THE ROLE OF SERINE-THREONINE KINASE TPL2 IN HOST DEFENSE AGAINST VIRAL INFECTIONS

by

TENEEMA KURIAKOSE

(Under the Direction of Wendy T. Watford)

ABSTRACT

Tumor progression locus 2 (Tpl2), also designated Cot or MAP3K8, is a serine-threonine kinase with critical functions in regulating innate and adaptive immune responses. Although Tpl2 is known to be important in the regulation of major immunomodulatory cytokines, including antiviral Type I and Type II interferons (IFNs), and in host protection against various bacterial and parasitic infections, remarkably little is known about how Tpl2 might contribute to host defense against viruses. In this study, Tpl2 activation during pathogen sensing as well as Tpl2 regulation of virus replication and antiviral immune responses were investigated. Even though Tpl2 is an integral component of pathogen sensing pathways, differential activation of Tpl2-ERK signaling was observed in response to model microbial ligands. While a subset of toll-like receptors (TLRs), including virus-sensing TLR7, directly activate the Tpl2-extracellular signal-regulated kinase (ERK) pathway, TLR3 and TLR9 activate ERK indirectly via autocrine

signaling by reactive oxygen species (ROS), which is generated in a Tpl2-dependent manner. Consistent with its activation during pathogen sensing, Tpl2 regulates Type I and Type III IFN production upon stimulation of TLR and RIG-I like helicases involved in virus sensing. However, only Type III IFN induction required Tpl2 during influenza virus infection *in vitro* and *in vivo*. In addition to the regulation of IFN production, Tpl2 is also important in transducing Type I IFN signals that promote the expression of antiviral genes to limit virus replication. Moreover, *Tpl2*$^{-/-}$ mice showed impaired expansion of virus-specific CD8^{+} T cells that facilitate viral clearance. Secretion of antigen-specific IFNγ was also decreased in *Tpl2*$^{-/-}$ cells. Experiments using *in vitro* cultured CD8^{+} T cells revealed a cell-intrinsic role for Tpl2 in promoting effector CD8^{+} T cell responses. Consistent with its critical role in facilitating both innate and adaptive antiviral responses, Tpl2 was necessary for controlling virus replication and restricting morbidity and mortality associated with influenza virus infection. Collectively, these studies establish Tpl2 as a host factor that promotes virus sensing and integrates antiviral innate and adaptive immune responses.

INDEX WORDS: MAP kinase cascades, Inflammation, Influenza virus, Interferons, Toll-like receptors, Virus sensing, Reactive oxygen species, CD8^{+} T cells

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With love and gratitude

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Virus infection activates the host immune system, which mounts an antiviral response aimed at controlling virus replication and eliminating the infectious virus. The immune system is broadly divided into innate and adaptive immune systems, and both these arms of immunity are important in host protection during viral infections. While the innate immune system recognizes pathogens early after infection via germline-encoded pattern recognition receptors (PRRs) (1), the adaptive immune system recognizes pathogen-associated antigens via a diverse repertoire of antigen receptors and mount virus-specific response at later stages of infection (2). Concerted action of both innate and adaptive immune system restricts virus replication and spread and also facilitates viral clearance. This review will focus on mechanisms of virus sensing and antiviral immune responses with particular emphasis on tumor progression locus 2 (Tpl2/MAP3K8), a serine-threonine kinase with critical functions in regulating both innate and adaptive immune responses (3).

PATTERN RECOGNITION RECEPTORS AND VIRUS SENSING

Viruses are obligate intracellular parasites, and viral invasion of cells initially activates the innate immune system. The innate immune response to virus infection is initiated by recognition of viral pathogen associated molecular patterns (PAMPs) by PRRs with broad specificities and expressed either on the cell surface or within specific intracellular compartments (1). Detection is directed against specific structures or motifs in viral genomes in order to distinguish viral nucleic

acids from self. Three major classes of PRRs involved in virus detection are Toll Like Receptors (TLRs), RIG-I Like Receptors (RLRs) and NOD Like Receptors (NLRs) (4). Even though cell surface proteins of some viruses are known to activate certain TLRs, innate immune sensing of viruses mainly relies on recognition of viral nucleic acids in endosomes or the cytoplasm (5,6). While virus sensing TLRs are primarily expressed by macrophages and dendritic cells (DCs) and recognize viral nucleic acids within the endosomal compartment, RLRs and NLRs are ubiquitously expressed and sense viral PAMPs within the cytoplasm of infected cells (2) (Figure 1.1).

Toll like receptors

Toll like receptors (TLR) are Type 1 transmembrane glycoproteins containing extracellular leucine rich repeats involved in pathogen recognition and a cytoplasmic toll-interleukin 1 receptor (TIR) domain for signal transduction. Thirteen TLRs named TLR1 to TLR13 have been identified in mice and humans. Upon ligand binding, TIR domain containing adaptors like Myeloid differentiation primary response gene 88 (MyD88), TIR domain containing adaptor-inducing interferon-β (TRIF), TIR domain containing adaptor protein (TIRAP) and TRIF–related adaptor molecule (TRAM) are recruited to the TIR domains of different TLR receptors (7). These adaptors facilitate downstream signaling via nuclear factor kappa B (NFκB), mitogen activated protein (MAP) kinases and interferon regulatory factor (IRF) transcription factors to induce secretion of antiviral interferons (IFNs) and proinflammatory cytokines.

Many pathogenic viruses are detected by various TLRs; viral PAMPs involved in recognition are surface proteins and nucleic acids of the viruses. Viral patterns recognized by PRRs include genomic DNA, single- and double-stranded genomic RNA as well as double-stranded RNA generated as a replication intermediate (8). TLR2 and TLR4 can detect surface proteins of some

viruses like respiratory syncytial virus, while endosomal TLRs (TLR3, 7 and 9) detect viral nucleic acids (9) (Figure 1.1). TLR3 detects double-stranded RNA (10), whereas single-stranded viral RNA is the ligand for TLR7 (11). TLR9 recognizes unmethylated CpG oligonucleotides present in viral genomic DNA (12). All TLRs except TLR3 recruit the adaptor protein MyD88 for downstream signaling; TLR3 signaling is MyD88–independent but relies instead upon the TRIF adaptor protein (7).

MyD88-dependent signaling: After recruitment by TLRs, MyD88 recruits interleukin-1 receptor associated kinase 4 (IRAK4) and IRAK1 through homotypic association between the death domains of both molecules. IRAK4 is autophosphorylated and activates IRAK1, which in turn, activates downstream molecules TNF receptor associated factor 6 (TRAF6) and Transforming growth factor β–activated kinase 1 (TAK1). These proteins activate the inhibitor of kappa B kinase (IKK) complex leading to ubiquitination and degradation of IκB, the inhibitor of NFκB. Active NFκB transcription factors translocate to the nucleus and induce transcription of target genes. The IRAK family of proteins can also activate the transcription factor activator protein 1 (AP-1) through MAP kinase signaling pathways. In plasmacytoid dendritic cells (pDCs), IRAK1 directly associates with and phosphorylates interferon regulatory factor 7 (IRF7) (13). Activated AP-1 and IRF-7 also translocate to the nucleus and initiate the transcription of various proinflammatory cytokines and Type 1 IFNs.

MyD88-independent signaling: MyD88-independent signaling is mediated through the adaptor protein TRIF downstream of TLR3 and TLR4 (14). TRIF signaling is initiated within endosomes where viral dsRNA is sensed by TLR3. TLR4 recognizes viral surface proteins, and two downstream signaling pathways are induced sequentially. MyD88-mediated signaling is initiated at the plasma membrane earlier than TRIF-mediated signaling from endosomes. After binding to

its ligand at cell surface, sorting adaptor TRAM controls endocytosis of TLR4 and associated pattern recognition receptor CD14 (15). Signaling from both TLR3 and TLR4 is similar downstream of the endosomal compartment, and both TLRs recruit the adaptor TRIF. TRIF recruits TRAF6 and receptor interacting protein 1 (RIP1) and activates TAK1. TAK1 facilitates NFκB activation, and thereafter downstream signaling from this complex is similar to that of MyD88-dependent pathway. TRIF also recruits TANK binding kinase-1 (TBK1) and IKKε via TNF receptor associated factor 3 (TRAF3), and these proteins phosphorylate interferon regulatory factor 3 (IRF3) (16). Activated IRF3 dimerizes and translocates into the nucleus to initiate Type I IFN transcription.

RIG-I like Receptors

The RIG-I like RNA helicases are cytoplasmic sensors of viral patterns, and they are essential for detection of a variety of RNA viruses (6). The three members of this family are retinoic acid inducible gene I (RIG-I), melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) (17). All three RLRs have an internal DExD/H-box RNA helicase domain. The helicase domain interacts with viral RNA, and the ATPase activity of this domain is necessary for downstream signaling. RIG-I and MDA5 have N terminal caspase recruitment domains (10) that recruit CARD-containing adaptors and link to downstream signaling pathways. LGP2 lacks the CARD domain, and its role in viral sensing is not well understood (18).

Like TLRs, RLRs can signal through IRF3 and IRF7 and induce transcription of antiviral IFNs. Activated RLRs interact with the downstream adaptor, IFNβ promoter stimulator-1 (IPS-1), also called mitochondrial antiviral signaling protein (MAVS), CARD adaptor inducing IFN-β (CARDIF), or virus-induced signaling adaptor (VISA) (19). This association with the adaptor is

mediated by the homotypic interaction of CARD domain of RIG-I/MDA5 with that of IPS-1. Activated IPS-1 associates with TRAF3 and Numb-Associated Kinase (20) associated protein 1, and this complex activates the IKK family kinases, TBK1 and IKKε. These kinases mediate the phosphorylation and activation of IRF3 and IRF7 to induce transcription of IFNs (18). IPS-1 can also mediate activation of NFκB and MAP kinase pathways through its association with Fas associated death domain (FADD) and the kinases RIP1 and TAK1.

Even though RIG-I and MDA5 share structural similarity and transduce signals via a common pathway, they are known to recognize distinct ligands. The uncapped 5' triphosphate of viral RNA is detected by RIG I (11), whereas MDA5 is involved in the recognition of viral double-stranded RNA in the cytoplasm (21,22) (Figure 1.1). They also play non-redundant roles in recognizing different RNA viruses. RIG-I recognizes different positive and negative strand viruses like Hepatitis C virus, respiratory syncytial virus, measles virus, vesicular stomatitis virus and influenza A virus; MDA5 senses Picornaviruses like encephalomyocarditis virus. Both RIG-I and MDA5 are involved in detection of reoviruses, West Nile virus and Dengue virus (17).

NOD-Like Receptors

NLRs are soluble proteins that survey the cytoplasm for the presence of PAMPs expressed by pathogens. The human genome consists of more than 23 NLR proteins. They are characterized by their tripartite domain structure with an N terminus pyrin domain (PYD), caspase recruitment domain and a nucleotide binding domain that mediates self oligomerization, and a series of leucine rich repeats (LRRs) in the C terminus that are involved in ligand binding (1). Nucleotide-binding oligomerization domain 1 (NOD1) and NOD2 are two NLR family members involved in intracellular sensing of peptidoglycan subcomponents (23). Ligand recognition by NOD2 initiates downstream signaling via the adapter protein, receptor interacting protein kinase 2

(RIP2), to activate NFκB and MAP kinase cascades. NOD2 also detects single-stranded viral RNA and facilitates IRF3 activation leading to IFN production (24). The significance of NOD2-RIP2 signaling in ameliorating immunopathology during influenza infection has been demonstrated (25,26).

Activation of some NLRs leads to their assembly into large multimeric protein complexes called inflammasomes (26). NLR family members, Nacht domain-, leucine-rich repeat-, and PYD-containing protein 1 (NLRP1), NLRP3, NLRP6, NLRP12 and NLRC4 have been found in association with different inflammasomes. Upon ligand binding the cytoplasmic NLRs oligomerize and recruits the adaptor apoptosis-associated speck-like protein containing a CARD (ASC). The CARD domain of activated ASC subsequently interacts with the CARD domain of pro-caspase 1, leading to its activation, which in turn facilitates cleavage of pro-IL-1β and -IL-18. The processing of pro IL-1β and pro IL-18 into mature, bioactive cytokines through regulation of caspase 1 activation is the best known effector mechanism of inflammasomes (26). Both IL-1β and IL-18 are important in protective immune responses against many viruses. Inflammasome-associated immune responses against viruses are mediated by the recognition of viral RNA in the cytoplasm (27). Multiple studies have demonstrated the critical role of inflammasomes in host protective antiviral immune responses (27-29).

INTERFERONS

Interferons (IFNs) are important mediators of antiviral resistance providing a major line of defense against viral infections. The IFN family is subdivided into Type I (IFNα/β), Type II (IFNγ) and Type III IFNs (IFNλs), and all three types are host protective during viral infection (30). Even though these different types of IFNs signal through distinct receptor complexes, all of them activate the JAK-STAT signaling pathway to induce similar biological responses (30). The

biological effects of IFNs are generated by cooperative signaling by diverse cascades that induce the expression of hundreds of genes collectively known as IFN-stimulated genes (ISGs). ISGs establish an antiviral state in infected tissues that inhibits cell-to-cell spread (31). Although all types of IFNs possess antiviral effects and contribute to the elimination of viral infections, only IFNα/β and IFNλ are directly induced in response to viral infection (32). IFNα/β and IFNλ are induced upon virus recognition by PRRs. In contrast, induction of IFNγ is regulated indirectly by Type I IFNs and cytokines secreted during the early innate response to pathogens (33).

