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Analysis of Toll-Like Receptor 4 Signal Transduction and IRF3 Activation in the Innate Immune Response: A Dissertation

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A Dissertation Presented

By

DANIEL C. ROWE

Submitted to the Faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of:

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Analysis of Toll-like receptor 4 signal transduction and IRF3 activation in the innate immune response

A Dissertation

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ABSTRACT

INTRODUCTION

METHODS & MATERIALS
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Parts of this dissertation have been presented in the following publications:


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First and foremost, I would like to thank my advisor, Dr. Douglas Golenbock, for allowing me to perform my dissertation research in his laboratory. Dr. Golenbock not only allowed me to pursue my interests in immunology but also allowed me to see the world by attending conferences all across the globe. To him I am very grateful.

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Finally, I would like to thank my family for being supportive in all aspects of my life. Most importantly, I would like to thank my parents, who sacrificed much of their lives to raise six successful children. Mom and Dad, all of your children are completely indebted to your love and devotion.
Over the last decade, the innate immune system has been the subject of extensive research. Often overlooked by the robustness and specificity of the adaptive immune system, the innate immune system is proving to be just as complex. The identification of several families of pattern recognition receptors (PRRs) has revealed an ancient yet multifaceted system of proteins that are responsible for initiating host defense. A wide array of pathogens, from virus to bacteria, is detected using this assortment of receptors. One such family, the Toll-like receptors (TLRs), has been at the forefront of this research. To date, 10 TLRs have been described in the human genome. Activation of TLRs leads to the induction of immune-related genes that ultimately control the response of the host. However, the signaling pathways emanating from activated TLRs and other PRRs are not fully understood. In particular, the pathway leading to the activation of interferon regulatory factor 3 (IRF3), a transcription factor crucial for the induction of type I interferon, remains undefined. IRF3 activation occurs as the consequence of viral infection and through the activation of TLRs 3 and 4 by dsRNA and lipopolysaccharide (LPS), respectively. The focus of this research is to describe components of the IRF3 activation pathway, partly through the analysis of TLR signal transduction.

IRF3 normally resides in the cytoplasm of cells. Upon infection with certain viruses and bacteria, IRF3 is activated though phosphorylation at its C-terminus. Phosphorylated IRF3 homodimerizes and associates with co-activators CBP-p300. After translocating to the nucleus, the activate IRF3 complex induces the activation of type 1 interferon and interferon related genes. Little is known about the pathways that lead to
the activation of IRF3, especially the kinases involved. In this study we report that the non-canonical IκB kinase homologues, IκB kinase epsilon (IKKe) and TANK-binding kinase-1 (TBK1), which were previously implicated in NF-κB activation, are also essential components of the IRF3 signaling pathway. In particular, mouse embryonic fibroblasts from TBK1 deficient mice fail to activate IRF3 in response to both viral infection and stimulation with LPS or poly (IC), a dsRNA analog. Thus, both IKKe and TBK1 play a critical role in innate immunity and host defense.

In addition to viral infection, IRF3 activation also occurs via the activation of TLR3 and 4. TLRs signal through a subfamily of Toll-IL-1-Resistance (TIR) domain containing adapter molecules. One such adapter, MyD88, is crucial for all TLRs, with the exception of TLR3. MyD88 participates in a signal transduction pathway culminating in the activation of the transcription factor NF-κB. Studies from MyD88-deficient mice reveal that both TLR3 and 4 still are capable of activating NF-κB, although with slightly delayed kinetics. Another aspect of the MyD88-independent signal transduction pathway is the activation of IRF3. A second TIR domain containing adapter molecule called Mal/Tirap was discovered and originally thought to mediate the MyD88-independent pathway. However, Mal-deficient mice were found to be defective in both TLR2 and 4 mediated NF-κB activation. We hypothesized that other TIR domain containing adapters could mediate this MyD88-independent pathway of TLR3 and 4 leading to the activation of IRF3. Two additional TIR adapters were discovered, TRIF and TRAM. TRIF was shown to mediate TLR3 signal transduction. In this study, we report that both TRIF and TRAM mediate the activation of the MyD88-independent pathway in response to LPS/TLR4 activation. Unlike any of the other known TIR domain containing adapters,
TRAM appears to be restricted to the LPS/TLR4 activation pathway while TRIF plays a role in both TLR3 and TLR4 pathways leading to IRF3 target gene expression.

Our studies revealed that TRAM could be acting upstream of TRIF in the LPS/TLR4 pathway. To this end, we sought to determine the localization of TRAM within the cell. We found that TRAM localizes to the plasma membrane. TRAM localization is the result of myristoylation since mutation of the predicted myristoylation site (G2A) resulted in the re-distribution of TRAM from the membrane into the cytoplasm. Reconstitution of TRAM-deficient macrophages with TRAM G2A is unable to rescue LPS/TLR4 signal transduction. Thus, myristoylation and membrane association of TRAM are critical for LPS/TLR4 signal transduction.

The data generated in this dissertation extends our understanding of the signaling pathways of the innate immune system. Indeed, the molecules and pathways described herein could prove to be beneficial targets for ameliorating symptoms of disease, both autoimmune and pathogen-associated. Finally, the research described here will spur further insight into the complex signaling pathways of a once ignored arm of the immune system.
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CHAPTER I:
INTRODUCTION
1.1 Innate Immunity

The ability of an organism to defend against invasion and disease caused by pathogens depends upon a functional immune system. The immune system of vertebrates consists of two components: innate and adaptive. These two “arms” of the immune system work closely together to combat disease, infection, and re-infection. The innate immune system is generally considered non-specific, quick acting, and represents the first line of defense against pathogens. In contrast, the adaptive immune system is extremely specific, exhibits immunologic memory and is triggered only if the pathogen escapes destruction by the innate immune system.

The innate immune system relies on several different modules working in unison to destroy and eliminate potential pathogens. Mediators of these modules are both cell-based and/or depend upon the direct action of effector molecules. Although a wide variety of cells are ultimately involved in innate immunity, in general, three key cell types can be described based upon their collective activities. These groups are 1) macrophages, dendritic cells (DCs), and neutrophils, 2) γδ T cells and B-1 B cells, and 3) Natural Killer (NK) cells.

**Macrophages, DCs, and neutrophils**

Resident tissue macrophages are one of the first cell types to contact a pathogen once it has crossed the epithelial barrier. The next events involve the recruitment of a large number of neutrophils to the site of infection from the circulation. Using a variety of surface bound receptors, e.g., mannose binding receptor, the macrophages and neutrophils are able to discriminate between self and non-self. Upon encountering a
pathogen, these cells begin the process of phagocytosis. Phagocytosis involves the engulfment of a pathogen into an intracellular vacuole known as a phagosome. As the phagosome matures, it becomes acidified. This maturation, combined with the release of lytic enzymes and powerful toxins such as nitric oxide, proceed to dismantle and destroy the pathogen. Phagocytosis provides two basic functions. First, it results in the elimination of most, if not all, of the pathogen at the site of infection. Second, it results in the production of signals and attractants (cytokines and chemokines) that are involved in the inflammatory response and recruitment of DCs. It is here, at the site of antigen uptake, that DCs also encounter microbial products that provide a “danger signal” (discussed below) that allows for effective T cell priming. After antigen uptake, DCs migrate into the lymphatic system, and interact with T cells to initiate the adaptive immune response.

**γδ T cells and B-1 B cells**

The γδ T cells and B-1 B cells are a second group of cells involved in the innate immune system. These cells represent more primitive versions of their adaptive counterparts, the T and B cells. However, unlike mature T and B cells, γδ T cells and B-1 B cells undergo limited receptor rearrangement. This leads to receptors of limited diversity. In addition, both cell types seem to have a distinct location in the body. γδ T cells are primarily found in most endothelial layers while B-1 B cells are generally found in the pleural cavities. What makes these cell types innate in nature is their early response to infection when compared to T and B cells. In addition, these cells are present early on
in ontogeny, and thus play a crucial role in preventing disease before the development of a fully functional adaptive immune system.

**Natural Killer cells**

The third and final group of cells described here are the Natural Killer (NK) cells. NK cells are derived from the common lymphoid progenitor cells in the bone marrow. They are larger than T and B cells and contain large cytotoxic granules in their cytoplasm. NK cells are thought to be involved in protection against both intracellular pathogens and cancer. NK cells possess two classes of receptors that control the release of their cytotoxic granules. These receptors are classified as either activating or inhibitory receptors. The activating receptors recognize certain glycoproteins present on cells. The inhibitory receptors recognize major histocompatibility complex (MHC) class I expressed on cells. This balance of positive and negative signals is normally skewed towards a negative signal on healthy cells that are expressing normal levels of MHC I. Under these circumstances, the target cells are left untouched. However if altered or lower expression of MHC I is expressed, as is often seen in infected or cancerous cells, the positive signal is stronger and cytotoxic granules are released by the NK cell thus killing the target cell. Although resting NK cells are capable of killing a target cell, they can become activated. An activated NK cell becomes more sensitive and gains enhanced cytotoxicity. The activation of NK cells is tightly linked to the cytokines and interferons produced by activated macrophages. IL-12 and type I interferons (IFNα/β) released by macrophages and dendritic cells activates NK cells, which helps control the infection...
before an adaptive immune response can be mounted. Moreover, activated NK cells produce larger amounts of the type II interferon, IFNγ.

In addition to the cellular based facets of the innate immune system there is an important component that is based upon effector molecules. These include the complement system, antimicrobial proteins and peptides, and “natural antibodies.” Innate effector molecules are extremely important to an organism’s health. In contrast to cytokines and chemokines, which act mainly to enhance cell-mediated immunity, these effector molecules provide immediate antimicrobial properties and assist in discerning pathogens from host constituents.

*Complement*

A key effector system of innate immunity involves the complement system. The complement system is a class of over twenty soluble and membrane-bound proteins. There are three major pathways leading to the activation of the complement system (Fig. 1.1). Activation results in the orchestrated enzymatic cleavage of many of these plasma proteins. Some complement protein products are directly involved in pathogen destruction, such as the C5-C9 membrane attack complex. Other proteins bind to and opsonize pathogens, thus enhancing the phagocytic abilities of phagocytes. Besides these direct effects upon pathogens, activated complement proteins and cleavage products can also act as chemoattractants causing the vasodilation of surrounding blood vessels and aiding in the recruitment of phagocytes.
**Antimicrobial proteins and peptides**

Other effector molecules of the innate immune system consist of antimicrobial proteins and peptides. These effector molecules are present in bodily secretions of the epithelial linings of the body and provide a means of defense against potential pathogens. A prime example of this can be seen in the lungs. The lungs represent an organ that is in high contact with the outside world. The vast surface area and amount of air exchanged over the airway epithelium exposes the lungs to a variety of microbes and pollutants. It is here that a plethora of antimicrobial proteins and peptides can be found. For example, the contents of the epithelial lining fluid (ELF) contain lysozyme and lactoferrin, both proteins with general bactericidal activities (1). Several cationic peptides of the defensin and cathelicidin families are also present in the ELF. These peptides have selective membrane permeabilizing activity. Resident alveolar macrophages work in conjunction with these antimicrobial proteins and peptides to provide immediate defense against pathogens in the lung.

**Natural antibodies**

A final category of innate effector molecules in the body is the so-called natural antibodies. These natural antibodies are generally of the IgM class and comprise a large amount of the circulating IgM found in humans. The exact origin of the antibodies remains unclear, but their low affinity for many microbial pathogens and lack of diversification by somatic hypermutation result in innate-like characteristics. For example, these antibodies have been described to bind to phosphatidylcholine on the
surface of bacteria, such as *Streptococcus pneumoniae*, and promote clearance before they can become harmful (2).

1.2 The Danger Hypothesis

The key to an effective immune system is the ability to discriminate between self and foreign antigens. The adaptive immune system must ensure that responses to self-antigens are kept to a minimum. This is mainly accomplished during the stages of lymphocyte development. Self-reactive T and B cells are eliminated during maturation in the thymus and bone marrow, respectively. It is in these compartments that T and B cells are exposed to self-antigens. Any self-reactive lymphocyte is programmed to self-destruct. Escaping from these developmental checkpoints are lymphocytes reactive towards antigens expressed exclusively in certain tissues and not in either the thymus or bone marrow. However, these potential autoreactive lymphocytes are usually quiescent. This is explained by the lack of a “second signal” required for lymphocyte activation. This lack of a second signal leads to tolerance and a state of anergy among the autoreactive lymphocytes.

The second signal or co-stimulatory signal comes from the detection of microbes or possibly tissue injury during the innate immune response. The mechanism of detection will be discussed later. For now, the second signal is dependent upon the upregulation of surface molecules, *e.g.*, CD80, CD86, (also called B7 molecules) and CD40, on the antigen presenting cells (APCs). This activated APC travels to the lymph nodes and presents the antigen on either MHC I or II to the cognate T cell. T cell receptor (TCR)
ligation (signal 1) and co-stimulatory CD28 or CD40L ligation (signal 2) results in the formation of an effector T cell.

It is this second co-stimulatory signal that provides the basis for the 'danger hypothesis'. The danger hypothesis, coined by Polly Matzinger, is the result of years of immunologic observations by a number of people (reviewed in (3). The danger hypothesis, in its simplest form, states that the immune system responds to challenge or antigen only if the threat is determined to be dangerous. At its basis, the danger hypothesis implies that the immune system is not concerned with discriminating between self and non-self, but is rather concerned with sensing the presence of danger. In the case of infectious disease, the danger signal comes from the pathogens themselves. To this end, the innate immune system has evolved a variety of receptors to detect pathogens or pathogen associated molecular patterns (PAMPs). These PAMPs are usually molecules that are crucial for the existence of the organism or its virulence.

1.3 Pattern Recognition Receptors (PRRs)

The ability of an organism to detect pathogens and their PAMPs is crucial for an effective immune response. Over the past decade, intense research has been underway to decipher the mechanism of this action. This research has led to the discovery of a variety of proteins that serve this function. Along with these discoveries, much interest has been focused on the signaling pathways that emanate from these receptors and ultimately lead to the transcription of pertinent molecules involved in host defense. Three such families will be discussed here: the NACHT (domain present in NAIP, CIITA, HET-E and TP1) –
LRR (leucine-rich repeat) family (the NLR/CATERPILLAR family), cytoplasmic RNA helicases, and the Toll-like receptors (TLRs).

**NLR Family**

The recently identified NLR family is responsible for detecting intracellular pathogens. The NLR family consists of many members in humans (reviewed in (4)). At its core, as with the TLR family, is the presence of a LRR domain. The LRR domain is thought to serve as the recognition site for the PAMP, although direct ligand binding has not yet been demonstrated for this family of PRRs. A second conserved domain is the NACHT/NOD (nucleotide oligomerization) domain. The NACHT domain is present in all NLR family members and serves as an oligomerization and activation domain. This allows two or more of the same NLR molecules to come together. Finally, the third domain, located at the C-terminus, determines what type of signal transduction pathway the individual NLR will activate. These effector domains occur in three varieties: a caspase recruitment and activation domain (CARD), a pyrin domain (PYD), or a baculovirus inhibitor of apoptosis repeat domain (BIR). The CARD and PYR domains are thought to initiate signaling cascades through homotypic interactions with other CARD or PYR domain-containing molecules. The only NLR to posses a BIR domain is NAIP. Recently, the signaling pathway initiated from the BIR domain of NAIP was shown to regulate caspase-1 activation (5, 6).
RNA helicases

The RNA helicase family represents another family of PRRs that also surveys the cytoplasm of cells. In this case they act to sense viral infection by the detection of dsRNA. The family consists of three members: retinoic acid-inducible gene (RIG-I), melanoma differentiation-associated gene-5 (Mda-5), and Lgp2 (7). All three of these proteins contain a DExD/H box RNA helicase domain which binds and unwinds dsRNA in an ATP-dependant manner. However, only RIG-I and Mda-5 contain CARD effector domains. As a result, these two RNA helicases are capable of inducing type 1 interferon (IFN) (7, 8). In fact, RIG-I is required for induction of type 1 IFN in response to Newcastle disease virus (NDV) and Sendai virus (SV), both ssRNA viruses. Lgp2, the newest family member, does not contain an effector domain. It is thought to act in a negative feedback loop by binding and sequestering dsRNA from RIG-I or Mda-5 (7, 9). In this manner, Lgp2 serves to limit the overproduction of IFNs, which could become harmful to the host.

Toll-like Receptors (TLRs)

TLRs were originally discovered using Drosophila melanogaster as a model organism (discussed below) (10). This ultimately led to the search for TLRs in other species. The mammalian TLRs were discovered as the genomic data base expanded during the mid 1990s (11, 12). In 1997, Medzhitov and colleagues reported that “hToll” (TLR4) was capable of initiating an inflammatory signal upon transfection into HEK293 cells (13). This was subsequently followed by a rapid number of publications linking TLRs to responses to numerous biological agents including lipopolysaccharide (LPS),
mycobacterial lipoarabinomannan, bacterial lipopeptides, CpG-rich DNA, polyI:C, flagellin and many more (14)). There are now at least 13 known TLRs; TLR11, 12 and 13 are present only in mice.

TLRs are characterized as being type 1 transmembrane proteins with extracellular LRR domains and an intracellular "Toll–Interleukin (IL)-1 Resistance" (TIR) domain (Fig. 1.2). As with the NLR family, the LRRs of TLRs are thought to serve as the ligand recognition domain. The intracellular TIR domain of TLRs, as with the IL-1R and IL-18R, represents the effector domain. Over the last five years, a great deal has been learned about the proximal signaling events of TLRs and will be discussed below.

The subcellular location of individual TLRs is also an important aspect of their biology. For instance, the nucleic acid sensing TLRs (3, 7, 8, and 9) all reside in the endoplasmic reticulum (ER) and analogous endosomal compartments. Bacteria and viruses that use this pathway to gain entry into the host or are taken up by phagocytosis are detected in these compartments. Other TLRs, such as 1, 2, 4, 5, and 6 can be found on the cellular membrane. Here these TLRs can sense products that are often shed from bacteria, such as lipoproteins, LPS, and flagellin, or viral fusion proteins such as the RSV F protein (Fig. 1.3).

The ability of individual TLRs to discriminate between invading pathogens is an important determinant of the unique gene expression profiles activated by different microorganisms. Whereas the specificity of microbe detection begins with the ligand-recognition features of one or more TLR, the discovery of a family of TIR domain-containing adapter molecules (defined below), including MyD88 (15), Mal/TIRAP (16, 17), TRIF/TICAM1 (18, 19), TRAM/TIRP/TICAM2 (20-22), and SARM (23), suggest
that the outcome of pathogen-induced recognition also depends on the TLR-restricted utilization of these molecules, alone or in combination, to drive a stimulus-specific response. Here I will focus primarily on TLR4-dependent recognition of LPS and receptor-proximal signaling events, since this TLR is the focus of my Ph.D. studies.

1.4 TLR4 and LPS

Recognition of LPS is mediated by TLR4, which functions in complex with MD-2 (see below). At the outset of my Ph.D. studies in 2002, transduction of LPS signaling was known to depend upon the constitutive association and/or the recruitment of at least three of these adapter molecules to the intracytoplasmic TIR domain present in both TLR4 and the associating adapter molecules, MyD88, Mal/TIRAP, and TRIF. These adapters provide a structural platform, to which various downstream kinases are recruited and downstream signals propagated, culminating in the induction of pathway-specific transcriptional responses.

CD14 and LBP

Until 1990, the prevailing wisdom was that LPS activated immune cells through a non-specific mechanism that involved the spontaneous intercalation of lipid A into the mammalian lipid bilayer. The discovery of lipopolysaccharide binding protein (LBP) ultimately led to the refutation of this concept. LBP is a blood-borne protein that is produced in the liver (24) and has the properties of an acute phase reactant (25). In addition to the liver, LBP is produced in the lung, where it has numerous proposed important physiological and pathophysiological activities, including a role in ARDS,
nosocomial Gram negative pneumonia, asthma, and pneumococcal disease. LBP has opsonic activity, but its importance as a protein involved in LPS responses lies with its abilities to accelerate the binding of endotoxin to CD14 (26). Indeed, LBP is probably best thought of as a lipotransferase that enhances LPS activity by moving LPS onto CD14. For example, studies in the LBP knockout mouse demonstrated that LBP enhances the sensitivity to LPS by approximately 300-fold (27). The importance of this observation is that in the absence of LBP, knockout animals are hypersusceptible to invasion by otherwise harmless numbers of Gram negative bacteria (28, 29). This sentinel function of LBP is shared by CD14 (30).

CD14 was initially discovered as the receptor for LBP-bound LPS, and is central to mammalian responses to endotoxin. CD14 is present in two forms: a GPI-linked form and as a soluble proteolytic fragment found in blood (referred to as “soluble CD14” or sCD14). Soluble CD14, functions to enhance LPS responses in cells that do not ordinarily express CD14. Over 1400 publications have documented the importance of CD14 in LPS responses. CD14-deficient mice, engineered by Goyert et al. (31) and Freeman et al. (30), are relatively insensitive to LPS. The presence of CD14 shifts the LPS response curve by 1000-fold. Golenbock et al. have found that the β2 integrins, CD11b/CD18, can partially substitute for CD14 (32). The ability of β2 integrins to replace CD14 as an endotoxin enhancing protein is nearly complete when macrophages encounter LPS as an insoluble aggregate, such as a whole bacterium (30).

