Syk might be playing a role in the exocytosis and subsequent secretion of synthesized TNFα. To investigate this possibility, control and Syk-deficient RAW cells were stimulated with CpG, and membrane lysates were measured by Western analysis and by ELISA. Both measurements revealed elevated levels of TNFα protein in membranes of Syk-deficient cells compared to control cells (Figures 2.10A-B). Because the membrane preparations included intracellular and plasma membranes, this approach cannot distinguish TNFα retention in organelles within the cell from TNFα residing at the cell surface. It is known that prior to secretion, TNFα translated at the endoplasmic reticulum membrane traffics through the Golgi apparatus and the endosomal compartment before reaching the plasma membrane (Murray et al., 2005b; Stow et al., 2009). At the plasma membrane, membrane TNFα is cleaved by TACE, allowing soluble TNFα to be secreted from the cell (Black et al., 1997). TNFα retention inside the cell would reflect a trafficking defect, while increased cytokine at the plasma membrane would suggest a defect in TACE activity. To distinguish between these possibilities, we assayed TACE activity in isolated membrane lysates and found no difference in TACE activity between the control and Syk-deficient cells, suggesting that both constitutive and CpG-induced TACE activity is intact in the absence of Syk (Figure 2.10C).

To investigate the role of Syk in TNFα trafficking to the plasma membrane, RAW cells and BMDCs were stimulated with CpG alone or with CpG in the presence of a TACE inhibitor (TAPI). TAPI allows TNFα to accumulate at the plasma membrane by blocking its cleavage and secretion, thus allowing us to measure trafficking of total TNFα to the cell surface, as reported previously (Low et al., 2010; Manderson et al., 2007). We observed similar amounts of surface TNFα on Syk-sufficient and Syk-deficient cells after CpG stimulation in the presence of TACE activity (Figure 2.10D, CpG alone). However, in the presence of TAPI, significantly less TNFα accumulated at the surface of the Syk-deficient RAW cells and BMDCs compared to control cells (Figure 2.10D, CpG/TAPI). The decrease in surface TNFα in the absence of Syk was almost two-fold, consistent with the two-fold reduction in TNFα secretion (Figure 2.3) and the two-fold increase in TNFα membrane protein (Figures 2.10A-B). Taken together, these data demonstrate that Syk is important for trafficking of TNFα to the cell surface downstream of CpG signaling.

Discussion

Here we demonstrate that in response to CpG, Syk is required for optimal trafficking of TNFα to the plasma membrane prior to secretion in macrophages and DCs. Mice harboring a DC-specific deletion of *Syk* showed a substantial reduction of serum TNFα levels following CpG injection. In vitro in cells lacking Syk, CpG stimulation resulted in activation of the canonical TLR9 signaling cascade resulting in normal increases in TNFα message and protein but diminished secretion of TNFα. Syk deficiency did not result in a global defect in cytokine secretion, however, as IL-6 secretion and IFNβ production were normal. Syk-deficient cells displayed significantly less TNFα at the cell surface compared to Syk-sufficient cells in response to CpG, consistent with the observation that total membrane TNFα levels were higher in the absence of Syk and that TACE activity was unaffected by Syk deletion. Together, these data suggest that Syk is important for trafficking of membrane-bound TNFα to the cell surface. Furthermore, the data demonstrate a novel mechanism for the differential secretion of cytokines downstream of CpG by way of Syk and suggest that CpG induces an active signal for cytokine secretion independent of production.

PRRs, including TLRs and Nod-like receptors, expressed by cells of the innate immune system can shape the immune response by bridging innate and adaptive immunity. Upon pathogen recognition, these receptors signal for the production of cytokines and for the upregulation of costimulatory molecules, both of which recruit and activate cells of the adaptive immune system. The type of cytokine released plays a key role in determining the appropriate effector cell response. TNFα is a potent pro-inflammatory cytokine. While its signaling is important for clearance of certain pathogens, including *L. monocytogenes* and *M. tuberculosis* (Flynn et al., 1995; Pfeffer

et al., 1993), dysregulated TNFα production and signaling can lead to inflammatory disease (Flavell, 2002). Therefore, to maintain immune homeostasis, TNFα production and release must be tightly regulated. Indeed, TNFα transcription and translation are controlled by several factors (Collart et al., 1990; Dumitru et al., 2000; El Gazzar et al., 2007; Han et al., 1990). Additionally, post-translational mechanisms of TNFα regulation have also been reported. Among these include cleavage of TNFα prior to secretion at the plasma membrane by TACE (Black et al., 1997) and regulation of its transport within the cell (Low et al., 2010; Murray et al., 2005a; Murray et al., 2005b; Stow et al., 2009). Here, we report a novel mechanism of TNFα regulation by way of exocytosis activated by PAMPs through the protein tyrosine kinase Syk in macrophages and DCs. This finding may have implications for additional immune cell types such as neutrophils, which were shown to have defective TNFα secretion in response to opsonized bacteria upon Syk deletion (Van Ziffle and Lowell, 2009). Similar to our data, these neutrophils generated TNFα protein normally, suggesting that Syk is also important for efficient exocytosis of TNFα downstream of PAMP signaling in this lineage.

IL-6 is a proinflammatory cytokine whose actions differ from that of TNFα. While both cytokines can recruit other cells of the immune system to the site of injury, such as monocytes, and promote inflammation, IL-6 can also influence the differentiation of T helper 17 cells (Ghoreschi et al., 2010; Veldhoen et al., 2006). Therefore, differential sorting and secretion of these cytokines is essential for tailoring the immune response to appropriately combat specific pathogens. Indeed, IL-6 and TNFα can segregate to different recycling endosome during exocytosis in macrophages, and these cytokines are delivered to different sites at the plasma membrane prior to secretion (Manderson et al., 2007; Murray et al., 2005a). We find that Syk-deficient cells secrete IL-6 normally in

response to CpG, which demonstrates that Syk is responsible for differential secretion of TNFα, perhaps at the level of endosomal sorting or plasma membrane sorting. Similarly, T cells also can differentially regulate cytokine secretion at the immunological synapse by polarizing certain cytokines at specific sites at the plasma membrane or by promoting more generalized secretion (Huse et al., 2006). Therefore, understanding the pathways involved in exocytosis of various cytokines and chemokines will provide insight into targeting specific effector molecules to treat inflammatory disease.

Our data demonstrate that Syk-deficient cells are defective in their ability to secrete MCP-1 in addition to TNFα in response to CpG. MCP-1 is a chemokine that can contribute to tissue injury through its recruitment of inflammatory cells, like monocytes, to the site of inflammation (Ajuebor et al., 1998). Similar to TNFα, MCP-1 has been implicated in inflammatory diseases including Rheumatoid arthritis and inflammatory bowel disease (Koch et al., 1992; Rantapaa-Dahlqvist et al., 2007; Spoettl et al., 2006). It is possible that these proteins are transported via a similar mechanism within immune cells involving Syk, but the trafficking intermediates involved in MCP-1 exocytosis are not clearly defined. Therefore, additional work is needed to determine the mechanism by which Syk affects MCP-1 secretion. Furthermore, it remains to be determined if Syk regulates IFNβ secretion, because our method of detection relied on its production via qPCR.

All TLRs signal through the transcription factor NF-κB which leads to transcription of a variety of cytokines and chemokines (Kawai and Akira, 2007). However, the release of such cytokines occurs differently upon stimulation of different receptors. For example, the amount of secreted TNFα varies upon TLR3, TLR4, or TLR9 ligation (Dearman et al., 2009; El-Hage et al., 2011), even though these TLRs all

activate NF-κB similarly. Indeed, our data demonstrate normal NF-κB activation but defective TNFα secretion following CpG stimulation in the absence of Syk, while IL-6 secretion was unaffected. Differential activation of trafficking molecules downstream of PAMP recognition may be a useful strategy for cells to affect the qualitative cytokine response of receptors and, therefore, tailor the immune response. Furthermore, an active signal distinct from that of NF-κB-induced transcription is critical for differentially sorting cytokines for appropriate release.

In summary, our data demonstrate that Syk is important for CpG-induced secretion of TNFα and MCP-1, but not IL-6, in DCs and macrophages. Differential sorting of proinflammatory cytokines and chemokines represents an important layer of regulation that can shape the immune response downstream of PAMP recognition. It will be important to determine other biochemical mediators involved in protein trafficking to uncover possible targets for therapeutic treatment of inflammatory disease. In the following chapter of this thesis, we will examine the biochemical mechanism by which Syk promotes CpG-induced TNFα exocytosis.

Figures

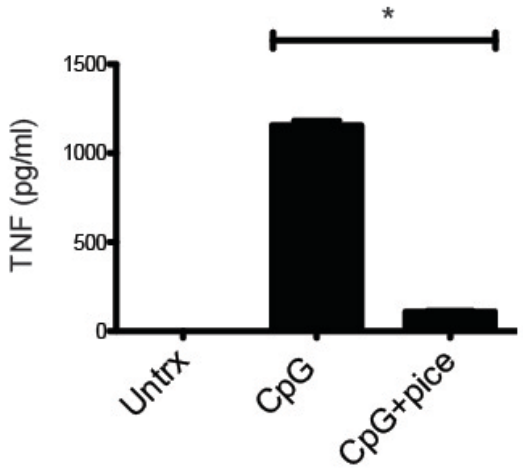

Figure 2.1 Syk inhibition results in decreased CpG-induced TNFα secretion in RAW cells. RAW cells were stimulated with CpG in the presence or absence of the Syk inhibitor piceatannol (pice) for 3 hours. TNFα was detected in the supernatants by ELISA. Graph is representative of at least 4 independent experiments performed in triplicate. Data were analyzed using an unpaired t test. **p<0.05*

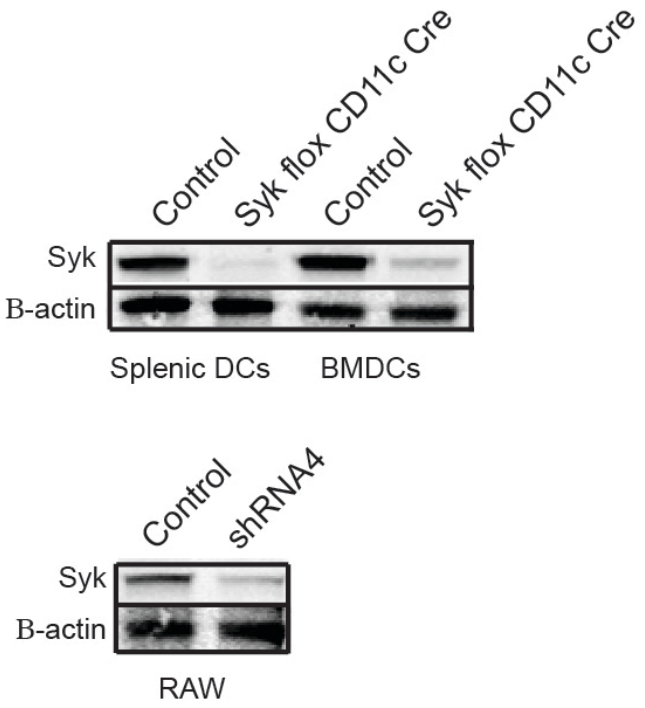

Figure 2.2 Syk is deleted in dendritic cells and RAW cells. DCs were isolated from the spleen or generated in vitro from the bone marrow of control and Syk flox CD11c Cre mice (top). RAW cells were transduced with lentivirus expressing *Syk* shRNA (bottom). Cells were lysed, and the presence of Syk protein was assessed by Western blot.

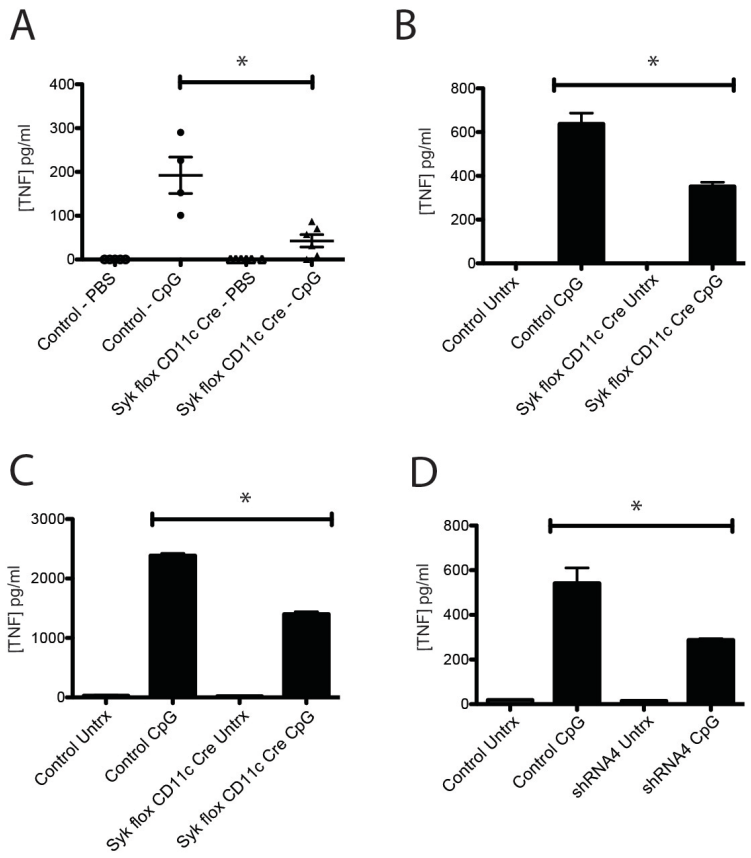

Figure 2.3 Syk deficiency results in less CpG-induced TNFα secretion in vivo and in vitro. (A) Control (Syk flox Cre negative, n=4) and Syk flox (Syk flox CD11c Cre, n=5) mice were injected with PBS or 50μg CpG through the intraperitoneal cavity. One hour following injection, serum was collected from blood and analyzed for the presence of TNFα by ELISA. Control and Syk-deficient splenic DCs (B), BMDCs (C), and RAW cells (D) were stimulated with 10μg/ml CpG in vitro for 3 hours. Supernatants were analyzed for the presence of TNFα by ELISA. Control RAW were transduced with empty vector. Graphs are representative of at least 8 independent experiments for RAW cells, splenic DCs, and BMDCs performed in triplicate. Data were analyzed using an unpaired t test. **p<0.05*

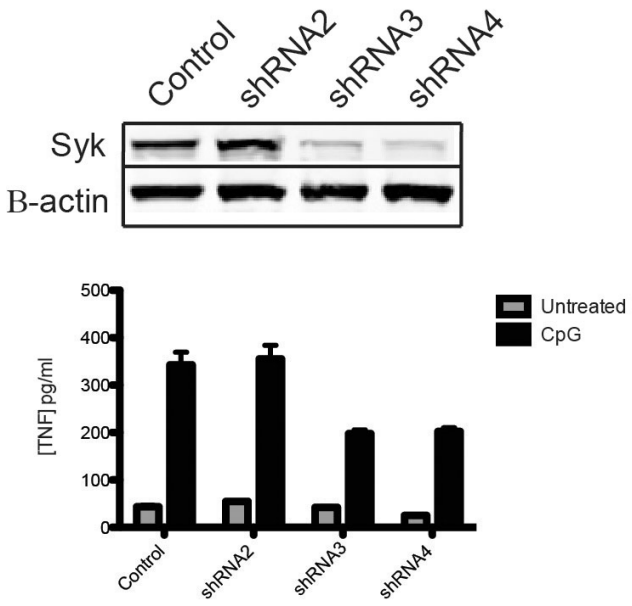

Figure 2.4 Syk knockdown results in decreased CpG-induced TNFα in the supernatants. RAW cells were transduced with lentivirus expressing *Syk* shRNA. Cells were lysed, and the presence of Syk protein was assessed by Western blot (top). Transduced RAW cells were stimulated with CpG for 3 hours, and TNFα was detected in the supernatants by ELISA (bottom). Blots were developed on Odyssey and analyzed by Licor software. Bar graph is representative of at least 4 independent experiments performed in triplicate.