ANTIVIRAL ADAPTIVE IMMUNE RESPONSES

Adaptive immunity helps in eliminating the pathogens that eludes innate defense mechanisms. Interferons and other innate cytokines and chemokines stimulate activation of antigen presenting cells (APCs) and facilitate initiation of adaptive immune responses (34). B and T lymphocytes are the effector cells of adaptive immunity, and both humoral and cell-mediated responses are important in eliminating infection and conferring long-term immunity (35). The adaptive immune response is initiated by antigen presentation on self-restricted major histocompatibility complex (MHC) molecules displayed on innate antigen presenting cells (APCs) (34). Antigen presentation on MHC II induces differentiation of $CD4^+$ T cells into T helper I (Th1), Th2 and Th17 cells with specialized functions, whereas MHC class I presentation activates $CD8^+$ cytotoxic T cells (CTLs) that are able to lyse infected cells (36). Effector T cells secrete various cytokines like IFNγ to further activate phagocytic cells. T helper cells also influence the humoral responses by facilitating affinity maturation and isotype switching in B cells leading to the secretion of neutralizing antibodies. The humoral response is directed mainly against the surface proteins of the viruses, whereas CTL responses are directed against immunodominant peptides (37). While the humoral response is usually subtype-specific, cell-mediated immunity can

provide some level of cross protection against viruses like influenza virus (37). The adaptive immune response not only controls acute and chronic viral infections, but also confers long-lived memory to combat re-infections (35).

TUMOR PROGRESSION LOCUS 2

Tumor progression locus 2 (Tpl2 or MAP3K8), also designated as Cancer Osaka Thyroid (*Cot*), is a serine-threonine kinase of the MAP kinase family. It was originally identified in a human thyroid cancer cell line in the early 1990s. Later the murine homolog of *Cot* was identified as a protooncogene, since provirus integration of Moloney murine leukemia virus in *Tpl2* locus induced T cell lymphomas in mice (3,38). *In vitro* over-expression experiments with cell lines as well as experiments with *Tpl2*$^{-/-}$ cells established that Tpl2 functions as a MAP kinase kinase kinase (MAP3K) (39). Initial characterization of Tpl2-deficient mice showed major defects in the induction of proinflammatory cytokines, particularly TNFα by APCs, that rendered *Tpl2*$^{-/-}$ mice resistant to endotoxin-induced septic shock (40). Tpl2 is now known to be activated by cytokine receptors, TLRs, antigen receptors and G protein-coupled receptors, and it regulates the production of a variety of immunomodulatory cytokines through the activation of MAP kinase pathways downstream of NFκB (3). Since Tpl2 regulates the production as well as cellular responses to important proinflammatory cytokines, it has been identified as a potential target for the development of anti-inflammatory drugs to treat chronic inflammatory diseases (41).

Tpl2 is constitutively associated with NFκB1 p105 in the steady state, and the interaction of Tpl2's C terminus with p105 is necessary for its stability. Direct interaction of the death domain of p105 with the kinase domain of Tpl2 prevents the MEK kinase activity of Tpl2 (42,43). Tpl2 stability is also regulated by A20-binding inhibitor of NFκB-2 (ABIN-2) that forms a ternary complex with Tpl2 and p105 (44). In addition to p105 and ABIN2, the p38 MAPK family

members p38γ and p38δ indirectly regulate Tpl2 stability by regulating steady state levels of ABIN-2 (45). Upon receptor engagement, the IkB kinase (IKK) complex is activated, and IKK-mediated phosphorylation and proteosomal processing of p105 releases active Tpl2 (Figure 1.2). Tpl2 signaling is also positively regulated by auto- and transphosphorylation. Among the two key residues, T290 is believed to be autophosphorylated, whereas Akt (46) and IκB kinase 2 directly phosphorylate S400 to activate Tpl2 (47-49).

Activated Tpl2 transduces signals to various downstream signaling pathways in a cell type- and stimulus-specific manner (50). In macrophages and DCs, Tpl2 is necessary for LPS mediated activation of MEK1/2 and extracellular signal-regulated kinase1/2 (ERK1/2), but it is dispensable for NFκB, p38 and JNK activation. Impaired ERK activation in *Tpl2*$^{-/-}$ macrophages resulted in abrogated TNFα production in response to LPS, however only a partial reduction in TNFα secretion was observed in DCs (51). Tpl2 signaling is also required for both ERK and JNK activation in response to TNFα and IL-1β in mouse embryonic fibroblasts (MEFs) (50). In addition, Tpl2 is necessary for ERK activation in B cells upon CD40 ligation (52).

Since Tpl2 is a regulator of important immune and inflammatory cytokines, multiple studies have investigated the role of Tpl2 in immune responses during inflammation and infection. As mentioned previously, Tpl2-deficient mice are resistant to endotoxin-induced shock due to impaired TNFα production (40). This is because Tpl2-mediated ERK signaling is necessary for post-transcriptional nucleocytoplasmic transport of TNFα mRNA (40). Moreover, Tpl2 signaling also regulates the cell surface expression and processing of pre-TNFα via the ERK-dependent phosphorylation of TNFα converting enzyme (TACE) (53). In a study using the TNF$^{\Delta ARE}$ mouse model, which constitutively over-expresses TNF due to the deletion of the 3' AU-rich posttranslational regulatory element, the development of Crohn's-like inflammatory bowel

disease was attenuated in the absence of Tpl2 (54). In this case, protection is attributed to defective TNF signaling in the absence of Tpl2. Therefore, Tpl2 is required for both TNF secretion and signaling through the TNF receptor. Consistent with these findings, another study demonstrated that genetic ablation or pharmacological inhibition of Tpl2 ameliorated intestinal inflammation using a dextran sulfate sodium (DSS)-induced model of colitis (55). In addition to TNF, Tpl2 regulates both the induction and signaling of IL-1β, another potent proinflammatory cytokine involved in many inflammatory disorders (50,51).

Tpl2 also shapes the adaptive immune response via regulation of T helper cell differentiation. Tpl2 is required for *in vivo* differentiation of T helper 1 (Th1) cells, and *Tpl2*$^{-/-}$ mice are defective in controlling *Toxoplasma gondii* infection due to impaired IFNγ production (56). Tpl2 regulates *in vitro* polarization and IFNγ production from CD4^{+} T cells in a cell intrinsic manner, and Tpl2 signaling is necessary for optimal expression of transcription factors T-bet and STAT4 that are necessary for Th1 polarization and IFNγ production. *Tpl2*$^{-/-}$ mice also show a Th2-biased response and increased lung inflammation in an ovalbumin-induced model of asthma (56). In contrast to the above studies, experiments using an independently generated *Tpl2*$^{-/-}$ mice strain showed an enhanced Th1 response following infection with *Leishmania major* (57). This Th1 skewing was reported to be due to increased IL-12 production in *Tpl2*$^{-/-}$ mice. These conflicting results could be due to differences in the knockout mouse strains or due to differences in host responses elicited against the two parasites used in these studies. In addition to Th1 and Th2 responses, Tpl2 might also play a role in Th17 differentiation, as the production of Th17 promoting cytokine IL-23 is impaired in Tpl2-deficient BMDMs (58). Several recent studies have demonstrated the significance of Tpl2 in Th17 cell differentiation and Th17 cell-mediated autoimmune diseases (20,59).

The role of Tpl2 in host defense against viruses is currently unclear. Previous studies have reported normal cytotoxic T cell responses against lymphocytic choriomeningitis virus (LCMV)-infected cells (40) and resistance to mouse cytomegalovirus infection (MCMV) in *Tpl2*$^{-/-}$ mice (60). Conflicting reports exist regarding the role of Tpl2 in antiviral Type I IFN production. One study demonstrated increased IFNβ production in Tpl2-deficient in bone marrow derived macrophages and myeloid DCs in response to TLR4 and TLR9 ligands, while decreased IFNα production was observed in response to TLR9 in pDCs (61). The negative regulation of IFNβ production was due to impaired Tpl2- and ERK-dependent induction of c-fos, a negative regulator of IFNβ. Tpl2 also positively regulates IL-10 production through a related mechanism, since c-fos is a positive regulator of IL-10 (61). However, the biochemical mechanism behind decreased IFNα production in pDCs is not yet known. In contrast to this, Xiao and colleagues showed impaired Type I IFN production in response to both TLR7 and TLR9 in *Sluggish* mice expressing a mutated Tpl2 gene (60). Another study aimed at identifying signaling components in virus sensing pathways implicated Tpl2 as a key regulator of both inflammatory and antiviral gene induction in response to model TLR ligands (62). A recent study also reported induction of Tpl2 in cell lines infected with influenza virus (63). They further demonstrated increased replication of vesicular stomatitis virus in Tpl2-deficient mouse embryonic fibroblasts (MEFs). Collectively, these reports suggest a key role for Tpl2 in antiviral host defense mechanisms, which warrant further studies.

CONCLUSION

Viruses utilize cellular machinery for their replication, and therefore host factors that regulate virus replication offer new avenues for therapeutic intervention. Targeting host factors involved in antiviral responses is an active area of research since these drugs can cross-protect

against diverse viruses and are less likely to develop resistance. Understanding cellular mechanisms that contribute to antiviral response and viral clearance is critical when designing immunotherapies for viral infections. Among various intracellular signaling cascades, MAP kinase cascades, in particular, are important in the regulation of antiviral cytokine production and are known to be important for the replication of many viruses (64,65). Although the MAP3K, Tpl2, has been identified as a critical regulator of antiviral IFNs and various other immunomodulatory cytokines (40,51,56,61), there is still limited and contradictory information about how Tpl2 contributes to host defense against viruses. Due to the conflicting literature and lack of definitive studies *in vivo*, we have systematically investigated the role of Tpl2 in host defense against viruses. In Chapter 2, in order to understand the significance of Tpl2 in virus sensing, we investigated Tpl2 activation and signaling downstream of PRRs involved in early innate sensing of pathogens. In Chapter 3, using various model ligands and a murine model of influenza virus infection, we investigated how Tpl2 regulates antiviral immune responses and viral clearance. In Chapter 4, Tpl2 regulation of effector $CD8^+$ T cell responses was investigated to determine how Tpl2 regulates antiviral adaptive immune responses. The overarching aim was to identify the contributions of Tpl2 in innate and adaptive antiviral immune responses.

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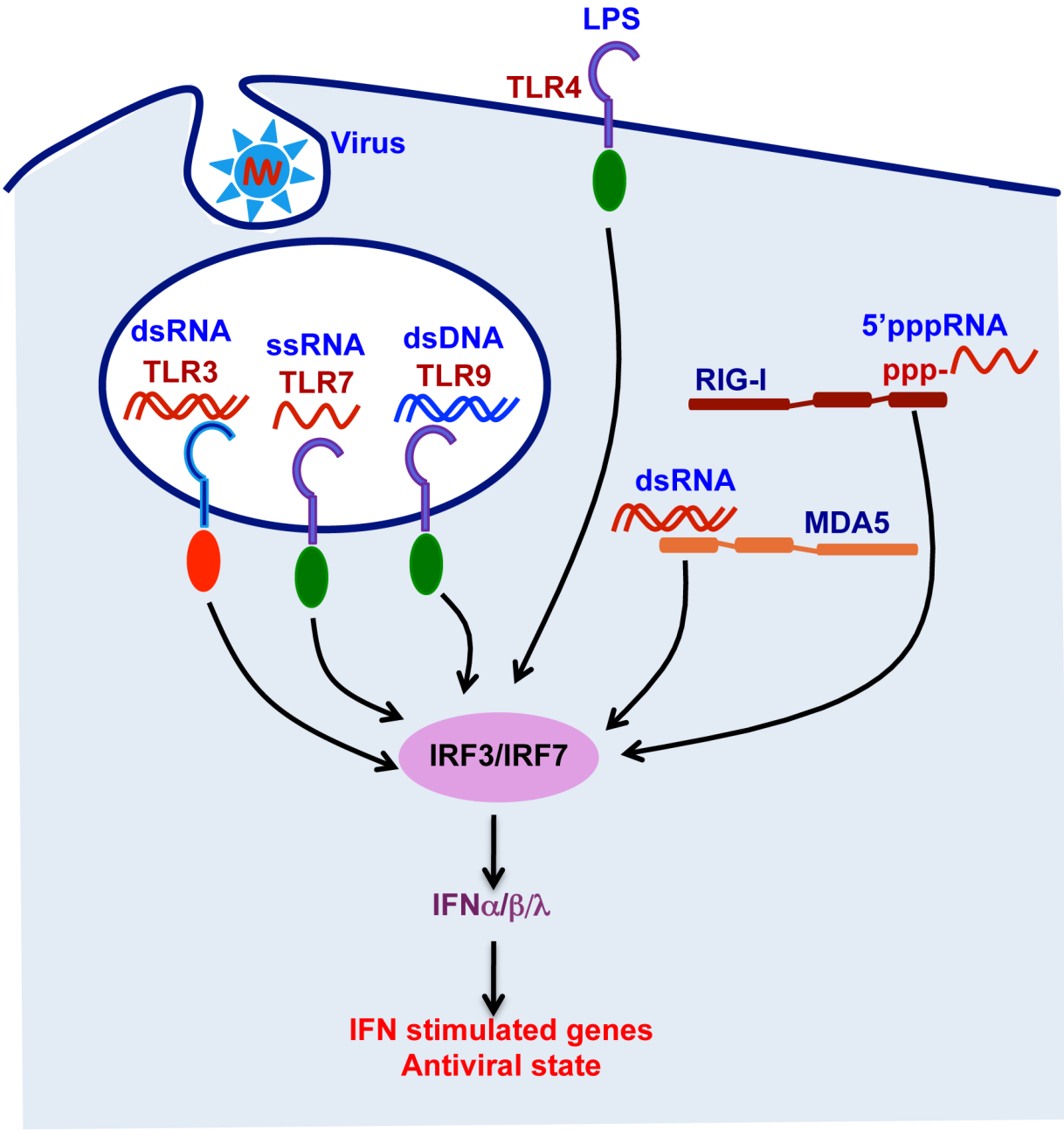

Figure 1.1: Virus sensing TLRs and RIG-I-like helicases. Receptors of both TLR and RLR families are involved in virus sensing. While cell surface TLRs like TLR4 are involved in sensing surface glycoproteins of viruses, the endosomal TLRs, TLR 3, 7 and 9 recognize viral nucleic acids within the endosomal compartment. The RLRs, RIG-I and MDA5 detect viral

patterns within the cytoplasm of virus-infected cells. Virus sensing by these receptors activate various downstream signaling cascades inducing the production of interferons, which in turn induce expression of interferon stimulated genes and an antiviral state in the infected tissue to limit virus replication and cell to cell spread.