CD14 has no intrinsic signaling capabilities, as it lacks both a transmembrane and cytoplasmic domain. Furthermore, a variety of studies have formally demonstrated that CD14 could not be responsible for some of the unique properties of the LPS receptor,
especially the ability to discriminate between closely related lipid A analogs (33). As a result, the existence of a transmembrane signal transducer, present on the surface of cells and as a major part of an LPS receptor complex, was hypothesized (33). It was believed to be the binding portion of the receptor, analogous to the IL-6 receptor and its associated gp130 signal transducer (34).

An alternate hypothesis that was developed during the 1990s was that the function of CD14 was to internalize LPS, which was subsequently shuttled to the Golgi apparatus. The nature of the movement of LPS to the Golgi seemed to represent a unique biological event, as none of the known membrane constituents, in particular CD14, were then thought to accompany LPS to the Golgi [(35); we have subsequently demonstrated that this is untrue]. The implication of this work was that signaling did not begin on the cell surface. This hypothesis has not stood the test of time, as LPS signal transduction has clearly been shown to be initiated on the cell surface in cells that express abundant CD14 and TLR4/MD-2 (36). However, in some cells, such as epithelium from the tracheobronchial tree, the concentration of TLR4/MD-2 on the surface may be minimal, and thus, LPS transit to the Golgi, where TLR4 and MD-2 also are localized, appears to have a correspondingly important function (37).

**Toll-like receptors**

The discovery of Toll, and hence the TLRs, came about as a consequence of a forward genetic screen in the Nüsslein-Volhard laboratory (10). Nüsslein-Volhard and her group were searching for developmental mutants in Drosophila, and identified a lesion that later proved to be involved in both early development and insect immunity.
Drosophila Toll itself is a type I transmembrane protein with an extracellular domain rich in leucine repeats, and a cytoplasmic domain with an area of high homology to the human interleukin 1 receptor (38). Flies that carried the Toll mutation proved to have severe defects in the production of antimicrobial peptides when challenged with either filamentous fungi or Gram positive bacteria (39). The completion of the Drosophila genome revealed 8 homologs. Remarkably, the recognition of Gram negative bacteria is probably through the recognition of Gram negative peptidoglycan by peptidoglycan recognition protein (PGRP) Lc (40-42), which has no structural similarities to Toll. Indeed, fly cells have no ability to respond to LPS (43).

One important issue is whether or not Toll (or TLRs) actually bind microbial products. In flies, Toll is activated indirectly after fungal invasion. The ligand for Toll is known as spätzle, a peptide that is cleaved from a propeptide as a result of the activation of a serine protease cascade by fungal elements. During development, proteolytic cleavage of spätzle by the serine protease Easter leads to proper dorsal-ventral patterning of the Drosophila embryo (44). In contrast, a different protease named “spätzle-processing enzyme” is responsible for spätzle cleavage during the Drosophila immune response (45). While the molecular genetic evidence that spätzle binds Toll has been strong for many years, actual data demonstrating high affinity binding of purified spätzle to Toll was not published until recently (46). This is characteristic of the entire progression of knowledge in the Toll field, where molecular genetics have identified probable receptors, but biochemical demonstrations of ligand binding have lagged far behind. Very few TLR ligands have been definitively shown to bind their cognate receptor. Indeed, the most obvious example of this gap in our knowledge is LPS, where a
direct demonstration of LPS binding has not been definitively made; leading some to speculate that a spätzle-like molecule must exist. This is supported indirectly by studies carried out in the early 1980's showing that treatment of cells with the serine protease, trypsin, enhanced LPS sensitivity while inclusion of the serine protease inhibitor, STI, diminished LPS signaling.

The first TLR to be identified as a putative LPS receptor was TLR2 (47, 48). Subsequent studies showed that these initial reports were flawed, because the LPS preparations used appeared to be contaminated with bacterial lipoproteins, which we now know can activate TLR2 (49). In fact, early studies established that TLR2-deficient hamsters responded to LPS, and we now know that the primary TLR responsible for (most) LPS responses is TLR4. The evidence for this conclusion began with the observation that two strains of LPS hyporesponsive mice had mutations in TLR4 (50). One of these strains, the C3H/HeJ mouse, had been widely studied for over three decades. The C3H/HeJ mouse contains a single point mutation in the "BB loop" of the TIR domain of TLR4: a pro712his that converted this mutant TLR4 into a dominant negative gene when coexpressed with a wild-type gene (50). Similar mutations in other TLRs and TIR-domain containing adapter molecules often result in dominant negative suppressing activity.

After the initial discovery that a point mutation in TLR4 accounted for the LPS response defect in C3H/HeJ mice, there was a brief pause in progress on the LPS receptor. Many groups had the frustrating experience of transfecting TLR4 into HEK293 cells and finding that the cells remained unresponsive to LPS, although there were some reports that expression of TLR4 in a TLR4-null cell resulted in LPS responses. The
discrepancies observed between labs that had performed nearly identical experiences were due to the unappreciated role of MD-2, a soluble TLR4 co receptor.

**MD-2**

Several observations helped resolve the initial confusion concerning the identification of TLR2 as the LPS signal transducer. First, was the discovery that Chinese hamsters were natural null mutants for TLR2 expression. Yet, cells from Chinese hamsters respond exquisitely well to LPS (51). Hirschfeld and colleagues clarified the exact basis of the problem when they found that when LPS was subjected to a second phenol extraction, which removes trace amounts of bacterial lipopeptides (49), all of the TLR2 activity, but none of the TLR4 activity, was lost.

Most important in characterizing the central role of TLR4 in LPS signal transduction was the discovery of MD-2 (52). MD-2 is an 18-25 kDa protein with a cleavable signal sequence that is both bound to TLR4 in the Golgi and is secreted as a soluble molecule from MD-2 expressing cells (53, 54). Miyake and co-workers found when MD-2 was co transfected into HEK293 cells, it bound to TLR4, and greatly enhanced the response of TLR4-transfected cells to LPS (52). A forward genetic screen of CD14-transfected CHO cells, performed by Golenbock et al., revealed that MD-2 is essential for LPS responses. Indeed, they found that MD-2 can be supplied as a soluble receptor component to TLR4 positive cells (54). It is now believed that earlier reports on TLR4 responsiveness in HEK cells is attributed to the presence of small quantities of soluble MD-2 in some lots fetal calf serum but not others.
**TLR4/MD-2 initiation of signal transduction**

TLR4 does not appear to bind LPS directly (36). But, evidence abounds that MD-2, which is bound to TLR4, binds LPS. Indeed, it has been suggested that MD-2/LPS complexes, and not LPS, may function like spätzle as a protein ligand for TLR4.

MD-2, at least when transfected into cells, is secreted as both a monomeric protein and a series of polymeric proteins of variable molecular weight. Studies by Re and Strominger (55), as well as Visintin and Golenbock (36), have demonstrated that only the monomeric form of MD-2 is biologically important for LPS signaling. There appear to be two reasons for this: first, only monomeric MD-2 binds to LPS. Secondly, only the monomeric form of MD-2 binds to TLR4.

The exact locations of the LPS binding site on MD-2, as well as the nature of the MD-2 binding site on TLR4, are not known. It has been hypothesized that the contact point of MD-2 with TLR4 involves amino acids 95 and 125 on MD-2, based upon the observation that a C95Y mutant of MD-2 failed to bind surface MD-2 (36), and the observation that a C105 mutant similarly lacked biological activity (56). The C95Y mutant clearly failed to immunoprecipitate with TLR4. Furthermore, a region of MD-2 that shares some sequence homology with bactericidal permeability increasing protein (BPI), another LPS binding protein, has been identified. Two amino acids in particular, lysines 128 and 132, have been proposed as cationic interaction sites for the 1 and 4' phosphates of lipid A. A model describing the events leading to LPS recognition by the TLR4/MD2 complex is shown in Figure 1.4.
**TLR4 Signal Transduction and the TIR domain containing adapter molecules**

Once LPS binds to TLR4/MD-2, the mechanism whereby the receptor becomes activated is unclear. Studies suggest that the aggregation of TLR4, either artificially using mAbs, or as a result of LPS binding, is sufficient to activate signal transduction (57). The next step in the signaling process involves the recruitment of TIR domain containing adapter molecules to the cytoplasmic face of the TLR4 cluster via homophilic interactions with the TIR domain of TLR4.

As of 2002, when I began my Ph.D. studies, three TIR domain containing adapter molecules were known to mediate TLR4 signaling. All three share significant amino-acid sequence similarity within their TIR domains, including (in order of their discovery): myeloid differentiation factor 88 (MyD88) (15); MyD88 adapter-like (Mal) (16), also called TIR domain-containing adapter protein (TIRAP) (17); TIR domain-containing adapter inducing interferon (IFN)-β (TRIF) (18), also called TIR domain-containing adapter molecule-1 (TICAM-1) (19). A fourth TIR domain-containing protein called SARM (for “Sterile alpha and HEAT/Armadillo motif protein”), which encodes an orthologue of *Drosophila* and *C. elegans* proteins, also exists in mammals. Similar to the other adapters, SARM contains a TIR domain in its C-terminus; however, its inability to activate either the NF-κB or IRF3 pathway (see below) makes its role in TLR signaling unclear at present (23). Other TLRs also recruit some of these same adapter molecules, but TLR4 is unique in that it requires at least three to develop a comprehensive immune response. A schematic representation of these adapters is shown in Figure 1.5.
MyD88

MyD88 was originally isolated as a gene induced during IL-6-stimulated differentiation of M1 myeloleukemic cells into macrophages (58). It was subsequently cloned as a TIR and death domain (DD)-containing protein recruited to the C-terminal TIR domain of the IL-1 receptor (IL-1R) (15). MyD88 recruits the serine/threonine kinase, IL-1R-associated kinase-1 (IRAK1) via its N-terminal DD (15) and forms both homodimers as well as heterodimers with other TIR domain containing adapters. In addition to its ability to interact with the IL-1R, MyD88 is also recruited to the TIR domain of other TLR family members, with the exception of TLR3. MyD88, therefore, functions as a shared component of IL1R/TLR signaling.

The importance of MyD88 in LPS-mediated TLR4 signaling in vivo was first demonstrated when MyD88-deficient mice were generated by Shizuo Akira and colleagues (59). MyD88-deficient mice were refractory to LPS-induced death and failed to secrete cytokines such as IL-6 and TNF-α in vivo, or in vitro in LPS-stimulated macrophage cultures (59). Intriguingly, NF-κB translocation and phosphorylation of mitogen-activated protein kinases (MAPK), although slightly delayed by ~10 minutes, were not eliminated, indicating the existence of a MyD88-independent pathway for TLR4 signaling (59). Other TLRs (60) (except TLR3) and IL-1R family members require MyD88 for signaling. TLR4 is unique, however, in its ability to activate both MyD88-dependent and MyD88-independent responses.

Two key features of MyD88-independent signaling is the induction of a DC maturation pathway and the induction of the type 1 interferon (IFN-β) and IFN-regulated genes (61, 62). There is some evidence to suggest that the DC maturation pathway is
dependent on IFN production (63). There is considerable interest in understanding how IFN is regulated. The transcription enhancer of the IFN-β gene is known to bind NF-κB, interferon regulatory factor (IRF) 3, and ATF-2–c-Jun. Whereas all TLRs activate NF-κB and ATF2–c-Jun, not all TLRs induce IFN-β because not all TLRs induce IRF3 activation. TLR3 and TLR4 activate IRF3 via the MyD88-independent pathway and, as such, appear to have diverged evolutionarily from other TLRs to activate gene expression programs and trigger antiviral responses by a mechanism involving the coordinate activation of NF-κB and IRF3. A recent microarray study performed by Bjorkbacka et al. has revealed that 76% of 913 genes induced in LPS-treated wild-type macrophages are still induced in LPS-treated MyD88 KO macrophages (64) suggesting that MyD88-independent signaling accounts for the majority of the LPS response.

*Mal/TIRAP*

MyD88 adaptor–like protein (Mal) (16), also called TIRAP (17), is a related TIR domain-containing protein, which was discovered based on its ability to mediate TLR4 but not IL-1R signaling. Mal possesses a C-terminal TIR domain, but lacks the N-terminal DD present in MyD88. Like MyD88, Mal is capable of both homo- as well as hetero-dimerization with other adapter molecules, at least by coimmunoprecipitation assays, presumably via TIR-TIR interactions. LPS-induced cytokine production is severely impaired in Mal-deficient macrophages (65, 66); however, responses to flagellin (TLR5), the immune response modifier resiquimod (R-848) (TLR7), CpG DNA (TLR9), IL-1, and IL-18 were normal in these macrophages. Unexpectedly, TLR2 signaling in Mal-deficient splenocytes and macrophages was also impaired. Mal-deficient
macrophages also showed delayed NF-κB translocation and MAPK phosphorylation induced by LPS, whereas TLR2-mediated NF-κB translocation was essentially eliminated (65, 66).

Mal had also been implicated in LPS-induced IFN-β induction in vitro and, as such, represented a likely candidate responsible for the regulation of MyD88-independent signaling (61). However, activation of IRF3 and induction of IFN-β and IFN-stimulated genes is intact in Mal-deficient cells (65). These observations prompted database searches for additional TIR domain containing adapter molecules, which might regulate MyD88-independent signaling.

**TRIF/TICAM-1**

The third TIR-containing adapter, called TRIF or TICAM-1 was discovered based on its contribution to TLR3 signaling (18, 19). TRIF is a much larger protein than either MyD88 or Mal (712 amino acids vs. 296 and 235, respectively) and possesses the conserved proline (\(^{334}\)P) found in the BB loop of other TIR domains (except that of TLR3 which has an alanine). Overexpression of TRIF results in NF-κB activation; however, only overexpressed TRIF, but neither MyD88 or Mal, leads to strong expression of IRF3-dependent reporters such as the IFN-β gene reporter (18, 19).

The role of TRIF in TLR signaling was confirmed using siRNA silencing technology (19, 20) and either TRIF-deficient (60) or chemically mutagenized (\(lps2\)) mice (67). TRIF-deficient macrophages are extremely deficient in their capacity to induce IFN-β, RANTES, IP-10 and MCP-1 gene expression, and their B cells can neither proliferate nor increase the expression of activation markers in response to TLR3
activation (60). Deficiency of TRIF also affects LPS-induced cytokine gene expression in macrophage cultures, but does not alter cytokine expression induced by peptidoglycan (via TLR2), R-848 (via TLR7), or CpG DNA (via TLR9) (60). In addition, TRIF-deficient fibroblasts and macrophages failed to activate IRF3 in response to either TLR3 or TLR4 activation. NF-κB is activated by LPS in these cells, but not by poly I:C, indicating roles for both MyD88 and TRIF in NF-κB activation through TLR4. This is supported by the observation that LPS fails to induce NF-κB activation in fibroblasts from TRIF/MyD88 double-deficient mice (60).

*Lps2*, a chemically induced mutation that led to LPS-challenged hyporesponsiveness in mice was identified as a single base-pair deletion in TRIF that is predicted to replace the C-terminal twenty-four amino acids with an unrelated eleven amino acid sequence (67). Nonetheless, the mutated TRIF gene still has the potential to encode a protein that contains a TIR domain and an N-terminal region. The *Lps2* mutant mice exhibited a similar phenotype to that seen with TRIF-deficient mice (60). Experiments in mice that harbor both the MyD88-deficiency and the *Lps2* mutation, revealed that both adapters normally contribute to LPS-induced TNF-α secretion. It remains unclear why TLR4-dependent signaling utilizes both MyD88-dependent as well as MyD88-independent pathways to induce inflammatory cytokines. These findings might indicate that the MyD88-independent/TRIF-dependent pathway regulates the expression of a factor or factors required for inflammatory gene expression.
1.5 Type 1 Interferon

PRRs have the important role of detecting and alarming the host to the presence of a pathogen. Upon ligand recognition of PAMPs, PRRs activate complex signal transduction pathways that lead to the activation of host transcription factors and the induction of immune related genes. Some of the most prominent transcription factors include NF-KB, ATF-2/c-Jun (AP-1), and interferon regulatory factors (IRFs). The transduction pathways of NF-κB and AP-1 activation have been well characterized. However the activation pathway of IRF family members was unclear.

The induction of type 1 Interferon

Distinct sets of genes that are induced upon infection are the type 1 interferons (IFN-α and IFN-β) and interferon inducible genes (ISGs). The induction of type 1 IFN and ISGs are instrumental in defending against viral challenge. Incidentally, defending against bacterial infection may also require induction of these genes as LPS and several bacterial pathogens and bacterial DNA have also been shown to induce type 1 IFN in certain cell types (62). Type 1 IFNs act in an autocrine and paracrine manner by signaling through the IFN-α/β receptor. Key signaling components of the IFN-α/β receptor are the signal transducers and activators of transcription (STATs). Phosphorylation of STATs by Janus kinases (JAKs) leads to their dimerization and nuclear translocation (reviewed in (68)). As a result, several hundred genes classified as ISGs are induced. Many of these genes directly help combat viral gene expression and replication (e.g., the double stranded RNA-dependent protein kinase, PKR). In addition, ISGs can also lead to other effects in the host that help control cell growth and/or apoptosis. IFN-β is a good example
of a gene induced upon infection. Its transcriptional control requires the specific activation of IRF3 along with NF-κB and AP-1 transcription factors.

**IRF3 activation**

Virus-induced IRF3 activation has been well documented. In addition, IRF3 activation can be achieved by stimulating cells with the synthetic TLR3 agonist poly (I:C) or the TLR4 agonist LPS. The molecular mechanisms regulating IRF3 activation were unclear until recently. IRF3 is a ubiquitously expressed gene, which normally resides in the cytoplasm of resting cells. Upon infection with virus, IRF3 is phosphorylated at its C-terminus. This phosphorylation is thought to induce a conformational change in IRF3 that leads to its homodimerization and/or heterodimerization with other IRFs resulting in nuclear translocation. In the nucleus IRF3 associates with co-activator proteins CBP and p300 and regulates the transcription of IFNβ and ISGs (reviewed in (69)).

A key unidentified component of the IRF3 activation pathway is the identity of the kinase(s) responsible for phosphorylating IRF3 on its C-terminus. This kinase was often referred to as the virus-activated kinase or VAK (70). Studies using small molecule inhibitors and site directed mutagenesis of serine/threonine residues suggested that VAK was a serine/threonine kinase and the targets were in the C-terminal cluster comprising serines 385, 386, 396, 398, 402, 405 and threonine 404 (71, 72). Defining the identity of VAK was a major goal of the IFN field. Whether VAK is also required for non-viral stimuli acting on IRF3 is also unclear.
1.6 Thesis Objectives

The following studies were aimed at addressing the molecular gaps in our understanding of TLR4 signal transduction, the activation of IRF3 and the induction of type 1 IFN and ISGs. In this regard, we were interested in defining these events in the context of LPS, but also in the broader sense of anti-viral immunity. Although much insight into how the innate immune system begins to function and interact with the adaptive immune system has been gained, many gaps still exist. The identification of the novel components in the signaling pathways discovered in the research that follows may lead to further breakthroughs and possibly into the development of new treatments for infectious disease.
Figure 1.1 Complement Pathways. Classic, lectin, and alternative complement pathways. Copyright © Lippincott Williams & Wilkins.

Figure 1.1
Figure 1.2 Toll-like Receptors. The mammalian Toll-like receptors are type 1 transmembrane proteins with extracellular leucine rich repeats. The intracellular TIR domain of TLRs is also present in the IL1R. Illustrated are 9 TLRs and the IL1R with a representation of their known ligands (Not shown: TLR10).
Figure 1.2

Bacterial lipoproteins, Peptidoglycan (PGN)
Lipoarrabinomannan (LAM)
Zymosan

LPS
F-protein (RSV)
dsRNA
Flagellin
Imiquimod R-848
HSV-2 DNA
CpG DNA

TLR2 TLR6
TLR3
TLR4
TLR5
TLR7 TLR8
TLR9

IL1R
Figure 1.3 The cellular localization of TLRs. Illustrated is the localization of TLRs within the cell. Surface TLRs (1, 2, 4, 5, and 6) recognize microbial components of the outer surface of pathogens or components that are often shed from pathogens. TLRs (3, 7, and 9) are localized to the endoplasmic reticulum and traffic to the endosomal compartments. Here they encounter ligands (nucleic acids) from digested pathogens (73).
Figure 1.3
Figure 1.4 Mediators of endotoxin recognition. The recognition of endotoxin occurs via a series of proteins that bind LPS and synergistically act to enhance the response to infection. See text for detail.
Figure 1.5 TIR-domain containing adapter molecules as of 2002. A schematic representation of MyD88, Mal/TIRAP, TRIF/TICAM1, and SARM is shown. The position of the TIR domain in all proteins is indicated. MyD88 contains a death domain in its N-terminus, Mal contains a PEST domain and SARM contains two SAM domains.
Figure 1.5

- Death - TIR - MyD88: 296 aa
- PEST - TIR - Mal/TIRAP: 235 aa
- TIR - TRIF/TICAM1: 712 aa
- SAM - SAM - TIR - SARM: 735 aa
CHAPTER II:

IKKε and TBK1 are essential components of the IRF3 signaling pathway.
Abstract

The transcription factors IRF3 and NF-κB are required for the expression of many innate immune response genes. Viral infection, the recognition of dsRNA by TLR3, or LPS recognition by TLR4 results in the coordinate activation of IRF3 and NF-κB. Activation of IRF3 requires signal-dependent phosphorylation, but little is known about the signaling pathway or kinases involved. Here we report that the non-canonical IκB kinase homologues, IκB kinase epsilon (IKKe) and TANK-binding kinase-1 (TBK-1), which were previously implicated in NF-κB activation, are also essential components of the IRF3 signaling pathway. In addition, we have also examined the expression of IRF3-dependent genes in mouse embryonic fibroblasts (MEF's) derived from TBK1−/− mice. We find that TBK1 is required for IRF3 activation and nuclear localization in primary mouse embryonic fibroblasts (MEFs). Moreover, TBK1−/− MEFs show marked defects in IFN-α/β, IP-10, and RANTES gene expression following infection with either Sendai or Newcastle Disease viruses, or engagement of the TLRs 3 and 4 by dsRNA and LPS, respectively. Finally, the TIR domain-containing adapter molecule, TRIF, fails to activate IRF3-dependent genes in TBK1−/− MEFs. Thus, IKKe and TBK1 play a pivotal role in coordinating IRF3 activation in the innate immune response.
Introduction

IRF3 is required for IFN-β and RANTES expression in response to viral infection (74-76). In unstimulated cells, IRF3 is present in the cytoplasm. Viral infection results in the phosphorylation of IRF3 leading to its dimerization and interaction with the co-activators CBP-p300 (74). The IRF3 complex then translocates to the nucleus where it activates promoters containing IRF3 binding sites. In spite of considerable effort, neither the components of the signaling pathway nor the kinases involved in the phosphorylation of IRF3 have been identified (69, 77-79).