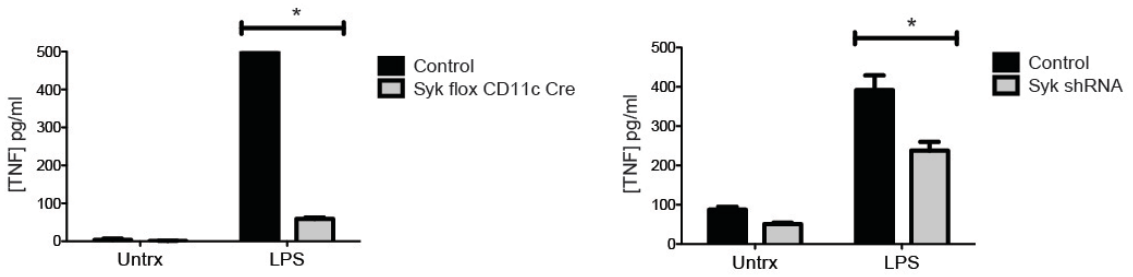

Figure 2.5 Syk deficiency results in less LPS-induced TNFα secretion in vitro. Control and Syk-deficient splenic DCs (left) and RAW cells (right) were stimulated with 100ng/ml LPS in vitro for 3 hours. Supernatants were analyzed for the presence of TNFα by ELISA. Graphs are representative of at least 2 independent experiments for each cell type performed in triplicate. Data were analyzed using an unpaired t test. **p<0.05*

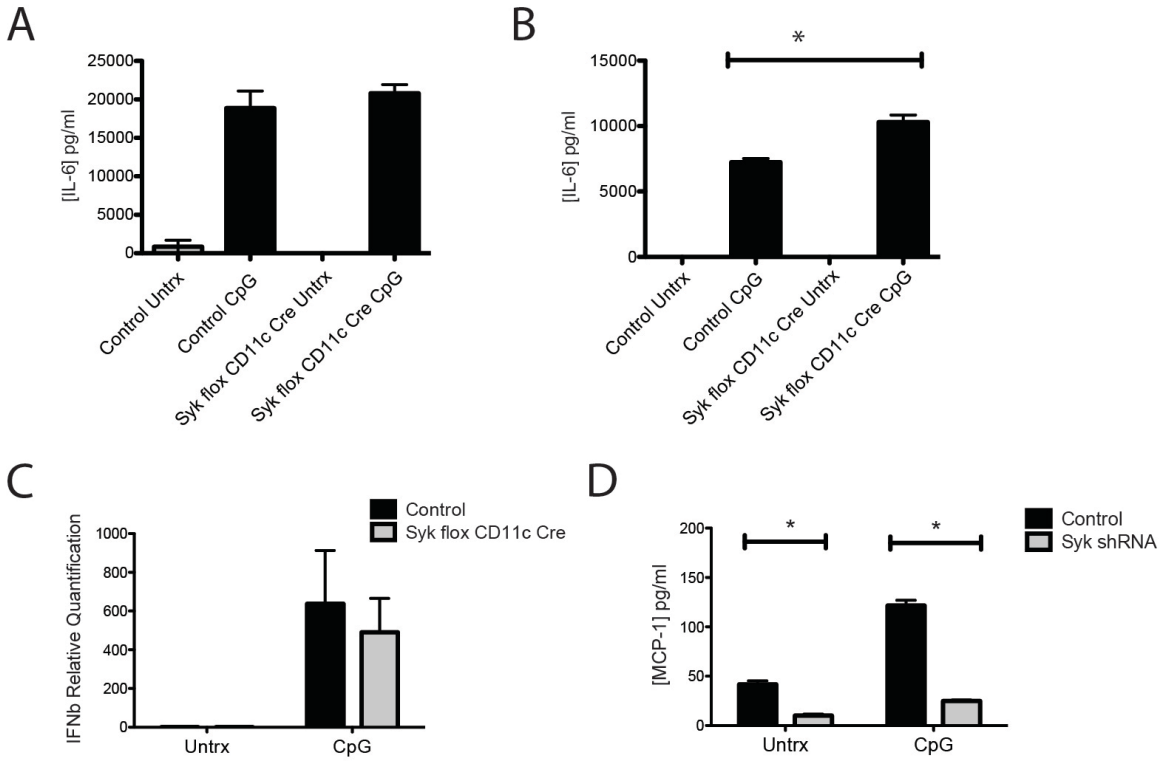

Figure 2.6 Syk deficiency results in differential CpG-induced cytokine responses. Control and Syk-deficient splenic DCs (A), BMDCs (B,C), and RAW cells (D) were stimulated with CpG in vitro for 3 hours. Supernatants were analyzed for the presence of IL-6 (A,B) and MCP-1 (D) by ELISA. (C) RNA was extracted from BMDCs stimulated with CpG. Quantitative RT-PCR was performed, and IFNβ message was normalized to GAPDH message. Bar graph is representative of 3 independent experiments performed in triplicate. ELISA graphs are representative of at least 3 independent experiments for RAW cells, splenic DCs, and BMDCs performed in triplicate. Data were analyzed using an unpaired t test. **p<0.05*

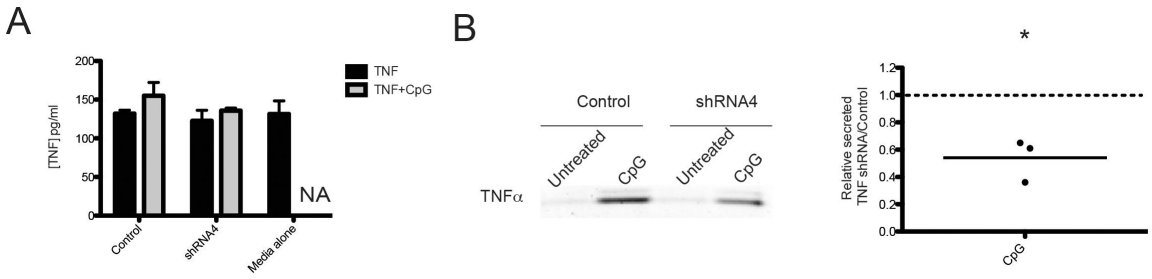

Figure 2.7 Secreted TNFα is not consumed or bound by soluble proteins abnormally in the absence of Syk. (A) CpG-stimulated RAW cells were treated with exogenous TNFα in the presence of TAPI to prevent secretion of endogenous, synthesized TNFα. Supernatants were harvested, and TNFα was detected by ELISA. Bar graph is representative of 3 independent experiments performed in triplicate. (B) TNFα was detected by Western blot from denatured supernatants of CpG-treated RAW cells. Quantification is expressed as a ratio of TNFα intensity of Syk-deficient cells to that of control cells over 3 independent experiments. Data were analyzed using a one sample t test against a theoretical mean of 1. *$p<0.05$*

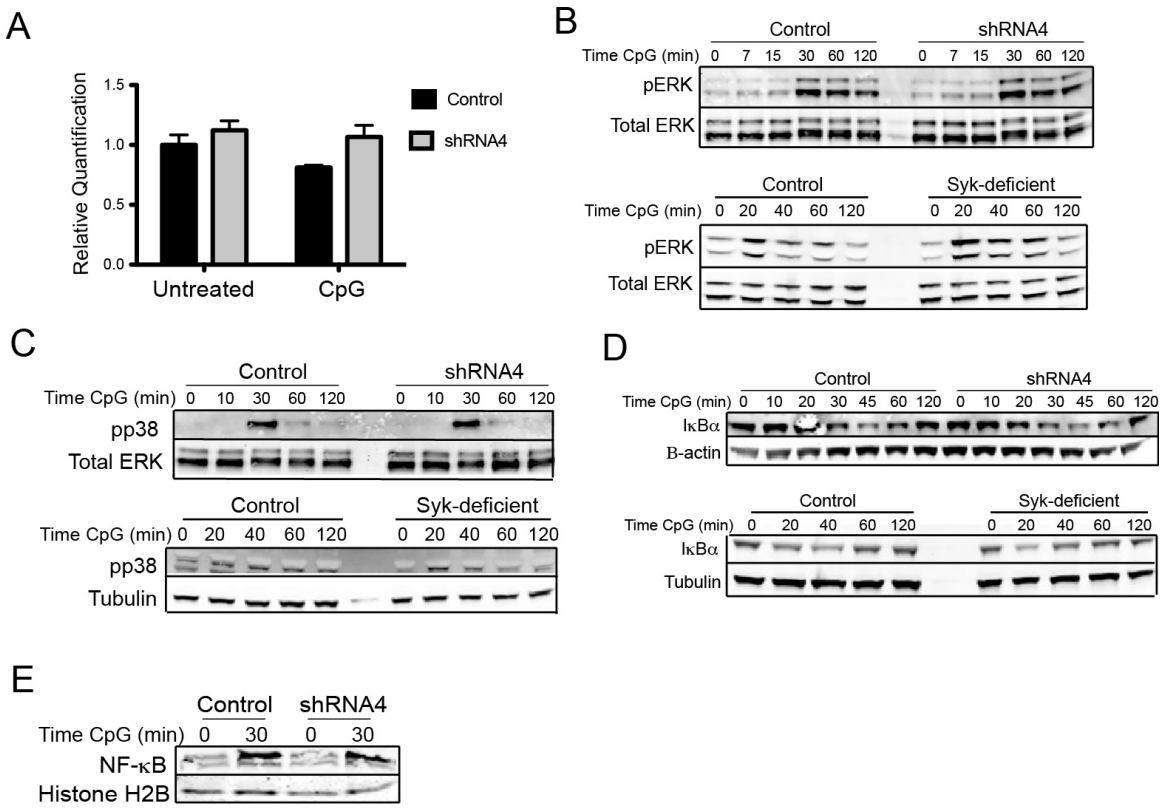

Figure 2.8 Canonical signaling downstream of TLR9 is intact in Syk-deficient cells. (A) RNA was extracted from RAW cells stimulated with CpG. Quantitative RT-PCR was performed, and TLR9 message was normalized to GAPDH message. Bar graph is representative of 3 independent experiments performed in triplicate. RAW cells were stimulated at various times with CpG, and cell lysates were analyzed by Western blot for phosphorylation of the MAP kinases ERK (B) and p38 (C) and degradation of IκBα (D). (E) Nuclear extracts were isolated from RAW cells stimulated for 30 minutes with CpG and analyzed by Western blot for nuclear translocation of NF-κB. Blots were developed on Odyssey and analyzed by Licor software and are representative of at least 4 independent experiments.

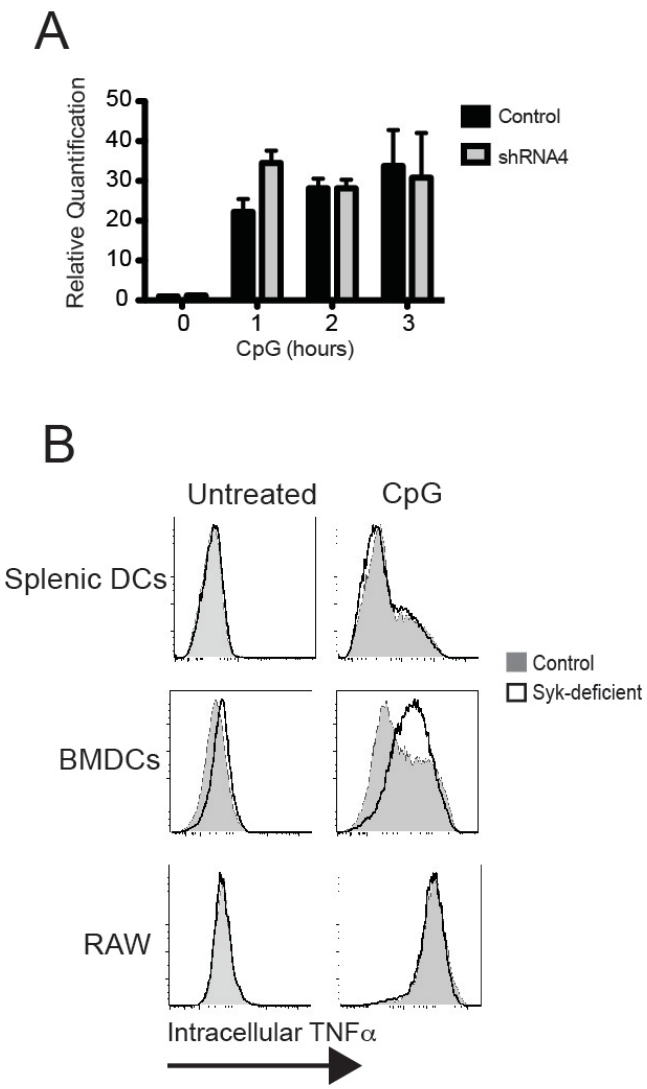

Figure 2.9 CpG-induced TNFα is transcribed and translated normally in Syk-deficient cells. (A) RAW cells were stimulated with CpG, and RNA was isolated and analyzed by quantitative RT-PCR. TNFα message was normalized to GAPDH message. Bar graph is representative of 3 independent experiments performed in triplicate. (B) RAW cells were stimulated for 3 hours with CpG in the presence of Brefeldin A, and cells were harvested, permeabilized and stained for TNFα for analysis by flow cytometry. Histograms are representative of at least 5 independent experiments performed for each cell type.