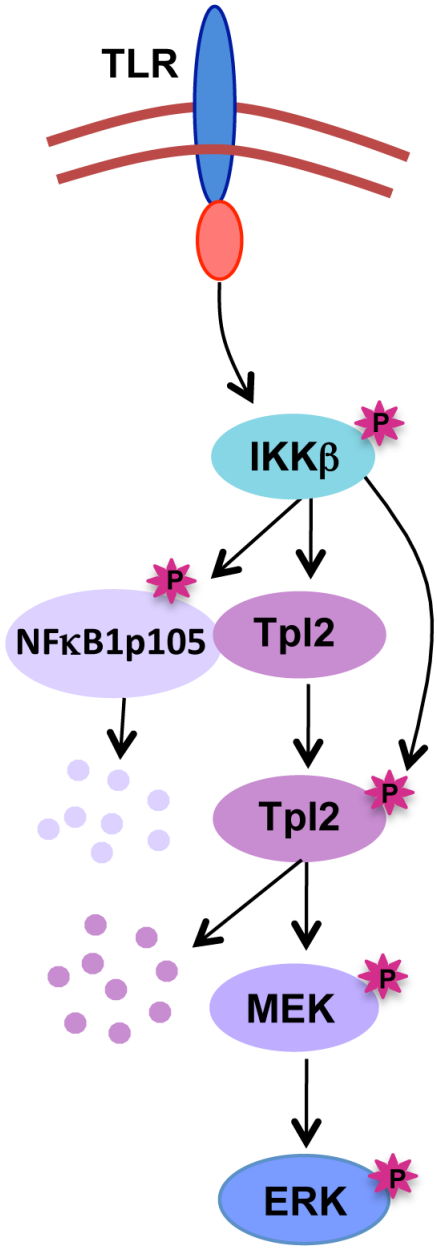

Figure 1.2: Model of Tpl2 activation during TLR signaling. In unstimulated cells**,** Tpl2 is associated with NFκB1 p105 and this association keeps Tpl2 in inactive form. Stimulation of TLRs leads to phosphorylation of the IKKβ kinase, and active IKKβ phosphorylates both p105 and Tpl2. Tpl2 then transduces downstream signals to activate MEK, which in turn activates ERK MAP kinase. Active Tpl2 is unstable and it undergoes rapid proteosomal degradation.

CHAPTER 2

TUMOR PROGRESSION LOCUS 2-DEPENDENT OXIDATIVE BURST DRIVES PHOSPHORYLATION OF EXTRACELLULAR SIGNAL-REGULATED KINASE DURING TLR3 AND 9 SIGNALING

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ABSTRACT

Signal transduction via NFκB and MAP kinase cascades is a universal response initiated upon pathogen recognition by Toll-like receptors (TLRs). How activation of these divergent signaling pathways is integrated to dictate distinct immune responses to diverse pathogens is still incompletely understood. Herein, contrary to current perception, we demonstrate that a signaling pathway defined by the inhibitor of κB kinase β (IKKβ), MAP3 kinase tumor progression locus 2 (Tpl2/MAP3K8) and MAP kinase ERK is differentially activated by TLRs. TLRs 2, 4 and 7 directly activate this inflammatory axis, inducing immediate ERK phosphorylation and early TNFα secretion. In addition to TLR adaptor proteins, IKKβ-Tpl2-ERK activation by TLR4 is regulated by the TLR4 co-receptor CD14 and the tyrosine kinase Syk. Signals from TLRs 3 and 9 do not initiate early activation of IKKβ-Tpl2-ERK pathway but instead induce delayed, NADPH-oxidase-dependent ERK phosphorylation and TNFα secretion via autocrine reactive oxygen species signaling. Unexpectedly, Tpl2 is an essential regulator of ROS production during TLR signaling. Overall, our study reveals distinct mechanisms activating a common inflammatory signaling cascade and delineates differences in MyD88-dependent signaling between endosomal TLRs 7 and 9. These findings further confirm the importance of Tpl2 in innate host defense mechanisms and also enhance our understanding of how the immune system tailors pathogen-specific gene expression patterns.

INTRODUCTION

Toll-like receptors (TLRs) are a major class of pattern recognition receptors that specifically detect conserved pathogen associated molecular patterns (PAMPs) and alarm the host of an infection. TLRs are expressed either on the cell surface or within specific intracellular compartments. Cell surface TLRs (TLR1, 2, 4, 5 and 6) detect outer membrane components of microbes, whereas endosomal TLRs (TLR3, 7, 8 and 9) sense microbial nucleic acids (1). Signals emanating from TLRs activate various intracellular signaling cascades including NFκB, mitogen-activated protein (MAP) kinases, and interferon regulatory factors that collectively induce the secretion of host protective proinflammatory cytokines and interferons (1). The magnitude and quality of this early response also regulates the initiation of adaptive responses (2). Despite extensive research, the precise molecular mechanisms that dictate specific cellular responses to TLRs are still incompletely understood.

NFκB and MAP kinase pathways are the two major signaling cascades initiated after recognition of specific PAMPs by TLRs (3). Engagement of all TLRs activates both of these pathways, and cross-talk between them coordinates the cellular responses to external stimuli (3,4). One of the key regulatory molecules known to coordinate the activation of both NFκB and MAP kinase pathways is the inhibitor of κB kinase β (IKKβ). IKKβ is activated in response to proinflammatory stimuli, including TLRs and cytokines, and it regulates activation of NFκB and MAP kinases by phosphorylating IκBα, NFκB1p105 and the MAP3 kinase, Tumor progression locus 2 (Tpl2) (5,6).

Tpl2 is a serine-threonine kinase originally identified as a proto-oncogene and expressed in both hematopoietic and non-hematopoietic compartments (7). Differential translation initiation of Tpl2 mRNA gives rise to 52 and 58 kDa isoforms expressed in equimolar levels in macrophages

(8). In unstimulated cells, Tpl2 is constitutively associated with NFκB1p105, and this interaction is necessary for Tpl2 stability but blocks Tpl2 kinase activity (9). Phosphorylation of p105 by IKKβ leads to Tpl2 release (10). IKKβ also mediates phosphorylation of Tpl2 at threonine 290 and serine 400, which regulates Tpl2 kinase activity (5,10-12). Once phosphorylated, Tpl2 transiently transduces signals but is unstable and undergoes rapid proteosomal degradation (9,13). The p58 isoform is preferentially released and degraded in LPS treated macrophages, since only this isoform undergoes IKKβ-mediated Thr^{290} phosphorylation (5,10).

Early studies on Tpl2 signaling established the non-redundant role of Tpl2 in LPS-mediated activation of ERK1/2 (14). *Tpl2*$^{-/-}$ mice are resistant to endotoxin-induced shock due to defective ERK-dependent TNFα secretion. Further studies demonstrated a cell type- and stimulus-specific role for Tpl2 in transducing signals leading to the production of a variety of immune mediators, including IL-1β IL-10, IL-12 and COX-2 (15-18). Because of its role in regulating expression, secretion and signaling of proinflammatory cytokines like TNFα and IL-1β, Tpl2 is considered an attractive target for immunotherapy of inflammatory conditions. Several studies have examined Tpl2 regulation of signal transduction and cellular responses to diverse TLR ligands (19). Tpl2 kinase activity and Tpl2-dependent ERK phosphorylation were demonstrated in macrophages in response to ligands of TLR2, 3, 4, 7 and 9 (19). Moreover, ERK phosphorylation in response to LPS, TNFα, CpG, Pam3CSK, poly I:C, flagellin and R848 were blocked in *Nfkb1*^{SSAA} macrophages which express a p105 mutant that cannot be phosphorylated by IKKβ (20). From these studies, it has been concluded that all TLRs similarly activate the Tpl2-ERK signaling pathway.

In order to better understand the molecular mechanisms utilized by different TLRs to distinguish their cellular responses, we examined the induction of proinflammatory genes and signal

transduction events by diverse TLR ligands, focusing on Tpl2 signaling. Contrary to prevailing thought, we demonstrate that the signaling pathway defined by IKKβ, Tpl2 and ERK, which helps to initiate and influence the nature of the innate immune response, is differentially regulated by TLRs. Among the MyD88-coupled TLRs, TLR4 uniquely requires CD14 and the tyrosine kinase Syk for Tpl2-ERK activation. TLRs 3 and 9 do not induce Tpl2-p58 phosphorylation or early ERK activation; instead they induce delayed ERK activation that is dependent upon autocrine signaling by reactive oxygen species (ROS) generated in a Tpl2-dependent manner. These findings demonstrate a differential mechanism of ERK activation by diverse TLRs and also identify divergent signaling pathways emanating from the MyD88-dependent endosomal TLRs 7 and 9. Overall, our study provides a better understanding of signaling pathways utilized by major TLRs and also demonstrate a major role for Tpl2 in eliciting host protective immune responses, including the generation of antimicrobial reactive oxygen species.

EXPERIMENTAL PROCEDURES

Mice

Wild type (C57BL/6J), *Myd88*$^{-/-}$, *Ticam*$^{lps2/lps2}$ and *Ifnar1*$^{-/-}$ mice were purchased from The Jackson Laboratory. *Tpl2*$^{-/-}$ mice backcrossed to C57B6/J were kindly provided by Dr. Philip Tsichlis (Tufts University) and Thomas Jefferson University. Femurs and tibiae from *Cd14*$^{-/-}$ mice were generously provided by Dr. Donald Harn (University of Georgia). Femurs and tibiae from M*yd88/Trif* double-knockout mice (21) were kindly provided by Dr. Alan Sher (NIAID, NIH). Animals were housed in sterile microisolator cages in the Central Animal Facility of the College of Veterinary Medicine. The Institutional Animal Care and Use Committee (IACUC) of the University of Georgia approved all animal experiments.

Generation of Bone marrow derived cells

Bone marrow derived macrophages (BMDMs) and dendritic cells (BMDCs) were generated from age- and sex-matched mice as described previously (16). The cells were cultured at a concentration of $2x10^6$/mL in DMEM low glucose medium containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine on sterile petri dishes for 7 days at 37°C supplemented with 10 ng/mL macrophage colony stimulating factor (M-CSF) (PeproTech). Fresh medium equal to half of the initial culture volume containing M-CSF was added on day 5 of the culture. On day 6, after removing the media and washing the cells with PBS, the adherent cells were incubated with cell dissociation buffer (Invitrogen) for 10 minutes at 37°C. The harvested cells were counted and replated in the same culture medium overnight before stimulation.

BMDCs and plasmacytoid DCs (pDCs) were generated by culture of bone marrow cells in complete RPMI (RPMI 1640 containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine and 50 μM 2-ME). Cells were cultured with 40 ng/mL GM-CSF (PeproTech) for 7 days or 100 ng/mL Flt3 ligand (PeproTech) for 10 days for BMDCs and pDCs, respectively. For BMDCs, nonadherent cells were harvested on day 7 and $CD11c^+$ cells were isolated using CD11c microbeads and MACS columns (Miltenyi Biotec). The purity of cell population was determined to be more than 95% by flow cytometry. $CD11c^+CD11b^-B220^+$ pDCs were sorted using a Beckman Coulter MoFlo XDP cell sorter to >98% purity.

Peritoneal Exudate Cell Isolation

Mice were injected intraperitoneally with 1 ml of 3% Brewer thioglycollate medium to recruit macrophages. After 72 h, mice were sacrificed, and the peritoneal cavity was lavaged three times

with 3 ml of sterile PBS to collect recruited cells. Cells were centrifuged at 1200 rpm for 10 min at room temperature and were resuspended in supplemented DMEM.