Two distinct pathways for the activation of the innate immune response by viral infection have been identified. In one pathway, viral nucleocapsid proteins trigger signaling cascades leading to IRF3 activation (80). The receptor and signaling components for this IRF3 signaling pathway remain to be identified (81). In the second pathway, NF-κB activation by virus infection or dsRNA requires the IκB kinase-β (IKKβ) component of the IKKαβγ complex (82). The IKKαβγ complex contains two closely related kinases (IKKα and IKKβ) and a third structural subunit, IKKγ (83). The dsRNA activated protein kinase (PKR) may also be involved but its in vivo significance is controversial (16, 82, 84).

The Toll-like receptor, TLR3, recognizes dsRNA generated during viral infection (85). Signal transduction through TLRs requires the conserved TIR domain, which facilitates recruitment of the TIR domain-containing adapter molecule, MyD88 (86). The association of MyD88 with receptor TIR domains results in recruitment of the IRAK-1 and -4 kinases (87), upstream components of the signaling pathway, which leads to
activation of the canonical IKKαβγ complex, and ultimately NF-κB activation (83).

TLR3 also activates IRF3 (88).

Recently, TLR3 (85, 88) and TLR4 (61, 62) were shown to signal independently of MyD88 to induce IFN-β. As presented in the introduction Mal/TIRAP (16, 17) also plays an essential role in the NF-κB activation pathway in response to lipopolysaccharide (LPS), but is not required for IFN-β induction (89). A third adapter protein, TRIF/TICAM-1 mediates TLR3-dependent IRF3 and NF-κB activation (18, 90). The signaling components regulating IRF3 and NF-κB activation downstream of TLR3-TRIF have yet to be identified (91).

In contrast, the canonical IKKαβγ complex is required for TLR3-TRIF mediated NF-κB activation (K.A.F. and D.T.G., unpublished). Activated IKKαβγ phosphorylates serines 32 and 36 of the NF-κB inhibitor protein IκBα, leading to its polyubiquitination and degradation by the proteosome (83). This, in turn, leads to the translocation of NF-κB to the nucleus where it activates the transcription of target genes (92).

Two related IκB kinase homologues, IKKe (93) (also called IKKi (94)) and TBK1 (also called NAK, for NF-κB-activating kinase (95) or T2K, for TRAF-2 associated kinase (96)) have also been implicated in NF-κB activation. NF-κB-dependent genes are not expressed in embryonic fibroblasts from TBK1-deficient mice, which die as a consequence of apoptotic liver degeneration (96). The target of TBK1 in the NF-κB activation pathway is unknown (97), but does not appear to be IκBα (96). A potential target of IKKe and TBK1 is the RelA protein, also called p65. Both kinases can phosphorylate p65 on serine 536 (S.M.M. and T.M., unpublished), an event, which is
essential for p65 transactivation (98). Mice with a targeted disruption of IKKe have yet to be reported.

In an effort to identify potential downstream targets of IKKe and TBK1, we conducted a detailed analysis of the role played by these kinases in regulating IFN-β and RANTES gene expression. We found that in addition to their role in the NF-κB pathway, these kinases are essential for the activation of the IRF3 pathway following viral infection, TLR3 stimulation by dsRNA, and TLR4 stimulation by LPS.
Materials and Methods

Reagents. Promoter elements from –110 IFNβ-CAT (99), PRD(III-I)3-CAT (99) and PRD(II)2-CAT (100) were amplified by PCR and cloned into pLuc-MCS (Stratagene) to generate pLuc-IFNβ, pLuc-PRD(III-I)3 and pLuc-(PRDII)2. PRD(IV)6-CAT is as described (100). The ISG54 ISRE was from Stratagene. The RANTES-luciferase, IRF3-5D and IRF3-GFP were from John Hiscott, (Montreal, Canada). The NF-κB-luc and the IκB super repressor (IκB SR) were as described (16). The ELAM-luc was from D. Golenbock (Worcester, MA). The IP-10 reporter construct was from A. Luster (Massachusetts General Hospital, Boston, MA). pCDNA3-Flag-IKKε, IKKβ, IKKε(K38A) and pCDNA3-T7-IKKε were as described (93). pCDNA3-Flag-TBK1 and TBK1(K38A) were from Makoto Nakanishi (Nagoya, Japan). pCDNA3-T7-TBK1 was generated from pCDNA3-Flag-TBK1. pEF-Bos-Flag murine TRIF was generated by PCR cloning from a RAW264.7 cDNA library. HA-IRF3ΔN was from T. Fujita (Tokyo, Japan). Gal4-IRF3 and Gal4-IRF3 A7 were as described (99, 101). IRF3ΔN was from Takashi Fujita (Tokyo, Japan). pEF-Bos-Flag Mal (TIRAP) and TRIF (TICAM-1) were generated by PCR cloning from a human PBMC cDNA library and pEF-Bos-TRIF-TIR (aa387-566) was derived from full length TRIF by PCR cloning (K.A.F. and D.T. G.).

Cell lines. HEK293 stable cell lines expressing fluorescent full length TLR3 or IRF3-GFP were engineered by calcium phosphate transfection, selection of bulk populations of cells by selection in the neomycin analog G418 (1mg of total drug/ml) and positive selection by fluorescence-activated cell sorting (BD Vantage, Becton Dickinson Immunocytometry) as described (57). Mouse embryonic fibroblasts (MEFs) from wild-
type and *TBKI*-deficient mice were provided by Wen-Chen Yeh (University of Toronto, Toronto) as described (96).

**Transfection assays.** Cells (1.5 x 10⁴ cells per well) were seeded into 96-well plates and transfected on the following day with 40 ng of luciferase reporter genes using FuGENE6 (Roche). The *Renilla*-luciferase reporter gene (Promega) (40 ng) was cotransfected for normalization. In other experiments, MEFs from TBK1⁺/⁺ and -/- mice (7500 cells/well) were plated in 24-well plates and transfected 24 h later with 0.25 µg of reporter genes as indicated using FuGENE6 (Roche, Indianapolis). 0.5 µg of murine TRIF was also cotransfected with reporter genes where indicated. 24h after transfection, some cells were infected with NDV or SV (200 HAU), stimulated with LPS (100 ng/ml) or poly IC (50 µg/ml) as indicated. In all cases, cell lysates were prepared and reporter gene activity measured using a Dual Luciferase Assay System (Promega). Data are expressed as mean relative stimulation ± S.D. for a representative experiment from a minimum of 3 separate experiments, each performed in triplicate. Whole cell lysates were also subjected to SDS-PAGE and immunoblotted to evaluate expression amounts of various epitope-tagged constructs (data not shown).

**RNA interference.** Oligonucleotides were cloned into pSuper (kindly provided by R. Agami) to express the sihIKKε, sihTBK1, sihIKKβ hairpins downstream of the human H1 RNA promoter as described (102). The following sequences were targeted: For sihTBK1, GACAGAAGTTGTGATCACA; for sihIKKε GAGCTATCTCACCAGCTCC; for sihIKKβ, GTACAGCGGCAAACCGAG; or for sihp53 (102). For reporter assays,
HEK293 or 293T cells (6 x 10^3 cells/well) in 96-well plates were transfected with the appropriate reporters and siRNA targeting vectors or pSUPER empty vector alone (EV) as indicated using FuGENE6 (Roche). 72 h following transfection, cells were infected with Sendi Virus (293T) or stimulated with dsRNA [50 μg/ml poly IC] as indicated for ~16 h before luciferase activity was measured. To determine efficiency of gene silencing, whole cell lysates from siRNA transfected 293T cells (12, well plates, 4 x 10^4 cells/well) were subjected to immunoprecipitation and western blotting for TBK1 (anti-TBK1 IMG-139, Imgenex) and western blotting for IKKβ (anti-IKKβ, IMG-159, Imgenex). Cell lysates from Flag-IKKε transfected cells were subjected to western blotting for IKKε using monoclonal anti-Flag (M2, Sigma).

**RANTES ELISA.** Cell supernatants were harvested from IKKε, TBK1, IKKβ transfected or virus-infected 293T cells or dsRNA treated TLR3-expressing HEK293 cells following siRNA targeting as indicated. Cell supernatants were analyzed for RANTES according to the manufacturer’s recommendations (R&D Systems).

**Immunofluorescence and confocal microscopy.** A HEK293-IRF3-GFP stable cell line was transiently transfected with Flag-tagged constructs as indicated. 48 h after transfection cells were fixed, permeabilized and stained with Cy3-conjugated anti-Flag antibody (clone M2, Sigma-Aldrich). DRAQ5 was added to counter stain nuclei. MEFs from TBK1++ and TBK1−/− mice were transiently transfected with an expression vector for IRF3-GFP as indicated. 24 h after transfection cells were infected with SV for 6 h and
imaged. All cells were imaged by confocal microscopy using a Leica TCS SP2 AOBS microscope.

Co-immunoprecipitation. IRF3-GFP-expressing HEK293 or 293T cells in 10 cm plates were transiently transfected using the FuGENE6 method with 4 μg of the indicated plasmids. Cells were lysed in 800 μl of lysis buffer (50mM Hepes, pH 7.5, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP40, and protease inhibitors), 24 h after transfection. Lysates were precleared with protein A sepharose for 1 h. Polyclonal anti-GFP antibody (Molecular Probes) or anti-Flag (M2, Sigma) was incubated with the cell lysates in Protein A sepharose overnight. The immune complexes were precipitated and washed thoroughly. The protein was eluted by adding sample buffer and was subsequently run on a 10% SDS-PAGE gel and visualised by immunoblotting for Flag- or T7- tagged kinases using monoclonal anti-Flag (M2, Sigma) or monoclonal anti-T7 (T7-Tag, Novagen).
Results

IKKe and TBK1 induce IFN β and RANTES

To investigate the functions of IKKe and TBK1, we examined their effects on the regulation of innate immune response genes. In particular, we found that the IFN-β and RANTES reporter genes were strongly activated by transfection of HEK293 cells with either IKKe or TBK1 expression vectors whereas neither gene was activated by transfection of cells with an expression vector for IKKβ (Fig. 2.1a and 2.1b). In addition, both kinases induced endogenous RANTES secretion, but transfection of IKKβ had little effect (Fig 2.1c).

A common feature of the IFN-β and RANTES genes is that multiple regulatory elements are required for their activation in response to virus infection. For example, the transcription enhancer of the IFN-β promoter contains four positive regulatory domains (PRD1-IV), that act cooperatively to activate IFN-β gene expression in response to virus infection (103). The transcription factors that bind to these elements include NF-κB, which binds to PRDII; IRF3, which binds to adjacent PRDIII and PRD1 sites, collectively referred to as PRDIII-I, and the heterodimeric transcription factor ATF-2-c-Jun, which binds to PRDIV.

To identify the targets of IKKe and TBK1 in the IFN-β enhancer, we tested the ability of these kinases to activate transcription from individual IFN-β regulatory elements. Each of these promoter elements, when present in multiple copies, can be activated by virus infection (99, 104). As expected from the previously demonstrated ability of IKKe and TBK1 to activate NF-κB (92, 97), all three kinases (IKKe, TBK1 and IKKβ) activated a reporter gene containing multiple copies of PRDII (Fig. 2.1d).
Remarkably, a reporter gene containing multiple copies of PRDIII-I was activated by IKKe and TBK1, but not by IKKβ (Fig. 2.1e). Consistent with these observations, another IRF3 dependent promoter element, the ISRE (for Interferon Stimulated Regulatory Element) of the ISG-54 gene, was activated by IKKe and TBK1 expression, but not by IKKβ (Fig. 2.1f). We were unable to detect significant PRDIV reporter activation by any of the IKKs (data not shown).

Together, these results indicate that all three kinases (IKKβ, IKKe and TBK1) activate NF-κB, while only IKKe and TBK1, but not IKKβ, activate IRF3.

**IKKe and TBK1 activate IRF3**

The observation that PRDIII-I and ISRE-containing promoters can be activated by IKKe or TBK1 suggests that these IκB kinase homologues can directly, or indirectly activate IRF3. To test this possibility, we employed an in vivo assay for IRF3 activation, which utilizes a hybrid protein consisting of the yeast Gal4DNA binding domain (DBD) fused to IRF3 lacking its own DNA binding domain (103). Reporter gene expression from the Gal4 upstream activation sequence in this assay requires IRF3 activation (99). The Gal4 reporter gene is activated by virus infection in the presence of the wild-type Gal4-IRF3 fusion protein, but not in the presence of a fusion protein in which the serine/threonine residues critical for IRF3 activation have been substituted by alanine (Gal4-IRF3-A7) (Fig. 2.2a) (71, 72).

The wild-type fusion protein was also activated by transfection with IKKe or TBK1, but not with IKKβ, and this effect was not observed with the mutant Gal4-IRF3-A7 protein (Fig. 2.2a). In addition, a dominant negative mutant of IRF3 (that lacks the
DNA binding domain) (99), suppressed the induction of IFN-β or the ISRE reporter genes by IKKe or TBK1 overexpression (Fig. 2.2b). Transfection of the IκBα dominant negative ("IκB super repressor" (IκB SR), IκBα<sup>S32/36A</sup>), which blocks NF-κB activation (83), had a similar effect on the intact IFN-β promoter but had no effect on IKKe- or TBK1-induced ISRE activation (Fig. 2.2b).

As a further test of IKKe and TBK1-dependent IRF3 activation, we examined their effects on the nuclear translocation of IRF3. Transfection of IKKe, TBK1, but not IKKβ, induced nuclear translocation of IRF3 in a HEK293 cell line stably expressing an IRF3-GFP fusion protein (Fig. 2.2c).

We hypothesized that IKKe and TBK1 could phosphorylate IRF3 directly or may activate an upstream IRF3 kinase. The former possibility predicts that IKKe and TBK1 interact with IRF3 directly, or as part of a complex. In this regard, we found that both IKKe and TBK1 associate with IRF3-GFP in co-immunoprecipitation experiments (Fig. 2.2d). When IRF3-GFP was immunoprecipitated with an anti-GFP antibody and immunoblotted for Flag-tagged IKKe, TBK1, or IKKβ, IKKe and TBK1, but not IKKβ, were detected. When HEK293 cells, which lack GFP-IRF3, were immunoprecipitated with anti-GFP antibody, neither IKKe nor TBK1 were detected (data not shown). These observations are consistent with, but do not prove that, IKKe and TBK1 directly phosphorylate IRF3.

**IKKe and TBK1 siRNA block IRF3 activation**

We carried out siRNA knockdown experiments to determine whether IKKe or TBK1 is required for the activation of a PRDIII-I reporter following virus infection.
Small interfering RNA (siRNA) targeting of \textit{IKKe}, \textit{TBK1}, or \textit{IKK\beta} decreased the amount of the corresponding protein (Fig. 2.3a). Reduction in either \textit{IKKe} or \textit{TBK1} protein resulted in a significant decrease in virus induction of the PRDIII-I reporter (Fig. 2.3b). A smaller effect was observed with siRNA targeting of \textit{IKK\beta} or the negative control \textit{p53}. When the intact \textit{IFN-\beta} promoter was tested, the effect of siRNA targeting of \textit{IKK\beta} was comparable to that of \textit{IKKe} and \textit{TBK1} (data not shown). Taken together, these data strongly support the conclusion that both \textit{IKKe} and \textit{TBK1} are required for IRF3 activation in response to viral infection, attempts to order \textit{IKKe} and \textit{TBK1} in the IRF3 signaling pathway with kinase inactive mutants and siRNA targeting vectors were inconclusive. Finally, endogenous RANTES expression was also reduced in cells targeted by \textit{IKKe} and \textit{TBK1} siRNA, but not by \textit{IKK\beta} siRNA (Fig. 2.3c).

As mentioned above, the \textit{IFN-\beta} gene is a target of the TLR3 signaling pathway following treatment of cells with dsRNA (105). Indeed, the \textit{IFN-\beta}, \textit{NF-\kappaB}, and the \textit{ISRE} reporters were activated by dsRNA in a HEK293 cell line stably expressing TLR3 (Fig. 2.4a). Similarly, the \textit{RANTES} promoter was also activated by dsRNA (data not shown). Finally, dsRNA activated the \textit{Gal4} reporter gene in the presence of the wild-type Gal4-IRF3 fusion protein (Fig. 2.4b), but not the Gal4-IRF3-A7 hybrid protein (data not shown) (71, 72). Therefore, we conclude that IRF3 plays an essential role in the activation of these genes in response to TLR3 stimulation.

We therefore examined the role of \textit{IKKe} and \textit{TBK1} in TLR3-dependent signaling. TLR3 induction of the \textit{ISRE} reporter following dsRNA stimulation was significantly inhibited when endogenous \textit{IKKe} and \textit{TBK1} but not \textit{IKK\beta} or \textit{p53} were targeted by siRNA (Fig. 2.4c). siRNA targeting of \textit{IKKe}, \textit{TBK1} and \textit{IKK\beta} also inhibited dsRNA induction of
the intact *IFN-β* promoter (data not shown). Endogenous RANTES expression was also reduced in cells targeted by *IKKe* and *TBK1* siRNA (Fig. 2.4d).

**TRIF induces IRF3 via IKKe and TBK1**

To investigate how signals are transmitted from TLRs to IKKe and TBK1, we examined the effect of the TLR adapter proteins, MyD88, Mal, and TRIF (16-18, 88, 106) on the activation of IRF3-dependent genes. Neither MyD88 nor Mal activated the *IFN-β* promoter or an *ISRE* reporter gene, whereas both adapters activated *NF-κB* reporters (Fig. 2.5a). As a positive control we showed that a constitutively active form of IRF3 (IRF3-5D) (79) potently induced *IFN-β* and *ISRE* reporters, but had no effect on the *NF-κB* reporter. Overexpression of TRIF strongly induced *IFN-β*, *ISRE*, and *NF-κB* reporter activity (Fig. 2.5b) as well as the Gal4-IRF3 fusion protein (data not shown). We conclude that TRIF activates IRF3 and NF-κB, both of which are essential for IFN-β induction.

To determine the role of IKKe and TBK1 in TRIF-dependent signaling, we examined the effect of kinase inactive mutants of IKKe and TBK1 on TRIF-induced *IFN-β*, *ISRE*, and *NF-κB* reporter activities. Kinase inactive IKKe (K38A) and TBK1 (K38A) inhibited the induction of the *IFN-β*, *ISRE*, and *NF-κB* reporters by TRIF (Fig. 2.6a). As expected, dominant negative IRF3 (IRF3ΔN) inhibited TRIF-induced *IFN-β* and the *ISRE* reporters, but had no effect on *NF-κB* reporter activity. In contrast, dominant negative IκBα (IκB SR) inhibited TRIF-induced IFN-β and NF-κB reporters, while no effect was observed with the *ISRE* reporter. siRNA targeting of IKKe and TBK1, but not
IKKβ or p53, significantly reduced TRIF-dependent activation of the ISRE reporter (Fig. 2.6b).

Moreover, IKKe and TBK1 associated with TRIF in co-immunoprecipitation experiments (Fig. 2.6c). When Flag-tagged TRIF was immunoprecipitated with an anti-Flag antibody, co-expressed T7-tagged IKKe and TBK1 were detected by immunoblotting with an anti T7 antibody. Furthermore, there was no detectable T7-tagged IKKe or TBK1 when only the TRIF TIR domain only was used. These overexpression experiments must be verified with endogenous proteins when appropriate antibodies are available. Nonetheless, we conclude that IKKe and TBK1 play an essential role in TRIF-mediated IRF3 activation.