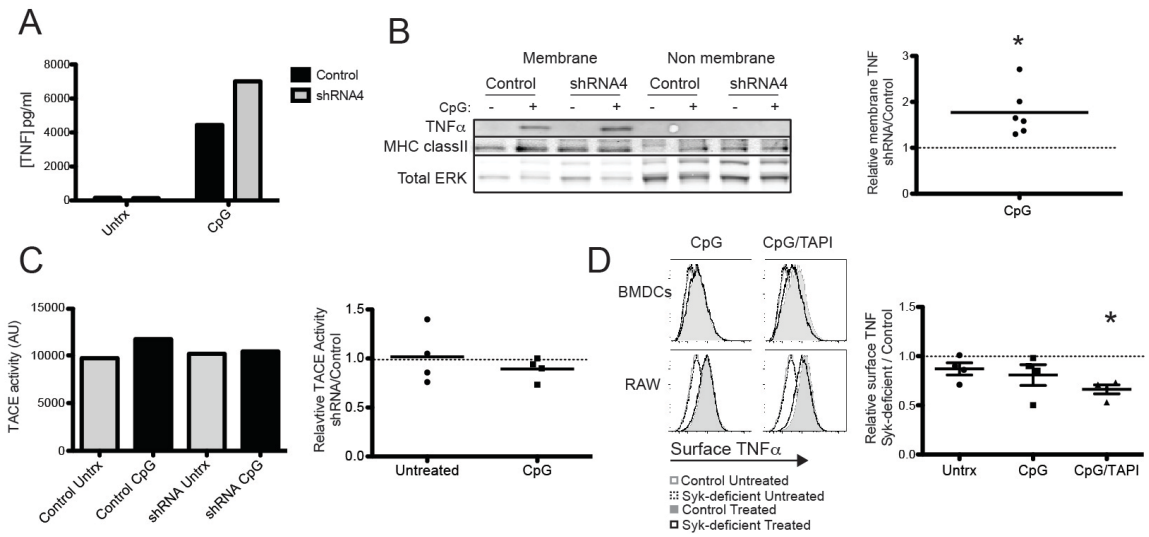

Figure 2.10 CpG-induced TNFα trafficking is impaired in Syk-deficient cells. Membrane lysates were prepared from RAW cells stimulated for 1 hour with CpG and analyzed by ELISA (A) and by Western blot for TNFα (B). For quantification of Western blot, TNFα was normalized to MHC class II, and that ratio was compared between control and Syk-deficient cells over 6 independent experiments. A ratio of 1 would demonstrate equality between control and Syk-deficient cells. (C) TACE activity assay was performed with membrane lysates from untreated and CpG-stimulated RAW cells. Quantification is expressed as a ratio of TACE activity (measured by fluorescence) of Syk-deficient cells to control cells over 4 independent experiments. (D) BMDCs and RAW cells stimulated with CpG in the presence of TAPI were fixed and stained for TNFα for analysis by flow cytometry. Quantification is expressed as a ratio of surface TNFα median fluorescence intensity of Syk-deficient cells to that of control cells over 4 independent experiments. Data were analyzed using a one sample t test against a theoretical mean of 1. **p<0.05*

Chapter III

Results

Syk-dependent calcium signals mediate efficient CpG-induced exocytosis of TNFα in innate immune cells

Introduction

We previously demonstrated that Syk is important for trafficking of TNFα to the plasma membrane following CpG stimulation in macrophages and DCs. The signals downstream of Syk orchestrating cytokine exocytosis, however, are not known. Understanding the mediators involved in secretion of potent proinflammatory cytokines, like TNFα, is crucial for identifying potential targets for manipulating the immune response downstream of PAMP recognition.

Syk is involved in many cellular processes. Downstream of engagement of immunotyrosine-based activation motif (ITAM)-containing receptors, including the B cell receptor and Fc receptors, Syk becomes tyrosine phosphorylated and initiates a signaling cascade. Syk can associate with the adaptor molecule SH2 domain containing leukocyte protein of 65 kDa (SLP-65) (Kulathu et al., 2008), PI3 kinase (Moon et al., 2005), and PLCγ (Law et al., 1996), downstream of which diverse signaling events occur, including activation of MAPKs, NF-κB, and calcium mobilization. Activation of these signaling intermediates leads to several cellular responses. Among them are cytokine release, proliferation, and survival. While Syk function has been elucidated downstream of these ITAM-bearing receptors (Mocsai et al., 2010), the mechanism by which Syk exerts its effects following PRR engagement to promote cytokine secretion is not clear.

Calcium mobilization, a known downstream effector of Syk, has been implicated in synaptic vesicle transport and exocytosis in neurons (Pang and Sudhof, 2010). Therefore, we sought to determine if calcium signaling downstream of Syk is important for CpG-induced TNFα secretion. Indeed, our data demonstrate that Syk orchestrates a calcium-dependent signaling event leading to the activation of calcium calmodulin kinase II (CaMKII). This process facilitates the efficient exocytosis of TNFα downstream of CpG signaling.

Results

Calcium signaling is impaired in the absence of Syk in response to CpG.

Known downstream effectors of Syk include PLCγ (Law et al., 1996) and cytosolic calcium (Kulathu et al., 2008; McVicar et al., 1998), and calcium signaling has been implicated in synaptic vesicle trafficking and exocytosis in neurons (Pang and Sudhof, 2010). We, therefore, sought to determine whether Syk dependent mobilization of intracellular calcium regulates TNFα secretion and accounts for the defect observed in Syk-deficient cells. Control RAW cells exhibited a rise in intracellular calcium levels in response to CpG in the presence of extracellular calcium (Figure 3.1A, 8 minutes). In contrast, Syk-deficient RAW cells failed to mobilize calcium upon CpG stimulation under these same conditions. This defect in calcium was selective, however, as cells lacking Syk were able to mobilize calcium in response to ATP, a ligand for G-protein coupled purinergic receptors that results in Syk-independent calcium flux (Surprenant and North, 2009), and to pharmacological release of calcium from internal stores with ionomycin. These data demonstrate that CpG-induced calcium mobilization is defective in the absence of Syk.

To test whether the increase in intracellular calcium we observed following CpG stimulation was important for TNFα secretion, we treated RAW cells with BAPTA-AM, an intracellular calcium chelator. Stimulation of BAPTA-treated cells with CpG failed to elicit secretion of TNFα (Figure 3.1B), suggesting that the Syk-dependent calcium response regulates TNFα secretion. To test this further, we designed a gain of function experiment reasoning that pharmacological mobilization of calcium should rescue the TNFα secretion defect in the Syk-deficient cells. Indeed, addition of ionomycin and CpG increased the total amount of TNFα secreted into the supernatants from the Syk-

deficient RAW cells to control levels (Figure 3.1C), suggesting that calcium signaling is important for efficient TNFα regulation and that Syk is mediating this process. The same was true for MCP-1 levels (Figure 3.1D). These results indicate that CpG mediated Syk-dependent calcium signals regulate efficient trafficking of TNFα to the plasma membrane for its secretion from the cell and regulate MCP-1 secretion.

PLCγ2 knockdown results in defective CpG-induced TNFα secretion.

PLCγ2 is a known target of Syk kinase activity, and its activation results in calcium release from the endoplasmic reticulum. We, therefore, reasoned that suppression of PLCγ2 should result in a similar phenotype as suppression of Syk. To test this hypothesis, we gene silenced PLCγ2 using shRNA in RAW cells and found that PLCγ2-deficient cells phenocopied Syk-deficient cells; TNFα protein was produced normally, but its secretion was impaired with a similar magnitude as Syk deficiency following CpG stimulation (Figure 3.2). Moreover, TNFα secretion was rescued upon addition of ionomycin. These data are consistent with PLCγ2 acting as the downstream effector of Syk and being required for the optimal calcium-induced exocytosis of TNFα.

CaMKII promotes CpG-induced exocytosis of TNFα downstream of Syk.

We next sought to identify the target of calcium that regulates TNFα secretion in macrophages and DCs. We focused on CaMKII, a serine threonine protein kinase whose phosphorylation has been reported following TLR stimulation (Liu et al., 2008). Binding of CaMKII to calcium induces a structural rearrangement resulting in its autophosphorylation (Griffith, 2004), a necessary step for activation of its downstream targets. In neurons, CaMKII has been reported to participate in the phosphorylation of

molecules involved in exocytosis (Hilfiker et al., 1999; Verona et al., 2000). Consistent with CaMKII being the target of CpG-induced Syk and PLCγ2 dependent calcium signaling, we found that Syk-deficient BMDCs exhibited defective CpG-induced CaMKII phosphorylation (Figure 3.3A). We next looked more directly at the role of CaMKII in TNFα secretion by suppressing its expression in RAW cells. We found that CaMKII-deficient cells, like those lacking Syk or PLCγ2, generated normal amounts of TNFα protein but secreted less cytokine in response to CpG (Figure 3.3 B-C). This secretion defect was not rescued by ionomycin, which is consistent with the notion that CaMKII is downstream of calcium mobilization. Thus, we have identified Syk-PLCγ2 dependent activation of calcium-induced CaMKII signaling as a novel pathway for the regulation of TNFα secretion (Figure 3.4). This suggests that in addition to activating the transcription and translation of cytokines, CpG provides an active signal to promote the trafficking and secretion of TNFα.

Discussion

In the second chapter of this thesis, we identified a role for Syk in trafficking and exocytosis of TNFα, but not IL-6, downstream of CpG signaling. Here we demonstrate that the signals required for this process downstream of Syk rely on calcium mobilization and subsequent CaMKII phosphorylation. Once activated, the Syk binding partner PLCγ cleaves PIP_2 into diacylglycerol and IP_3. IP_3 in turn binds to its receptors located on the endoplasmic reticulum membrane, and these receptors serve as calcium channels and thus allow an influx of calcium from ER stores. PLCγ2-deficient DCs cannot mobilize calcium in response to the TLR4 ligand LPS (Zanoni et al., 2009) providing a precedent for activation of this pathway by TLR ligands. We found that knockdown of PLCγ2 resulted in decreased CpG-induced TNFα secretion, which was rescued by ionomycin. Knockdown of the calcium-responsive kinase CaMKII also resulted in decreased CpG-induced TNFα secretion. Similarly, Syk-deficient cells showed defective CpG-induced calcium mobilization and CaMKII phosphorylation. These data demonstrate that a Syk-dependent pathway signaling through a PLCγ-calcium-CaMKII axis is responsible for optimal TNFα secretion downstream of CpG activation.

Calcium signaling is a well-established pathway for exocytosis, particularly for the transport of synaptic vesicles in neurons (Pang and Sudhof, 2010). For example, intracellular membrane fusion events necessary for cytokine secretion involve the assembly of Soluble NSF Attachment Protein Receptors (SNAREs), the activation of which has been linked to calcium mobilization. CaMKII was reported to phosphorylate proteins important for exocytosis in neurons, including the SNARE-interacting molecule Synaptotagmin, which initiates membrane fusion events (Hilfiker et al., 1999; Verona et al., 2000). In macrophages, SNAREs including VAMP3, Syntaxin6, and Vti1B were

upregulated following LPS stimulation, and VAMP3 colocalized with TNFα in recycling endosomes (Murray et al., 2005a; Murray et al., 2005b). It is plausible, therefore, that Syk is orchestrating a calcium-dependent signaling event that triggers exocytosis by way of Synaptotogamin and SNARE activation downstream of CpG stimulation. IL-6 trafficking and exocytosis were also reported to be dependent on the SNARE machinery, but it is possible that IL-6 relies on a different set of CaMKII-independent trafficking intermediates for its secretion. Therefore, in addition to signaling for activation of NF-κB and cytokine transcription, CpG must also provide distinct signals for activation of trafficking machinery to ensure exocytosis and secretion of cytokines. This provides a useful way for tightly coordinating the differential secretion of potent proinflammatory cytokines in order to adequately direct the effector cell response. Furthermore, because similar trafficking intermediates are employed for cytokine release downstream of PRRs in innate immune cells and for synaptic vesicle exocytosis in neuronal cells (Jahn and Fasshauer, 2012), understanding the signaling pathways that regulate these processes have broad implications for release of cellular contents in different tissues throughout the body.

In summary, we have demonstrated a novel pathway involving a PLCγ2-calcium-CaMKII axis downstream of Syk important for the exocytosis of TNFα, but not IL-6, following CpG stimulation. These findings establish Syk as an important signaling intermediate for the differential release of cytokines downstream of PAMP recognition to elicit an appropriate cytokine response. The regulation of exocytosis by PAMPs marks a new area to explore in the control of cytokine release by PRRs. There likely exist other biochemical mediators activated by PRRs that regulate the secretion of cytokines other than TNFα. Understanding in which vesicular compartments these cytokines reside and

which pathways regulate their secretion will provide greater insight into how particular cytokine responses are generated.

Figures

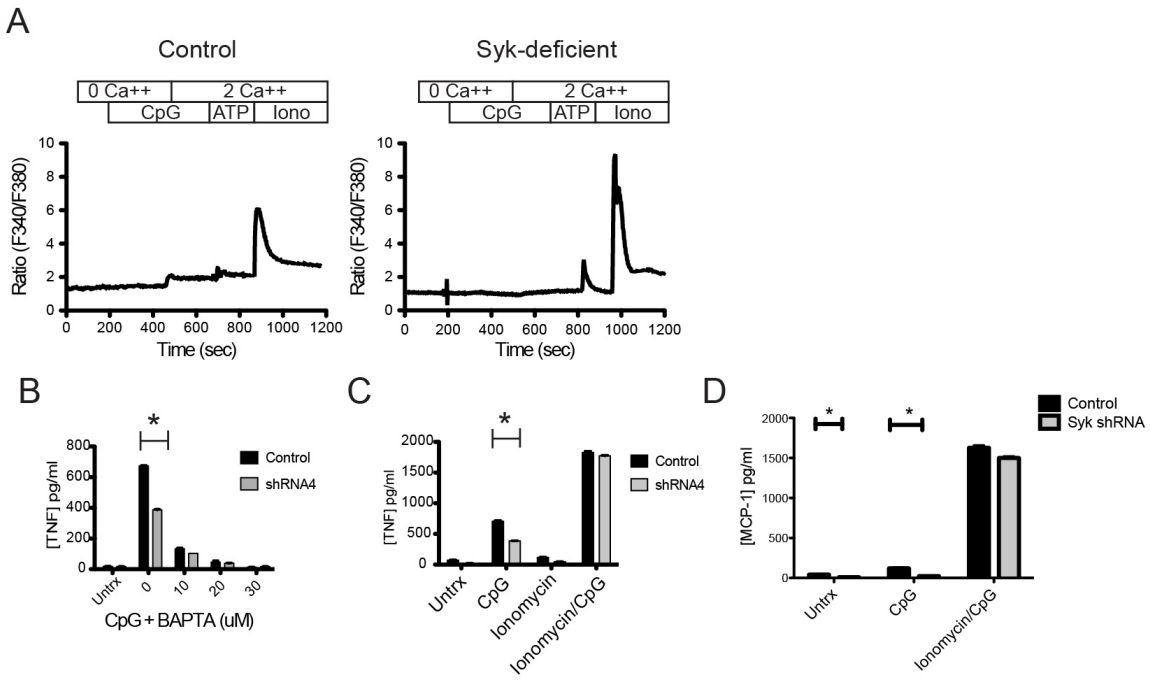

Figure 3.1 Calcium signaling is important for TNFα secretion downstream of Syk. (A) RAW cells were loaded with the calcium sensitive dye Fura-2, and calcium mobilization was detected in response to CpG, ATP, and ionomycin. Plots are representative of 5 independent experiments. RAW cells were stimulated with CpG and BAPTA (B) and with CpG and ionomycin (C-D) for 3 hours. Culture supernatants were analyzed by ELISA for TNFα (B-C) and MCP-1 (D). Bar graphs are representative of at least 3 independent experiments performed in triplicate. **p<0.05*