Antibodies and other reagents

The following antibodies were used for immunoblotting - Tpl2 (Cot M-20), ERK1 and ERK2, β-actin (Santa Cruz Biotechnology), phospho-ERK1/2 (Thr202/Tyr204), pIKKα/β (Ser176/180), IKKβ, pIκBα (Ser32), IκBα, pNFκBp105, NFκB-p65 and pSTAT1 (Tyr701) (Cell Signaling Technology). In some experiments cells were pretreated with 10 μg/mL cycloheximide (Sigma-Aldrich), 100 ng/mL pertussis toxin (Sigma-Aldrich) or 20 μM diphenyleneiodonium (DPI) (Sigma-Aldrich) for 30 min or 10 mM glutathione (GSH) (Sigma-Aldrich) for 10 min. To investigate ROS signaling, cells were treated with 1 or 10 mM H_2O_2 for 1h. To block cytokine signaling 1 μg/mL of anti-TNFα (R&D Systems) or anti-IL-1β (BD Pharmingen) was used.

Cell stimulation and measurement of cytokines – Bone marrow derived cells or PECs at $1x10^6$/mL concentration were stimulated with Pam3CSK4 (1 μg/mL), poly I:C (10 μg/mL), ultrapure LPS from *E. coli* 0111:B4 (1 μg/mL), R848 (1 μg/mL), CpG ODN2395 (10 μg/mL) or CpG ODN1668 (0.5 -1.5 μM). All TLR ligands were purchased from Invivogen. Cell culture supernatants were collected at different time points after stimulation, and TNFα, IL-10 and IFNβ levels were measured by ELISA (eBioscience, PBL Interferon Source). Alternatively, BMDMs were directly stimulated with the following cytokines: rmTNFα (10 ng/mL; eBioscience), rmIL-1β (10 ng/mL; eBioscience), rmIFNα A (2000 IU/mL; R&D Systems), or rmIFNβ (10 ng/mL; Peprotech).

Analysis of mRNA expression

BMDMs stimulated with various ligands were washed with PBS after collecting supernatants, and cell lysates were prepared using TRK lysis buffer (Omega Bio-Tek). RNA was extracted

using a Total RNA Kit (Omega Bio-Tek). Real time PCR was performed after synthesizing cDNA using High capacity cDNA Reverse Transcription kit (Applied Biosystems). The expression of *il12b* (Mm00434174_m1), *il6* (Mm00446190_m1), *tnfα* (Mm00443258_m1), *il10* (Mm01288386_m1), *ccl2* (Mm00441242_m1), *ccl5* (Mm01302427_m1), *nos2* (Mm00440502-m1) and *actinb* (4352341E-1112017) were determined by real-time PCR (Applied Biosystems). RT-PCR reactions were performed in microAmp Fast plates (Applied Biosystems) using SensiFAST Probe Hi-ROX kit (Bioline) and a StepOnePlus RT-PCR machine (Applied Biosystems). Relative gene expression levels were calculated by normalizing the Ct levels of target gene to both endogenous actin levels and an unstimulated WT control using the ΔΔCt method.

Protein Analysis

Cell lysates were prepared in protein lysis buffer (0.5% Triton X-100, 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2 mM EDTA, 0.4mM Na_3VO_4, 2.5mM aprotinin, 2.5mM leupeptin, and 2.5 μM nitrophenyl p-guanidinobenzoate) and protein concentrations were measured using a BCA kit (Pierce). Approximately 20 μg of denatured proteins were separated on 4-12% gradient gels (Invitrogen) under reducing conditions and were transferred to PVDF membranes using the iBlot Gel Transfer system (Invitrogen). Membranes were probed with various antibobies followed by horseradish peroxidase-labeled secondary antibodies. Protein bands were visualized by enhanced chemiluminescent reagent (Lumigen) and Amersham Hyperfilm ECL (GE Healthcare). In some experiments, images were acquired using a Flourchem Hd2 imaging system (Alpha Innotech Corporation).

Confocal microscopy

BMDMs plated on chamber slides (Lab-Tek) were treated with poly I:C (10 μg/mL), CpG ODN2395 (10 μg/ml) or LPS (1 μg/mL) for 1 h. Cells were washed and fixed in 3.7% paraformaldehyde solution at 37^0C for 30 min. Fixed cells were incubated in blocking buffer (1% BSA in PBS) for 30 min, followed by a 1 h incubation with primary antibody (NFκBp65, 1:100 dilution, Cell Signaling Technology) followed by a fluorochrome-conjugated secondary antibody and a DAPI nuclear counterstain. Images were acquired using a Nikon A1R confocal microscope.

Measurement of intracellular ROS

$1.5X10^5$ BMDMs plated in clear bottom, white plates were washed, and 100 μL prewarmed (37 °C) assay medium containing HBSS, 100 μg/mL superoxide-specific lucigenin and stimuli were added. Cells were either left untreated or stimulated with poly I:C (25 μg/mL), CpG ODN2395 (25 μg/mL), LPS (1 μg/mL) or phorbol myristate acetate (PMA) (50 ng/mL). Chemiluminescence was followed for 60 min in Varioskan Flash microplate luminometer. Integrated luminescence units indicative of superoxide production during the entire measurement were calculated and expressed as relative units (RU).

Statistical Analysis

Data are represented as the mean ± SD or SEM, as indicated. *p* values were determined by two-tailed Student's *t* test or paired Student's *t* test.

RESULTS

Early induction of proinflammatory gene expression and secretion of TNFα and IL-10 are restricted to a subset of TLRs

To better understand how Tpl2 regulates early innate responses induced by diverse TLR ligands, we measured the expression of various proinflammatory genes including *il12b*, *il6*, *tnfα*, *ccl2* and *ccl5* as well as anti-inflammatory *il10* in WT and *Tpl2*$^{-/-}$ BMDMs stimulated with Pam3CSK4 (TLR2), poly I:C (TLR3), LPS (TLR4), R848 (TLR7) and CpG (TLR9) for 1 h. Even at this early time point, induction of most genes was observed in response to TLR2, 4 and 7 ligands, but not in response to TLR3 and 9 ligands (Figure 2.1A and Supplementary Figure 2.8). Of these cytokines, only TNF and IL-10 were secreted by 1 h after stimulation (Figure 2.1B). TLR2, 4 and 7 stimulation induced high levels of TNFα and IL-10 production, and their secretion was significantly less or undetectable in Tpl2-deficient BMDMs (Figure 2.1B). On the contrary, neither TLR3 nor 9 stimulation induced secretion of TNFα or IL-10, despite modest induction of both at the mRNA level. To determine whether cytokine secretion occurred with delayed kinetics in poly I:C- and CpG-treated cells, TNFα levels were measured at different time points. Significant induction of TNFα was observed in response to poly I:C and CpG by 2 h post stimulation, although the levels were less compared to LPS-treated cells (Figure 2.1C). However, by 24h, both CpG and poly I:C induced high levels of TNFα, comparable to that induced by LPS stimulation. TNFα secretion was significantly impaired in *Tpl2*$^{-/-}$ cells at all time points confirming the critical role of Tpl2 in regulating TNFα production (Figure 2.1C). This differential induction of early proinflammatory gene expression and secretion of TNFα and IL-10 suggested the possibility of immediate activation of an inflammatory signaling cascade downstream of a restricted set of TLRs.

Signaling by TLR3 and 9 fails to induce Tpl2-p58 or early ERK phosphorylation

The MAP kinase ERK is activated in response to TLRs and mediates both transcriptional and post-transcriptional regulation of many immune mediators, including TNFα and IL-10 (14,17). Tpl2 plays a critical role in TLR-mediated ERK activation and regulates TNFα production by promoting both nucleocytoplasmic transport of TNF mRNA and processing of pre-TNFα by TNFα converting enzyme (14,22). Since early TNFα secretion was abolished in WT BMDMs in response to TLR3 and 9 ligands despite transcriptional induction, we investigated whether the Tpl2-ERK TNF-processing pathway was differentially activated by these TLRs. BMDMs from WT and *Tpl2*$^{-/-}$ mice were stimulated with Pam3CSK4, poly I:C, LPS, R848 and CpG, and both Tpl2 activation and ERK phosphorylation were assessed by immunoblotting. We observed decreased mobility of Tpl2-p58, consistent with phosphorylation (10), at early time points following Pam3CSK4, LPS and R848 stimulation (Figure 2.2A). Tpl2-p58 was completely degraded by the proteosome within 1 h of stimulation with the same ligands that induced Tpl2 phosphorylation (Figure 2.2B). Surprisingly, neither the TLR3 ligand poly I:C nor the TLR9 ligand CpG induced a mobility shift or Tpl2-p58 degradation (Figure 2.2A-B). Furthermore, the lack of Tpl2 degradation in response to TLR3 or TLR9 ligation was independent of the ligand dose or type of CpG ODN used, since we did not observe any Tpl2-p58 mobility shift in response to a high dose of poly I:C or CpG or in response to class B CpG ODN (Figure 2.2C). Interestingly, no mobility shift or degradation of the Tpl2-p52 isoform occurred in response to any of these ligands. Consistent with previous studies (19), all TLR ligands, including poly I:C and CpG, induced strong ERK1/2 phosphorylation in WT BMDMs by 60 min (Figure 2.2B), regardless of Tpl2 mobility shift or degradation. Notably, in WT BMDMs ERK phosphorylation by either CpG or poly I:C was delayed compared to that induced by Pam3CSK4, LPS or R848

since it was evident at 60 min but not 15 min (Figure 2.2A-B). Stimulation of WT BMDMs with poly I:C over a longer time course up to 2 h also did not lead to Tpl2 degradation, despite maximal ERK activation by 1 h that was comparable to that induced by LPS, followed by a decline in ERK activation by 2 h (Figure 2.2D). Consistent with an essential, non-redundant role for Tpl2 in activating ERK (19), none of these ligands induced ERK1/2 phosphorylation in Tpl2-deficient BMDMs (Figure 2.2B). Thus, even though Tpl2-dependent ERK activation was observed following stimulation of all TLRs examined, only TLR2, 4 and 7 signaling induced Tpl2-p58 phosphorylation, degradation and early ERK1/2 activation. These findings demonstrate that Tpl2-p58 phoshorylation, degradation and early ERK activation are restricted to a subset of TLRs.

Although Tpl2 signaling is essential for TNFα production in macrophages, previous studies have reported distinct requirements for Tpl2 in response to different pattern recognition receptors in a cell-type specific manner (15,16). To investigate whether differential Tpl2-ERK activation occurs in other cell types in a ligand-dependent manner, we assessed Tpl2-p58 shift and ERK phosphorylation in peritoneal macrophages (PMs), BMDCs and pDCs. Similar to BMDMs, Tpl2-p58 mobility shift, degradation and ERK phosphorylation were observed in PMs in a stimulus-specific manner (Figure 2.2E). Interestingly, complete degradation of both p52 and p58 isoforms of Tpl2 was observed within 15 min in pDCs treated with R848 (Figure 2.2E). Similar to other cell types examined, CpG stimulation did not have any effect on Tpl2 in pDCs. In contrast, Tpl2-p58 mobility shift was not observed in BMDCs even upon LPS stimulation, despite early (15 min) ERK phosphorylation in response to both CpG and LPS stimulation (Figure 2.2E). Collectively these studies demonstrate that the differential degradation of Tpl2

isoforms observed in response to LPS versus poly I:C or CpG in BMDMs is recapitulated in both PMs and pDCs. However, they also reveal cell type-specific differences in Tpl2 activation.

Either the MyD88 or TRIF adaptor protein is required for activation of Tpl2-ERK pathway during TLR4 signaling

Ligand binding to TLRs initiates recruitment of specific adaptor molecules necessary for intracellular signal transduction. All TLRs except TLR3 transduce signals via the MyD88 adaptor, whereas TLR3 utilizes the TRIF adaptor (1). TLR4 is the only TLR that can transduce signals via both MyD88 (from the plasma membrane) and TRIF (from endosomes) (1). Lack of Tpl2 p58 phosphorylation by either TLR3 or 9 was surprising, especially because these two TLRs utilize different adaptor proteins for downstream signaling. To further investigate the role of TLR adaptors in Tpl2-ERK activation, we used cells deficient in MyD88 or TRIF adaptor proteins. WT, *Myd88*$^{-/-}$ and *Ticam*$^{LPS2/LPS2}$ (23) BMDMs were stimulated with different TLR ligands, and immunoblotting was performed to assess Tpl2 degradation and ERK activation. Tpl2 degradation was absent in *Myd88*$^{-/-}$ macrophages in response to all TLR ligands tested except LPS, confirming the requirement for Myd88-dependent signaling in Tpl2-ERK activation during TLR2 and 7 signaling (Figure 2.3A). Similar to *Myd88*$^{-/-}$ cells, no impairment in Tpl2-p58 degradation was observed in *Ticam*$^{LPS2/LPS2}$ BMDMs in response to LPS (Figure 2.3B). This pattern of Tpl2 degradation by ligands was also mirrored by the Tpl2-p58 mobility shift observed at 15 min. (Supplementary Figure 2.9 A-B). Consistent with the absolute requirement for either the MyD88 or TRIF adaptor protein for downstream signaling from TLR4 (23), Tpl2 mobility shift, degradation and ERK activation was completely abrogated in the *Myd88*$^{-/-}$/*Trif*$^{-/-}$ cells (Figure 2.3C). Thus, signaling via either TLR adaptor protein is necessary for activation of the Tpl2-ERK pathway by TLR4.