**TBK1 is required for IRF3-dependent gene expression in MEFs.**

The role of TBK1 in IRF3 activation was investigated by using a previously reported TBK1 knockout mouse (96). Previous studies showed that TBK1 deficient mice die at ~E14.5 of liver degeneration due to massive TNF-α induced apoptosis (96). Based on the similarity of this phenotype with that observed with RelA−/− mice, TBK1 was proposed to play an essential role in the NF-κB pathway (96). We therefore carried out studies to determine the effect of TBK deficiency on IRF3 activation in mouse embryonic fibroblasts (MEFs). As shown in Figure 2.7, induction of an IFN-β reporter gene in response to infection with SV was completely defective in the TBK1-deficient MEFs. When Newcastle Disease Virus (NDV), another well-characterized inducer of type I interferon expression was examined, IFN-β reporter gene expression was also completely abrogated in TBK-deficient cells (Fig. 2.7). We also investigated the role of TBK1 in
signaling via TLR3 and 4, following stimulation with poly IC or LPS, respectively. Induction of the IFN-β reporter genes was significantly reduced in TBK1 deficient cells following stimulation with either poly IC or LPS (Fig. 2.7). Furthermore, induction of both the RANTES and IP-10 reporter genes also showed similar defects in TBK1-deficient cells with all stimuli tested (data not shown). Together, these results show that type I interferon and related genes require TBK1 for their expression.

**TBK1 is essential for IRF3 activation.**

The inhibition of IFNβ-PRDIII-I and ISRE in TBK1-deficient mice suggested that in agreement with our previous observations (107), TBK1 mediates its effects via IRF3. We therefore examined the activation of IRF3 in TBK1-deficient cells. Viral infection leads to the phosphorylation of IRF3 followed by its dimerization, nuclear translocation and interaction with the co-activators CBP-p300 (74). Embryonic fibroblasts from wild type and TBK1-deficient mice were transfected with a vector encoding an IRF3-GFP fusion protein and nuclear translocation of IRF3 was examined following virus infection by confocal microscopy. In uninfected cells, IRF3-GFP was expressed in the cytoplasm of both wild-type and TBK1-deficient cells. In both cases, the nucleus was almost completely free of GFP fusion protein (Fig. 2.8). However, when cells were infected with SV for 6 h, IRF3-GFP clearly translocated to the nucleus in wild type cells. In contrast, IRF3-GFP remained in the cytoplasm in TBK1-deficient cells and no nuclear translocated IRF3-GFP could be detected following virus infection (Fig. 2.8).
**TBK1 is essential for IRF3 activation mediated by TRIF.**

TRIF has recently been shown to activate IRF3 and induce IRF3 target gene expression such as IFN-β, RANTES, and IP-10 (18, 88, 107). Recent studies with TRIF-deficient mice confirm its essential role in type I interferon induction following engagement of TLR3 and 4 by poly I:C and LPS, respectively (60, 108). Furthermore, TRIF-mutant mice, derived by chemical mutagenesis, are hypersusceptable to murine cytomegalovirus infection, due to a failure to produce type I interferons (108). Taken together, these observations suggest that TRIF is an essential shared component of the pathways by which TLRs and viruses induce type I interferon. While some viruses appear to signal via specific TLRs (109-111), the precise relationship between TRIF, individual TLRs, and all viruses is still unclear.

We have previously demonstrated using siRNA silencing of IKKε or TBK1 that the induction of type I interferon and related genes by TRIF is dependent on these kinases (107). Furthermore, immunoprecipitation studies suggested that TRIF interacted with IKKε or TBK1 and this interaction was abolished by deletion of the N or C-terminal non-TIR domain portion of the molecule (107). IKKε and TBK1 have also been shown to phosphorylate TRIF (112). Overexpression of TRIF induces IFN-β, IP-10, and RANTES reporter gene expression in HEK293 cells (107). We therefore examined the induction of IFN-β, IP-10 and RANTES reporter genes in wild-type and TBK1-deficient cells. The induction of IFN-β, IP-10 and RANTES reporter genes by TRIF was completely abrogated in TBK1-deficient MEFs, confirming the importance of TBK1 in mediating TRIF signaling to these target genes (Fig. 2.9). In contrast, TRIF-induced ELAM reporter gene activity was normal in either wild-type or TBK1-deficient cells (Fig. 2.9, right).
Discussion

Activation of innate immunity requires the integration of complex networks of signal transduction pathways, leading to the induction of specific sets of genes in response to a wide range of infectious microorganisms (113). Specificity is achieved by the coordinate activation of multiple transcription factors that assemble on enhancers located upstream from target genes. A well-characterized example of this is the human IFN-β gene which requires IRF3, NF-κB and at least one other transcription factor to be activated in response to viral infection (103).

An essential component of the NF-κB signaling mechanism is the canonical IκB kinase complex (IKKαβγ) (83), which is activated in response to a vast array of signals. The upstream activators and the downstream target of this complex are well characterized (83). By contrast, relatively little is known about the related kinases IKKe and TBK1. In this study, we confirm earlier observations showing that the expression of either IKKe (94) or TBK1 (95, 114) can activate NF-κB reporters; however, the mechanism by which these kinases activate NF-κB is unclear. Although IκBα does not appear to be a target of these kinases, the physiological target in the NF-κB pathway has yet to be identified (93-95, 97, 114).

The studies reported here reveal an unexpected role for IKKe and TBK1 in IRF3 activation, which is an essential component of the IFN-β enhanceosome (103, 104). IRF3 activation by virus, dsRNA, or LPS requires phosphorylation on a cluster of serine/threonine residues in the C-terminus of IRF3 (between residues 385-405) (78). Substitution of certain serine/threonine residues within this cluster with phosphomimetic amino acids results in a constitutively active protein, while substitution of the same
residues with alanine prevents signal-dependent activation (69, 77-79). A recent study has shown that substitution of serine 396 with aspartic acid, thus mimicking phosphorylation, is sufficient to activate IRF3 (115). Moreover, serine 396 was shown to be phosphorylated in vivo in response to viral infection or treatment with dsRNA (115). However, the importance of additional serine residues within the C-terminal cluster remains controversial (69, 77-79). Although overexpressed IKKe and TBK1 interact with IRF3, additional studies are required to unequivocally demonstrate the in vitro specificity and in vivo significance of this observation.

We have shown that TLR3 and TRIF function upstream in the signaling pathway leading to the activation of IKKe and TBK1 by dsRNA. It is known that IKKe and TBK1 can promote the interaction between a protein called TANK and IKKγ (116). TANK was identified by virtue of its interaction with the tumor necrosis factor (TNF) receptor associated factor, TRAF. Thus, IKKe-TBK1 and IKKγ may all be recruited to the intracellular domains of cell surface receptors through TRAFs or other adapter proteins. Activated IKKe is present in a high molecular weight complex in Jurkat T-cells (93), and recent studies have shown that this complex contains IKKαβγ (S.M.M. and T.M., unpublished). Although the functional importance of these multiple protein-protein interactions has not been established, it is possible that IKKe and TBK1 function in a multi-kinase complex recognizing downstream targets.

Although IKKe and TBK1 share significant sequence homology and have indistinguishable activities, they do not appear to be redundant in our cell culture system. Depletion of either IKKe or TBK1 by siRNA inhibits IRF3 activation by virus infection, dsRNA treatment, or TRIF overexpression. As shown in studies presented here, IRF3
dependent gene expression is defective in TBKI-deficient MEFs. The relative roles of these kinases in primary cell types, and their functions in the IRF3 signaling pathway will require further studies in single- or double-deficient mice.

Based on the data presented here and previous reports implicating IKKe and TBK1 in NF-κB activation, we propose that these non-canonical IκB kinases play a pivotal role in coordinating the activities of IRF3 and NF-κB, which in turn, synergize to regulate the expression of certain innate immune response genes.
Figure 2.1 Both IKKε and TBK1 induce IFN-β and RANTES. HEK293 or HEK293T cells were transfected with 40 ng of the following reporter genes, *IFN-β* (a), *RANTES* (b), *IFN-β PRDII* (d), *IFN-β PRDIII-I* (e) or *ISG-54 ISRE* (Stratagene) (f) and cotransfected with expression vectors for IKKε, TBK1 or IKKβ. Twenty-four hours after transfection, some cells were infected with Sendi Virus (Charles River Laboratories, 200 HAU/ml, ~16 h), as a positive control. In all experiments, luciferase reporter gene activity was measured and data normalized for transfection efficiency with a *Renilla* luciferase. (c) HEK293T cells were transfected with expression vectors for IKKε, TBK1, or IKKβ and twenty-four hours later supernatants were analyzed for endogenous RANTES by ELISA (R&D Systems).
FIGURE 2.1

(a) IFN-β

(b) RANTES

(c) RANTES (pg/ml)

(d) IFN-β PRDII

(e) IFN-β PRDII

(f) ISG-54 ISRE
Figure 2.2 IKKe and TBK1 activate IRF3. (a) HEK293T cells were transfected with 40 ng of a luciferase reporter gene containing the Gal4 upstream activation sequence and cotransfected with Gal4 DBD, Gal4-IRF3 or Gal4-IRF3-A7 (IRF3 serine/threonine 385, 386, 396, 398, 402, 404, and 405 have been substituted by alanine) and cotransfected with IKKe, TBK1 or IKKβ as indicated. 24 h after transfection, some cells were infected with Sendi Virus (~16 h), as a positive control. (b) HEK293 cells transfected with 40 ng of the IFN-β or an ISG-54 ISRE reporter and cotransfected with 40 ng of IKKe, TBK1 or empty vector in the presence of IRF3ΔN or IκB SR as indicated. 24 h following transfection, cell lysates were prepared and luciferase reporter gene activity measured. (c) IRF3-GFP-expressing HEK293 cells were plated on 35 mm glass-bottom sterile tissue culture dishes (Mattek Co., Ashland, MA) and transiently transfected with 1 µg Flag-tagged IKKe, TBK1, IKKβ, and visualized 48 h later by confocal microscopy. (d) HEK293 IRF3-GFP cells in 10 cm plates were transiently transfected with 4 µg Flag-tagged IKKe, TBK1, IKKβ. 48 h later, lysates were immunoprecipitated with polyclonal anti-GFP antibodies, and western analysis performed using monoclonal anti-Flag antibody or anti-GFP as shown. The relatively low amount of TBK1 is likely a consequence of its low level of expression. A Flag-expressing lysate was used as a positive control for western blotting.
Figure 2.2

a. Relative stimulation

b. IFN-β and ISG-54 ISRE

b. IRF-3-GFP Anti-Flag-Cy3 Overlay
Figure 2.3 IKKe and TBK1 are required for viral activation of PRDIII-I. (a) siRNA targeting vectors (100 ng) for IKKe, TBK1, IKKβ or targeting vector alone (EV) were transfected into HEK293T cells. After 72 h, total cell lysates were analyzed by Western blotting for endogenous TBK1 (A), IKKβ (B), or co-transfected Flag-tagged IKKe (50 ng) (C). (b) HEK293T cells were transfected with 40 ng of the PRDIII-I IFN-β promoter and cotransfected with siRNA targeting vectors as indicated for 72 h before infection with virus. Approximately 16 h later, luciferase reporter gene activity was measured, (*= significantly different from virus alone (Students t-test, p<0.01)). (c) Cell supernatants were also harvested following siRNA silencing for 72 h and endogenous RANTES production measured after virus infection by ELISA, (*= significantly different from virus alone (Students t-test, p<0.05)).
Figure 2.3

a. EV IKKe IKKβ TBK1: siRNA

IP: α TBK1, WB: α TBK1

EV IKKe TBK1 IKKβ: siRNA

WB: α IKKβ

b. IFN-β PRD III-I

Relative stimulation

Medium Virus 10 30 100 10 30 100

IKKe siRNA * * * * * * *
IKKβ siRNA * * * * * * *
p53 siRNA * * * * * * *
Vector (ng)

40 30 20 10

0

4000 3000 2000 1000

RAIITES (pg/ml)

Medium Virus 10 30 100 10 30 100

p53 siRNA * * * * * * *
IKKe siRNA * * * * * * *
TBK1 siRNA * * * * * * *
IKKβ siRNA * * * * * * *
siRNA Vector (ng)
Figure 2.4 IKKe and TBK1 are required for IRF3 activation by dsRNA. (a) TLR3-expressing HEK293 cells were transfected with an IFN-β promoter, an NF-κB reporter (16) or an ISG-54 ISRE reporter gene (b) or 40 ng of a luciferase reporter gene containing the Gal4 upstream activation sequence and cotransfected with 20 ng of Gal4 DBD or Gal4 IRF3. 24 h after transfection, cells were stimulated with dsRNA [50 μg/ml poly I:C] or left untreated for ~8 h and luciferase reporter gene activity was measured. (c) TLR3–expressing HEK293 cells were transfected with an ISRE reporter and cotransfected with siRNA targeting vectors as indicated for 72 h. Cells were then stimulated for 8 h with dsRNA [50 μg/ml poly I:C] before luciferase reporter gene activity was measured, (*= significantly different from dsRNA alone (students t-test, p<0.01). (d) Cell supernatants were also harvested following siRNA silencing for 72 h and endogenous RANTES production measured after dsRNA stimulation [50 μg/ml poly I:C] by ELISA, (*= significantly different from dsRNA alone (students t-test, p<0.05).
Figure 2.5 TRIF activates IRF3. (a) HEK293 cells were transfected with 40 ng of the IFN-β, the ISG-54 ISRE or NF-κB reporter (16) constructs and cotransfected with the indicated amounts of plasmids encoding MyD88, Mal or constitutively active IRF3 (IRF3-5D) or (b) TRIF. 24 h after transfection, luciferase reporter gene activity was measured.
Figure 2.5

a. IFN-β

b. IFN-β

NFKB
Figure 2.6 Both IKKe and TBK1 are required for activation of IRF3 by TRIF. (a) TLR3-expressing HEK293 cells were transfected with 40 ng of a plasmid encoding TRIF and 40 ng of the IFN-β, the ISG-54 ISRE or NF-κB reporter constructs. The same cells were cotransfected with dominant negative IRF3 (IRF3ΔN), IκB SR, IKKe (K38A) or TBK1 (K38A) as indicated. Twenty-four hours after transfection, luciferase reporter gene activity was measured. (b) TLR3-expressing HEK293 cells were transfected with 40 ng of the ISG-54 ISRE reporter and cotransfected with siRNA targeting vectors as indicated for 72 h before a second transfection with 40 ng of a TRIF expression plasmid. Twenty-four hours later, luciferase reporter gene activity was measured, (*= significantly different from TRIF only (Students t-test, p<0.05)) (c) HEK293T cells in 10 cm plates were transiently transfected with 4 μg of Flag-tagged TRIF or TRIF-TIR domain vectors alone or with T7-tagged IKKe, TBK1 or empty vector. Twenty-four hours later, lysates were immunoprecipitated with anti-Flag and Western analysis performed using monoclonal anti-T7 or anti-Flag as shown. The relatively low amount of TBK1 is likely a consequence of its low expression.
Figure 2.6

a. 

b. 

---

Figure 2.6

a. 

b. 

c.
Figure 2.7 Reduced IFNβ reporter gene activity in the absence of TBK1. MEFs from TBK1+/+ (filled bars) and TBK1−/− (empty bars) mice were transfected with an IFN-β luciferase reporter gene. Cells were left untreated (none) or infected with NDV or SV, or stimulated with poly IC or LPS. Lysates were assayed for luciferase activity.
Figure 2.7
Figure 2.8 Deficiency of TBK1 leads to defective virus-induced nuclear translocation and phosphorylation of IRF3. MEFs from TBK1+/+ and TBK1−/− mice were transfected with 1.0 μg of an IRF3-GFP-fusion protein. Samples were left untreated (none) or infected with SV (100 HAU) and visualized by confocal microscopy.
Figure 2.8

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Figure 2.9 TRIF induces IFNβ and RANTES reporter genes by means of TBK1. MEFs from TBK1^{+/+} (filled bars) and TBK1^{+/−} (empty bars) mice were transfected with IFN-β (Left), RANTES (Center) or ELAM (Right) luciferase reporter genes and cotransfected with a murine TRIF expression vector or left untreated (none). Lysates were assayed for luciferase activity.
Figure 2.9

- ■ TBK1 +/-
- □ TBK1 +/-

**IFN-β**

- Relative Simulation
  - none
  - TRIF

**RANTES**

- Relative Simulation
  - none
  - TRIF

**ELAM**

- Relative Simulation
  - none
  - TRIF
CHAPTER III:

LPS/TLR4 signaling to IRF3/7 and NF-κB involves the Toll adapters

TRAM and TRIF
Abstract

TRAM is the fourth TIR domain containing adapter protein to be described that participates in TLR signaling. Like TRIF, TRAM activates IRF3 and NF-κB dependent signaling pathways. TLR3 and 4 activate these pathways to induce IFN-α/β, RANTES, and IP-10 expression independently of the adapter protein MyD88. Dominant negative and siRNA studies performed here demonstrate that TRIF functions downstream of both the TLR3 (double stranded RNA) and TLR4 (LPS) signaling pathways, while the function of TRAM is restricted to the TLR4 pathway. TRAM interacts with TRIF, Mal/TIRAP, and TLR4 but not with TLR3. These studies suggest that TRIF and TRAM both function in LPS/TLR4 signaling to regulate the MyD88-independent pathway during the innate immune response to LPS.
**Introduction**

The TLR family is the essential recognition and signaling component of mammalian host defense (117-119). At least ten TLRs have been cloned in mammals, which recognize molecular products derived from all the major classes of pathogens (117-119). TLR signaling to NF-κB originates from the conserved TIR domain, which mediates recruitment of the TIR domain-containing adapter molecule, MyD88 (106), a critical adapter molecule utilized by all TLRs except TLR3 (86)). The recruitment of MyD88 to proximal TIR domains of activated TLRs allows for the interaction and activation of IRAK-family members (87, 120), and the subsequent activation of tumor necrosis factor receptor-associated factor-6 (TRAF-6 (121)). These events, at a minimum, result in NF-κB activation via the IKKαβγ complex (83).

While most of the TLRs seem to be absolutely dependent on the expression of MyD88 for all of their functions, TLR3 and TLR4 are unique in their ability to activate MyD88-independent responses as well (59, 61, 88, 122). A feature of MyD88-independent signaling is the induction of a dendritic cell maturation pathway, and the induction of the type I interferon IFN-β (18, 61, 62, 88, 123). The transcription enhancer of the IFN-β promoter binds NF-κB, IRF3, and ATF-2-c-Jun. While all TLRs activate NF-κB and ATF2-c-Jun, not all TLRs induce IFN-β, because not all TLRs activate IRF3. Thus TLR3 and TLR4 appear to have diverged evolutionarily from other TLRs to activate gene expression programs and trigger anti-viral responses by a mechanism involving the coordinate activation of NF-κB and IRF3 (124).

MyD88-adapter-like (Mal) (16), also called TIRAP (17), is a related MyD88-like protein, that was discovered based on its ability to mediate TLR4 signaling. Mal/TIRAP
has been implicated in LPS-induced IFN-β induction *in vitro* (61, 125). However, studies with Mal/TIRAP gene targeted mice show that Mal/TIRAP functions in the MyD88-dependent NF-κB activation pathway following LPS stimulation, as well as engagement of TLR2 by its ligands (66, 89). A third adapter protein, TRIF (19, 60), interacts with TLR3 and mediates the TLR3-dependent induction of IFN-β via NF-κB and IRF3 activation as presented in Chapter II.

Constitutively expressed IRF3 has been implicated in the induction of IFN-β (74, 126, 127), RANTES (75, 128), and ISG-54/56 expression (129). IRF3 is activated following phosphorylation on a cluster of specific C-terminal serine residues (74, 78, 79), facilitating its dimerization and interaction with the co-activators CBP and p300 (71, 72, 99, 130). The activated IRF3 complex then translocates to the nucleus where it regulates the transcription of target genes (75, 99). IRF7 is a related transcriptional regulator that is expressed mostly in lymphoid cells and is essential for IFN-α gene expression (131, 132). The transcription of IRF7 is induced by IFN and post-translationally activated by phosphorylation on its C-terminal serine residues, some of which are conserved with IRF3 (131, 133). IKKe (93, 94) and TBK1 (95, 96, 114) are key regulators of the IRF3 and IRF7 activation pathways in cells that have been exposed to some viruses and/or activated by double stranded RNA via TLR3 (70, 107). IKKe and TBK1 are also required components of the TRIF signaling pathway resulting in IRF3 activation (107).