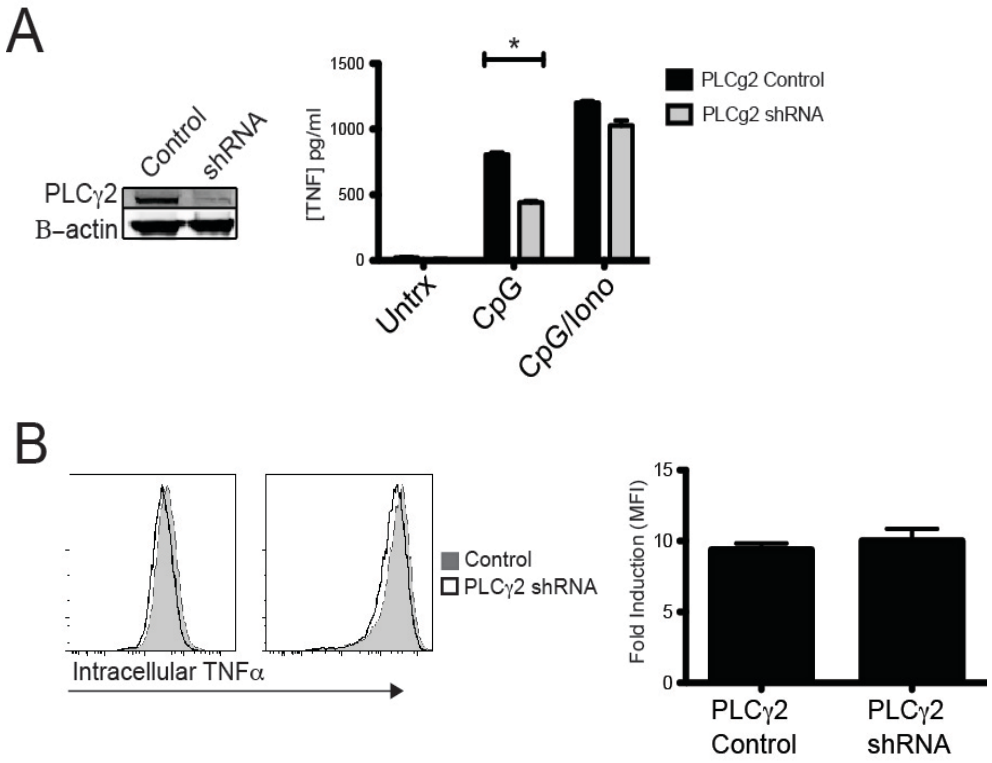

Figure 3.2 PLCγ2 deficiency results in impaired CpG-induced TNFα secretion. (A) RAW cells transduced with PLCγ2 shRNA delete PLCγ2 protein (Western blot). PLCγ2 control cells were transduced with an irrelevant shRNA sequence. PLCγ2-deficient RAW cells were stimulated with CpG or with CpG and ionomycin for TNFα detection by ELISA. (B) PLCγ2-deficient RAW cells were stimulated with CpG in the presence of Brefeldin A for detection of intracellular TNFα by flow cytometry. ELISA data are representative of 4 independent experiments performed in triplicate. Fold induction of intracellular TNFα with CpG was calculated based on median fluorescence intensity. Bar graph (B, right panel) represents summary data from 3 independent experiments, +/- SEM. **p<0.05*

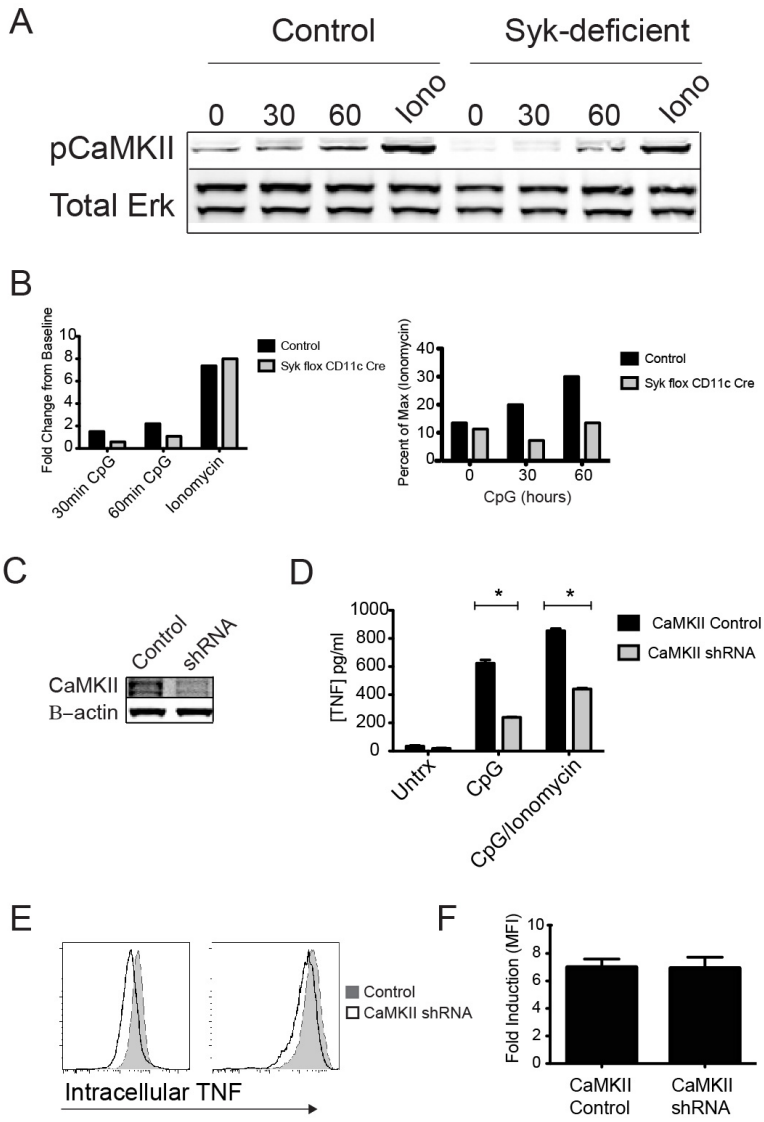

Figure 3.3 CaMKII is important for CpG-induced TNFα secretion downstream of Syk. (A) BMDCs were stimulated with CpG or ionomycin at various times, and lysates were harvested and analyzed by Western blot for phospho-CaMKII. (B) For quantification, pCamKII was normalized to total ERK, and that ratio, at each time of stimulation, was compared to the signal at baseline (left panel). Normalized pCamKII from CpG-stimulated cells was also compared to the maximum signal achieved with ionomycin stimulation (right panel). Blot is representative of 3 independent experiments. (C) RAW cells transduced with CaMKII shRNA delete CaMKII protein (Western blot). CaMKII control cells were transduced with an irrelevant shRNA sequence. (D) CaMKII-deficient RAW cells were stimulated with CpG or with CpG and ionomycin for TNFα detection by ELISA. (E) RAW cells were stimulated with CpG in the presence of Brefeldin A for detection of intracellular TNFα by flow cytometry. (F) Fold induction of intracellular TNFα with CpG was calculated based on median fluorescence intensity. ELISA data are representative of 4 independent experiments performed in triplicate. Bar graph represents summary data from 3 independent experiments, +/- SEM. **p<0.05*

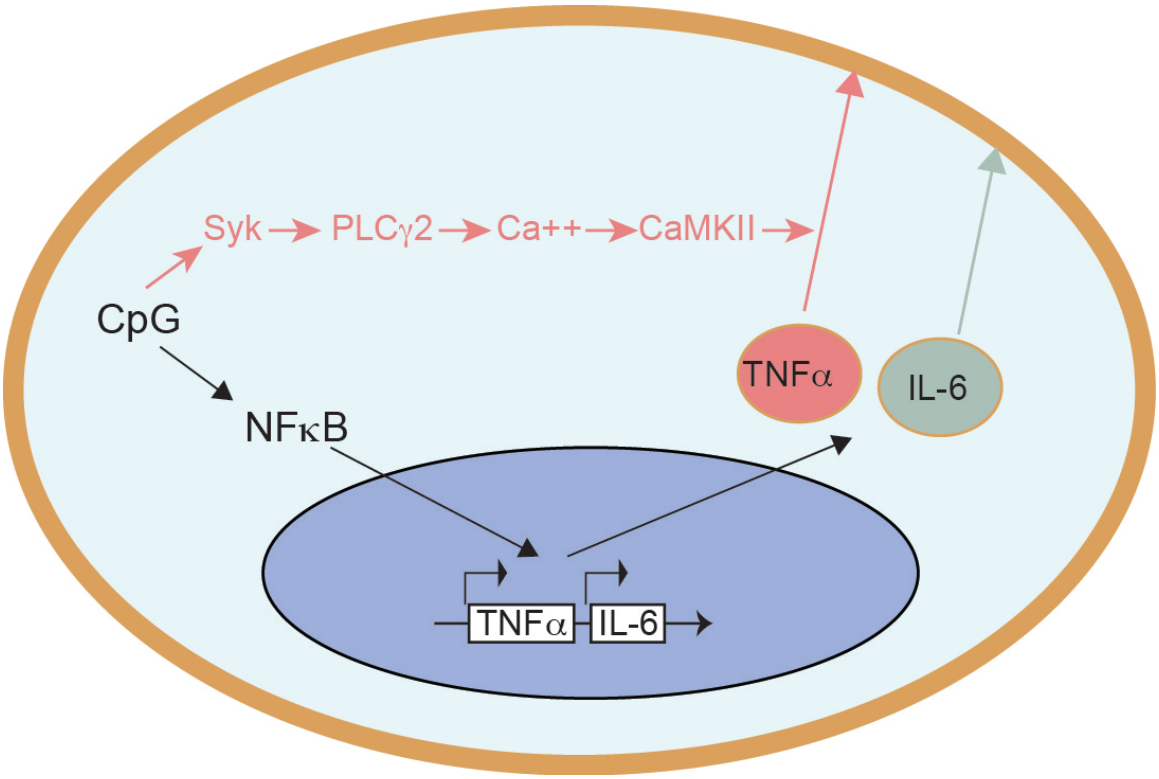

Figure 3.4 Syk dependent calcium signals mediate efficient CpG-induced exocytosis of TNFα. Upon CpG stimulation, the canonical TLR9 machinery activates NF-κB, resulting in Syk-independent transcription of proinflammatory cytokines like IL-6 and TNFα. These cytokines are packaged into secretory vesicles after synthesis. CpG also activates a Syk-dependent signaling cascade resulting in calcium release through PLCγ2. Calcium binds to CaMKII, leading to its autophosphorylation, a step necessary for activation of its downstream targets. This signaling pathway is important for the trafficking of intracellular TNFα, but not IL-6, to the plasma membrane. Thus, Syk activation downstream of CpG stimulation results in differential trafficking of cytokines.

Chapter IV

Results

TLR9 tyrosine 870 mediates efficient receptor maturation

Introduction

The pattern recognition receptor TLR9 binds CpG DNA from pathogenic stimuli such as viruses and bacteria in the endosomal compartment. Upon ligand recognition, the receptor can recruit MyD88 to initiate signaling for the induction of NF-κB-dependent transcription of proinflammatory cytokines and interferon regulatory factor (IRF)-dependent transcription of type I IFNs. Together, these cytokines contribute to the immune response by recruiting and activating other cells of the immune system to aid in pathogen clearance.

Prior to ligand recognition, a functional receptor must be generated to initiate signaling. From the endoplasmic reticulum, TLR9 is escorted to the endosome by its physical interaction with the membrane protein UNC93B1 (Brinkmann et al., 2007; Kim et al., 2008). In the absence of UNC93B1 TLR9 fails to traffic to the endosome, and CpG-induced signaling is abolished. Once in the endosome, the receptor is proteolytically processed to generate its mature form (Park et al., 2008). While the unprocessed receptor is capable of binding CpG, only the processed form can interact with MyD88 to initiate downstream signaling. This ensures that inappropriate signaling is prevented.

All TLRs contain a relatively conserved TIR domain that facilitates interactions with other TLRs and other TIR domain-containing proteins, including the adaptor molecules Trif and MyD88 (Jiang et al., 2006; Li et al., 2005; Ohnishi et al., 2009). This domain is comprised of about 160 amino acids and is located in the cytoplasmic portion

and is essential for downstream signaling. The domain contains three different short conserved regions, termed box 1 (F/Y)DA, box 2 RDXXPG, and box 3 FW, all of which were reported to be important for receptor function based on a series of alanine mutations (Slack et al., 2000). Interestingly, the tyrosine residue of box 1 is conserved among all TLRs except, TLRs 1, 6, and 12. These receptors instead contain a phenylalanine in that position. Moreover, these are the only TLRs that do not homodimerize. TLRs 1 and 6 heterodimerize with TLR2, and TLR12 forms heterodimers with TLR11. Thus, these observations raise the question of whether tyrosine phosphorylation of that residue is important for full receptor activation or perhaps whether there exists a structural requirement at that position for activation. To investigate these possibilities, we generated TLR9 mutants containing either phenylalanine or alanine at residue 870. Although preliminary, we observed defective receptor maturation with both mutations. The alanine mutation completely abolished receptor activation, while the phenylalanine mutation partially diminished TLR9 maturation. These data suggest that perhaps there are both phosphorylation and structural requirements at residue 870, but additional experiments are needed to determine the mechanism by which tyrosine 870 affects TLR9 maturation.

Results

Mutation of TLR9 tyrosine 870 results in decreased receptor maturation.