In *Myd88*$^{-/-}$ BMDMs, LPS signals only via the TRIF adaptor, similar to TLR3 (23). Notably, we observed distinct capabilities of TLR3 and TLR4 to induce Tpl2 degradation in *Myd88*$^{-/-}$ BMDMs, suggesting the possibility for different Tpl2-dependent consequences of TRIF signaling by these two ligands. In order to evaluate TRIF-dependent responses we measured the production of the TRIF-regulated cytokine, IFNβ, in response to poly I:C and LPS. While IFNβ levels were higher in Tpl2-deficient cells in response to LPS as previously reported (17), poly I:C induced IFNβ was significantly reduced in *Tpl2*$^{-/-}$ BMDMs compared to WT cells (Figure 2.3D). Therefore, despite the lack of Tpl2-p58 mobility shift or degradation, there was still evidence of Tpl2-dependent regulation of poly I:C-mediated responses, suggesting a different mode of Tpl2 activation in response to this ligand.

Activation of the Tpl2-ERK pathway by TLR4 requires CD14 and the tyrosine kinase Syk

CD14 is a cell surface pattern recognition receptor that binds directly to LPS and serves as a co-receptor, delivering LPS to TLR4. Similar to *Tpl2*$^{-/-}$ mice, *Cd14*$^{-/-}$ mice, as well as Heedless mice harboring a mutation in the *Cd14* gene, are defective in TNFα production and consequently resistant to LPS-induced shock (24,25). Importantly, CD14 co-receptor functions for TLR4 are dispensable at high LPS concentrations of greater than 100 ng/mL and MyD88-dependent MAP kinase and NFκB activation occurs normally in *Cd14*$^{-/-}$ cells (26). To investigate the contribution of CD14 to Tpl2-ERK signaling, WT and *Cd14*$^{-/-}$ BMDMs were stimulated with TLR ligands, and Tpl2 activation was assessed. In contrast to WT cells treated with LPS, both phosphorylation-induced mobility shift and degradation of Tpl2-p58 was absent in *Cd14*$^{-/-}$ cells (Figure 2.3E and Supplementary Figure 2.9C). These results demonstrate that, despite normal MyD88-dependent signaling, the TLR4 co-receptor CD14 was required for Tpl2 activation and degradation in response to LPS. Consistent with the lack of Tpl2 activation, ERK

phosphorylation was abrogated in *Cd14*$^{-/-}$ macrophages at earlier time points (Supplementary Figure 2.9C), whereas similar levels of ERK phosphorylation were observed 1 h after stimulation (Fig. 3E). This delayed ERK activation in the absence of Tpl2 degradation was reminiscent of the phenotype observed in WT BMDMs treated with TLR3 and 9 ligands, suggesting an inability of *Cd14*$^{-/-}$ BMDMs to induce early Tpl2-dependent ERK phosphorylation.

CD14-mediated TLR4 endocytosis depends on the enzyme PLCγ2 and the tyrosine kinase Syk (26). Syk is known to regulate Tpl2 activation and release from NFκB1p105 in response to TNFα (27). Moreover, LPS-induced Syk phosphorylation is also CD14-dependent (26). This prompted us to investigate whether Syk is involved in Tpl2 activation by TLR4. The well-characterized Syk inhibitor piceatannol blocked LPS-induced mobility shift of Tpl2-p58 as well as ERK phosphorylation in a dose-dependent manner (Figure 2.3F). Notably, only early ERK phosphorylation by LPS was regulated by Syk, as piceatannol did not block LPS-induced ERK phosphorylation at 1h (Supplementary Figure 2.10). These data confirmed the significance of CD14-dependent Syk activation, in addition to MyD88/TRIF signaling, in the regulation of Tpl2-ERK signaling by TLR4.

Early induction of Tpl2-ERK signaling correlates with IKKβ phosphorylation

One signaling event that regulates activation of Tpl2 and its release from inhibitory NFκB1p105 is IKKβ phosphorylation (5,10). To investigate whether the observed differences in phosphorylation and degradation of Tpl2-p58 were due to differential activation of IKKβ, we examined phosphorylation of IKKβ and its downstream target IκBα in response to various TLR ligands. Consistent with Tpl2-p58 degradation, only TLR2, 4 and 7 stimulation induced phosphorylation of IKKβ early after stimulation (15 min) (Figure 2.4A). A reduction in IKKβ mobility was also observed in response to the ligands that induced its phosphorylation. IKKβ

immunoprecipitation followed by Western blotting confirmed that IKKβ was the IKK species being phosphorylated (Supplementary Figure 2.11). Since IKKβ is also known to regulate NFκB signaling, we examined whether NFκB activation is also differentially regulated by diverse TLR ligands. The phosphorylation of NFκB1-p105 as well as degradation of IκBα were observed in both poly I:C- and CpG-treated cells, although with different magnitude and kinetics compared to LPS (Figure 2.4B). Moreover, NFκB-p65 was almost exclusively localized to the nuclei in cells treated with both poly I:C and CpG, confirming their activation of NFκB (Figure 2.4C). Notably, although poly I:C and CpG induced phosphorylation of IKKβ by 30 – 60 min (Figure 2.4B), we did not observe Tpl2-p58 mobility shift or degradation at later time points. Collectively, these findings support the conclusion that differential activation of IKKβ by diverse TLRs controls early induction of Tpl2-ERK signaling.

IKKβ phosphorylation in response to LPS was present in *Myd88*$^{-/-}$ and *Ticam*$^{LPS2/LPS2}$ cells, but absent in *Myd88*$^{-/-}$/*Trif*$^{-/-}$ and *Cd14*$^{-/-}$ BMDMs, demonstrating that either one of the adaptors as well as CD14 is required for IKKβ activation (Figure 2.4D and Supplementary Figure 2.12). In addition, IKKβ phosphorylation was significantly reduced in cells pretreated with the Syk inhibitor piceatannol (Figure 2.4E). Under these conditions, IκBα degradation was not dramatically affected in piceatannol treated samples suggesting minimal off-target effects of the inhibitor (Figure 2.4E). These results demonstrate that IKKβ is likely the convergence point through which TLR adaptor proteins, CD14 and Syk regulate the Tpl2-ERK pathway during TLR4 signaling.

TLR3 and 9 activate ERK indirectly via NADPH-oxidase-dependent autocrine ROS signaling

In order to explain TLR3- and 9-induced ERK activation in the absence of Tpl2-p58 phosphorylation, we considered the possibility of Tpl2-dependent ERK activation by autocrine signaling. Both TLR3- and 9-mediated ERK phosphorylation were abolished when BMDMs were treated with the translation inhibitor cycloheximide (CHX), suggesting that new protein synthesis was required for ERK activation by these TLRs (Figure 2.5A). Since Tpl2 is also essential for TNFα– and IL-1β–mediated signal transduction leading to ERK phosphorylation (15), we investigated whether an indirect cytokine feedback loop via TNF or IL-1 receptors was responsible for the delayed ERK activation observed in the absence of Tpl2 degradation. As expected, TNFα and IL-1β induced ERK phosphorylation in WT BMDMs (Supplementary Figure 2.13A). However, unlike poly I:C or CpG, TNFα, but not IL-1β, also induced Tpl2-p58 degradation. Furthermore, neutralizing antibodies against TNFα and IL-1β did not inhibit delayed ERK phosphorylation in response to TLR3 and 9 ligands (Supplementary Figure 2.13B). TLR3- and 9-induced ERK phosphorylation was also evident in *Ifnar1*$^{-/-}$ BMDMs as well as in BMDMs pretreated with pertussis toxin, demonstrating that neither type 1 interferons nor chemokines are responsible for the indirect ERK phosphorylation in response to TLR3 and 9 ligands (Supplementary Figure 2.13C-D).

Inhibition of protein synthesis is known to inhibit accumulation of ROS and oxidative stress by increasing cellular glutathione concentrations (28). In addition, several studies have demonstrated that ROS can induce activation of MAP kinases, including ERK (29,30). Because both poly I:C and CpG are known inducers of ROS (31,32), we tested the possibility that ERK was phosphorylated via a ROS-dependent mechanism in BMDMs stimulated with these ligands.

Indeed, ERK phosphorylation in response to both poly I:C and CpG was completely abrogated in BMDMs pretreated with the NADPH oxidase (NOX) inhibitor diphenyleneiodonium (DPI), demonstrating that activation of ERK by TLR3 and 9 is dependent on NOX activity (Figure 2.5B). Scavenging ROS with the antioxidant glutathione (GSH) also clearly reduced ERK phosphorylation in response to poly I:C and CpG, supporting the conclusion that autocrine ROS signaling is responsible for indirect, delayed ERK activation by TLR3 and 9 (Figure 2.5C). Although DPI can also inhibit nitric oxide synthase, the involvement of nitric oxide (NO) in the autocrine signaling loop was excluded because expression of nitric oxide synthase 2 (NOS2), a secondary response gene that drives NO production (33), was not induced in poly I:C and CpG treated cells during the 1 h stimulation (Figure 2.5D). If autocrine ROS signaling is responsible for ERK phosphorylation downstream of TLRs 3 and 9, then DPI treatment should also inhibit delayed ERK-dependent TNFα secretion observed in BMDMs treated with poly I:C and CpG (Figure 2.1C). Indeed, DPI treatment significantly decreased TNFα secretion in response to poly I:C and CpG, but not LPS (Figure 2.5E). Collectively, these data delineate a ROS-dependent autocrine loop that mediates ERK phosphorylation and TNFα secretion during TLR3 and 9 signaling.

Tpl2 is essential for ROS production during TLR signaling

The observation that inhibiting NADPH oxidase can abolish ERK phosphorylation in response to poly I:C and CpG was unexpected. Acidification of the endosomal compartment, a pre-requisite for both TLR3 and 9 signaling (34,35), is coupled to rapid induction of ROS (32). Since ROS-mediated ERK phosphorylation is also Tpl2-dependent, it is necessary to determine whether Tpl2 regulates ROS production and/or signaling. The involvement of Tpl2 in ROS signaling was excluded since treatment with the oxidizing agent H_2O_2 induced ERK phosphorylation in both

WT and *Tpl2*$^{-/-}$ BMDMs (Figure 2.6A). Despite inducing strong ERK phosphorylation, H_2O_2 did not induce Tpl2 degradation even at the non-physiological concentration of 10 mM (Figure 2.6A). Having demonstrated that Tpl2 is dispensable for ROS-induced ERK phosphorylation, we next sought to determine whether Tpl2 regulates ROS production during TLR signaling. To determine whether Tpl2 regulates the generation of intracellular ROS, we measured superoxide production in WT and *Tpl2*$^{-/-}$ BMDMs treated with various TLR ligands. As previously reported (31,32,36), poly I:C, CpG and LPS all induced ROS production (Figure 2.6B-C). As expected based on our previous results, superoxide generation in response to multiple TLR ligands was significantly less in Tpl2-deficient BMDMs compared to WT cells (Figure 2.6B-C). In contrast, there was no intrinsic defect in the capacity of *Tpl2*$^{-/-}$ cells to generate ROS in response to phorbol myristate acetate (PMA), indicating a ligand-specific defect. This unanticipated role for Tpl2 in ROS production explains Tpl2- and ROS-dependent ERK phosphorylation observed during TLR3 and 9 signaling.

DISCUSSION

Activation of NFκB and MAP kinases are key features of all TLR signaling pathways initiating a proinflammatory response (3). In this study, we made several important discoveries regarding differential mechanisms activating a common inflammatory signaling cascade during TLR signaling as summarized in Figure 2.7. First, we demonstrated an indirect, delayed mechanism of ERK activation by a subset of TLRs that limits early innate responses, including early TNFα and IL-10 secretion, to TLR3 and TLR9 ligands. This pathway is distinguished by the lack of Tpl2-p58 phosphorylation and degradation despite evidence of NFκB activation, including IκBα phosphorylation and degradation, and despite Tpl2-dependent biological responses to these ligands. Second, we delineated an inflammatory pathway controlled by CD14 and the tyrosine

kinase Syk in the activation of the IKKβ-Tpl2-ERK axis during TLR4 signaling. Third, we identified a ROS-dependent autocrine loop responsible for the delayed, indirect ERK phosphorylation during TLR3 and 9 signaling. Finally, we demonstrated the critical role of Tpl2 in ROS generation during TLR signaling.

Activation of ERK in response to diverse TLR ligands and the critical role of Tpl2 in transducing ERK activation signals are well documented (19). Our results are in agreement with previous studies demonstrating that stimulation of all major TLRs induce Tpl2-dependent ERK activation in BMDMs. Consistent with the data reported by Kaiser *et al.*, we observed delayed ERK phosphorylation in both CpG- and poly I:C-treated cells (17). We further linked this reduced ERK activation to lack of Tpl2 phosphorylation and degradation.