Studies with IRF3-deficient mice have established an essential role for IRF3 in LPS-induced IFN-β gene expression and endotoxin shock (134). However, the molecular mechanisms regulating the MyD88-independent LPS/TLR4 pathway to IRF3 and NF-κB activation are unknown. Here, we have identified a fourth TIR domain containing
adapter molecule, which we have named TRAM (TRIF-Related Adapter Molecule). TRAM, like all of the TIR-domain containing adapter molecules, activated NF-κB. In addition, TRAM, like TRIF, activated IRF3 and IRF7. Unlike any of the other known TIR domain containing adapters, TRAM appears to be restricted to the LPS activation (TLR4) pathway while TRIF plays an essential role in both TLR3 and TLR4 pathways leading to IRF target gene expression. Our findings suggest that TRAM may have evolved to mediate TLR4 specific signals resulting in a gene expression profile that is not shared by TLR3.
Materials and Methods

Reagents. The IRF3-DN, Gal4-IRF3 and Gal4-luciferase reporter gene were from T. Fujita (Tokyo, Japan). IKKe-k38a and TBK1-k38a were as described (93, 107). IRF7, IRF7DN and Gal4-IRF7 were from P. Pitha (Baltimore, Maryland). The RANTES-reporter construct was as described (128). The IP-10 reporter construct was from A. Luster (Massachusetts General Hospital, Boston, MA). The NF-κB-luciferase construct (16), pEF-Bos-Flag Mal and Flag-TRIF were as described (107). The plasmids pEF-Bos-Flag-TRAM, TRAM-CFP, TRIF-CFP and Mal-CFP were generated by PCR cloning from a human PBMC cDNA library. pEF-Bos-TRAM-TIR (amino acids 63-235), pEF-Bos-TRAM-C117H, TRAM-P116H and TRIF-P434H were generated using the Quick-Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Polyclonal antibodies to IRF3 were from Zymed (San Francisco, CA) and CBP were from Santa Cruz (Santa Cruz, CA). pCMV-TRIFDNDC and MyD88-deficient mice were gifts from S. Akira (Osaka, Japan); the MyD88 knockout mice used were backbred onto a C57BL6 background for five generations. LPS derived from Escherichia coli strain 011:B4 was purchased from Sigma, dissolved in deoxycholate and re-extracted by phenol:chloroform as described (49). Poly IC was from Amersham Pharmacia (Piscataway, NJ).

Stable cell lines. We engineered clonal stable cell lines by transfecting HEK293 cells with chimeric fluorescent protein TLR constructs as described (57). A HEK293 cell line stably expressing both TLR4 and MD-2 was generated by retroviral transduction of HEK/TLR4 cells with a retrovirus encoding human MD2 (53). HEK/TLR3, HEK/IRF3-GFP (107) and U373/CD14 cells (135) were generated as described.
**Electrophoretic mobility shift assays.** Bone marrow-derived macrophages were cultured from C57BL/6 mice or age and sex-matched MyD88−/− mice for 8 days in M-CSF (10 ng/ml). Nuclear extracts from 5 x 10⁵ cells were purified after LPS (10 ng/ml), Malp-2 (1 nM), or Poly IC (50 mg/ml) stimulation for the times indicated. The extracts were incubated with a specific probe for the ISRE consensus sequence (Promega, Madison, WI), electrophoresed, and visualized by autoradiography (136). Supershift analysis was performed with antibodies to mouse IRF3, p65 or IgG control.

**ELISA.** Macrophages (5 x 10⁴ cells per well) were seeded into 96-well plates for 24 hrs prior to stimulation with LPS, poly I:C or medium for 12 h. Cell culture supernatants were removed and analyzed for the presence of RANTES, IP-10, or TNF-α by ELISA following the manufacturer's instructions (R&D Systems).

**Transfection assays.** Cells were seeded into 96-well plates at a density of 1.5 x 10⁴ cells per well and transfected 24 h later with 40 ng of the indicated luciferase reporter genes using Genejuice (Novagen). The thymidine kinase Renilla-luciferase reporter gene (Promega) (40 ng/well) was co-transfected in order that the data could be normalized for transfection efficiency. Cell lysates were prepared and reporter gene activity was measured using the Dual Luciferase Assay System (Promega). Data are expressed as the mean relative stimulation ± S.D. All of the experiments described were performed a minimum of three occasions and gave similar results.
**Immunofluorescence and confocal microscopy.** A HEK293-IRF3-GFP stable cell line was transiently transfected with Flag-tagged constructs as indicated. After allowing two days for protein expression to occur, the transfected cells were fixed, permeabilized and stained with Cy3-conjugated anti-Flag antibody (clone M2, Sigma-Aldrich). DRAQ5 was added to counter stain nuclei. Cells were imaged by confocal microscopy using a Leica TCS SP2 AOBS microscope.

**RNA interference.** siRNA duplexes targeting the coding region of TRAM and Lamin A/C were from Dharmacon (Lafayette, CO). TRAM-siRNA sequences: GGAAGAAAGTCGTGGATT (product#: D-004334-01™); Lamin A/C: CTGGACTTCCAGAAGAACA. siRNA duplexes targeting the 3' UTR of TRIF were as described (88). In order to determine the efficiency of gene silencing, 293T cells (24 well plates, 4 x 10⁴ cells/well) were transfected with 0.5 mg of plasmids encoding TRAM-CFP, TRIF-CFP or Mal-CFP expression vectors. These cells were co-transfected with TRAM or Lamin A/C siRNA duplexes (50nM) using Mirus TransIT® TKO and TransIT-LT1® transfection reagents in a combination protocol exactly according to the manufacturers recommendations (Mirus, Madison, WI). CFP fluorescence was quantified by flow cytometry (Becton Dickinson, LSR) 24 hours later. For reporter assays, U373/CD14 cells or TLR3-expressing HEK293 cells (4 x 10⁴ cells/well) were transfected with 0.5mg of the RANTES reporter gene and 0.25mg of a thymidine kinase-renilla reporter gene and cotransfected with 50 nM of siRNA targeting vectors as described above in 24-well tissue culture dishes. 36 h following transfection, cells were stimulated with LPS or dsRNA for ~8 h before luciferase activity was measured.
Co-immunoprecipitation. HEK293T cells or TLR-expressing cells (10 cm plates) were transfected using GeneJuice (Novagen) with 4 mg of the indicated plasmids. Cells were lysed in 1 ml of lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 137 mM NaCl, 0.5% TX-100, Glycerol 10%, with protease inhibitors) one to two days after transfection. Polyclonal anti-GFP (Molecular Probes), anti-IRF3 or anti-CBP antibodies were incubated with the cell lysates in Protein A sepharose overnight. The immune complexes were precipitated and subjected to 4-15% SDS-page and immunoblotted for Flag- or CFP/YFP- tagged adapters using the anti-Flag mAb M2 (Sigma), or anti-GFP mAb (Clontech), which also recognizes the spectral variants of GFP.
Results

LPS and dsRNA activate IRF3 and IRF7

The promoters of RANTES and IP-10, like that of IFN-β, contain transcription factor binding elements for NF-κB and IRF3 (75, 128). The expression of RANTES and IP-10 represent downstream targets of TLR receptors that are entirely independent of MyD88 expression following stimulation by LPS or dsRNA. Stimulation of mouse bone marrow-derived macrophages with LPS or dsRNA induced RANTES secretion, an effect that was observed comparably in bone marrow macrophages deficient in MyD88 (Fig. 3.1a). This was also true for IP-10 levels as measured by ELISA (data not shown). In contrast, TLR2 signaling via lipopeptides absolutely requires MyD88 and did not lead to RANTES expression. TLR2-mediated production of TNF-α was entirely absent in MyD88-deficient macrophages (data not shown), in agreement with published reports (59, 85, 137).

We next examined the effect of LPS and dsRNA on IRF3 DNA binding activity. IRF3 DNA binding activity was induced in both wild-type and MyD88-deficient macrophages following LPS and dsRNA stimulation (Fig. 3.1b). The presence of IRF3 in this ISRE DNA binding complex was confirmed by depletion ("gel shift") analysis using antibodies to IRF3 (Fig. 3.1b, lower panel). Stimulation of cells with the TLR2 ligand, Malp-2, did not result in IRF3 activation. NF-κB was activated in wild-type cells by all stimuli and in MyD88-deficient macrophages following LPS or dsRNA stimulation (data not shown).

We next addressed the question of whether the related transcriptional regulator IRF7 was a target of TLR signaling. We employed an in vivo assay for IRF7 activation,
that utilizes a hybrid protein consisting of the yeast Gal4DNA binding domain (DBD) fused to IRF7 lacking its own DNA binding domain (103). Reporter gene expression from the Gal4 upstream activation sequence in this assay requires IRF7 activation (99). IRF3 activation was also measured in this assay using a Gal4 IRF3 fusion protein. Stimulation of TLR3- or TLR4/MD2-expressing HEK293 cells with dsRNA or LPS, respectively, but not IL-1β, activated both IRF3 and IRF7 (Fig. 3.1c). IRF7 plays a critical role in regulating IFN-α expression. Exogenously expressed IRF7 increased the activation of an IFN-α reporter construct when TLR4/MD2- or TLR3-expressing HEK293 cells were stimulated with LPS or dsRNA, respectively, while a dominant negative IRF7 mutant inhibited the effect (data not shown). These observations provide strong evidence that TLR3 and TLR4 activate IRF3 and IRF7 and, as a result, induce target genes such as RANTES and IFN-α/β.

*Discovery of a fourth TIR domain containing adapter molecule, TRAM*

A search of the human genome for additional TIR domain containing adapter molecules resulted in the identification of a small protein fragment that shares sequence similarity with other TIR domain containing proteins, most notably with TRIF/TICAM-1. A set of overlapping EST sequences were subsequently identified and used to clone the full-length cDNA of human and mouse TRAM, which shares 75% sequence identity (Genbank Accession number AY232653 and AY268050, respectively). The TRAM gene is located on human chromosome 5 (ENSEMBL ID: ENSG00000164226). TRAM is a 235 amino acid protein with a C-terminal TIR domain. Figure 3.2a shows a multiple sequence alignment of human and mouse TRAM with other human and murine adapters
and TLRs. The crystal structure of the TIR domain of TLR2 has been resolved. The TIR-domain 'BB-loop' is an essential part of its structure, and this portion of the molecule appears to engage downstream elements such as adapter molecules or other TLRs (119, 138). Most TIR-domain BB-loop sequences contain a conserved proline residue in the BB-loop. When this residue is mutated to histidine, the mutant protein is typically unable to signal, and may even function as a dominant suppressing mutation (16, 17, 139). Unlike the other known adapter proteins, both human and mouse TRAM contain a cysteine residue at this position (denoted by a # in Fig. 3.2a). A proline residue resides directly adjacent to this residue in TRAM, at position 116.

Because of the similarity in the sequence of the TIR domain of TRAM and TRIF, we first compared the effect of TRIF and TRAM on IRF3 and IRF7 activation. Overexpression of TRAM activated both the IRF3 and IRF7 responses (Fig. 3.2b). TRIF also activated both transcription factors (Fig. 3.2b). As a consequence, TRAM and TRIF also induced the IFN-β, RANTES, IP-10 and IFN-α1/α2 promoters, all of which contain ISRE elements required for IRF binding (data not shown). These data imply that TRAM and TRIF also activate NF-κB, as some of these promoters (IFN-β, RANTES, and IP-10) also require NF-κB for full activity (see below).

As a further test of TRAM- and TRIF-dependent IRF3 activation, we examined their effects on the nuclear translocation of IRF3. Overexpression of TRAM and TRIF in a stable cell line that expresses a green fluorescent protein chimera of IRF3 resulted in the nuclear translocation of this IRF3-GFP fusion protein (Fig. 3.3a). TRIF has been shown recently to co-immunoprecipitate with IRF3 (18). We were interested in determining if TRAM might also associate with IRF3. When HEK293 cells were
transfected with Flag-tagged TRAM and immunoprecipitated with antibody to endogenous IRF3, Flag-tagged TRAM could be detected in the immunoprecipitated complex (Fig 3.3b, top panel). Immunoprecipitation with an anti-Flag antibody confirmed this interaction; endogenous and co-transfected IRF3 could be detected in the immunoprecipitated complexes (Fig 3.3b, second panel). TRIF also interacted with endogenous and transfected IRF3, in agreement with published reports (data not shown). There were no non-specific associations detected in cells lacking the transfected adapter constructs. Although not shown, we also performed similar experiments with IRF7. IRF7 also interacted with TRAM and TRIF and vice versa.

Activated IRF3 must associate with the co-activators CBP and p300 in order to enhance target gene expression (71, 72, 99, 130). When endogenous CBP was immunoprecipitated from cell lysates expressing transfected Flag-tagged TRAM, TRAM could be detected in these immunoprecipitated complexes (Fig 3.3b, third panel). This was also true for transfected TRIF (data not shown).

The non-canonical IκB kinases, IKKe (93, 94) and TBK1 (95, 96, 114) are key regulators of the IRF3 activation pathway resulting from viral exposure and activation of TLR3 or TRIF signaling cascades (70, 107). IKKe has also been implicated in LPS signaling (140). We next examined the effect of dominant negative mutants of IKKe and TBK1 on TRAM signaling. We used the RANTES reporter gene construct to address this issue. Cells were co-transfected with TRAM, which activates downstream molecules as a result of overexpression, and the kinase inactive mutants of IKKe (IKKe-k38a) or TBK1 (TBK1-k38a). Both mutants inhibited TRAM-induced RANTES reporter activation in a dose-dependent manner, suggesting that these two kinases may also
function downstream of TRAM. Taken together, these observations, provide strong evidence that TRAM and TRIF are important components of the IRF3 signaling pathway, and suggest that these adapter proteins form a multi-protein complex with IRF3/7, CBP, and the IRF3/7 kinases (IKKε and TBK1) during signal transduction.

**TRAM activates the IRF pathway in the TLR4 but not the TLR3 signaling pathway**

We next generated a series of TRAM mutants and examined their ability to activate the *RANTES* reporter gene. Transfection of HEK293 cells with a plasmid encoding the TIR domain alone of TRAM (TRAM-TIR) induced the RANTES reporter, although this response was considerably less than that observed with the full length TRAM cDNA (Fig. 3.4a). TRAM contains a cysteine residue (C117) in the BB-loop with an adjacent proline residue (P116). Mutation of the proline residue to histidine (TRAM-P116H) significantly impaired the RATES inducing activity of TRAM, while mutation of the cysteine residue at position 117 (TRAM-C117H) completely abrogated all activity (Fig. 3.4a). This suggested that either TRAM-C117H or TRAM-P116H might function as a dominant interfering mutant of TRAM activity. The effect of these TRAM constructs was similar when an IP-10 promoter-based reporter construct was assessed (data not shown).

We subsequently examined the effect of these TRAM mutants on TLR-mediated signaling that culminates in RANTES promoter activation or the activation of the transcription factors IRF3 and IRF7. We focused on TLR3 and TLR4 because of their unique abilities to activate both NF-κB and IRF3. Neither the TRAM-TIR domain nor the TRAM-P116H mutants had any dominant negative inhibitory activity on either TLR-
dependent IRF3 pathway tested (data not shown). Transfection of HEK/TLR3 cells with TRAM-C117H had no inhibitory effect on dsRNA-induced RANTES response (Fig. 3.4b). In contrast, LPS-induced activation of the RANTES reporter via TLR4 was impaired by the TRAM-C117H mutant (Fig 3.4b, left panel). The LPS-dependent induction of the RANTES reporter gene was considerably less potent than that observed following TLR3 stimulation. The TRAM-C117H mutant also inhibited the TLR4-, but not the TLR3-dependent activation of IRF3 and IRF7 (Fig. 3.4c). The TRAM-C117H mutant also inhibited the TLR4- but not the TLR3-dependent activation of an IP-10 reporter construct (data not shown). We also examined the role of TRIF in the TLR3- and TLR4-dependent pathways in parallel, by expressing a dominant negative mutant of TRIF lacking both the N-terminal and C-terminal regions surrounding the TIR domain (TRIFΔNΔC (18)). As expected, this mutant completely suppressed the TLR3-dependent response (Fig. 3.4d). The TRIFΔNΔC mutant also inhibited the TLR4-response, although the effect was less potent than that observed in the TLR3 pathway under identical experimental conditions (Fig. 3.4d, right panel). Taken together, these observations suggest that TRIF regulates the TLR3 and TLR4 pathways to IRF-3/7target genes, while TRAM appears to be TLR4-specific.

**TRAM also activates NF-κB**

We subsequently addressed the role of TRAM in the NF-κB activation pathway. Transfection of HEK293 cells with TRAM resulted in a potent NF-κB activation response (Fig. 3.5a). The isolated TIR domain of TRAM also induced a robust NF-κB response, though this was considerably less than that observed with the full-length gene.
(Fig. 3.5a). Neither the TRAM-P116H nor the TRAM-C117H mutants induced NF-κB activation. Thus, like all of the other known adapters, TRAM is also an NF-κB activator.

The TRAM-C117H negative interfering mutant was next tested for its ability to inhibit TLR-dependent signaling to NF-κB. TLR2-, TLR3-, TLR4/MD2-, TLR7- and TLR8-expressing HEK293 cells were transfected individually with an NF-κB reporter gene and co-transfected with increasing concentrations of TRAM-C117H. Following stimulation with their cognate TLR agonists, luciferase reporter gene activity was measured. NF-κB activation induced by the TLR2 agonist Malp-2, the TLR3 agonist dsRNA, the TLR7 and TLR8 agonist R-848, IL-1β, or TNF-α were all unaffected when cells were co-transfected with the suppressing TRAM-C117H mutant (Fig. 3.5b). In striking contrast to these negative results, the TRAM-C117H mutant inhibited LPS-induced NF-κB activity in HEK/TLR4/MD2 cells. The TRAM-P116H had no inhibitory activity on any TLR- pathway to NF-κB, including the TLR4 pathway, confirming the importance of the C117 residue for this response (data not shown). These observations suggest that TRAM regulates NF-κB as well as IRF3/7 in the LPS/TLR4 signaling pathway.

**TRIF and TRAM cooperate in the IRF3 activation pathway**

We examined the effect of the TRIFΔNΔC mutant on TRAM-induced RANTES promoter activation in order to define the relationship between TRIF, TRAM, and the TLR4 signaling pathway. The TRIFΔNΔC construct inhibited the TRIF-induced RANTES reporter gene response (Fig. 3.6a, hatched bars). The TRIFΔNΔC mutant completely abrogated the TRAM-induced RANTES reporter gene response (Fig. 3.6a).
The TRAM-C117H mutant also abrogated the induction of the RANTES reporter gene in response to TRAM overexpression (Fig. 3.6a, far right), but had no effect on the response to TRIF overexpression (hatched bars). The observation that a TRIF dominant-negative construct blocked TRAM activity but not vice versa suggests that TRAM signaling requires TRIF.

Subsequently, we performed co-immunoprecipitation experiments on cells that heterologously expressed both of these gene products, as well as the related adapter molecule Mal/TIRAP. These immunoprecipitation studies demonstrated that TRAM interacted with both TRIF and Mal/TIRAP (Fig 3.6b, left panel). TRIF also interacted with Mal (right panel). Finally, both TRIF and TRAM interacted with the Mal-P125H (dominant negative) mutant. The stronger interaction of TRIF and TRAM observed with the Mal-P125H mutant does not reflect a higher avidity for this interaction, but rather, was a consequence of the higher expression level of the MAL-P125H mutant in whole cell lysates, compared to the expression level of Mal or TRIF (Fig 3.6b, middle panel). These data may explain a previously unexplained finding, i.e., that the Mal/TIRAP dominant negative mutant powerfully inhibited LPS-induced signaling to NF-κB (16, 17) and IFN-β expression (61, 125), while the Mal/TIRAP knockout mouse both retained the ability to induce NF-κB (66, 89) and IFN-β expression (89). It is likely that the more profound effect of the dominant negative construct is due to its ability to limit the function of other adapter molecules involved in LPS signaling such as TRAM and TRIF. Furthermore, these data suggest that TRIF and TRAM interact with Mal at a site distinct from the TLR4 interaction site of Mal (17).
Co-immunoprecipitation studies were next performed to determine if TRAM interacts with TLR4. Stable TLR3- or TLR4-expressing cell lines were transiently transfected with Flag-tagged expression vectors for TRAM, TRAM-C117H and Mal and co-immunoprecipitation experiments performed. These experiments indicated that TRAM interacts with TLR4, but not with TLR3 (Fig. 3.6c), one more indication of the specificity of TRAM for the TLR4 pathway. The dominant negative mutant TRAM-C117H, failed to immunoprecipitate with TLR4, suggesting that the C117 residue is critical for this interaction. Mal also interacted with TLR4 but not TLR3, providing additional evidence that Mal has a role in the TLR4, but not the TLR3, signaling pathway.

**TRIF and TRAM are essential for TLR4 signaling**

The data obtained by testing dominant negative constructs and assessing protein:protein interactions suggest that TRIF and TRAM both function in the TLR4 signal transduction pathway. Dominant negative constructs, when highly expressed, have the potential to bind (e.g., as seen in Fig 3.6b) and interfere with proteins that might otherwise not be related to a specific signal transduction pathway. We therefore performed siRNA silencing experiments as an additional methodology to delineate the relationship between TRIF and TRAM in the TLR4 and TLR3 signaling pathways.

In order to assess the gene silencing activity of siRNA duplexes we selected, cells were transiently transfected with a fluorescent chimeric construct of TRAM (TRAM-CFP) and cotransfected with siRNA duplexes targeting the coding region of TRAM or a control siRNA, Lamin A/C. These siRNA duplexes can therefore be used to assess the
silencing effect of a fluorescent chimeric construct of TRAM. This methodology has been used extensively to assess siRNA efficiency and provides a quantitative assessment of silencing efficiency (102). We found that siRNA duplexes targeting the TRAM coding region completely ablated the expression of the TRAM-CFP chimeric fusion protein while lamin A/C siRNA duplexes were without effect (Fig. 3.7a). We also examined the effect of these TRAM siRNA duplexes on TRIF and Mal expression in order to insure the specificity of the TRAM siRNA duplexes. This is particularly important as TRIF and TRAM are most closely related in sequence. TRAM siRNA duplexes had no effect on chimeric constructs of TRIF or Mal expressed as CFP fusion proteins (Fig 3.7a).