To investigate the effects of tyrosine 870 on CpG-induced TLR9 signaling, we generated retrovirus expressing either WT HA-tagged TLR9, HA-tagged receptor in which tyrosine 870 has been mutated to phenylalanine (Y870F), or HA-tagged receptor in which tyrosine 870 has been mutated to alanine (Y870A). Transcription of HA-tagged TLR9 is followed by an internal ribosomal entry site leading to expression of green fluorescent protein (GFP). WT TLR9, based on HA expression, was expressed in TLR9 deficient BMDCs and rescued cytokine secretion following CpG stimulation (Figure 4.1). The full-length glycoysylated TLR9 is proteolytically processed to generate a mature, functional receptor (Park et al., 2008). We observed expression of both the unprocessed (160 kilodaltons) and processed (80 kilodaltons) receptor in TLR9 deficient bone marrow transduced with WT TLR9 (Figure 4.1). BMDCs also expressed the full-length receptor when transduced with either TLR9 Y870F or Y870A (Figure 4.2A-B). However, the Y870A-expressing cells did not generate the mature form of TLR9, and the Y870F-expressing cells demonstrated reduced expression of processed receptor. Furthermore, we observed similar transduction efficiency of each construct, based on GFP expression, but diminished HA expression when 870 was mutated to F or A (Figure 4.2C-D). Collectively, these data suggest that tyrosine 870 is important for receptor maturation.

Because tyrosine 870 is located in the TIR domain, we next considered whether this residue is also important for TLR9 signaling. CpG stimulation of TLR9 KO cells expressing the Y870F mutant demonstrated impaired secretion of TNFα and IL-6 (Figure 4.3A). However, when we normalized the amount of CpG-induced cytokine in the supernatant to the expression of the processed form of the receptor, we observed

comparable levels of TNFα and IL-6 between WT TLR9-expressing cells and Y870F-expressing cells (Figure 4.3B). Moreover, HA+ cells produced similar amounts of CpG-induced cytokine whether WT TLR9 or Y870F was stimulated (Figure 4.3C), indicating that the processed form of the receptor is functional when there is a phenylalanine present at the 870 position. Together, these data suggest that tyrosine 870 is important for TLR9 maturation but dispensable for CpG-induced signaling.

Discussion

Although preliminary, our data suggest that a tyrosine at position 870 is important for receptor maturation of TLR9. The Y870A mutation abolished TLR9 maturation, and transduction with the Y870F mutant resulted in diminished expression of the processed form. However, the remaining mature Y870F receptor was able to signal appropriately upon CpG stimulation. This indicates that once processed, mature TLR9 does not rely upon tyrosine 870 for ligand recognition and downstream signaling.

TLR9 binds UNC93B1 in the endoplasmic reticulum for its transportation to the endosomal compartment (Brinkmann et al., 2007; Kim et al., 2008). Once in the endosome, the receptor is cleaved to generate functional TLR9 that can interact with MyD88 and initiate downstream signaling (Park et al., 2008). It is unclear from our data if the receptor mutated at residue 870 is impaired in its ability to bind UNC93B1 and is, therefore, not trafficking to the endosome, or whether TLR9 is present in the endosome but unable to be processed into the functional form. The predicted cleavage site for TLR9 is between amino acids 441 and 470, a site flanked by two leucine rich repeat domains (Park et al., 2008). Therefore, it is unlikely that residue 870 is involved in receptor cleavage, but it is possible that the Y870A mutant is misfolded such that the cleavage site is no longer accessible to proteases. It was reported that amino acids 812 and 813, located between the transmembrane domain and box 1 of the TIR domain, were important for UNC93B1 binding (Kim et al., 2013). Because of the proximity of this region to tyrosine 870, it is possible that the structure of the mutants has changed sufficiently to affect UNC93B1 binding and subsequent transport of TLR9 out of the endoplasmic reticulum to the endosome. We are pursuing studies investigating the localization of TLR9 Y870F and Y870A. If tyrosine 870 is important for receptor trafficking, it is likely that the Y870A mutant and to a lesser extent, the Y870F mutant,

will be stuck in the endoplasmic reticulum and absent from the endosome. For these studies, we can take advantage of the glycosylation pattern of TLR9. Furthermore, investigating localization of GFP-tagged receptor will be useful for determining how tyrosine 870 affects TLR9 trafficking.

Because the Y870F receptor results in a phenotype different from that of the Y870A receptor, it is possible that phosphorylation of that residue is important for TLR9 activation. Studies are underway to determine the phosphorylation status of tyrosine 870. The Y870F mutant demonstrated only a partial defect in the ability of TLR9 to mature, so perhaps phosphorylation of a different tyrosine residue is sufficient to allow limited receptor activation. Tyrosine kinase inhibition could be a useful strategy to determine whether tyrosine phosphorylation is necessary for generation of a functional receptor, but to answer the question more definitively, a mass spectrometry approach is necessary to determine if tyrosine 870 is phosphorylated.

In summary, our data suggest that the conserved tyrosine residue located in box 1 of the TIR domain is important for TLR9 activation. The mechanism, phosphorylation and/or structural requirements, by which this residue exerts its effects is currently unknown. It will be interesting to investigate how this residue affects trafficking and localization of the other TLRs. Additionally, it is possible that the tyrosine of TIR box 1 is important for receptor dimerization. Most TLRs homodimerize. TLR1 and TLR6 form dimmers with TLR2, and TLR11 heterodimerizes with TLR12, suggesting that perhaps the presence of a tyrosine at position 870 allows for efficient receptor dimerization. Additional work is needed to investigate how the conserved tyrosine of TIR domain box 1 influences receptor activation.

Figures

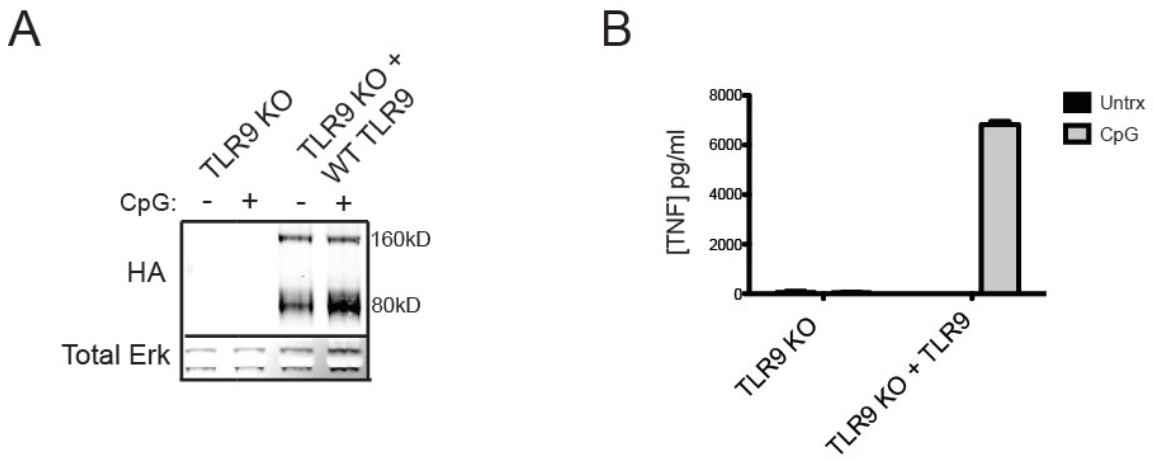

Figure 4.1 WT TLR9 is expressed and rescues signaling in TLR9 deficient BMDCs. TLR9 KO bone marrow was transduced with MigR retrovirus expressing HA-tagged WT TLR9. (A) Western blot analysis of whole cell lysates demonstrated expression of WT TLR9 in TLR9-deficient BMDCs. (B) BMDCs were stimulated for 3 hours with CpG (1μg/ml). Supernatants were harvested, and TNFα was detected by ELISA.

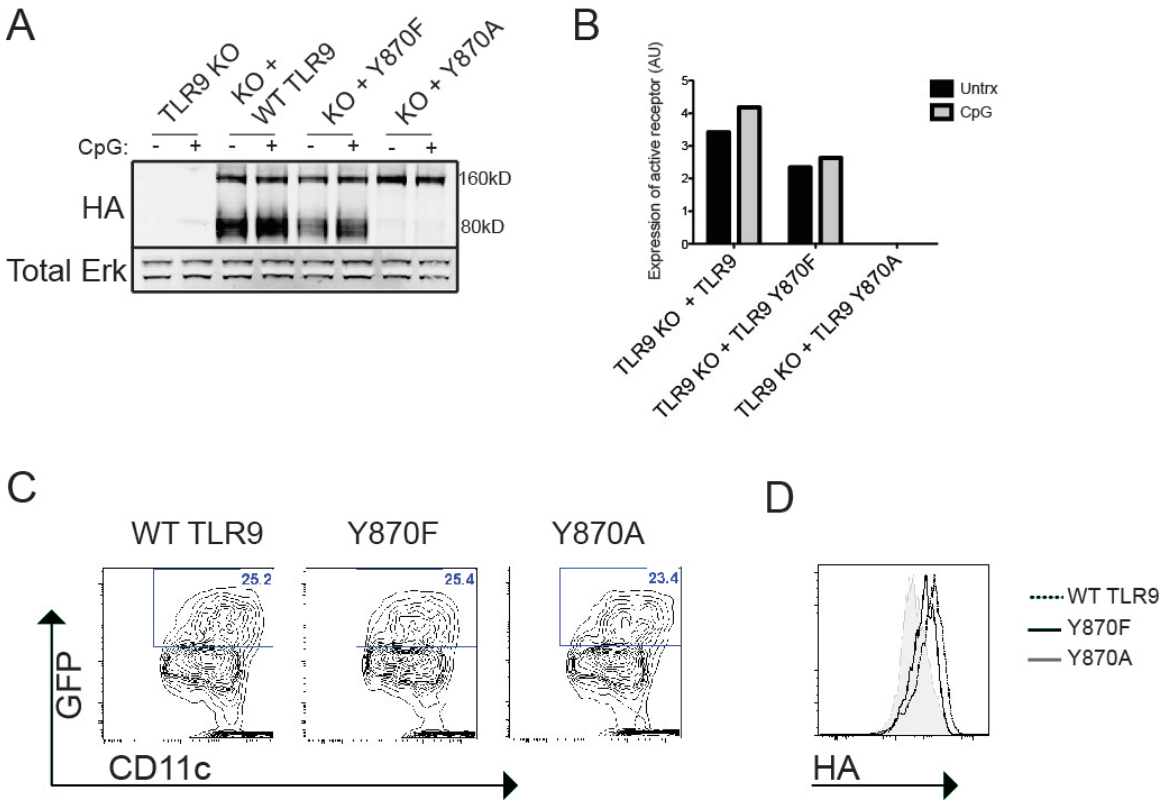

Figure 4.2 Decreased expression of mature receptor when Tyr870 is mutated. (A) Cells were lysed, and the presence of TLR9 was assessed by blotting with HA. (B) Expression of the unprocessed form of TLR9 (160kD) was normalized to Total Erk, and the expression of processed receptor (80kD) was normalized to the resulting ratio. Blots were developed on Odyssey and analyzed by Licor software. (C-D) BMDCs were harvested and stained with antibodies for CD11c and HA. (C) GFP expression was determined for live, CD11c+ cells by flow cytometry. (D) HA expression was determined for GFP+CD11c+ cells by flow cytometry.

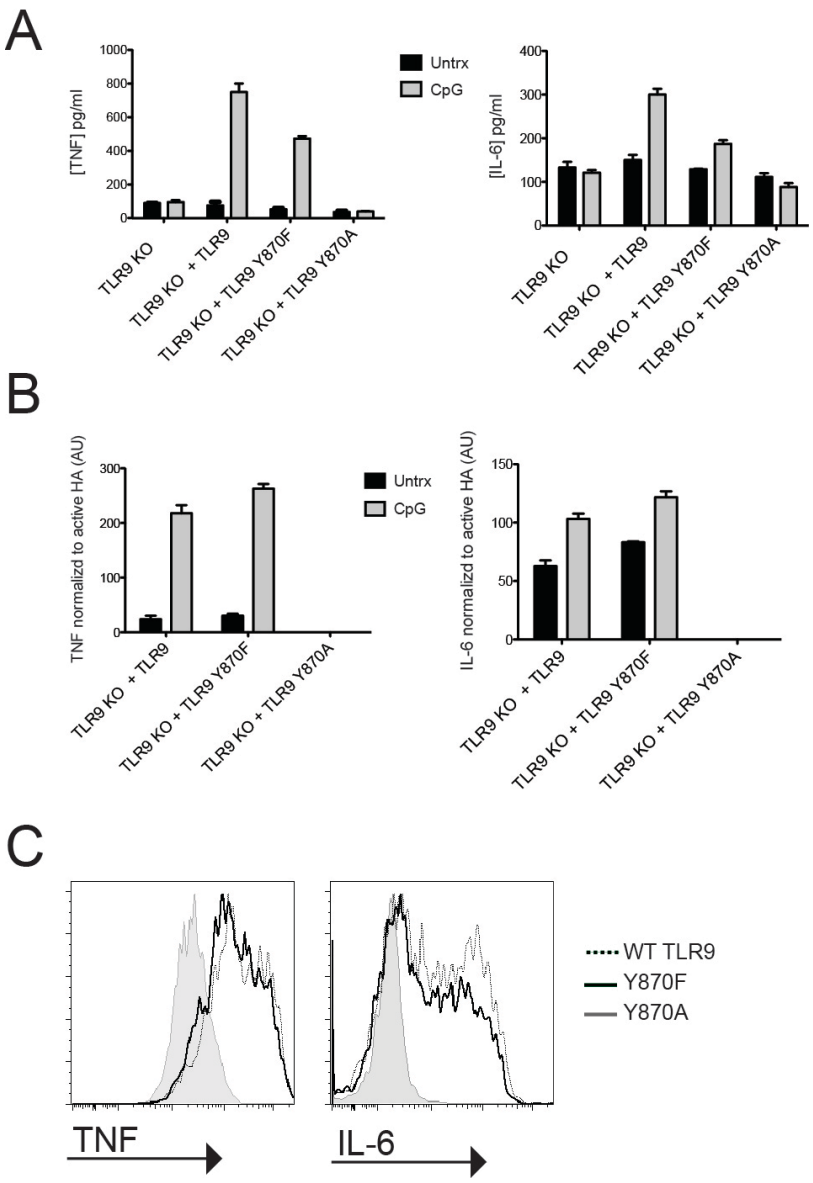

Figure 4.3 TLR9 Y870F signals appropriately for cytokine secretion and production. (A) TLR9 KO BMDCs expressing WT TLR9, Y870F, or Y870A were stimulated for 3 hours with CpG (1μg/ml). Supernatants were harvested, and TNFα and IL-6 were measured by ELISA. (B) TNFα and IL-6 levels from B were normalized to the active (80kD) receptor, as shown in Figure 4.2. (C) CpG-induced intracellular levels of TNFα and IL-6 were determined for HA+GFP+CD11c+ cells by flow cytometry.