Conflicting reports regarding CpG-induced ERK phosphorylation in different cell types exist (17,37,38). Our findings clarify a controversy and demonstrate that TLR3 and 9 signaling do not directly couple to ERK activation. Instead, the observed ERK phosphorylation by TLR3 and 9 is due to NADPH oxidase-dependent, autocrine ROS signaling. The significance of ROS as second messengers during innate immune responses and in regulating the production of various inflammatory mediators is well appreciated (39). For example, ROS-dependent activation of MAP3K5/ASK-1 and MAP kinase p38 was shown to be necessary for TLR4 mediated innate responses (36). In addition to ROS-mediated ERK phosphorylation during TLR3 and 9 signaling, our study identified Tpl2 as a critical regulator of ROS production during TLR signaling. The requirement of Tpl2 in ROS production may contribute in part to the defective induction of IL-1β in *Tpl2*$^{-/-}$ macrophages, since ROS is important for IL-1β expression in response to LPS (29). The signaling events linking Tpl2 to NOX enzymes are currently unknown. Therefore, further studies are needed to determine the precise mechanisms by which Tpl2 regulate ROS production.

Tpl2-p58 mobility shift and degradation, while excellent predictors of Tpl2-dependent MEK/ERK activation, are poor indicators of overall Tpl2 biological activity. For example, Tpl2 is required for TNF processing and secretion in response to both poly I:C and CpG (Figure 1C), both of which fail to induce Tpl2-p58 phosphorylation-induced mobility shift, degradation or early ERK activation. Tpl2 is also required for normal IFNβ production in response to poly I:C (Figure 3D). Similarly, IL-1β also utilizes Tpl2 to transduce signals, but fails to induce Tpl2-p58 degradation (data not shown). These findings raise the possibility of either phosphorylation-independent functions for Tpl2-p58 or Tpl2-p52 isoforms in cell signaling. Thr^{290} phosphorylation occurs only on the Tpl2 p58 isoform, whereas both p52 and p58 isoforms undergo phosphorylation on Ser^{400} in LPS-treated macrophages (12). Despite the fact that no functional differences between Tpl2-p58 and p52 have been reported so far, it is tempting to speculate that Tpl2-p52 transduces signals from receptors that do not induce IKKβ-mediated Thr^{290} phosphorylation and p58 degradation, such as poly I:C, CpG and IL-1β.

Cell type-specific requirements for Tpl2 in transducing TLR signals has been demonstrated previously (15,16). However cell type-specific differences in Tpl2 phosphorylation is a novel finding. While LPS induced Tpl2-p58 Thr^{290} phosphorylation and mobility shift in macrophages, a decrease in Tpl2-p58 mobility was not observed in BMDCs. This difference in Tpl2 activation could account for the partial requirement of Tpl2 for TNFα secretion in BMDCs compared to BMDMs (16). Notably, cell type specific differences between BMDMs and BMDCs in the requirement for CD14 during TLR4 signaling have been reported (26). Unlike macrophages and BMDCs, both isoforms of Tpl2 were completely degraded in pDCs early after stimulation, further supporting the uniqueness of signaling pathways in pDCs (17).

CD14 is a GPI-anchored protein without intrinsic signaling potential (40), however CD14 functions are necessary for Myd88-independent signaling by TLR4 (25). In their elegant study demonstrating the role of CD14 in TLR4 endocytosis, Zanoni *et al.* commented that all LPS responses actually initiate with CD14 (26). Our data confirming the necessity of CD14 in IKKβ-Tpl2-ERK signaling support their placement of CD14 as the 'king of all LPS responses' although either one of the TLR adaptor proteins is also necessary for this response. An inflammatory endocytosis pathway regulated by Syk was proposed for endocytosed receptors like TLR4, Dectin-1 and FcγRI (26). Interestingly, a recent study reported Tpl2-mediated ERK activation during FcγR signaling (41). Thus, regulation of IKKβ-Tpl2-ERK signaling by CD14 and Syk supports the existence of this proposed inflammatory pathway.

Although differences in biological responses upon TLR7 and 9 stimulation have been reported (42,43), the molecular basis for these differences has remained enigmatic. Herein, we demonstrate the direct coupling of TLR7, but not TLR9, to the IKKβ-Tpl2-ERK signaling pathway. To our knowledge, differences between TLR7 and 9 signaling *per se* have not been demonstrated. This finding was surprising, as both of these endosomal TLRs transduce signals via the same MyD88 adaptor (44). However, a recent study did report differences in UNC93B1-mediated trafficking of TLR7 and 9 (45). Identification of discrete trafficking pathways suggests the possibility of distinct signaling compartments for TLR7 and 9 that may correlate with their activation of distinct signaling cascades and cellular responses. Since cell surface expression of TLR3 has been reported (46,47), a trafficking route similar to that of TLR9 was proposed for this receptor. Hence, the differential activation of the IKKβ-Tpl2-ERK pathway could correlate with the involvement of distinct signaling compartments for these endosomal TLRs.

In addition to the new insights into TLR signaling pathways, our findings have many implications regarding the role of Tpl2 in innate immune responses during infections. We and others have previously demonstrated the critical role of Tpl2 in host defense against intracellular bacteria like *Listeria monocytogenes* and *Mycobacterium tuberculosis* (16,48). Defective ROS production in *Tpl2*$^{-/-}$ mice may contribute to decreased bacterial clearance, increased susceptibility to infection or altered redox-sensitive signaling (39,49). In addition to its bactericidal functions, ROS is also required for RIG-I-mediated antiviral responses (50). Moreover, direct and immediate activation of Tpl2 and ERK during TLR7 signaling suggests that Tpl2 is likely to play a preferential role in host defense against RNA viruses that trigger TLR7. In this regard, a recent study reported increased replication of vesicular stomatitis virus (VSV) in Tpl2-deficient mouse embryonic fibroblasts (51). This is especially interesting because, in addition to TLR7, VSV is known to signal via the CD14-TLR4 axis, and increased replication of VSV was also reported in macrophages from CD14 mutant mice (25). These findings suggest a role for Tpl2 in controlling virus replication and warrant further studies to assess the contribution of Tpl2 in antiviral host responses. Overall, our study provides a better understanding about key events that distinguish signal transduction by diverse TLRs and further underscores the significance of Tpl2 in eliciting host protective immune responses against diverse pathogens.

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FIGURES

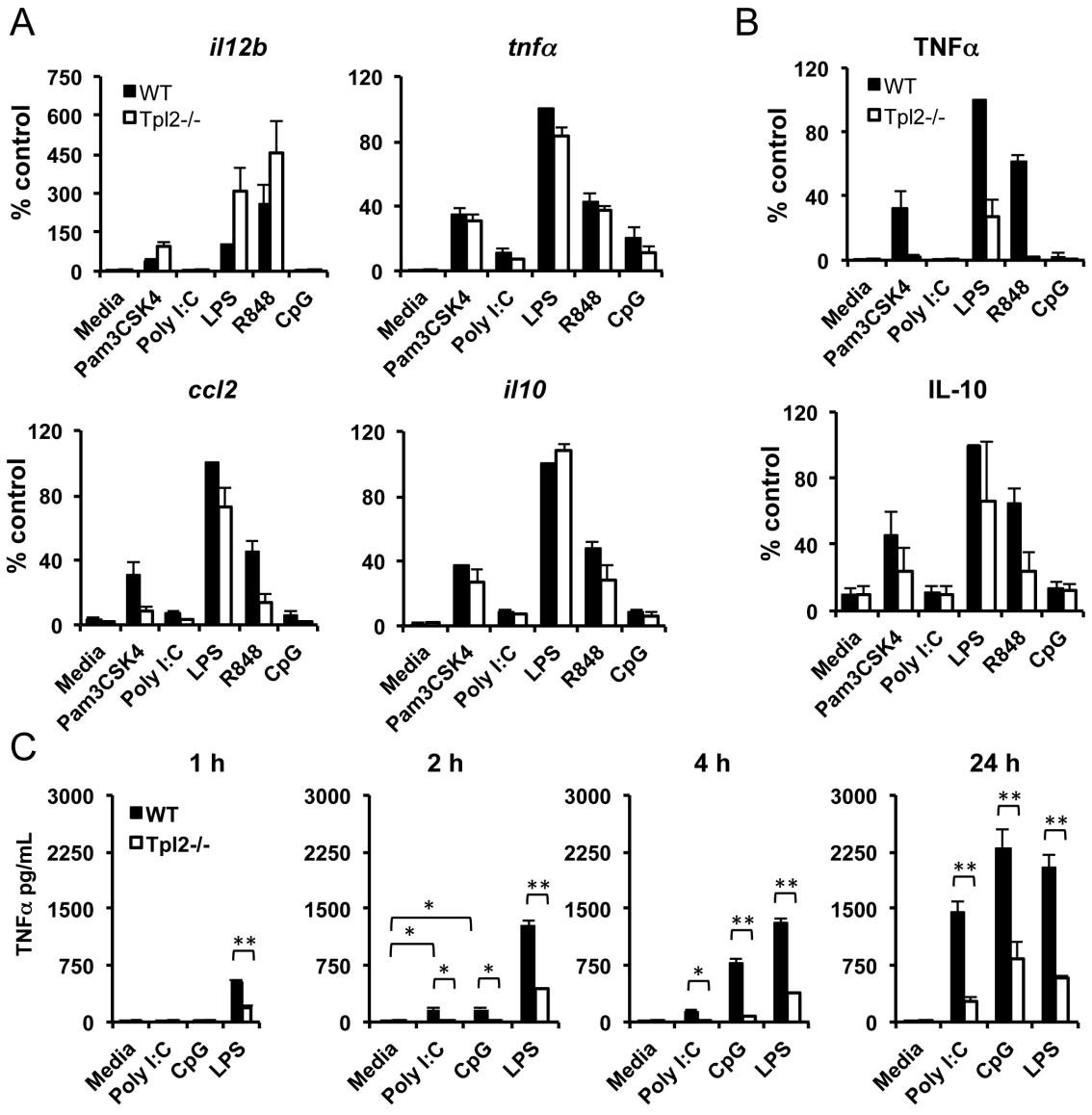


Figure 2.1: Expression of proinflammatory cytokines and early TNFα and IL-10 secretion are restricted to a subset of TLRs. (A) BMDMs from WT and *Tpl2*$^{-/-}$ mice were either left untreated or stimulated with Pam3CSK4, poly I:C, LPS, R848 or CpG for 1 h. Cells were lysed after collecting supernatants, and *il12b*, *tnfa*, *il10 and ccl2* mRNA expression were measured by real-time PCR relative to an actin control. (B) TNFα and IL-10 secretion at 1 h were measured by ELISA. Values are relative to WT cells treated with LPS, which was considered 100% in

individual experiments. (C) BMDMs from WT and *Tpl2*$^{-/-}$ mice were either left untreated or stimulated with poly I:C, LPS or CpG for 1, 2, 4 or 24 h. TNFα levels in cell culture supernatants were measured by ELISA. ** indicates $p<0.01$, *indicates $p<0.05$. For A-C, bars indicate mean ± SEM from 3 - 4 independent experiments.