Having determined that the siRNA duplexes chosen for TRAM effectively and specifically suppressed TRAM expression, we examined the effect of these siRNA duplexes on the LPS and dsRNA signaling pathways. Native macrophages and macrophage cell lines are extraordinarily difficult to transfect with siRNA. In contrast, U373/CD14 cells resemble central nervous system macrophages, are easily transfectable, and are highly inducible by treatment with LPS. We thus tested the effect of TRAM siRNA duplexes on the LPS-response in U373-CD14 cells. In comparison, we used HEK/TLR3 cells to test the effects of TRAM and TRIF in poly I:C stimulated RANTES expression. The response of each of these cell lines to these TLR ligands is comparable.

TRAM siRNA duplexes inhibited the LPS-dependent induction of the RANTES reporter gene in U373/CD14 cells, while siRNA targeting of Lamin A/C had no effect (Fig. 3.7b, top panel). We also examined the effect of published TRIF siRNA duplexes, which target the 3’ untranslated region of TRIF in this response. These TRIF siRNA
duplexes have been shown to completely silence endogenous TRIF mRNA expression (88). TRIF siRNA duplexes also inhibited the LPS response to RANTES induction (Fig 3.7b, top panel). In striking contrast to LPS, when the TLR3-mediated response to dsRNA was analyzed, the TRAM siRNA duplexes had no inhibitory effect on the dsRNA response, while TRIF siRNA duplexes inhibited dsRNA-dependent RANTES induction, in agreement with published reports (88). As with RANTES, siRNA silencing of TRAM prevented LPS, but not poly I:C, induction of the IP-10 promoter (data not shown). These observations confirm the studies with TRIF and TRAM dominant negative mutants and demonstrate that both adapter molecules are required for full LPS/TLR4 signaling to IRF target genes.
Discussion

The ability of individual TLRs to discriminate between invading pathogens is an important determinant of the unique gene expression profiles activated by different microorganisms. While the specificity of microbe detection begins with the ligand recognition features of one or more TLR, the discovery of a family of TIR-domain containing adapter molecules, including MyD88 (106), Mal (16, 17), TRIF (18, 88), and TRAM, suggest that the outcome of induced pathogen recognition also depends on the TLR-restricted utilization of these molecules, alone and in combination, to drive a stimulus-specific response. The TLR3- and TLR4-restricted utilization of IRF-inducing adapter molecules such as TRAM and TRIF induces not only the cytokines, co-stimulatory molecules, and anti-microbial peptides that are induced by all TLRs, but also anti-viral type I interferons and specific chemokines including IP-10 and RANTES.

The dominant negative, siRNA, and protein:protein interaction data presented herein demonstrate that TRAM is specifically required for LPS signaling, while TRIF is required for signaling by both TLR3 and TLR4. Both MyD88 and Mal/TIRAP also have a role in more than one TLR. Thus, the activity of TRAM, unlike any of the other known adapter molecules, may be restricted to a single TLR.

Defining the constituents of the TLR4 pathway activated by LPS has proven to be a complicated process. First, it was believed that MyD88 was the only adapter molecule needed for the full extent of the LPS response (59). However, data rapidly emerged that showed that at least part of the LPS response also involves Mal/TIRAP (66, 89). Thus, both MyD88 and Mal/TIRAP are involved in LPS signal transduction but cannot account for all, or even most, of the observed signaling traffic. The existence of TRAM and the
potential cooperativity of TRAM and TRIF in the TLR4 pathway, may explain how double MyD88/Mal-null cells are still capable of responding to endotoxin. It is intriguing that the TLR4 pathway requires TRIF, TRAM, MyD88, and Mal/TIRAP while TLR3 signaling appears to require only TRIF. The gene expression profiles activated following dsRNA and LPS stimulation of cells, though similar, are not identical (124, 141). The utilization of TRAM by TLR4 and not TLR3 may allow LPS/TLR4 to induce signaling pathways and gene expression programs not possible by TLR3/TRIF-mediated signaling. Thus, the combinatorial utilization of TRIF and TRAM by TLR4 may allow a specific tailoring of the immune response to the pathogens that activate TLR4.

Both functional and direct biochemical studies indicate that the adapter molecules used for the LPS response are interacting with one another as well as with TLR4. One obvious question that needs to be addressed is how these types of observations, which were made in a few types of transfected cell lines and primary bone marrow-derived macrophages, apply to the myriad of cell types that respond to LPS. The response to LPS is not uniform, an observation that is generally attributed to the cell surface density of the LPS receptor components. But, simple receptor density clearly does not always explain these differences and many different mechanisms are undoubtedly at work. Based upon scanning electron micrographs of LPS exposed cells, it is likely that activated TLR4 forms a large ‘signalosome’ involving multiple molecules of TLR4 (A.V. and D.T.G., data not shown). The cytoplasmic face of the activated LPS receptor is likely to have a large surface upon which these adapter molecules may sit. We propose that cell-specific differences in the response to LPS may also involve differences in the number and the composition of adapter molecules that assemble on TLR4. Co-crystallization of the
cytoplasmic face of TLRs in complexes with the various adapter molecules or combinations of adapter molecules will be necessary to understand fully the physical basis of how an activated TLR actually collaborates with these molecules to propagate an intracellular signal. We predict that TRAM will be a portion of a large platform of adapter molecules bound to TLR4 upon which a variety of kinases and other molecules can effectively function to initiate LPS responses.

While this manuscript was in preparation, Shu and colleagues reported on the identification of TRAM, to which they gave the name TIRP, as an adapter molecule that interacts with the IL-1R, Mal, IRAK and TRAF-6. These authors reported that TRAM/TIRP functions exclusively in IL-1 signaling (21). In agreement with Shu and colleagues, we have also detected TRAM in association with Mal (Fig. 3.4b), TRAF6 and the IL1RAcP (data not shown). Furthermore, we detected no inhibition of the IL1-induced NF-κB response when cells were transfected with the TRAM-C117H mutant (Fig. 3.6). Since submission of this paper, two manuscripts have reported on the role of TRIF in LPS signaling (60, 108). One of these manuscripts (108), postulates that there may be another TLR4 adapter molecule, which was designated as "X". Based on my data, TRAM very likely represents "X", although the precise mechanism whereby the four known LPS adapters interact and contribute to MyD88-dependent and MyD88-independent signaling remains to be determined.
Figure 3.1 LPS and dsRNA activate IRF3 and IRF7. (a) Bone marrow-derived macrophages from wild-type and MyD88-deficient mice were stimulated with LPS (0.1-100 ng/ml), Malp-2 (5 nM) and dsRNA (1-100 µg/ml) for 12 hours. The concentration of RANTES was measured by ELISA. (b) Nuclear extracts were isolated from wild-type and MyD88-deficient macrophages stimulated with LPS (10 ng/ml), Malp-2 (5 nM), and dsRNA (50 µg/ml) for 1 h and subjected to EMSA using a $^{32}$P-labeled ISRE consensus sequence (ISG-15) as a probe. Activated complexes were visualized by autoradiography. Activated ISRE DNA binding complexes were preincubated with polyclonal antibody to IRF3 or two control antibodies prior to incubation with the ISRE probe (right panel). (c) TLR3- and TLR4/MD2-expressing HEK293 cell lines were transfected with a luciferase reporter gene containing the Gal4 upstream activation sequence and with Gal4 DBD, Gal4-IRF3, or Gal4 IRF7 (40 ng). After 24 h, cells were stimulated with LPS (10 ng/ml), dsRNA (50 µg of poly I:C/ml), IL-1β (10 ng/ml) or left untreated for ~8 h and luciferase reporter gene activity measured.
Figure 3.1

a.

b.

c.
Figure 3.2 TRAM activates IRF3 and IRF7. (a) Alignment of TIR domains of TRAM, TRIF, MyD88 and Mal with TLR1, TLR2, TLR3 and TLR4. The amino acid colors are based on their physico-chemical properties where yellow = small, green = hydrophobic, turquoise = aromatic, blue = positively charged and red = negatively charged. (b) HEK293 cells were transfected as in (c) above and cotransfected with 40 ng of TRAM or TRIF. After 24 h, luciferase reporter gene activity was measured.
Figure 3.2

a. TRAM-C117 of 'BB-loop'

b.
Figure 3.3 TRAM interacts with IRF3 and CBP and signals via IKKe and TBK1. (a) IRF3-GFP-expressing HEK293 cells were plated on 35 mm glass-bottom sterile tissue culture dishes and transiently transfected with 1 mg of Flag-tagged TRAM, TRIF or empty vector and visualized 24 h later by confocal microscopy. (b) 293T cells were transfected with 4 μg of Flag-TRAM with or without a plasmid encoding IRF3 (untagged) as indicated. Twenty-four hours later, whole cell lysates were immunoprecipitated with anti-IRF3, anti-Flag or anti-CBP and the immunoprecipitated complexes immunoblotted for Flag-tagged TRAM and IRF3. Whole cell lysates (WCL) were also analyzed for Flag-tagged proteins. (c) HEK293 cells were transfected with the RANTES luciferase reporter gene and TRAM (20 ng) and cotransfected with increasing concentrations of IKKe-k38a, TBK1-k38a or IRF3-DN from 10, 20, 30, 40, 60, 80 ng. Luciferase reporter gene activity was measured 24h after transfection.
Figure 3.3

a. IRF-3-GFP α-Flag-Cy3 overlay overlay/nuclear

TRAM

TRIF

pCDNA3

b. TRAM-Flag IRF3
IP: anti-IRF3 WB: anti-Flag
IP: anti-Flag WB: anti-IRF3
IP: anti-CBP WB: anti-Flag
WCL: anti-Flag

IRF3 (transfected)
IRF3 (endogenous)

IRF3 (endogenous)

TRAM

TRAM

TRAM

TRAM

TRAM

20
15
10
5
0

Relative stimulation

RANTES

pCDNA3.1 TRAM + IRF3 ΔN + IKKe k38a + TBK1 k38a (ng) (ng) (ng)
Figure 3.4 TRAM mediates the TLR4 pathway to IRF3 and IRF7. (a) HEK293 cells were transfected with 40 ng of a RANTES reporter construct and cotransfected with TRAM, TRAM-TIR, TRAM-C117H or TRAM-P116H. (b, c) TLR4/MD2- and TLR3-expressing HEK293 cell lines were transfected with a luciferase reporter gene containing the Gal4 upstream activation sequence and cotransfected with Gal4 DBD, Gal4-IRF3, or Gal4-IRF7 or (d) the RANTES luciferase reporter gene as well as TRAM-C117H or TRIFΔNΔC as indicated. On the following day, cells were stimulated with LPS (10 ng/ml), dsRNA (50 μg/ml poly I:C) or left untreated for ~8 h and luciferase reporter gene activity measured.
Figure 3.4

a. 

![Graph showing relative stimulation with TRAM constructs.]

b. 

**HEK293-TLR3**

- **Medium**
- **poly IC**
- **RANTES**

![Bar graph showing relative stimulation with TRAM C117H (ng).]

**HEK293-TLR4/MD2**

- **Medium**
- **LPS**
- **RANTES**

![Bar graph showing relative stimulation with TRAM C117H (ng).]

c. 

- **pCDNA**
- **TRAM-C117H**

![Bar graph showing relative stimulation with poly IC, Gal4-IRF3, and Gal4-IRF7.]

d. 

- **RANTES**

![Bar graph showing relative stimulation with TRIF-ΔNAC, TRAM-C117H, Poly IC, and LPS.]

- **Medium**
- **Poly IC**
- **LPS**

![Bar graph showing relative stimulation with TRIF-ΔNAC, TRAM-C117H, and LPS.]

Figure 3.5 TRAM activates NF-κB and is specific to the TLR4 pathway. (a) HEK293 cells were transfected with 40 ng of an NF-κB reporter construct and cotransfected with TRAM, TRAM-TIR, TRAM-C117H and TRAM-P116H. (b) TLR-expressing HEK293 stable cell lines were transfected with 40 ng of an NF-κB reporter gene and co-transfected with increasing concentrations of TRAM-C117H. One day after transfection, TLR-expressing cells were stimulated with Malp-2 (2 nM), dsRNA (100 μg/ml poly I:C), LPS (10 ng/ml), R-848 (10 mM), IL1β (10 ng/ml), TNF-α (10 ng/ml) or left untreated for 8 h and luciferase reporter gene activity was measured.
Figure 3.5

a.

b.

HEK293- TLR2

HEK293- TLR3

HEK293+ TLR8

HEK293- TLR4/MD2

HEK293-pCDNA3

HEK293-pCDNA3
Figure 3.6 TRAM signaling requires the expression of TRIF. (a) HEK293 cells were transfected with the RANTES luciferase reporter gene, TRAM or TRIF (40 ng) and TRIFΔNΔC or TRAM-C117H. Luciferase reporter gene activity was measured 24 h later. (b) HEK293T cells were transfected with 4 μg of TRAM-CFP or TRIF-CFP and co-transfected with Flag-Mal, Flag-Mal-P125H or Flag-TRIF. Whole cell lysates were harvested 48h later, and immunoprecipitated with anti-GFP antibody (which also immunoprecipitates cyan fluorescent protein; CFP or yellow fluorescent protein; YFP). Immunoprecipitated complexes were resolved by SDS-PAGE and immunoblotted for Flag-tagged adapters. Whole cell lysates (WCL) were also analyzed for CFP- and Flag-tagged proteins by immunoblotting. (c) Stable TLR4YFP or TLR3YFP-expressing HELA cells were transfected with 4 μg of plasmid encoding Flag-Mal, Flag-TRAM or Flag TRAM-C117H. Forty-eight hours later, whole cell lysates were immunoprecipitated with anti-GFP antibody and immunoprecipitated complexes immunoblotted for Flag-tagged adapters. Western blotting of lysates demonstrates expression of stable TLRs and transfected adapter proteins.
Figure 3.6

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c. 

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| Mal-Flag     |
| TRAM-Flag    |
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| WB: anti-Flag|
| WCL: Anti-Flag|
| WCL: Anti-GFP|
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Figure 3.7 TRAM and TRIF are required for RANTES activation by LPS. (a) 293T cells plated in 24-well plates were transfected with 1μg of plasmids encoding TRAM-CFP, TRIF-CFP or Mal-CFP and cotransfected with 50 nM siRNA-TRAM or Lamin A/C as indicated. 24h later, CFP fluorescence was measured by flow cytometry using a 405 nm laser for excitation of CFP. The siRNA-TRAM nearly completely abolished expression of the subpopulation of cells that express CFP. (b) U373/CD14 or TLR3-expressing HEK293 cells were transfected with a RANTES reporter gene and co-transfected with siRNA duplexes as indicated for 36 h. Cells were then stimulated for 8 h with LPS or dsRNA and luciferase reporter gene activity was measured.
Figure 3.7

a. 

b. 

- No siRNA
- siRNA Lamin A/C
- siRNA TRAM
CHAPTER IV:

The Myristoylation of TRIF-Related Adapter Molecule is Essential for Toll-Like Receptor 4 Signal Transduction
Abstract

TRAM is the fourth TIR domain-containing adapter to be described that participates in TLR signaling. TRAM functions exclusively in the TLR4 pathway. Here we show by confocal microscopy that TRAM is localized to the plasma membrane and the Golgi apparatus, where it colocalizes with TLR4. Membrane localization of TRAM is the result of myristoylation since mutation of a predicted myristoylation site in TRAM (TRAM-G2A), resulted in the dissociation of TRAM from the membrane and its relocation to the cytosol. Further, TRAM, but not TRAM-G2A, was radiolabeled with $^3$H-myristate in vivo. Unlike wild type TRAM, overexpression of TRAM-G2A failed to elicit either IRF3 or NF-KB signaling. Moreover, TRAM-G2A was unable to reconstitute LPS responses in bone marrow-derived macrophages from TRAM-deficient mice. These observations provide clear evidence that the myristoylation of TRAM targets it to the plasma membrane where it is essential for LPS responses through the TLR4 signal transduction pathway, and suggests a novel manner in which LPS responses can be regulated.
Introduction

TLRs recognize microbial products derived from all the major classes of pathogens and initiate a complex immune response designed to eliminate invading pathogens. A key structural motif involved in the signal transduction of TLRs is the TIR domain (reviewed in (119)). TIR domains can be found in the cytoplasmic portions of all TLRs and the IL-1 receptor family, as well as a third subgroup of TIR-domain containing adapter proteins. The initial events of TLR signal transduction are thought to involve the recruitment of pertinent adapter molecules, which in turn, provide a scaffold to enable the recruitment and activation of additional signaling molecules. To date, four adapter molecules have been associated with TLR signaling: MyD88 (15, 142); Mal/TIRAP(16, 17, 66); TRIF/TICAM-1(18, 19, 60, 67); TRAM/TICAM-2 (20, 22, 143) (all reviewed in (144)). Exactly how the adapter molecules initiate signaling once a TLR is triggered by its ligand is still unclear. This is particularly complicated in the case of TLR4, where all four adapters are required for a complete LPS response.

LPS is the major constituent of the outer membrane of Gram negative bacteria (145, 146). The recognition of LPS by host phagocytes stimulates the release of inflammatory mediators and cytokines from a variety of target cells. In conjunction with CD14 and its co-receptor, MD-2, TLR4 binds LPS and serves to elicit a “danger” signal, thus initiating the host immune response (for a complete review see (147). The cytoplasmic face of TLR4 is quite unique amongst TLRs, as it utilizes all four TIR domain-containing adapter molecules: MyD88, Mal, TRIF and TRAM (148). In recent years, considerable progress has been made delineating the requirement for each of these adapter molecules in the TLR4-activated immune response. The recruitment of MyD88 to
proximal TIR domains of activated TLRs allows for the interaction and activation of the IRAK-family members, IRAK1 and IRAK4 (87, 120), and the subsequent activation of TRAF-6 (121), a RING finger domain-containing protein with ubiquitin ligase activity. TAK1 kinase, in turn, activates the IKK complex (149, 150), which phosphorylates IκBα. This phosphorylation marks IκB for ubiquitination and degradation by the proteosome. NF-κB is then released from IκBα, translocates to the nucleus, and regulates NF-κB-dependent target genes including inflammatory cytokines. TLR4 also activates the MyD88-independent responses that lead to the activation of IRF3, the induction of IFN-β, and IFN-inducible genes, and the upregulation of co-stimulatory molecules (62, 122, 148, 151). Mal/TIRAP appears to also regulate inflammatory cytokine genes suggesting that Mal may co-operate with MyD88 to control these responses (16, 17, 65, 66).

Using an siRNA approach or gene targeting, several reports have suggested that TRAM is uniquely required in the TLR4 signal transduction pathway and, together with, TRIF coordinates the activation of IRF3 and the MyD88-independent responses outlined above (20, 67, 152, 153). The TRAM-TRIF module also controls MyD88-dependent inflammatory responses suggesting that TRAM is a master regulator of both arms of the TLR4 signaling pathway. Our earlier studies using overexpression systems (Chapter III) suggested that TRAM functioned upstream of TRIF in TLR4 signaling (20). This conclusion was supported by the observation that TRAM binds TRIF directly and recruits it to TLR4 (22), as well as the observation that TRIF dominant negative constructs eliminated the direct activation of the MyD88-independent pathway by TRAM, but not
vice versa (22). In the case of TLR3 signaling, where TRAM is not required, TRIF binds directly to the TLR3 TIR domain (19).

To characterize further the unique role of TRAM in the initiation of TLR4 signaling, we analyzed the subcellular localization of TRAM and the consequences of TRAM localization for efficient signal transduction. Our study shows that TRAM is localized to the plasma membrane and Golgi apparatus via N-terminal myristoylation, where it co-localizes with TLR4. In fact, TRAM contains a putative N-terminal myristoylation site, similar to that found in mammalian Src kinases (154). Mutation of this predicted myristoylation site (TRAM-G2A) leads to a redistribution of TRAM from the plasma membrane to the cytosol. TRAM-G2A did not signal upon overexpression and was unable to reconstitute LPS responses in macrophages deficient for wild-type TRAM. These results indicate that myristoylation and plasma membrane localization of TRAM are critical for responses to LPS and indicate the potential for a hitherto unappreciated mechanism of regulation of LPS responses.
Materials and Methods

Reagents. Unless otherwise stated, all reagents were purchased from Sigma Chemicals (St. Louis, MO). E. coli 0111:B4 LPS was subjected to a second phenol extraction in order to remove contaminating lipopeptides (49). Lipopeptides were purchased from EMC Microcollections (Tuebingen, Germany).