Chapter V

Discussion and future directions

In this thesis, we examined the role of tyrosine phosphorylation in signaling downstream of TLRs. We first investigated the function of the protein tyrosine kinase Syk using genetic knockdown in macrophages in vitro and DC-specific genetic deletion in vivo. We uncovered a novel role for Syk in the promotion of TNFα and MCP-1 exocytosis in response to CpG in DCs and macrophages. We extended these studies by investigating the mechanism by which Syk exerts its function of TNFα regulation and found that Syk-dependent calcium mobilization activates CaMKII, which promotes TNFα exocytosis. It will be important to investigate where along the trafficking pathway CaMKII mediates TNFα exocytosis and whether a similar pathway exists for MCP-1. Shifting focus from downstream signaling events, we focused on the receptor itself as a mediator of CpG-induced signaling. TLRs contain a relatively conserved cytoplasmic TIR domain harboring a tyrosine in the box 1 region. Preliminary results with mutational analysis suggest that this tyrosine is important for receptor maturation but dispensable for downstream signaling. It is still unknown whether tyrosine phosphorylation is the mechanism mediating TLR9 maturation, and future experimentation will address this hypothesis. The data presented in this thesis generate interesting questions to explore for future investigations.

Syk-dependent "secretome"

Our data demonstrate that in response to TLR9 stimulation, Syk-deficient macrophages and DCs are impaired in their ability to secrete TNFα and MCP-1, while

IL-6 secretion is intact. Syk-deficient cells also demonstrate defective TNFα secretion downstream of TLR4 stimulation, suggesting the possibility that Syk-dependent regulation of cytokines is not specific to one receptor. This was a very unexpected finding, because tyrosine kinases have been described in other cellular systems as regulators of proximal signaling events ultimately affecting gene expression. For example, downstream of B cell receptor ligation, Syk is recruited to the phosphorylated ITAMs present in the receptor. This recruitment leads to activation of downstream effectors SLP-65, PLCγ, and MAPKs, ultimately leading to an increase in BCR-induced transcription. However, our data suggest that TLR9-induced activation of MAPKs and NF-κB and TNFα production is normal in the absence of Syk. Rather, Syk is functioning outside of the canonical TLR9 signaling machinery to promote TNFα secretion. These data demonstrate a previously underappreciated role for Syk specifically for TNFα trafficking in response to a non-ITAM bearing receptor, and they expand upon the results reported from Cliff Lowell's group. Lowell showed that Syk-deficient neutrophils had reduced TNFα secretion, but intact message, in response to opsonized bacteria (Van Ziffle and Lowell, 2009). Our data point the defect specifically to trafficking, and we include a biochemical mechanism that contributes to this process. Our findings also have implications for cytokine sorting. Jennifer Stow's group reported that IL-6 and TNFα can be sorted into different vesicles at the Golgi complex (Manderson et al., 2007). It is possible that Syk-dependent signals participate in the differential sorting of these cytokines at the Golgi complex, because we observe defective TNFα but normal IL-6 secretion in the absence of Syk. It will be useful to investigate the localization of TNFα in the Syk-deficient cells to confirm that the unsecreted cytokine is stuck at the *trans*-Golgi network. Furthermore, our data suggest that CpG must provide an additional signal

distinct from canonical activation of TLR9-induced cytokine transcription to initiate the activation of cytokine exocytosis and release.

Additional work is needed to determine the extent to which Syk deficiency affects cytokine responses globally. We are currently investigating Syk-dependent secretion in response to various stimuli, including ligands for TLR2/6, TLR3, TLR5, TLR7, and TLR8. We anticipate defective TNFα and MCP-1 secretion in the absence of Syk in response to these stimuli, which would suggest that Syk activity is not limited to just TLRs 4 and 9. Therefore, Syk could be a potential target for therapeutic intervention during infections and inflammatory diseases for which several PRRs are activated. Furthermore, we are investigating the secretion of a wide array of inflammatory mediators downstream of these receptors to determine the existence of a Syk-dependent "secretome." It is possible that there are cytokines whose secretion is more affected by Syk deletion than TNFα (as we saw with MCP-1), and there are likely cytokines whose secretion is completely Syk-independent (as we observed with IL-6). More work is needed to determine the mechanism by which Syk affects MCP-1 exocytosis. It is possible that MCP-1 traffics in a manner similar to TNFα and that Syk regulates a shared mediator of cytokine trafficking. Therefore, it will be important to determine the cytokines affected by Syk deletion and how these cytokines traffic in response to PRR engagement. Moreover, because we find that Syk deletion results in a two-fold decrease in TNFα secretion, we speculate that Syk can modulate different "pools" of cytokines. Perhaps there exists a Syk-independent pool of TNFα that relies on a different set of trafficking molecules. This level of regulation can be advantageous for a cell trying to secrete a fixed amount of cytokine in response to an insult. Cells can qualitatively determine the cytokine response by either engaging or not engaging Syk. Furthermore, modulating Syk activity might

represent a way to selectively diminish cytokine responses without abolishing the entire TLR pathway. It is also possible that different cell types utilize Syk for all existing TNFα pools or perhaps for no TNFα pools. Syk-deficient neutrophils displayed almost a 75% reduction in TNFα release in response to opsonized bacteria (Van Ziffle and Lowell, 2009), suggesting that Syk regulates the majority of TNFα in this lineage. Utilizing lineage-specific deletion of Syk in vivo will be important for determining the extent to which different cell types utilize Syk for cytokine secretion following PRR engagement.

All TLRs signal through the transcription factor NF-κB which leads to transcription of a variety of cytokines and chemokines (Kawai and Akira, 2007). However, the release of such cytokines occurs differently upon stimulation of different receptors. For example, the amount of secreted TNFα varies upon TLR3, TLR4, or TLR9 ligation (Dearman et al., 2009; El-Hage et al., 2011), even though these TLRs all activate NF-κB similarly. Indeed, our data demonstrate normal NF-κB activation but defective TNFα secretion following CpG stimulation in the absence of Syk, while IL-6 secretion was unaffected. Differential activation of Syk and/or trafficking molecules downstream of PAMP recognition may be a useful strategy for cells to affect the qualitative cytokine response of receptors and, therefore, tailor the immune response by regulating exocytosis. Furthermore, an active signal distinct from that of NF-κB-induced transcription is critical for differentially sorting cytokines for appropriate release.

Based on the pathogenic stimulus, a cell can determine the amount of cytokine, like TNFα, to release by either engaging Syk or not engaging Syk following receptor ligation. Because different cytokines exert different effects on the immune system, this strategy allows for appropriate release of cytokines to achieve the desired response. For example, TNFα and IL-6 both promote inflammation in response to PRR engagement,

but IL-6 can additionally facilitate differentiation of T cells into the T helper 17 lineage (Ghoreschi et al., 2010; Veldhoen et al., 2006). Therefore, following infection with *Listeria monocytogenes*, an intracellular pathogen for which a T helper 17 cell response is not useful, it is likely more advantageous for responding cells like macrophages to engage Syk following PRR engagement to induce maximal TNFα release. In response to a fungal infection, the PRRs might not need to engage Syk, thus allowing for reduced TNFα release. The limited TNFα release might be a strategy for the immune system to promote inflammation for pathogen clearance but to limit the amount of cytokine that could damage host tissues. Once a Syk-dependent "secretome" has been identified, it will be important to challenge mice harboring lineage-specific deletions of Syk with various pathogens to understand how Syk deletion affects the immune response.

Constitutive versus regulated exocytosis of CpG-induced TNFα

The literature on cytokine exocytosis defines cytokines secretion by regulated or constitutive exocytosis. During regulated exocytosis, cytokines packaged into vesicles in a resting cell are rapidly released upon some stimulus. During constitutive exocytosis, a stimulus signals for de novo synthesis of cytokines, and these cytokines are then immediately packaged and shuttled out of the cell. However, exocytosis of molecules other than cytokines, including hormones and membrane components, is described differently (Arvan and Halban, 2004). For example, constitutive exocytosis of insulin refers to the rapid movement of proteins packaged in small vesicles budding from the *trans*-Golgi network for spontaneous discharge. Conversely, the regulated secretory pathway involves packaging of cargo proteins in vesicles distinct from the small secretory vesicles involved in the constitutive pathway. The cargo is then delivery to

granules in a highly regulated fashion for subsequent release. The distinction between these two pathways for cytokine secretion is the presence or absence of de novo cytokine transcription; conversely, the distinction for non-cytokine cargo is the type of vesicle and the presence or absence of regulatory proteins. While our data suggest that Syk differentially regulates *cytokine* exocytosis, the description of the secretory pathways of non-cytokine cargo can offer an explanation for the Syk-dependent and the Syk-independent secretion of TNFα. That is, Syk regulates the vesicular movement of TNFα independent of its transcription.

Insulin release from pancreatic beta cells has been described as the result of a biphasic secretion pattern (Hou et al., 2009). The constitutive secretion of a rapidly released pool of hormone provides for a strong burst of insulin release upon some stimulus. The reserved pool can be released at a later time contributing to the second wave of insulin secretion. The immune response might benefit from a biphasic release of TNFα. A strong discharge of TNFα early in the immune response would promote the recruitment of additional immune effector cells and would participate in control of pathogen clearance. Because this cytokine also imposes harmful damage to host cells (see discussion below), it would be beneficial for the host to turn off the initial strong burst of TNFα release and, instead, allow a slow, constitutive secretion to combat lingering pathogen. By engaging Syk in response to microbial insults, the immune system can regulate this strong release of cytokine to disrupt pathogen replication. Turning Syk off would allow cells to secrete less TNFα once the "workload" of the immune system decreases. We measured TNFα secretion from one to five hours of CpG stimulation and observed a fifty percent reduction in cytokine levels in that time frame in the absence of Syk. It's possible that later during the response, the secreted TNFα

levels would be similar between control and Syk-deficient cells. It will be useful to perform a kinetic study on TNFα secretion in the response to CpG in the presence and absence of Syk.

Additionally, it will be interesting to investigate the Syk-independent and the Syk-dependent pools of TNFα in our cells. Peter Arvan described clathrin-coated vesicles for the delivery of insulin to the plasma membrane during regulated exocytosis (Arvan and Halban, 2004). Conversely, the small percentage of insulin secreted constitutively is packaged into secretory vesicles in the absence of clathrin. These vesicles can be distinguished by electron microscopy. If Syk-dependent signaling participates in the regulated exocytosis of TNFα, we might be able to observe an absence of these clathrin-coated TNFα vesicles in the Syk-deficient cells.

Syk modulates TNFα exocytosis in response to TLR9 stimulation

TNFα is a potent proinflammatory cytokine exerting both beneficial and detrimental effects for the host. TNFα signaling is known to be essential for the clearance of several intracellular pathogens, including *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Leishmania*, and *M. avium* (Hehlgans and Pfeffer, 2005). This cytokine can recruit other inflammatory cells, like monocytes and neutrophils, and enhance the killing of bacteria. However, when present in excess, TNFα can elicit damage to host tissues. Therefore, achieving the appropriate balance of TNFα production and signaling is crucial for maintaining the health and fitness of the host. Moreover, tight regulation of this molecule is necessary to control the balance between resistance and tolerance to infection.

In order to maintain fitness, an infected host must adequately control an invading pathogen while allowing minimal damage to its own tissues. For example, overstimulation of inflammatory pathways can lead to severe immunopathology even after the pathogen has been cleared (Medzhitov et al., 2012). Therefore, inflammatory pathways must be downregulated while expression of mediators contributing to pathogen clearance without detrimental effects on the host must remain high to limit damage to the host while still mounting an adequate immune response. Medzhitov demonstrated that LPS-pretreated bone marrow derived macrophages decreased expression of IL-6 following LPS stimulation in vitro. Antimicrobial peptide expression remained active during stimulation (Foster et al., 2007). These data suggest that cells are capable of "turning off" proinflammatory responses after continued TLR stimulation in vitro. In vivo, TNFα expression in the lamina propria of the small intestine was necessary to control *T. gondii* replication, but excessive stimulation resulted in TNFα-induced necrosis of the small intestine (Liesenfeld et al., 1999). Administration of a TNFα blocking antibody diminished intestinal pathology, but parasite burdened remained high. Because of the dichotomous role of TNFα, completely abolishing its actions does not entirely protect the host. Perhaps administration of anti-TNFα in the later stages of infection would allow ample time for TNFα-induced control of parasite replication while preventing TNFα-induced pathology. However, if parasite burden is large enough to induce a strong proinflammatory response early in infection, then TNFα-induced necrosis might occur concurrently with parasite replication control.

A recent study linking TNFα and immunopathology highlights the dichotomous nature of this cytokine. Humans infected with *Puumala hantavirus* (PUUV) develop hemorrhagic fever and renal failure, associated with high levels of TNFα, even after

pathogen clearance. Interestingly, rodents infected with this virus developed chronic infection with limited host damage (Guivier et al., 2010). Infected animals exhibited no TNFα upregulation. Therefore, these animals are tolerant to PUUV infection likely due to lack of TNFα expression.

In our system, we find that TNFα secretion was reduced two-fold in the absence of Syk following CpG-induced stimulation of TLR9. Interference of Syk could result in half the amount of serum TNFα, potentially enough to interfere with pathogen replication but not enough to elicit immunopathology. It will be interesting to infect the Syk flox CD11c Cre mice or Syk flox LysM Cre mice, resulting in myeloid-specific deletion of Syk, with various intracellular pathogens to investigate the physiological relevance of diminished, but not abolished, TNFα levels. We observe slightly greater than two-fold decrease in serum TNFα from CpG-injected Syk flox CD11c Cre mice compared to control mice (Figure 2.3). Interestingly, some groups have asked this question using TNFα heterozygous mice. These mice controlled replication of *L. monocytogenes* better than mice with a homozygous deletion of TNFα, but not as well as wild type mice (Amiot et al., 1997). This suggests that less than wild type levels of TNFα are still capable of interfering with bacterial replication. However, the degree of pathology on the host was not investigated in this study.

The dual nature of TNFα behavior was also demonstrated in mycobacterial infection. TNF knockout mice or wild type mice infected with high doses of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) that secreted TNFα died despite being able to control bacterial replication. Conversely, wild type mice infected with BCG alone or with low doses of BCG-TNF controlled and survived pathogen infection (Bekker et al., 2000). These results suggest that immunopathology induced by TNFα is dose

dependent. Therefore, the absence of Syk-dependent TNFα secretion could be a strategy by which the host can limit pathology while still combating pathogenic stimuli. In this system, one might expect Syk flox CD11c Cre (or Syk flox LysM Cre) mice to control *Mycobacterium* replication similar to wild-type mice, but perhaps to limit the deleterious effects of TNFα signaling on the host.