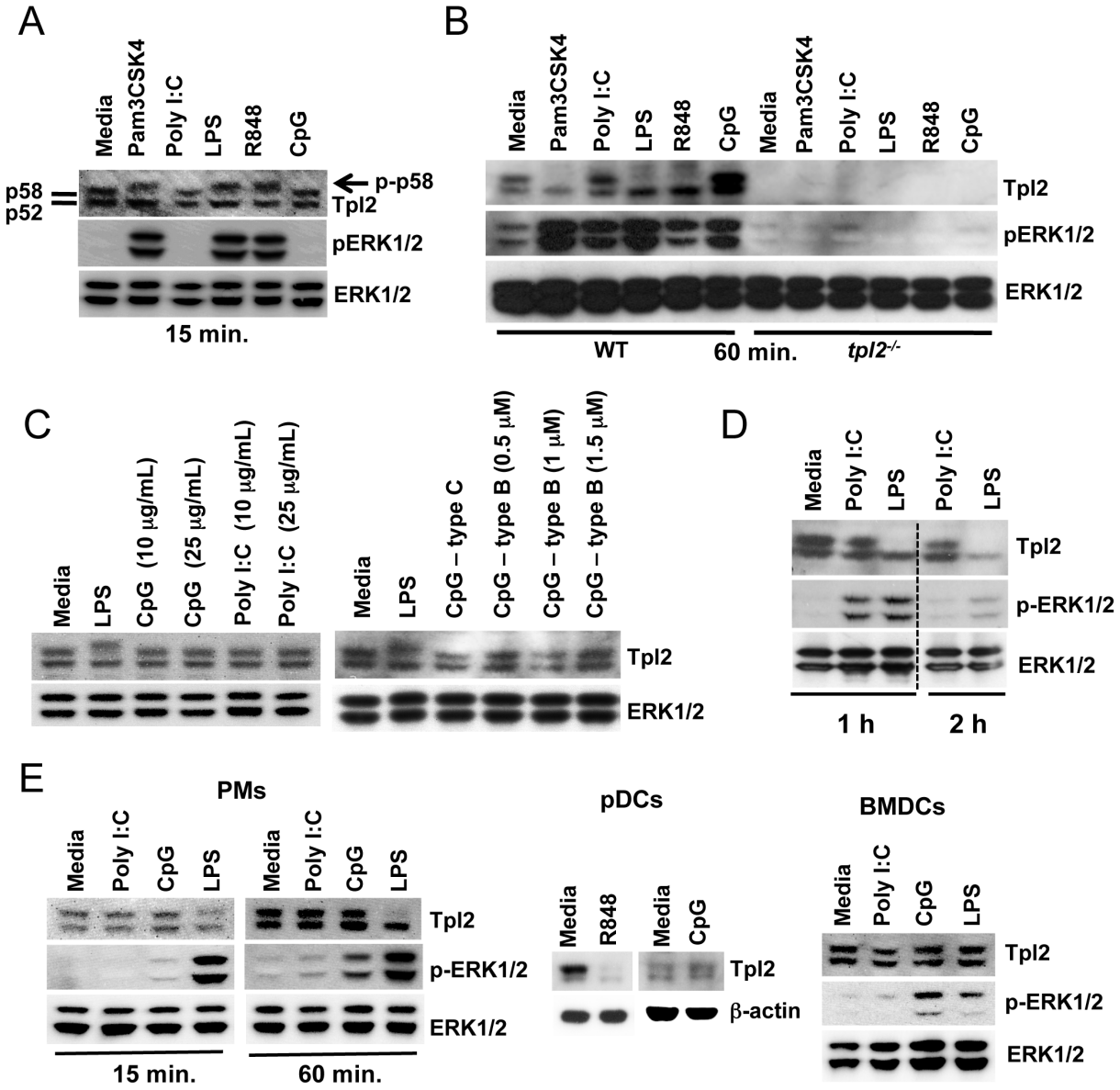


Figure 2.2: Signaling by TLR3 and 9 fails to induce Tpl2-p58 or early ERK phosphorylation. (A) BMDMs from WT mice were left untreated or stimulated with Pam3CSK4, poly I:C, LPS, R848 or CpG ODN for 15 min. **(B)** BMDMs from WT and *Tpl2*$^{-/-}$ mice were left untreated or stimulated with Pam3CSK4, poly I:C, LPS, R848 and CpG ODN for 1 h. (C) BMDMs from WT mice were treated with 10 or 25 μg/mL poly I:C and CpG or 1 μg/mL LPS for 15 min. (left panel) or stimulated with CpG-C ODNs, increasing doses of CpG-B

ODNs or LPS for 15 min. (right panel). (D) BMDMs from WT mice were left untreated or stimulated with poly I:C or LPS for 1 to 2 h. (E) Peritoneal macrophages (PMs), plasmacytoid dendritic cells (pDCs), or bone marrow-derived dendritic cells (BMDCs) from WT mice were left untreated or stimulated with poly I:C, LPS or CpG for 15 min. (A-E) Whole cell lysates (WCL) were immunoblotted with antibodies recognizing Tpl2, pERK1/2 , ERK1/2 and β-actin. Dashed line indicates that within a single exposure intervening lanes were removed. Data are representative of 2-4 independent experiments.

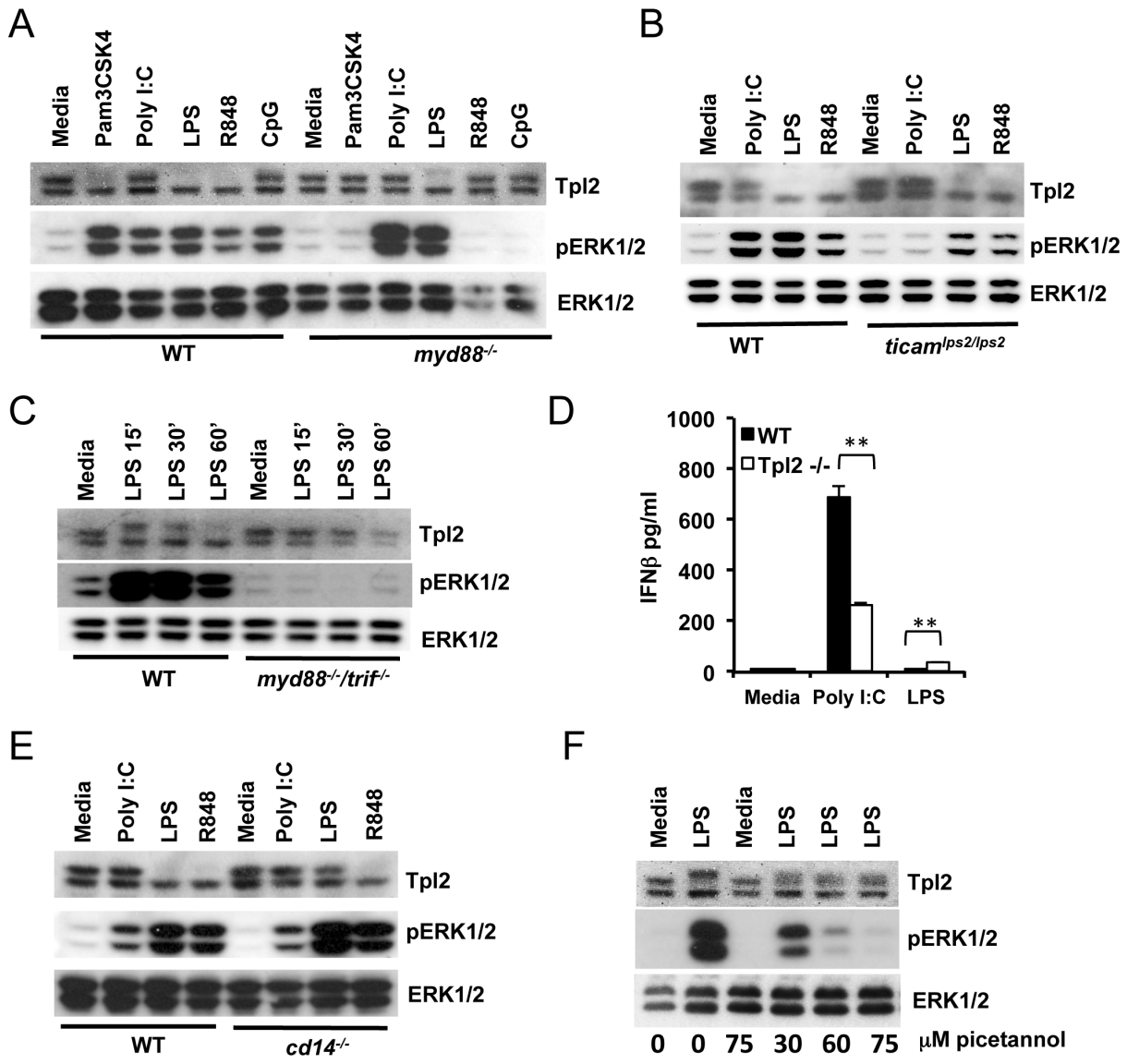


Figure 2.3: Activation of the Tpl2-ERK pathway by TLR4 requires either the MyD88 or TRIF adaptor protein and is regulated by the TLR4 co-receptor, CD14, and the tyrosine kinase Syk. (A) BMDMs from WT and *Myd88*$^{-/-}$ mice were stimulated with Pam3CSK4, poly I:C, LPS, R848 or CpG for 1 h. (B) BMDMs from WT and *Ticam*$^{lps2/lps2}$ mice were stimulated with poly I:C, LPS or R848 for 1 h. (C) BMDMs from WT and *Myd88*$^{-/-}$/*Trif*$^{-/-}$ mice were stimulated with LPS for 15, 30 and 60 min. (D) WT and *Tpl2*$^{-/-}$ BMDMs were treated with poly

I:C or LPS for 24 h. IFNβ levels in cell culture supernatants were measured by ELISA. Bars indicate mean ± SD (E) BMDMs from WT and *Cd14*$^{-/-}$ mice were stimulated with poly I:C, LPS or R848 for 1 h. (F) BMDMs from WT mice were pretreated with piceatannol for 1h before stimulating with LPS for 15 min. WCL were immunoblotted with antibodies recognizing Tpl2, pERK1/2 and ERK1/2. Data are representative of 2-4 independent experiments. ** indicates $p<0.01$.

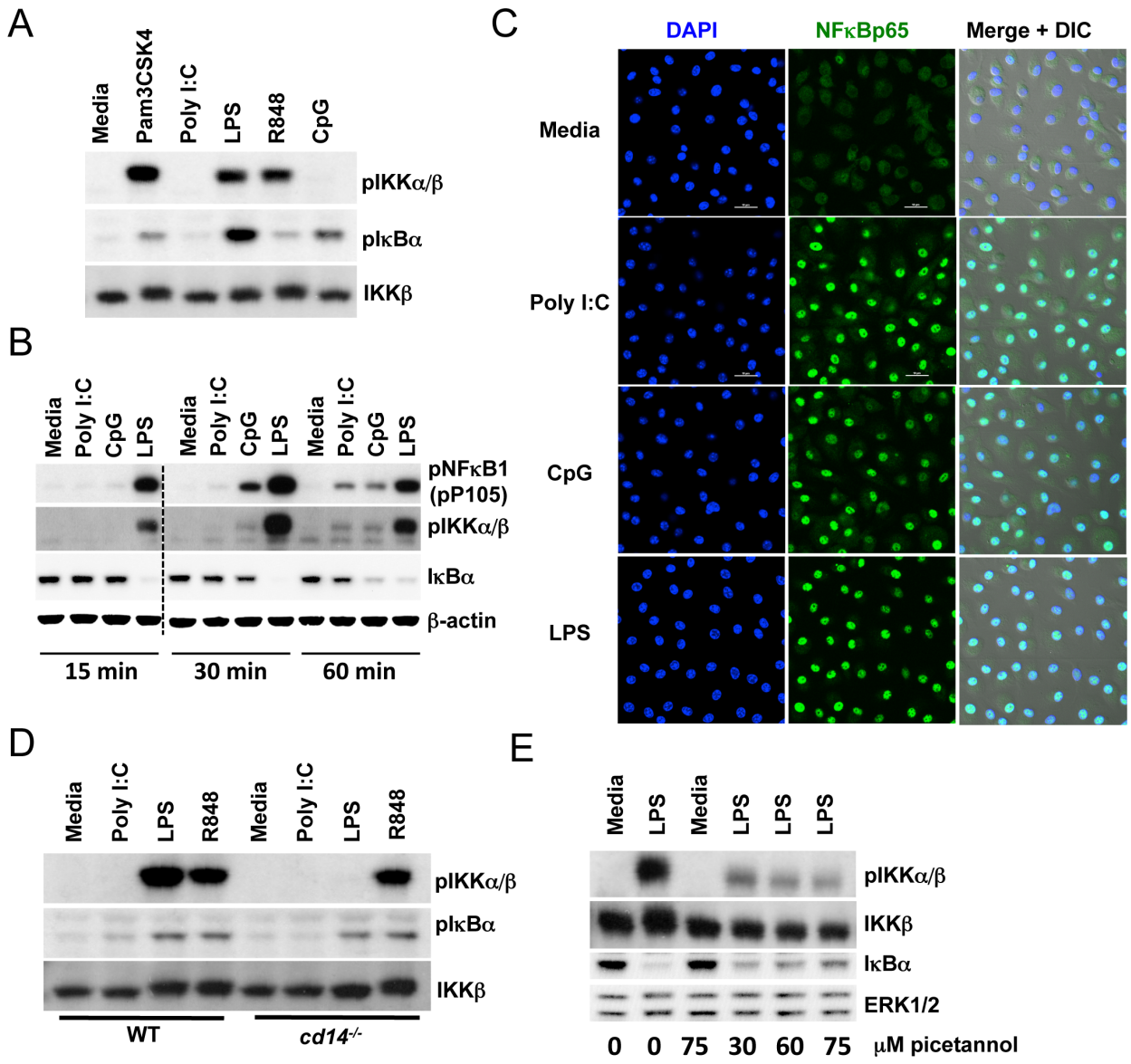


Figure 2.4: Diverse TLRs differentially activate IKKβ. (A) BMDMs from WT mice were left untreated or stimulated with Pam3CSK4, poly I:C, LPS, R848 and CpG for 15 min (B) BMDMs from WT mice were left untreated or stimulated with poly I:C, CpG ODN or LPS for 15 min, 30 min or 1h. WCL were immunoblotted with antibodies recognizing pIKKα/β, IKKβ, pIκBα, pNFκBp105, IκBα, and β-actin. Dashed line indicates that within a single exposure intervening lanes were removed. (C) BMDMs from WT mice were left untreated or stimulated with poly I:C, CpG ODN or LPS for 1 h. Nuclear translocation of NFκB-p65 was assessed by confocal

microscopy. (D) BMDMs from WT and *Cd14*$^{-/-}$ mice were stimulated with poly I:C, LPS or R848 for 30 min. (E) BMDMs from WT mice were pretreated with piceatannol for 1 h before stimulating with LPS for 15 min. (D-E) WCL were immunoblotted with antibodies recognizing pIKKα/β, IKKβ and pIκBα. Data are representative of 2-4 independent experiments.