Plasmids. Most of the constructs described in this paper have been described elsewhere. These include: pCDNA3-TRAM\textsuperscript{CFP}, pEF-BOS-TRAM\textsuperscript{Flag}, pEF-BOS-TRAM-C117H\textsuperscript{Flag}, pEF-BOS-TRIF\textsuperscript{Flag} (20); pCDNA3-MyD88\textsuperscript{CFP} (155); Myr\textsuperscript{YFP} (156); β-galactosyltransferase\textsuperscript{YFP} (Golgi\textsuperscript{YFP}) (57); NF-κB-luciferase, RANTES-luciferase, ISRE-luciferase and Renilla-luciferase (20); the retroviral vector pMSCV2.2-IRES-GFP (157). pCDNA3-TRAM-G2A and pEF-BOS-TRAM-G2A were generated by site directed mutagenesis using a kit (Stratagene, CA). The plasmid pCDNA3-Fyn was subcloned by PCR using cDNA obtained from E. Kurt-Jones (UMASS, MA); pCDNA3-Fyn-G2A was generated by site directed mutagenesis. pMSCV2.2-IRES-GFP-murine TRAM, murine TRAM-G2A and murine TRAM-C117H were subcloned from pEF-BOS vectors expressing murine constructs. pCDNA3-T7-MyD88\textsuperscript{CFP} was generated by PCR. The packaging vector for the retroviruses was the proviral clone of Moloney Murine Sarcoma Virus \textsuperscript{Ψ}Ecu from O. Witte (UCLA, CA).

Cell lines. HEK293 cells stably expressing TLR2\textsuperscript{YFP}, TLR4\textsuperscript{YFP} and TLR9\textsuperscript{YFP} were as described (57, 155).
Confocal Microscopy. HEK293 cells stably expressing TLR4\textsuperscript{YFP} and TLR9\textsuperscript{YFP} or Myr\textsuperscript{YFP} were transfected with TRAM\textsuperscript{CFP}, TRAM-G2A\textsuperscript{CFP}, MyD88\textsuperscript{CFP}, T7-MyD88\textsuperscript{CFP} where indicated. Twenty-four hours after transfection, cells were imaged by confocal microscopy using a Leica TCS SP2 AOBS microscope.

Subcellular fractionation. HEK293 cells stably expressing TLR4\textsuperscript{YFP} and TRAM\textsuperscript{CFP}, TRAM-G2A\textsuperscript{CFP} or MyD88\textsuperscript{CFP} were seeded in 10 cm dishes at a density of 1 x 10^6 cells per dish. Twenty-four hours later the media was removed, the cells were washed in PBS and then scraped into fractionation buffer (20 mM Tris-HCl pH 7.5, 10 mM MgCl$_2$, 1 mM EDTA, 250 µM sucrose, 200 µM PMSF). The samples were subjected to 20 strokes of a dounce homogenizer (Wheaton, USA) and spun at 100,000xg for 1 h. The supernatant (cytosolic fraction) was removed to a fresh tube and the pellet (membrane fraction) was resuspended in 50 µl SDS sample buffer (50 mM Tris-Cl, pH 6.8, 10 % glycerol (v/v), 2 % SDS (w/v), 0.1 % bromophenol blue (w/v) and 5 % β-mercaptoethanol). The fractions were run on a 10 % SDS-PAGE gel, transferred onto nitrocellulose and blotted with the appropriate antibody.

Luciferase reporter assay. Cells were seeded into 96-well plates at a density of 40,000 cells per well and transfected 24 h later with 80 ng of the indicated luciferase reporter genes and the indicated amounts of the TRAM constructs, MyD88 or TRIF, using 0.8 µl of GeneJuice (EMD Biosciences, CA) per well. The thymidine kinase Renilla-luciferase reporter was also cotransfected (40 ng) in order that the data could be normalized for transfection efficiency. Cells were either left untreated or treated with LPS (1µg/ml) or
Pam3Csk4 (2μg/ml) as indicated for 6 hours. Cell lysates were then prepared, and reporter gene activity was measured using the Dual Luciferase Assay System (Promega). Data are expressed as the mean relative stimulation ± SD.

**Radiolabeling.** HEK293T cells were transfected using GeneJuice with the CFP-tagged constructs as indicated. Eighteen hours post-transfection, cells were incubated in media containing 250 μCi of [3H] myristate for 4 hours. Cells were then lysed in .5 ml of lysis buffer (20mM TRIS-HCL, 2 mM EDTA, 137 mM NaCl, 0.5% Triton X-100, glycerol 10%, with protease inhibitors). Polyclonal anti-GFP (Molecular Probes, OR) was incubated with the cell lysates in protein-A sepharose for 2 hours. The immune complexes were precipitated and subjected to 4-15% SDS-PAGE. The gel was then dried and developed using Amplify (Amersham, UK) according to the manufacturers instructions.

**Reconstitution of bone marrow-derived macrophages.** The retroviral vector pMSCV expressing TRAM, TRAM-G2A, TRAM-C117H and GFP from the IRES element were used to generate high-titer helper-free retroviral stocks (using the packaging plasmid PECu) by transient co transfection of 293T cells (157). Twenty-four to seventy-two hours later, cell supernatants were harvested, filtered and used to transduce target cells. BM-derived macrophages were cultured from C57Bl6 mice or age- and sex-matched TRAM-/- mice for 8-10 days. Conditioned supernatant from L929 cells comprised 20% of the total volume as a source of M-CSF. Cells were transduced with virus encoding the indicated versions of TRAM or empty vector on day two, while cells were actively dividing. On
day ten, cells were sorted for GFP and seeded into 96-well plates at a density of 35,000 cells per well. Cells were stimulated with LPS overnight after being allowed to recover from cell sorting for 24h. Cell culture supernatants were analyzed for the presence of IL-6 and RANTES by ELISA (R&D Systems, Minneapolis, MN).
Results

Subcellular localization of TRAM

To further understand the role of TRAM in TLR4 signaling, we examined its subcellular localization. Using a C-terminal CFP-tagged version of TRAM (TRAM\textsuperscript{CFP}), we discovered that in resting cells, TRAM resided at the plasma membrane and also in the Golgi apparatus of HEK293 cells. Similar localization patterns were observed in TLR4\textsuperscript{YFP}-expressing stable cell lines. TLR4 was expressed both at the cell surface as well as localized to the Golgi apparatus, consistent with previous reports (57). In fact, TRAM and TLR4 appeared to colocalize on the plasma membrane (Fig. 4.1a. right panel, overlay). There was no co-localization of TRAM with TLR9 (Fig. 4.1a), which resides in the ER (155). In addition to the plasma membrane, TRAM appeared to localize in the Golgi apparatus as seen in Figure 4.1a (bottom). Cell fractionation studies in TRAM-expressing HEK293 cells revealed that TRAM was exclusively found in the membrane fraction in sharp contrast to the localization of MyD88, which resided in the cytosolic fraction (Fig. 4.1b).

TRAM contains a putative myristoylation site

This localization pattern of TRAM in living cells indicated that TRAM was either anchored to the membrane via direct interaction with a membrane-associated molecule (such as TLR4), by fatty acid modification, or both. To examine the possibility that TRAM was anchored by acylation, we subjected the TRAM sequence to N-myristoyltransferase (NMT) program analysis (http://mendel.imp.univie.ac.at/myristate/SUPLpredictor.html), which revealed that
TRAM contained a putative myristoylation consensus sequence consisting of Met-GlyX-X-X-Ser and Lys (Fig. 4.2a). Similar myristoylation sequences have previously been identified in members of the Src kinase family.

To investigate if TRAM was indeed a myristoylated protein, we took a number of complementary approaches. We generated a mutant form of TRAM in which the glycine at position 2 was mutated to alanine and examined its localization by confocal microscopy. As a comparison, we compared the localization of Myr\textsuperscript{YFP}, a synthetic construct containing an acylation sequence derived from the mammalian Src kinase, Lyn (156). Myr\textsuperscript{YFP} was clearly membrane localized. In fact, Myr\textsuperscript{YFP} and TRAM\textsuperscript{CFP} co-localized as indicated by the yellow overlay of the confocal image (Fig. 4.2b). Mutation of glycine at position 2 to alanine (TRAM-G2A) resulted in the re-localization of TRAM from the cell membrane to the cytosol (Fig. 4.2b). This was confirmed by sub-cellular fractionation, with TRAM-G2A only occurring in the cytosolic fraction (data not shown).

As a second approach, we compared the cellular localization of MyD88, a TLR adapter used by all TLRs except TLR3, which localized to the cytosol (Fig. 4.2b). In contrast to TRAM, MyD88\textsuperscript{CFP} did not co-localize with Myr\textsuperscript{YFP}. Next, we performed a "gain of function" experiment and generated a mutant form of MyD88, whereby the first 7 amino acids of MyD88 were replaced with those from TRAM (T7-MyD88). This resulted in a construct that, in theory, encoded a myristoylated form of MyD88. T7-MyD88 was also capable of localizing to the plasma membrane (Fig. 4.2c). Together, these results suggested that the putative N-terminal myristoylation site was sufficient for targeting TRAM to the plasma membrane.
To determine further if TRAM was a myristoylated protein, we monitored TRAM myristoylation in vivo. HEK293T cells were transfected with wild type or G2A mutant versions of TRAM, Mal, MyD88 or T7-MyD88. Cells were metabolically labeled with [³⁵S] myristate. Fyn, a Src kinase family member involved in T cell receptor (TCR) signal transduction, was used as a positive control for myristoylation. Likewise, Fyn G2A served as a negative control for the incorporation of [³⁵S] myristate. The indicated proteins were transfected into HEK293T cells, immunoprecipitated with anti-GFP, run on an SDS-PAGE gel, and examined for incorporation of [³⁵S] myristate by autoradiography (Fig. 4.3). TRAM, but not TRAM-G2A, was clearly myristoylated in vivo. Furthermore, neither Mal nor MyD88 were labeled with [³⁵S] myristate. In contrast, T7-MyD88 efficiently incorporated [³⁵S] myristic acid. These results provided clear evidence that TRAM was indeed a myristoylated protein. Despite the fact that TRAM and Mal have similar molecular weights, 232 and 235 amino acids, respectively, these two adapters resolved differently on SDS-PAGE (Fig 4.3, lower panel), possibly due to different post-translational modifications.

**TRAM-G2A does not signal upon overexpression**

As previously reported, transient expression of TRAM induced both NF-κB and IRF3 dependent reporter activation in HEK293 cells (20). Furthermore, mutation of the cysteine residue in the predicted TIR domain BB loop of TRAM, at position 117 (TRAM-C117H), a mutation equivalent to the C3H/HeJ mutation in the TLR4 TIR domain BB loop, abolished the ability of TRAM to signal (20). To determine if TRAM-G2A was compromised in its ability to signal upon overexpression, we monitored the
ability of TRAM-G2A to induce NF-κB or IRF3-dependent reporter gene activation in HEK293 cells. Wild-type TRAM, but neither the C117H nor the G2A mutant could drive NF-κB or RANTES reporters (Fig. 4.4a and b). Moreover, TRAM-G2A modestly inhibited LPS-induced IRF3 reporter gene induction, while wild-type TRAM potentiated this response (Fig 4.4c). Similar results were obtained when LPS-induced NF-κB activation was examined in cells transfected with TRAM, or the TRAM mutants (Fig 4.4d). As anticipated, TRAM-G2A had no effect on lipopeptide-induced NF-κB activation in TLR2 expressing HEK293 cells (Fig. 4.4e). We also examined the ability of TRAM-G2A to interfere with signaling by other TIR adapters. For example, transient expression of MyD88 induced NF-κB reporter gene expression, as was also true in the case of TRAM and TRIF, albeit to a lesser extent. TRAM-G2A had no effect on either MyD88- or TRIF-induced NF-κB activation, but inhibited the activation of NF-κB induced by overexpressing wild-type TRAM (Fig. 4.4f). These results suggested that TRAM-G2A cannot signal efficiently and, in fact, can act as a dominant negative interfering mutant. Together, these results suggested that the membrane localization of TRAM was critical for its ability to initiate efficient signaling.

Myristoylation of TRAM is critical for LPS responses in macrophages

To investigate if membrane localization of TRAM was critical for LPS responses in vivo, we reconstituted bone marrow-derived macrophages from TRAM-deficient mice. To this end, we generated retroviruses expressing either wild type TRAM, TRAM-G2A, or TRAM-C117H in tandem with an internal ribosomal entry site (IRES)-encoded GFP. This construct was designed to allow for the expression of a bicistronic mRNA that
would give rise to the translation of TRAM at a 1:1 molar ratio with GFP. Transduced bone marrow-derived macrophages from TRAM-deficient mice were sorted by flow cytometry for GFP fluorescence. As seen in Figure 4.5a, the level of GFP fluorescence, and hence TRAM reconstitution, was comparable in the cells transduced with either the wild-type TRAM, the TRAM-G2A, or the TRAM-C117H retroviruses. We were unable to detect TRAM or the mutants in transduced and GFP-sorted cells with Western blotting, presumably because of the relatively low-level expression of TRAM from the construct in these cells (data not shown). However, when we compared the expression level of the three proteins in transfected HEK293 cells, we found no significant difference in expression levels. We therefore believe that the levels of the wild type TRAM and the mutants were similar.

Wild-type or TRAM knockout macrophages transduced with an IRES vector that encoded only GFP were also sorted for productive infection (data not shown). The CC-chemokine RANTES is a downstream target gene of the MyD88-independent pathway following LPS stimulation (20). IL-6 production in response to LPS was reported to be severely abrogated in TRAM-deficient macrophages (143). Therefore, we examined IL-6 and RANTES production following LPS stimulation of wild-type and TRAM-deficient macrophages that had been transduced with either the control vector-expressing retrovirus or the TRAM-expressing retroviruses. As seen in Figure 4.5b, GFP-positive, wild-type, empty vector-expressing macrophages produced IL-6 and RANTES following LPS stimulation, while GFP-positive, TRAM-deficient, empty vector-expressing macrophages failed to induce either IL-6 or RANTES under identical experimental conditions. Remarkably, TRAM-deficient macrophages transduced with a virus encoding
the wild-type TRAM completely restored responsiveness to LPS challenge. As predicted, TRAM-C117H was unable to restore LPS responses, consistent with our earlier in vitro studies. Importantly, TRAM-G2A was also incapable of restoring LPS responses (Fig. 4.5b). Cells transduced with the TRAM-G2A mutant were unable to produce either RANTES or IL-6 in response to LPS challenge. These results provide clear evidence that the myristoylation and subsequent membrane localization of TRAM was critical for responses to endotoxin.
Discussion

A diverse range of viral and cellular proteins are known to be covalently modified by lipophilic moieties, including protein kinases, guanine nucleotide binding proteins, transmembrane receptors, and viral structural proteins (reviewed in (158)). The attachment of lipid groups to these molecules influences protein-protein interactions, membrane binding affinity and cellular signal transduction. Here we provide the first evidence that the TIR domain-containing adapter molecule, TRAM, is colocalized with TLR4 at the plasma membrane as the result of myristoylation and our findings implicate fatty acid modification as a critical event in TLR4 signaling.

Because TRAM does not contain a signal peptide sequence, its presence on the membrane could be the result of either protein:protein interactions, prenylation or fatty acid modification. TRAM does not contain a predicted prenyl modification site as it does not contain a CAAX box at or near its C-terminus. There are two distinct types of fatty acid modification: myristoyl and palmitoyl (154, 158, 159). In this study, we identified in TRAM a putative N-terminal myristoylation sequence. For myristoylation to occur, the initiating methionine is usually removed by methionine amino peptidase during translation and the glycine at position 2 becomes the N-terminal amino acid. The requirement for glycine at the N-terminus is absolute; no other amino acid can substitute. Such proteins are labeled with myristate on these N-terminal glycines in an irreversible, co-translational manner by N-myristoyl transferase. This type of covalent modification occurs in many signal transduction components and is central to their function (154). A well-characterized myristoylated protein is the Src kinase pp60v-src from Rous sarcoma virus. A myristoylation mutant of this Src kinase (G2A) does not bind to the membrane
and is incapable of mediating cellular transformation (154). Myristoylation is also required for membrane association and virion formation by Gag polyproteins of mammalian C- and D-type retroviruses. Non-myristoylated mutants of murine leukemia C virus Pr65gag and HIV-1 Pr55gag are predominantly cytosolic, and infectious viral particles are not produced (160).

Although myristoylation is clearly necessary for membrane binding of N-myristoylated proteins, myristoylation alone is not sufficient to confer stable membrane binding properties to proteins (161). It is thought that a second signal within the N-myristoylated protein is required for efficient membrane binding. The second signal is most often either palmitoylation or the presence of a polybasic cluster of amino acids. The latter is the mechanism used by the Src kinase pp60v-src for membrane anchoring (154, 159). A basic amino acid cluster in Src kinase, downstream from the glycine at position 2, forms electrostatic interactions with acidic phospholipid headgroups in the membrane. At present, it is unclear what the nature of the second signal that tethers TRAM to the membrane might be. TRAM has a number of lysine residues close to the myristoylation site, which may serve as a polybasic cluster. A combination of both myristate and basic motifs in TRAM would allow the hydrophobic and electrostatic forces to synergize and facilitate membrane binding (161). TRAM also has a number of cysteine residues, however, which could potentially become palmitoylated. A third possibility for membrane tethering might involve association with membrane anchored molecules, which in the case of TRAM could be TLR4. This latter explanation seems less likely, as when TRAM<sup>CFP</sup> was transfected into HEK293 cells, which ordinarily lack expression of TLR4, TRAM localization to the cell surface and Golgi were observed.
nevertheless (data not shown). Of course, other cell surface TLRs are theoretically capable of serving this function, but cells from TRAM-deficient animals appear to have normal responses to a variety of TLR ligands, including those for TLR2, TLR5, TLR7 and TLR9 (data not shown), suggesting that TRAM selectively binds to the cytoplasmic domain of TLR4.

Proper localization of signaling molecules to specific cellular membranes is critical for their function. It is tempting to speculate that clustering of myristoylated proteins into specialized membrane microdomains, such as lipid rafts or caveolae, enhances particular protein-protein interactions important for subsequent signal transduction. LPS has been reported to result in the redistribution of TLR4 into lipid rafts (162). Acylation of TRAM might aid in this redistribution. Similarly, in the case of TRAM, co-localization with TLR4 in lipid rafts may be critical for signaling.

A key question that remains to be answered is why TRAM must be constitutively surface localized at the plasma membrane in order to allow cells to signal through TLR4. One possible explanation is that membrane-localized TRAM must leave the membrane and dissociate from TLR4 in order to propagate signals downstream. Indeed, preliminary experiments suggest that TRAM leaves the membrane after LPS stimulation. In this scenario, the dissociation of TRAM from the membrane could be a regulated event (e.g. de-myristoylation, de-palmitoylation, or phosphorylation). N-myristoylation is usually a permanent modification. However, there is evidence in particular situations, e.g., brain synaptosomes, that a de-myristoylase activity modifies myristoylated MARCKS, promoting its release from the membrane (163). De-palmitoylation is a more common mechanism of protein translocation because the thio-ester linkage between palmitate and
the polypeptide is quite labile. Another possible mechanism involves protein phosphorylation. Electrostatic interactions between polybasic residues and phospholipids headgroups could be destabilized by phosphorylation events adjacent to polybasic clusters. Ligand induced phosphorylation of TRAM could potentially elicit its dissociation from the membrane. Recent evidence from O’Neill and colleagues suggest that TRAM is indeed phosphorylated by protein kinase C epsilon (PKCε) following LPS signaling. Myristoylated proteins are often phosphorylated by PKC near their N-termini, leading to their dissociation from the membrane (164-167). Phosphorylation of TRAM by PKCε is critical for LPS signaling (O’Neill personal communication), consistent with the possibility that TRAM phosphorylation could facilitate it to leave the membrane following LPS signaling.

In conclusion, the data described herein shows that TRAM is the only adapter in TLR signaling to be myristoylated, conferring upon it membrane localization that is a prerequisite for LPS signaling. Defining the functional significance of membrane-localized TRAM in LPS signaling remains to be completely elucidated, but the data strongly suggest that the acylation of TRAM is one means by which cells regulate their responses to bacterial endotoxin.
Figure 4.1 TRAM is associated with the cell membrane. a. Stable TLR4<sup>YFP</sup>-or TLR9<sup>YFP</sup>-expressing HEK293 cells were transiently transfected with TRAM<sup>CFP</sup>. Twenty-four hours after transfection, cells were visualized by confocal microscopy. b. Membrane fractionation was carried out on HEK293 cells stably expressing TLR4<sup>YFP</sup> and either TRAM<sup>CFP</sup> or MyD88<sup>CFP</sup>. The fractions were resolved on a 10% SDS-PAGE gel prior to transfer to nitrocellulose and immunoblotting.
Figure 4.1

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b. 