TNFα blockade in autoimmune disease

Given the potency of TNFα's damaging effect on host tissues, it is no surprise that this cytokine has been implicated in inflammatory disease. Rheumatoid arthritis (RA) is a disease characterized by chronic inflammation, joint damage, and bone erosion. Pathogenesis of RA is associated with numerous proinflammatory cytokines and chemokines, including TNFα, IL-6, IL-1β, and IL-8, and the production of autoantibodies (Moelants et al., 2013). A mouse model of collagen-induced arthritis revealed elevated levels of TNFα in the knee joints even before disease onset, and TNFα expression remained high during disease course (Palmblad et al., 2001). Blockade of TNFα early in disease onset ameliorated joint pathology (Joosten et al., 2008). It is evident, therefore, that TNFα contributes to inflammation and disease pathogenesis, so blocking its actions might be advantageous for controlling autoimmune disease. However, there is emerging evidence for the susceptibility of human autoimmune patients to infection when treated with TNFα blocking agents. Appropriate control of TNFα levels is crucial for balancing immunosuppression to combat inflammatory disease and the immunostimulatory effects of the immune system to control pathogenic stimuli.

There have been several cases reported in which RA patients receiving anti-TNFα therapy developed leishmaniasis (Guedes-Barbosa et al., 2013). Therapeutic

intervention for the infection, in addition to the RA treatment, was undertaken in order to control *Leishmania*. There has also been correlation between RA patients treated with the TNFα blocker infliximab and development of tuberculosis (Weisman, 2002), and blocking TNFα in mice in a model of latent *Mycobacterium* infection resulted in reactivation of tuberculosis (Mohan et al., 2001). These studies demonstrate the potential harm that can ensue for the host by blocking TNFα entirely. Interfering with Syk activity in RA patients could result in about a fifty percent reduction in TNFα levels, potentially leaving behind enough cytokine to combat infection while blocking enough to ameliorate autoimmune pathogenesis. Therefore, clinically available Syk inhibitors might work best in patients who already respond to anti-TNFα therapy; instead of completely blocking TNFα signaling, one can reduce the amount of circulating TNFα by half. It will be important to test TNF heterozygous mice or the Syk flox CD11c Cre mice in mouse models of arthritis to establish the effects of diminished TNFα levels on disease pathogenesis and potential susceptibility to infection. Indeed, Cliff Lowell's group demonstrated that neutrophil or myeloid-specific deletion of Syk blocked the progression of arthritis in a serum transfer model, based on joint inflammation (Elliott et al., 2011). However, susceptibility to pathogenic infection was not investigated in this study, but the results suggest that Syk deletion is effective in preventing disease. It will be useful to determine whether Syk blockade after disease onset is a successful therapeutic in this model.

Interestingly, two human trials of Syk inhibition for the treatment of RA yielded opposite results. The first trial reported significant improvement of patients receiving Syk inhibitor (Weinblatt et al., 2010), while a later trial revealed no significant differences between patients receiving the Syk inhibitor and patients receiving the placebo

(Genovese et al., 2011). The differences in these outcomes could be attributable to the patients' treatments. The first study, in which patients reported improvements using the Syk inhibitor, enrolled any patients who had been on long-term immunosuppressive drugs. The trial in which Syk inhibition did not work only enrolled patients who had already failed TNFα-blocking treatments. Perhaps the patients more likely to respond to Syk inhibitors are those who respond to TNFα blockers, since we have now demonstrated that one of the effects of Syk inhibition is a 50% reduction in secreted TNFα. In the case of the successful trial, TNFα blockade responsive patients would have been included in enrollment and, therefore, would likely respond to Syk inhibition. In contrast, all the patients enrolled in the second trial were, by definition, TNFα blockade unresponsive. Therefore, Syk inhibition and the resultant decrease in TNFα would not be expected to have a therapeutic effect. Thus, our data may provide a basic biologic explanation for the divergent results of these two trials.

Diabetes is a disease characterized by obesity that progressively leads to inflammation in the visceral adipose tissue. The inflammatory environment here contributes to insulin resistance and disease progression. Interestingly, mice heterozygous for the enzyme that cleaves membrane TNFα, TACE, were resistant to high fat diet induced obesity (Serino et al., 2007). *Tace+/-* mice on a high fat diet had significantly lower blood glucose levels compared to wild type mice on the same diet. Moreover, this group reported a fifty percent reduction in conversion of full-length TNFα to soluble, secreted TNFα in liver cells of *Tace*+/- mice compared to control mice. However, because TACE has substrates other than TNFα, including the IL-6 receptor and epidermal growth factor (EGF) receptor, it cannot be ruled out that a decrease in these molecules contributes to obesity prevention. Similarly, this study did not

investigate the effects of a high fat diet on TNFα knockout mice or TNFα heterozygous mice. Nonetheless, the data reported suggest that a decrease in TNFα signaling could contribute to a reduction in diabetes pathogenesis. Because TACE heterozygosity was enough to limit disease pathology, perhaps the fifty percent reduction in serum TNFα due to Syk deletion in the Syk flox CD11c Cre mice will be enough to lower blood glucose levels in a model of diet-induced obesity and diabetes. Moreover, it will be interesting to investigate the effects of reduced TNFα in such a model on accompanying pathogenic infection.

Syk mediates CpG-induced MCP-1 secretion

MCP-1 is a chemokine upregulated early during pathogen infection and can contribute to inflammation through its recruitment of inflammatory cells like monocytes to the site of infection (Ajuebor et al., 1998). We observe that Syk deletion impairs the secretion of MCP-1 in macrophages following CpG stimulation. Similar to the dichotomous nature of TNFα, MCP-1 can also exert both positive and negative effects on the host during the course of an immune response. Through its recruitment of inflammatory cells into the inflamed tissue, MCP-1 can contribute to pathogen replication control and clearance. However, excessive amounts of chemokine can result in host tissue damage. Blocking MCP-1 in a model of influenza infection resulted in decreased pathology in the lungs (Damjanovic et al., 2011). Furthermore, MCP-1 levels remained elevated during respiratory syncytial virus (RSV) infection and lead to lung tissue damage (Culley et al., 2006). Inhibiting the actions of Syk could be a mechanism by which to decrease MCP-1 levels without completely abolishing its expression, thereby allowing this chemokine to function appropriately during pathogenic infection.

Broad tyrosine kinase inhibition to diminish TLR signaling

In this thesis, we have investigated the role of tyrosine phosphorylation in TLR9 biology. The canonical signaling molecules described in TLR function have been serine/threonine kinases. Investigating the role of tyrosine kinases will uncover new biochemical mechanisms mediating TLR function and will provide a new class of molecules to target for therapeutic intervention. As discussed above, our data demonstrate that the protein tyrosine kinase Syk modulates TNFα and MCP-1 exocytosis in macrophages and DCs in response to CpG. In Chapter IV, we investigated how a conserved tyrosine in box 1 of the TIR domain affects TLR9 signaling. Though preliminary, our data suggest that tyrosine 870 mediates receptor maturation. Because mutation to phenylalanine resulted in a partial defect of TLR9 maturation, it is possible that phosphorylation of that tyrosine is important for this process. However, future studies are needed to confirm this hypothesis. If tyrosine phosphorylation mediates receptor maturation, then broad tyrosine kinase inhibition would result in abrogation of TLR responses. Conversely, inhibition of Syk would have a more specific effect, diminished secretion of a subset of cytokines.

Because tyrosine kinases are associated with such diverse cellular processes, including cell proliferation, metabolic function, and differentiation, dysregulation of this class of molecules is associated with malignant and non-malignant disorders (Grimminger et al., 2010). Indeed, several drugs have been developed to inhibit these molecules for treatment of a variety of disorders, including malignancy, pulmonary vascular disease, lung fibrosis, sclerosis, and kidney disease, among others (Grimminger et al., 2010). Tyrosine kinase inhibition also prevented lethality in a mouse model of LPS-induced sepsis (Novogrodsky et al., 1994). Treatment was associated with

reduced levels of TNFα and reactive oxygen species in vivo, providing another link between tyrosine phosphorylation events and TLR function. It is evident that disruption of the activity of these molecules can lead to a variety of physiological outcomes that can result in therapeutic relief. However, because pharmacological inhibition of kinases has been linked to off-target effects and undesired side effects, it is imperative that the function of these kinases are scrutinized in various cellular processes.

As noted above, broad tyrosine kinase inhibition would result in disruption of TLR9 signaling, if tyrosine phosphorylation is involved in receptor maturation. Therefore, treatment of a condition like RA, for example, with a broad tyrosine kinase inhibitor would likely result in a complete loss of TLR signaling, whereas Syk inhibition would lead to diminished TNFα secretion, a cytokine implicated in pathogenesis of this disease, while preserving other TLR functions. Cytokines impart different effector functions on the developing immune response. Therefore, more selective inhibition is useful for disrupting a specific cytokine response. Understanding how these responses are regulated is critical for the development of more specific drugs. Additionally, broad tyrosine kinase inhibition could potentially disrupt other signaling cascades required for normal homeostasis in the host. In the case of cytokine storm, however, broader inhibition was necessary to prevent signaling of a variety of inflammatory mediators that contribute to host destruction (Novogrodsky et al., 1994). Excessive TLR9 signaling induced cytokine storm in a mouse model of macrophage activation syndrome (MAS), while TLR9 KO mice were protected from disease (Behrens et al., 2011). Tyrosine kinase inhibition might represent a way to limit the pathology induced by the numerous cytokines, including IL-6, IL-12, and IFNγ, associated with disease and offer therapeutic relief. Furthermore, we can test if the TLR9 Y870F or Y870A mutants reduce disease severity.

For these experiments, we can generate bone marrow chimeras by reconstituting TLR9 KO mice with TLR9 deficient bone marrow transduced with either TLR9 Y870F or Y870A and by subjecting the recipient mice to CpG-induced MAS.

TLR9 tyrosine phosphorylation has been reported previously, and this phosphorylation was abrogated with Src family and Syk kinase inhibitors (Sanjuan et al., 2006). However, because of the off-target effects reported of these drugs (Geahlen and McLaughlin, 1989), more rigorous investigation is needed to determine the mechanism by which TLR9 is phosphorylated. Because Syk deletion results in normal CpG-induced MAPK and NF-κB activation, canonical TLR9 signaling intermediates (see Figure 2.8), it is unlikely that Syk is the kinase responsible for TLR9 phosphorylation in our system. To confirm this, we can express TLR9-HA in bone marrow from the Syk flox CD11c Cre mice and assess receptor phosphorylation and maturation in differentiated DCs. Furthermore, our data demonstrate reduced expression of mature TLR9 when tyrosine 870 is mutated to phenylalanine. It is likely, therefore, that this mutant exhibits abrogated activation of the canonical signaling pathway compared to the wild type receptor, in contrast to what was observed with the Syk-deficient cells. This will be important to confirm experimentally. Additional work is needed to determine the kinase mediating TLR9 phosphorylation.

Conclusions

The work presented here describes the regulation of TLR9 function by tyrosine kinases. Because TLR recognition triggers powerful immune responses against invading pathogens, understanding the biochemical mechanisms by which these responses are generated will provide insight into how these receptors meditate pathogen recognition

and host defense. Tyrosine kinases represent a class of molecules that can be targeted to control the immune response. Understanding their functions will enable the design of effective therapies with limited side effects.

Appendix I

Abbreviations

A

Ala, alanine

B

BCG, *Mycobacterium bovis* bacillus Calmette-Guerin

BMDC, bone marrow derived dendritic cell

BTK, Bruton's tyrosine kinase

C

Ca^{2+}, calcium

CaMKII, Ca^{2+}/calmodulin-dependent kinase II

D

DC, dendritic cell

E

EGF, epidermal growth factor

ER, endoplasmic reticulum

Erk, extracellular signal-related kinase

G

GPI, glycosylphosphatidylinositol

H

HSV-1, herpes simplex virus-1

I

IFN, interferon

IKK, IκB kinase

IL, interleukin

IRAK, interleukin-1 receptor associated kinase

IRF, interferon regulatory factor

ITAM, immunoreceptor tyrosine-based activation motif

J

JNK, c-Jun terminal kinase

K

KO, knockout

L

LPS, lipopolysaccharide

M

MAPK, mitogen activated protein kinase

MAS, macrophage activation syndrome

MCMV, mouse cytomegalovirus

MCP-1, monocyte chemoattractant protein-1

MD-2, myeloid differentiation factor-2

MHC, major histocompatibility complex

MIP-1α, macrophage inflammatory protein-1 alpha

mmLDL, minimally oxidized low-density lipoprotein

MyD88, myeloid differentiation factor-88

N

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NSF, *N*-ethylmaleimide-sensitive factor

P

PAMP, pathogen-associated molecular pattern

Phe, phenylalanine

PLCγ, phospholipase C gamma

PI3K, phosphoinositide kinase-3

PRR, pattern recognition receptor

Pyk2, proline rich kinase-2

PUUV, *Puumala hantavirus*

R

RA, Rheumatoid arthritis

ROS, reactive oxygen species

RSV, respiratory syncytial virus

S

SCAMP5, secretory carrier membrane protein-5

SLP-65, SH2 domain containing leukocyte protein of 65 kilodaltons

SNARE, soluble NSF attachment protein receptor

Syk, spleen tyrosine kinase

T

TACE, TNFα-converting enzyme

TAK1, transforming growth factor-β-activated protein kinase-1

TIR, toll interleukin-1 receptor and resistance protein

TIRAP, TIR domain-containing adaptor protein

TLR, toll-like receptor

TNF, tumor necrosis factor alpha

TRAF6, tumor necrosis factor receptor-associated factor-6

TRAM, TRIF-related adaptor molecule

TRIF, TIR domain-containing adaptor inducing IFNβ

Tyr, tyrosine

V

VAMP, vesicle associated membrane protein

W

WT, wild type

Appendix II

Materials and Methods

Chapter II

Mice

$Syk^{flox/flox}$CD11c Cre+ and $Syk^{flox/flox}$ CD11c Cre negative mice were housed in our AAALAC certified animal facility. Mice were used in experiments between 7 and 10 weeks of age. All experiments were performed with approval of the The Children's Hospital of Philadelphia IACUC.

Antibodies and reagents

The following Western blot antibodies were used from Cell Signaling: TNFα (#3707), phospho-ERK (clone D13.14.4E), total ERK (clone L34F12), phospho-p38 (clone 28B10), NF-κB p65 (clone C22B4), and IκBα (clone 44D4).

The following Western blot antibodies were purchased from Santa Cruz: β-actin (clone C-11), Syk (clone N-19), and MHC classII (clone M5/114). Secondary antibodies (mouse, goat, rat, and rabbit IgG) were purchased from Licor. Antibodies used for flow cytometry from BD Biosciences include TNFα (clone MP6-XT22) conjugated to AF-700 or Pe-Cy7 and CD11c (clone HL3) conjugated to APC or Pe-Cy7.

TAPI-0 (20μM; EMD Millipore) was used to inhibit TACE activity and thus prevent cleavage of surface TNFα.

Cell culture and lentiviral transduction

The mouse RAW264.7 macrophage cell line was cultured in DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals) and antibiotic (penicillin, streptomycin, and glutamine; Invitrogen) at 37 degrees in a 5% CO_2 incubator. For experiments, cells were stimulated with 10μg/ml CpG1826 (IDT).