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<td>MyD88&lt;sup&gt;CFP&lt;/sup&gt;</td>
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Figure 4.2 TRAM contains a putative myristoylation sequence. a. Comparison of the consensus sequence for protein N-terminal myristoylation to amino acids 1-18 of TRAM. Blue characters indicate amino acids that match myristoylation consensus sequences. b, Myr$^{YFP}$ stable HEK293 cells were transiently transfected with TRAM$^{CFP}$ or TRAM-G2A$^{CFP}$ and visualized by confocal microscopy. c. Myr$^{YFP}$ stable HEK293 cells were transiently transfected with MyD88$^{CFP}$ or T7-MyD88$^{CFP}$ and visualized by confocal microscopy 24 hours later.
Figure 4.2

a. Consensus Sequence: Met-Gly-X-X-X-Ser/Thr and Lys/Arg

\[
\text{TRAM-} \quad \text{MGIGKSKINSCPILSSLWG}
\]

b. TRAM\textsuperscript{CFP}

\[
\begin{array}{ccc}
\text{Myr-YFP} & \text{Overlay} \\
\end{array}
\]

TRAM G2A\textsuperscript{CFP}

\[
\begin{array}{ccc}
\text{Myr-YFP} & \text{Overlay} \\
\end{array}
\]

c. MyD88\textsuperscript{CFP}

\[
\begin{array}{ccc}
\text{Myr-YFP} & \text{Overlay} \\
\end{array}
\]

T7-MyD88\textsuperscript{CFP}

\[
\begin{array}{ccc}
\text{Myr-YFP} & \text{Overlay} \\
\end{array}
\]
Figure 4.3 TRAM is myristoylated in vivo. HEK293T cells were transiently transfected with CFP-tagged versions of the indicated constructs. Eighteen hours after transfection, cells were incubated in media containing $^3$H myristic acid (250 μCi/well) for 4 hours. Cells were lysed, immunoprecipitated with anti-GFP polyclonal Ab, and immune complexes resolved by SDS-PAGE, followed by autoradiography. Whole cell lysates were immunoblotted with anti-GFP mAb.
Figure 4.3

$^{3}H$-Myristic Acid

Lysates anti-GFP

UT  Fyn  Fyn G2A  TRAM  TRAM G2A  Mal  MYD88  T7/MYD88

105kd  75kd  50kd

50kd
Figure 4.4 TRAM-G2A fails to activate the RANTES promoter and NF-kB. *a and b*, HEK293 cells were transiently transfected with the RANTES, NF-κB, or ISRE luciferase reporters as indicated. Flag-tagged versions of TRAM, TRAM-G2A, and TRAM-C117H or empty vector were cotransfected as indicated. *c, d and e*, TLR2- or TLR4/MD2-expressing HEK293 cells were transfected as indicated and 24 hours later were stimulated with LPS (2 μg/ml) or Pam3Csk4 (2 μg/ml) for 6 hours. *f*, HEK293 cells were transfected with the NF-κB-luciferase reporter and cotransfected with wild-type TRAM, TRIF, or MyD88 in the presence or absence of TRAM-G2A, as indicated. In all cases, the thymidine kinase Renilla-luciferase reporter was used to control for transfection efficiency. Twenty-four hours after transfection, cell lysates were generated and luciferase activity was assayed. All the data are representative of three independent experiments.
Figure 4.4

a.  
\[ \text{NFκB-Luciferase} \]
- WT TRAM
- TRAM G2A
- TRAM C117H

b.  
\[ \text{RANTES-Luciferase} \]
- WT TRAM
- TRAM G2A
- TRAM C117H

c.  
\[ \text{ISRE} \]
- unstimulated
- LPS 1\muM

d.  
\[ \text{NFκB} \]
- unstimulated
- LPS 1\muM

e.  
\[ \text{NFκB} \]
- unstimulated
- PanCys 2\muM

f.  
\[ \text{NFκB} \]
- TRAMG2A
- Myc88
- TRAM
Figure 4.5 TRAM myristoylation is critical for optimal LPS responses.  

*a* Bone marrow-derived macrophages from C57BL/6 or TRAM−/− mice were cultured in 20% conditioned supernatant from L929 cells as a source of M-CSF. On day 2, cells were transduced with viral supernatant derived from either empty vector, WT TRAM, TRAM-G2A, or TRAM-C117H viral constructs. On day 10, cells were sorted for GFP and seeded in 96-well plates at 40,000 cells/well.  

*a* Transduced and GFP-sorted cells were examined for GFP fluorescence by FACS analysis.  

*b* GFP-positive cells were stimulated with LPS as indicated, and cell supernatants were assayed for IL-6 and RANTES by ELISA.
Figure 4.5

a.

- \( \text{TRAM}^{+/+} \) with TRAM WT
- \( \text{TRAM}^{+/+} \) with TRAM G2A
- \( \text{TRAM}^{+/+} \) with TRAM C117H

b.

- \( \text{TRAM}^{-/-} \) with Empty Vector
- \( \text{TRAM}^{+/+} \) with Empty Vector
- \( \text{TRAM}^{+/+} \) with TRAM WT
- \( \text{TRAM}^{+/+} \) with TRAM G2A
- \( \text{TRAM}^{+/+} \) with TRAM C117H
CHAPTER V:

DISCUSSION
The results of my Ph.D. studies have provided insights into the molecular signal transduction pathways of the innate immune system. In particular, my work has focused on the regulation of the IRF3-dependent innate immune response. Chapter 2 focused on the identification of IKKe and TBK1 as key regulators of the IRF3 activation pathway. Importantly, these kinases serve as a point of convergence for different pathways activating on the same transcriptional regulator. TBK1 is common to both the anti-viral response and the LPS response. Chapters 3 and 4 focused on the characterization of a new molecular player in the IRF3 response, TRAM, which is a critical component of the LPS/TLR4 signaling cascade. Elucidating the roles of IKKe, TBK1, and TRAM in these signal transduction pathways may provide new targets for drug intervention. This could lead to new cures or treatments for both pathogen-borne illnesses and autoimmune-related diseases.

Viral infection leads to the induction of IFNα/β. Specifically, this has been shown for a variety of viral families, including both RNA and DNA viruses. Hosts have developed a variety of ways to detect viral infection. As discussed in the introduction, both TLRs and RNA helicases have a role in this detection. As a result, the subsequent activity of type I IFNs is crucial in controlling the spread of the infection (reviewed in (168)). In fact, viruses attempt to inhibit the induction of type I IFN in a number of ways, most of which are unknown (reviewed in (169)). For example, the NS3/4A protease of hepatitis C virus can cleave TRIF, resulting in a blockage of IFN production upon infection (170). Thus, understanding the signals and pathways that lead to the induction of type I IFN is critical to understanding viral pathogenesis. The TLR4 agonist LPS also
induces type I IFN. It seems plausible that bacteria have also evolved ways to combat the induction of the IFN response although, to date, no such mechanism has been reported.

The identification of the kinases responsible for phosphorylating IRF3 increases our understanding of the IFN response. The work illustrated in this dissertation suggests that TBK1 and IKKε are key kinases involved in the signaling pathways leading to the transcription of IFNβ. A key question that remains is the relative contribution of these two kinases in the immune response to different bacteria and viruses. Since the completion of these studies, Akira et al. and Cheng et al. have made an effort to analyzed the requirement of the kinases in innate immunity by studying cellular responses of either singly- or doubly-deficient mice (171, 172). It appears that TBK1 is the predominant kinase in TLR3- and 4-mediated IFN responses, while both kinases have roles in virus-mediated IFN responses, at least in embryonic fibroblasts from doubly-deficient mice (172). Due to the embryonic lethality of TBK1 deficient mice, the generation of IKKε/TBK1-deficient mice under a TNFα deficient background is needed to analyze the relative roles of these kinases in various cell types as well as in the whole organism.

TLRs 7, 8 and 9 also induce antiviral responses. However, these responses are primarily mediated through IFNα. Transcription of IFNα requires the activation of IRF7. Hiscott et al. reported that IKKε and TBK1 could phosphorylate both IRF3 and IRF7 (70). Do either IKKε and/or TBK1 play a role in IFNα induction through TLRs 7, 8, and 9? It appears that the answer to this question is no. Akira et al. showed that TLR9-induced IFNα production is not abrogated in cells from either IKKε- or TBK1-deficient mice (173).
The continued research of TLRs has revealed a complex network of signal transduction pathways. Some of these pathways are shared by all TLRs, while others are unique to just a few. Activation of TLRs is thought to occur through the dimerization and/or clustering of TLRs upon engagement with cognate ligand. This occurs through TIR-TIR interactions. The next step is the recruitment of TIR domain containing adapter molecules. TLR4 is unique among the TLRs in that it utilizes four TIR domain-containing adapter molecules: TRAM, TRIF, Mal, and MyD88. The signaling cascade of TLR4 has often been described as consisting of two distinct pathways and readouts based upon the involvement of MyD88. Historically, the MyD88-dependent pathway was ascribed the function of inducing pro-inflammatory cytokines such as TNF-α and IL-6. In contrast, the MyD88-independent pathway was attributed to the activation of IRF3 and induction of IFNβ. This simple dissection of the LPS/TLR4 signaling pathway is incorrect, especially concerning pro-inflammatory cytokine induction. The phenotype of Mal and MyD88 deficient macrophages is similar in terms of LPS signaling (59, 65). Both Mal and MyD88 deficient macrophages are deficient in pro-inflammatory cytokine induction but are still capable of IRF3 activation/IFNβ induction. Conversely, TRAM and TRIF deficient macrophages are defective in IRF3 activation/IFNβ induction upon LPS stimulation. An often-overlooked phenotype of TRAM and TRIF deficient mice is their inability to induce pro-inflammatory cytokines in response to LPS as well. Thus, there is a clear distinction between the two arms of the pathway concerning IRF3 activation/IFNβ induction but it appears that both arms are important for pro-inflammatory cytokine induction. Moreover, a recent study by Thomas et al. illustrates the importance of IFNβ for normal MyD88-dependent gene expression. Specifically,
IFNβ	extsuperscript{-} macrophages exhibit diminished basal levels of essentially all MyD88-dependent genes examined (submitted).

Another important observation made from these TIR domain-containing adapter knockout mice is the detection of two distinct phases of NF-κB activation upon LPS stimulation. Mal and MyD88-deficient macrophages display a delay in NF-κB activation (~10-20 minutes). A different phenomenon can be seen with both TRAM- and TRIF-deficient macrophages (60, 153). TRAM and TRIF knockout macrophages have a normal early phase NF-κB activation. However, they are deficient in activating NF-κB during later time points (~20-45 minutes). Thus, it appears that Mal and MyD88 coordinate the early phase NF-κB activation while TRAM and TRIF coordinate the late phase NF-κB activation. Either of these phases alone is enough to induce TNF-α mRNA message but is insufficient to produce detectable amounts of TNF-α protein (K.A.F personal communication). Thus, a sustained NF-κB (0-45 minutes) activation provided by both MyD88-dependent and -independent pathways seem to be required for the optimal production of TNF-α protein and other pro-inflammatory cytokines.

Recently, Baltimore et al. (174) described a possible mechanism for the observance of these two phases of NF-κB activation. In this model, the activation of IRF3 leads to the induction of small amounts TNF-α. Autocrine signaling through the TNF receptor then accounts for the late phase of NF-κB activation. These studies also indicated that the late phase NF-κB activation was dependent upon protein synthesis and independent of TRAF6. This model, if correct, would then predict that the induction of other pro-inflammatory cytokines such as IL-6 and IL-12 in response to LPS would be
entirely dependent upon TNF-α expression. This specific aspect of the model has yet to be tested.

Another interesting aspect of the LPS/TLR4 signal transduction pathway is the requirement of four TIR domain-containing adapters. Exactly how these adapters assemble onto LPS-induced ligated TLR4 molecules is unknown. Moreover, the nature of the stoichiometry of the activated TLR4 receptor is not presently known. Is the active receptor simply a homodimer or an aggregated complex of multiple TLR4 molecules? The latter is supported by scanning electron microscopy studies suggesting that a huge signalsome forms shortly after TLR4 stimulation in HEK/TLR4/MD-2 cells (36).

Co-immunoprecipitation assays can reveal associations of molecules in a complex. However these associations are often not indicative of direct binding. A superior approach is yeast two-hybrid. A positive readout from data collected from yeast two-hybrid relies upon the direction interaction between two proteins. Several groups have performed yeast two-hybrid studies involving the TIR domains of TLR3 and 4 in combination with the adapter proteins. These data are summarized in Table 5.1 (16, 19, 22). Results from these studies indicate that TRAM can interact with both the TIR domain of TLR4 and TRIF. In contrast, TRIF does not directly bind to the TIR domain of TLR4. Unfortunately, there is no published yeast two-hybrid data concerning interactions of either MyD88 or Mal with the TIR domain of TLR4. Confocal studies of fluorescently tagged adapters performed by our lab indicate that TRAM (Fig. 4.1) and Mal (see Fig. 5.1) localize to the membrane of HEK293 cells, while MyD88 (Fig. 4.2) and TRIF (data not shown) appear to be cytosolic. These data support a hypothesis where Mal serves to recruit MyD88 to the plasma membrane while TRAM serves to recruit TRIF to the
plasma membrane upon activation of TLR4 (16, 19, 175). Hence, TRAM and Mal may play the role of bridging adapters while MyD88 and TRIF may act as signaling adapters in TLR4 signal transduction.

Preliminary research was undertaken to determine the membrane binding mechanism of Mal. Using a confocal approach, the membrane binding activity could be attributed to the first 74 amino acids of Mal (N-term Mal). Analysis of this N-terminal region of Mal revealed two stretches of positively charged lysines and arginines (region A (12-20); region B (30-36)). Replacement of these charged amino acids with asparagine, either alone or in combination (AB- Mal), resulted in a Mal construct that no longer displayed membrane localization properties, as seen by confocal microscopy (Fig. 5.1). These two charged regions could interact with the negative charge of the inner leaflet of the plasma membrane to facilitate membrane binding of Mal.

During the preparation of this dissertation, Medzhitov et al. reported that Mal contains a phosphatidylinositol 4,5-biphosphate (PIP2) binding domain (176). The PIP2 binding domain was localized to the first 40 amino acids of Mal. Specifically, mutation of lysines 15, 16, 31 and 32 to alanines (Mal 4x) abolished membrane binding of Mal. Interestingly, these four lysines are present in the two regions defined in Figure 5.1. Much like the inability of TRAM G2A to reconstitute LPS responses in TRAM-deficient macrophages, Medzhitov et al. showed that Mal 4x was incapable of reconstituting LPS responsiveness in Mal-deficient macrophages. The authors hypothesized that the main function of Mal was to recruit MyD88 to PIP2-containing membranes. This was experimentally tested by reconstituting Mal-deficient macrophages with a version of MyD88 that was capable of binding PIP2 on the cell surface. Indeed, a MyD88 construct
with a phospholipase C PIP2 binding domain attached at the C terminus was capable of reconstituting LPS responses in both MyD88 and Mal-deficient macrophages. Taken together, these observations suggest that the main function of Mal is to recruit MyD88 to PIP2-containing membranes where it can interact with TLR4. It would be interesting to test whether an analogous situation occurs with the interaction of TRAM and TRIF. First, does TRAM localize to a particular micro-domain of the membrane? Moreover, can a membrane-localized version of TRIF reconstitute LPS responses in TRAM- or TRIF-deficient macrophages?

Again, given the yeast two-hybrid data in Table 5.1 (e.g., neither TRAM nor TRIF directly bind to either Mal or MyD88) and the subcellular localization data one can speculate that a single homodimer of TLR4 can accommodate a TRAM-TRIF interaction as well as a Mal-MyD88 interaction, as detailed in Figure 5.2. If this is the case, are these two pathways separate signaling events or are they linked somehow? To determine unequivocally the relationship of the adapters and TLR4, co-crystallization of the cytoplasmic portion of TLR4 with the adapter molecules is needed. Another less technical approach could be to perform overexpression/dominant negative experiments, similar to Figure 3.6. For example, we can ask whether a TRIF or TRAM dominant negative can block the activation of NF-κB by Mal or MyD88 overexpression. Results from these types of experiments may answer the question of whether the two pathways are linked or separated.

Over the last decade, lipid rafts have gained significant attention in the field of signal transduction (177). Upon ligand binding, cell-surface receptors and their associated intracellular signaling molecules are thought to enter lipids rafts thus creating distinct
receptor clusters, which provide stable focal points for cellular activation (177). The LPS receptor appears to follow this hypothesis. LPS treatment of HEK/TLR4/MD-2 cells results in the aggregation of TLR4 on the cell surface as seen by scanning electron microscopy (36). Furthermore, Triantafilou et al. (162) showed that TLR4 and MyD88 are recruited to lipid rafts in a LPS-dependent manner. In addition, pretreating cells with raft-disrupting drugs, such as nystatin, significantly inhibited LPS-induced TNF-α secretion (162). Thus, it appears LPS/TLR4 signal transduction involves lipid rafts. As previously indicated both TRAM and Mal localize to the plasma membrane through myristoylation and PIP2 binding sites, respectively. As discussed in Chapter IV, the hypothetical second signal that localizes TRAM to the membrane is not known. In addition to myristoylation, TRAM could become palmitoylated. This dual acylation of TRAM could result in its localization into lipid rafts and be a requirement for LPS/TLR4 signal transduction. Further examination of the membrane localization mechanism of TRAM will answer many questions related to LPS/TLR4 signal transduction (see below).

Other important questions remain. For instance, why does TRAM need to be localized to the plasma membrane and not simply have the ability to be recruited as is the case with MyD88? A clue to the answer of this question may be the apparent role of Protein kinase C epsilon (PKCε) in LPS/TLR4 signal transduction (178). Quiescent PKC isoforms normally reside in the cytosol of resting cells. Upon activation, as in the case of phorbol 12- myristate 13-acetate (PMA) stimulation, PKC translocates to the cellular membrane where it phosphorylates relevant substrates, some of which are myristoylated proteins. For example, Myristoylated Alanine-rich C Kinase Substrate (MARCKS) is a myristoylated protein that is phosphorylated by PKC isoforms (179, 180).
PKCε has been shown to play a key role in the immune-detection of the Gram negative bacterial product, LPS (178). However, the target for PKCε in LPS/TLR4 signaling is unknown. In collaboration with Luke O’Neill (Trinity College, Dublin, Ireland), we have found that TRAM is transiently phosphorylated by PKCε on Serine 6 and 16 in an LPS-dependent manner. Activation of IRF3 and induction of the chemokine RANTES, which are both TRAM-dependent, were attenuated in PKCε-deficient cells (data not shown). Furthermore, TRAMS16A, which eliminates the PKCε phosphorylation site, is inactive when overexpressed and is attenuated in its ability to reconstitute signaling in TRAM-deficient cells. Collectively, these observations suggest that TRAM is a target of PKCε in LPS/TLR4 signal transduction, thus identifying a key therapeutic target for ameliorating conditions of bacterial sepsis.

PKCδ mediated phosphorylation of MARCKS results in the translocation of MARCKS from the plasma membrane to the cytosol (181). This event occurs through the disruption of a region of positively charge amino acids by the negatively charged phosphate group. This process, called and “electro-static switch”, is crucial for the activity of MARCKS (181). No equivalent stretch of positively charged amino acids can be seen in the primary amino acid sequence of TRAM. However, upon three-dimensional folding, TRAM may contain an equivalently charged area. Phosphorylation of TRAM on serines 6 and 16 may result in a destabilization of TRAM in the membrane and result in its relocation into the cytosol. This scenario would dramatically alter the current views of LPS/TLR4 signal transduction. Instead of an adapter complex as illustrated in Figure 5.2, activated TRAM, and presumably TRIF, would dissociated from the active TLR4 receptor into the cytoplasm to either propagate or deactivate the signal.
In conclusion, continued research in the area of innate immunology holds the promise to identify many novel proteins and pathways that could someday be useful in the development of specific immuno-modulating therapies. Indeed, many pharmaceutical companies and academic laboratories are making progress in this area. Future therapies may be quickly realized in the decades to come with the advent of new technologies and the deciphering of the human genome. It is my hope that the research performed herein adds to the knowledge of our species.
Figure 5.1 Positively charged regions within the N-terminus of Mal facilitate membrane localization. a. The N-terminus of Mal (amino acids 1-74). Amino acids shown in green represent positively charged amino acids. Region A (12-20); Region B (30-36). b. Confocal analysis of various Mal<sup>CFP</sup> fusion constructs. Full-length Mal and the N-terminus of Mal (a.a 1-74) localize to the membrane in HEK293 cells. Replacement of the charged residues in both regions A and B both alone or in combination (AB- Mal<sup>CFP</sup>) with alanine results in a cytoplasmic localization of Mal.
Figure 5.1

a.

\[
\begin{align*}
\text{A} & : \quad \text{MASSTSLPAP GSRPKPLGK MADWFRQTTLL} \\
\text{B} & : \quad \text{KKPKKRPNSP ESTSSDASQP TSQDSFLPSS} \\
& \quad \text{LSSVTSFSLP PTHA}
\end{align*}
\]

b.

- Mal\textsuperscript{CFP}
- N-term Mal\textsuperscript{CFP}
- AB-Mal\textsuperscript{CFP}
Figure 5.2 The potential arrangement of the TIR adapters on an activated TLR4 homodimer. A hypothetical adapter complex consisting of two separate modules on an LPS activated homodimer: a TRAM-TRIF module binding to one side of the TLR4 homodimer interface; a Mal-MyD88 module binding to the second TLR4 homodimer interface.
Figure 5.2

TLR4

Mal

MyD88

TRAM

TRIF
Table 5.1

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Yeast two-hybrid interactions among adapter molecules and TLR4

(16, 19, 22). ND= No Data.
References


72. Lin, R., C. Heylbroeck, P.M. Pitha, and J. Hiscott. 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation,


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protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proc Natl Acad Sci USA* 102:2992-2997.