Lentivirus containing the pLKO.1 vector expressing *Syk* shRNA (Open Biosystems) was made using the calcium phosphate method of transfection of HEK293 T cells and then transduced into RAW cells. Briefly, 0.2 million RAW cells were plated on 24-well sterile tissue culture treated plates (Cell Star) and allowed to attach overnight. On day 1 after plating, 1ml of viral supernatant was added to each well in the presence of polybrene (4μg/ml), and plates were centrifuged for 2 hours at 2000rpm at 32 degrees, and cells were returned to the incubator with fresh DMEM. Transduction was repeated on day 2. On day 4, puromycin (2μg/ml; Sigma) was added to the culture to select for virally-transduced cells. Protein knockdown was assessed by Western blotting.

Preparation of mouse BMDCs

Bone marrow was flushed from the tibias and femurs of control and Syk flox mice and cultured for 8 days in IMDM medium (Invitrogen) containing antibiotics (penicillin, streptomycin, and glutamine), fetal bovine serum, and GM-CSF (3.3ng/ml; Peprotech). On day 8, cells were stimulated with CpG for various assays.

Preparation of splenic DCs

Control and Syk flox mice were injected sub-cutaneaously with 5 million B16-Flt3L cells. This melanoma cell line is genetically engineered to secrete Flt3L. Fourteen days post-injection, mice were sacrificed and spleens were treated with DNase (50μg/ml; Roche)

and Collagenase (100μg/ml; Roche) for 30 minutes at 37 degrees. Splenocytes were harvested, and DCs were selected with magnetic CD11c beads (Miltenyi Biotec) run through a MACS LS column (Miltenyi Biotec). Purity was confirmed using flow cytometry. CD11c+ splenic DCs were cultured in complete IMDM medium containing 10ng/ml GM-CSF.

Cytokine Secretion Measurements

After stimulation of cultured cells with CpG, supernatants were collected and centrifuged for 10 minutes at 13,200rpm at 4 degrees. Cytokine concentrations were measured in the supernatants using TNFα, IL-6, and MCP-1 ELISA kits (BD OptEIA, BD) according to the manufacturer's instructions. For detection of serum TNFα from CpG-injected control and Syk flox mice, blood was collected from the cheek vein and centrifuged for 20 minutes at 13,200rpm. Serum was tested using an ELISA kit.

Flow Cytometry

For detection of surface TNFα, cultured cells stimulated with CpG and TAPI-0 were harvested, pelleted, and resuspended in FACS buffer (PBS containing 2% fetal bovine serum and 0.01% sodium azide). Cells were then resuspended sequentially in 4% paraformaldehyde (20 min) and Pe-Cy7-conjugated rat anti-TNF antibody (BD). For detection of intracellular TNFα, cultured cells were stimulated with CpG in the presence of Brefeldin-A, which prevents transport of newly synthesized proteins from the endoplasmic reticulum. After harvesting, cells were fixed and permeabilized for 20 minutes with 1x cytofix/cytoperm (BD). Cells were washed in 1x Permwash (BD) and stained with Pe-Cy7 or AF700-conjugated rat anti-TNF antibody (BD). All steps were

performed at 4 degrees. Cells were analyzed on a LSR II (BD) using FACSDiva software, and data were analyzed using FlowJo software (TreeStar).

Western blotting and TACE activity assay

For preparation of whole cell lysate, cells were washed in cold PBS and lysed in 1% NP40 buffer containing 5M NaCl, 1M Tris (pH 7.4), complete protease inhibitor cocktail (Sigma), 100mM NaF, 100mM NaVanadate, and 100mM phenylmethanesulfonylflouride. Lysates were centrifuged for 10 minutes at 13,200 rpm and stored at -20 degrees. For preparation of membrane lysates, cells were washed in cold PBS and resuspended in PBS containing protease and phosphatase inhibitors. Cells underwent 5 cycles of freeze/thaw lysis, frozen in an ethanol/dry ice bath and thawed in a 37 degree water bath. Lysates were centrifuged at 20,000*g* for 15 minutes. Pellets (membrane fraction) were washed in PBS and centrifuged again and resuspended in 1% NP40 lysis buffer. To obtain nuclear lysates to investigate nuclear translocation of NF-κB, CpG-stimulated cells were lysed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific) according to the manufacturer's instructions. All lysates were boiled and separated on SDS-PAGE (4% Bis-Tris gel; Invitrogen) and analyzed by Western blotting. Licor software of analysis was used to analyze blots developed on Odyssey.

Membrane lysates were also plated on a black 96-well plate for the Sensolyte 520 TACE Activity Assay (Anaspec) according to the manufacturer's instructions. Briefly, a peptide substrate, the fluorescence of which was quenched in the intact peptide, was added to lysates. In the presence of TACE, the peptide was cleaved, and fluorescence, which was directly proportional to the amount of TACE activity, of the cleaved substrate was measured.

Preparation of RNA for Real-time PCR

RNA was isolated from cultured cells using the RNeasy Kit (Qiagen) according to the manufacturer's instructions, and cDNA was synthesized using the SuperScript III kit (Invitrogen) from 1μg RNA. qPCR was performed on the cDNA using TaqMan Gene Expression Assays (Applied Biosystems), with probes for mouse TNFα (Mn00443258_m1), mouse TLR9 (Mn00446193_m1), and mouse GAPDH (Mn99999915_g1). Samples were run and analyzed using Sequence Detection System 7500 PCR machine from Applied Biosystems. TNFα message levels and TLR9 message levels were normalized to that of GAPDH.

Statistical Analysis

Graphs and statistical tests were generated and analyzed using Prism Version 5.0. In general, the unpaired t test was used to compare groups. To analyze membrane TNFα data, membrane TNFα was normalized to MHC class II for each cell type. The relative amount of membrane TNFα was computed by dividing normalized TNFα of the Syk-deficient cells to that of the control cells. The resulting ratio was compared to the value of 1 representing no change using a one sample t test. Similarly, the MFI of surface TNFα was compared between Syk-deficient and control cells, and the resulting ratio was analyzed against the value of 1.

Chapter III

Antibodies and reagents

The following Western blot antibodies were used from Cell Signaling: phospo-CaMKII (#3361), pan CaMKII (clone D11A10), and PLCγ2 (#3872). β-actin (clone C-11) was purchased from Santa Cruz. Secondary antibodies (mouse, goat, rat, and rabbit IgG) were purchased from Licor. Antibodies used for flow cytometry from BD Biosciences include TNFα (clone MP6-XT22) conjugated to AF-700.

Ionomycin (Molecular Probes) was used at 1μg/ml. BAPTA-AM was purchased from Sigma.

Cell culture and lentiviral transduction

The mouse RAW264.7 macrophage cell line was cultured in DMEM (Invitrogen) containing 10% heat-inactivated fetal bovine serum (Atlanta Biologicals) and antibiotic (penicillin, streptomycin, and glutamine; Invitrogen) at 37 degrees in a 5% CO_2 incubator. For experiments, cells were stimulated with 10μg/ml CpG1826 (IDT).

Lentivirus containing the pLKO.1 vector expressing *PLCγ2* shRNA or *CaMKII* shRNA (Open Biosystems) was made using the calcium phosphate method of transfection of HEK293 T cells and then transduced into RAW cells. Briefly, 0.2 million RAW cells were plated on 24-well sterile tissue culture treated plates (Cell Star) and allowed to attach overnight. On day 1 after plating, 1ml of viral supernatant was added to each well in the presence of polybrene (4μg/ml), and plates were centrifuged for 2 hours at 2000rpm at 32 degrees, and cells were returned to the incubator with fresh DMEM.

Transduction was repeated on day 2. On day 4, puromycin (2μg/ml; Sigma) was added to the culture to select for virally-transduced cells. Protein knockdown was assessed by Western blotting.

Preparation of mouse BMDCs

Bone marrow was flushed from the tibias and femurs of control and Syk flox mice and cultured for 8 days in IMDM medium (Invitrogen) containing antibiotics (penicillin, streptomycin, and glutamine), fetal bovine serum, and GM-CSF (3.3ng/ml; Peprotech). On day 8, cells were stimulated with CpG for various assays.

Flow cytometry

For detection of intracellular TNFα, cultured cells were stimulated with CpG in the presence of Brefeldin-A, which prevents transport of newly synthesized proteins from the endoplasmic reticulum. After harvesting, cells were fixed and permeabilized for 20 minutes with 1x cytofix/cytoperm (BD). Cells were washed in 1x Permwash (BD) and stained with Pe-Cy7 or AF700-conjugated rat anti-TNF antibody (BD). All steps were performed at 4 degrees. Cells were analyzed on a LSR II (BD) using FACSDiva software, and data were analyzed using FlowJo software (TreeStar).

Intracellular Calcium Measurements

RAW cells were loaded with fura-2 AM (3μM; Invitrogen) for 45 minutes at room temperature, and intracellular calcium was detected in response to CpG by digital imaging as previously described (Liu et al., 2002). The fluorescence emission ratio of fura-2 was detected at 510nm in a solution containing no calcium to establish a baseline

reading. CpG was added before the cells were returned to a bath solution containing 155mM NaCl, 4.5mK KCl, 2mM $CaCl_2$, 1mM $MgCl_2$, 5mM glucose, 10mM HEPES, pH 7.4. Cells were then stimulated with ATP (10μM) and Ionomycin.

Western blotting

For preparation of whole cell lysate, cells were washed in cold PBS and lysed in 1% NP40 buffer containing 5M NaCl, 1M Tris (pH 7.4), complete protease inhibitor cocktail (Sigma), 100mM NaF, 100mM NaVanadate, and 100mM phenylmethanesulfonylflouride. Lysates were centrifuged for 10 minutes at 13,200 rpm and stored at -20 degrees. All lysates were boiled and separated on SDS-PAGE (4% Bis-Tris gel; Invitrogen) and analyzed by Western blotting. Licor software of analysis was used to analyze blots developed on Odyssey.

Statistical Analysis

Graphs and statistical tests were generated and analyzed using Prism Version 5.0. In general, the unpaired t test was used to compare groups. For studies involving calcium signaling, pCaMKII was normalized to total ERK for each cell genotype. Data were analyzed based on fold induction from baseline (comparison of normalized pCaMKII at each timepoint of stimulation to that at baseline) and based on percentage of Ionomycin (comparison of untreated, 30 minutes CpG, and 60 minutes CpG to Ionomycin).

Chapter IV

Mice

TLR9 KO mice were housed in our AAALAC certified animal facility. Mice were used in experiments between 7 and 10 weeks of age. All experiments were performed with approval of the University of Pennsylvania IACUC.

Antibodies and reagents

Rabbit HA (clone C29F4) and mouse total Erk (clone L34F12) were purchased from Cell Signaling. Secondary antibodies (mouse and rabbit IgG) were purchased from Licor. Antibodies used for flow cytometry from BD Biosciences include TNFα (clone MP6-XT22) conjugated to AF-700, IL-6 (clone MP5-20F3) conjugated to PE, and CD11c (clone HL3) conjugated to PeCy7. HA (clone 6E2) conjugated to Alexa Fluor 647 was purchased from Cell Signaling.

Cloning

pUNO-mTLR9-HA was purchased from InvivoGen. Tyrosine 870 of TLR9 was mutated to either a phenylalanine (Y870F) or alanine (Y870A) using the QuikChange Lightning Site-Directed Mutagensis Kit (Agilent Technologies) according to the manufacturer's instructions. The following primers were used. Y870F Forward: 5'-CGCCCAAACTCTCCCTTTTGATGCCTTCGTGG-3'; Y870F Reverse: 5'-CCACGAAGGCATCAAAAGGGAGAGTTTGGGCG-3' ; Y870A Forward: 5'-GCAGCGCCCAAACTCTCCCTGCTGA-3' ; Y870A Reverse: 5'-CACGAAGGCATCAGCAGGGAGAGTT-3. The primer used for sequencing TLR9 was 5'-TGCTTTGGCCTTTCACTCTT-3'. WT-TLR9-HA, TLR9 Y870F-HA, and TLR9 Y870A-

HA were cut from the pUno vector with the restriction enzymes Age1 and Hpa1 (New England Biolabs) and sub-cloned into the MigR retrovirus multiple cloning site (MCS) upstream of the internal ribosomal entry site. For this, Age1 was inserted into the MCS of MigR using a BglII-Age1-Hpa1 linker.

Culture and retroviral transduction of mouse BMDCs

Bone marrow was flushed from the tibias and femurs of TLR9 KO mice and cultured for 10 days in IMDM medium (Invitrogen) containing antibiotics (penicillin, streptomycin, and glutamine), 10% fetal bovine serum, and GM-CSF (3.3ng/ml; Peprotech). MigR retrovirus expressing WT TLR9, TLR9 Y870F, or TLR9 Y870A was constructed using the calcium phosphate method of transfection into HEK293 T cells. On days 2 and 3 of bone marrow culture, cells were transduced with retrovirus. Briefly, viral supernatant containing 4μg/ml polybrene was added to cells in a 24 or 6-well plate, and plates were centrifuged for 2 hours at 2000rpm at 32 degrees Celsius. Transduction was assessed by GFP expression (flow cytometry) or by HA expression (flow cytometry and Western blot).

Cytokine Secretion Measurements

After stimulation of cultured BMDCs with CpG (1μg/ml), supernatants were collected and centrifuged for 10 minutes at 13,200rpm at 4 degrees. Cytokine concentrations were measured in the supernatants using TNFα and IL-6 ELISA kits (BD OptEIA, BD) according to the manufacturer's instructions.

Western blotting

For preparation of whole cell lysate, cells were washed in cold PBS and lysed in 1% NP40 buffer containing 5M NaCl, 1M Tris (pH 7.4), complete protease inhibitor cocktail (Sigma), 100mM NaF, 100mM NaVanadate, and 100mM phenylmethanesulfonylflouride. Lysates were centrifuged for 10 minutes at 13,200 rpm and stored at -20 degrees. All lysates were boiled and separated on SDS-PAGE (4% Bis-Tris gel; Invitrogen) and analyzed by Western blotting. Licor software of analysis was used to analyze blots developed on Odyssey.

Flow cytometry

For detection of surface CD11c, cells were washed, pelleted and resuspended in FACS buffer (PBS containing 2% fetal bovine serum and 0.01% sodium azide). Cells were then incubated with CD11c antibody conjugated to PeCy7. For detection of intracellular cytokines and HA, cells stimulated with CpG in the presence of Brefeldin-A were fixed and permeablized with 1x cytofix/cytoperm (BD) for 20 minutes. Cells were washed with 1x permwash (BD) and stained with TNF antibody conjugated to AF-700, IL-6 antibody conjugated to PE, and HA antibody conjugated to AF-647. All steps were performed at 4 degrees. Cells were analyzed on a LSR II (BD) using FACSDiva software, and data were analyzed using FlowJo software (TreeStar).

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