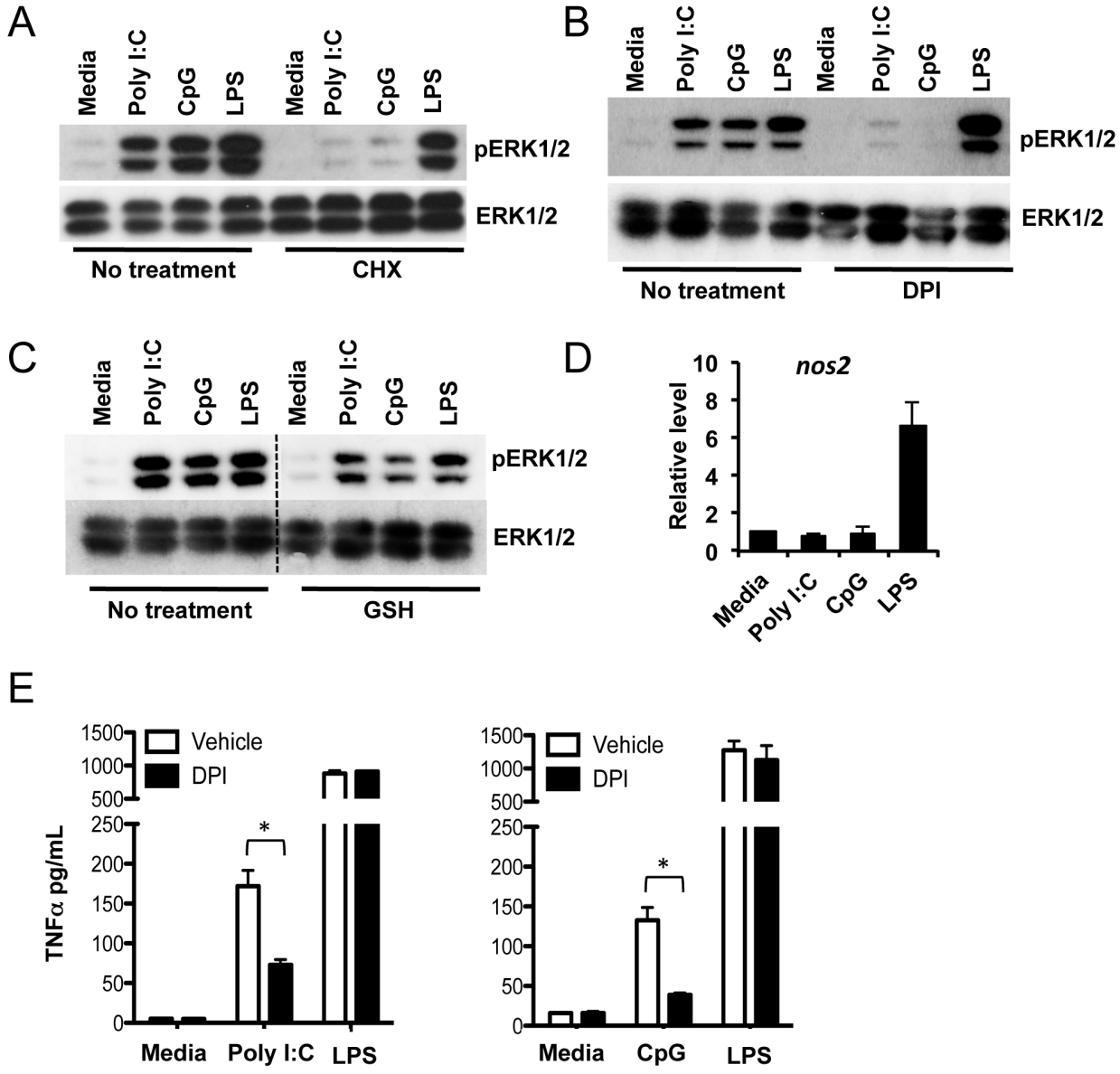


Figure 2.5: TLR3 and 9 activate ERK indirectly via NADPH-oxidase-dependent autocrine ROS signaling. WT BMDMs were pretreated (A) for 30 min with 10 μg/mL cycloheximide (CHX), (B) for 30 min with 20 μM diphenyleneiodonium (DPI), or (C) for 10 min with 10 mM glutathione (GSH) prior to 1 h stimulation with poly I:C, CpG or LPS. Dashed line indicates that within a single exposure intervening lanes were removed. WCL were immunoblotted with antibodies recognizing Tpl2, pERK1/2 and ERK1/2. Data are representative of 3 independent

experiments. (D) BMDMs from WT mice were either left untreated or stimulated with poly I:C, CpG or LPS for 1 h. Cells were lysed and *nos2* mRNA expression was measured by real-time PCR relative to an actin control. (E) WT BMDMs were pretreated for 30 min with 20 μM DPI before stimulation with poly I:C, CpG or LPS for 1 h. TNFα levels in cell culture supernatants were measured by ELISA. Bars indicate mean ± SEM from 3 independent experiments. * indicates $p<0.05$.

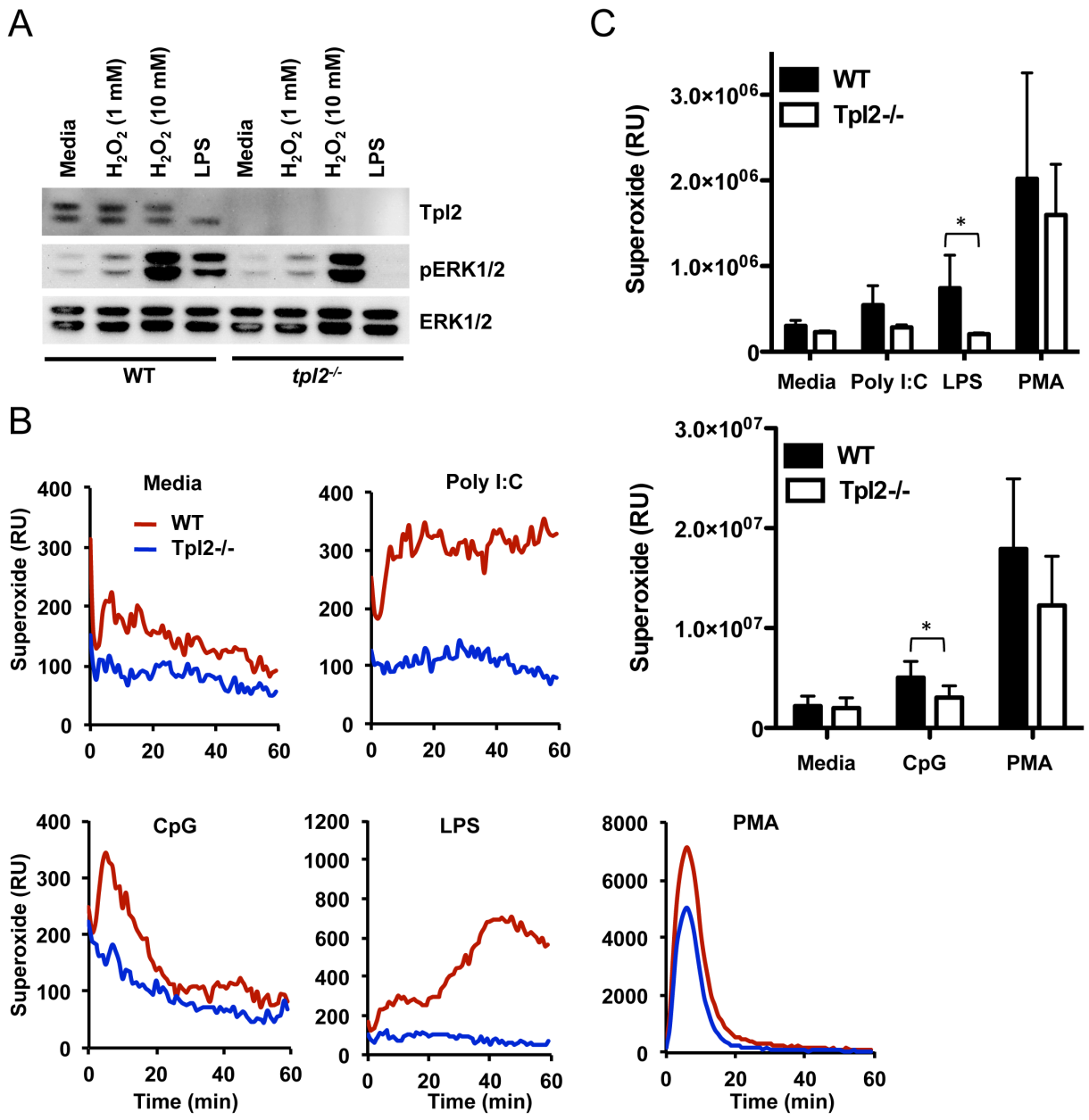


Figure 2.6: Tpl2 is essential for ROS production during TLR signaling. (A) BMDMs from WT and *Tpl2*$^{-/-}$ mice were treated with H_2O_2 (1 or 10 mM) or LPS for 1 h. WCL were immunoblotted with antibodies recognizing Tpl2, pERK1/2 and ERK1/2. Data are representative of 3 independent experiments. (B-C) BMDMs from WT and *Tpl2*$^{-/-}$ mice were treated with poly

I:C, LPS, CpG or PMA, and superoxide production was measured by a lucigenin-based chemiluminescence assay. Kinetics (B) and integrated luminescence data (C) from 3 independent experiments are represented. Bars indicate mean ± SEM. * indicates $p<0.05$.

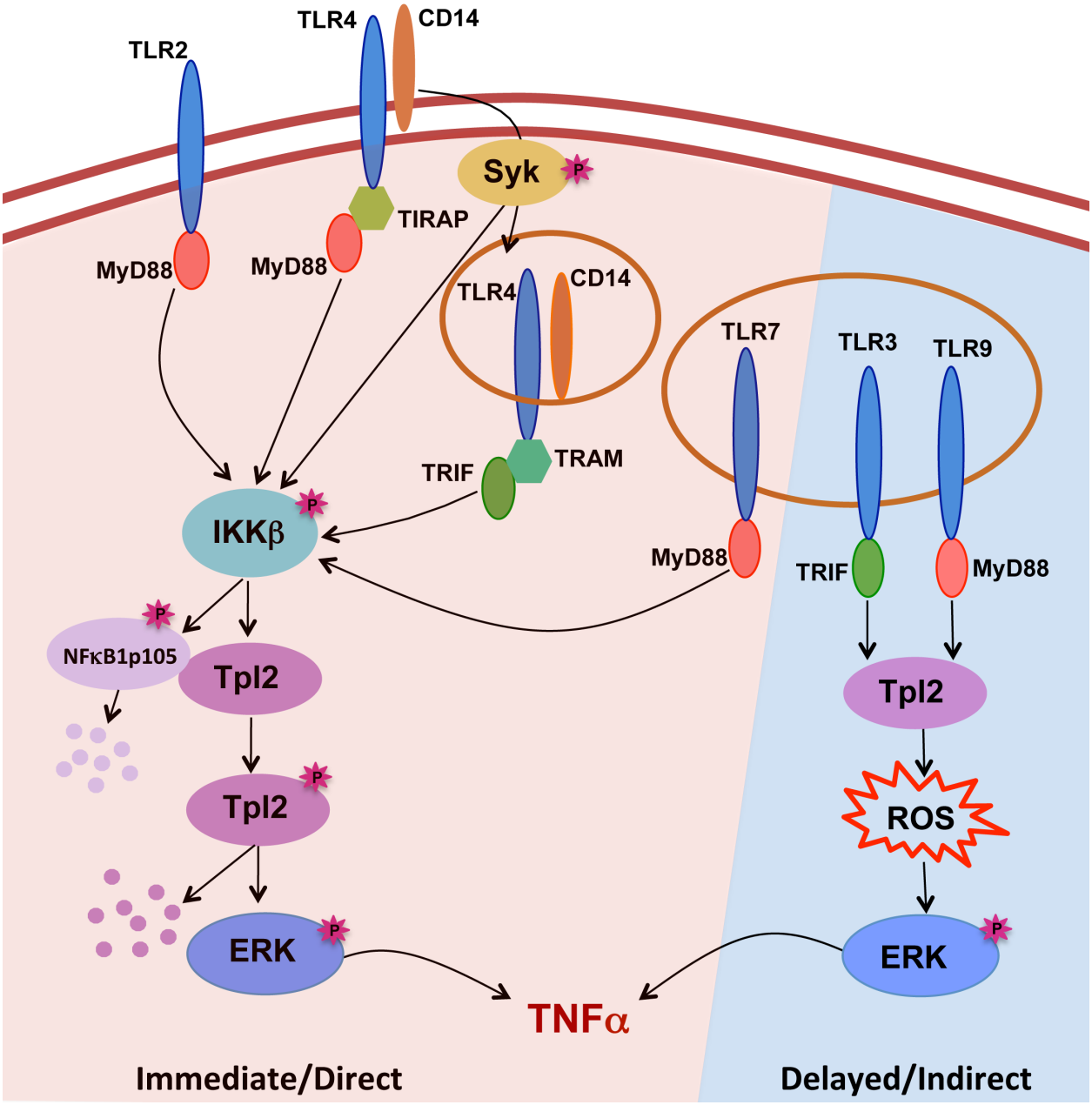
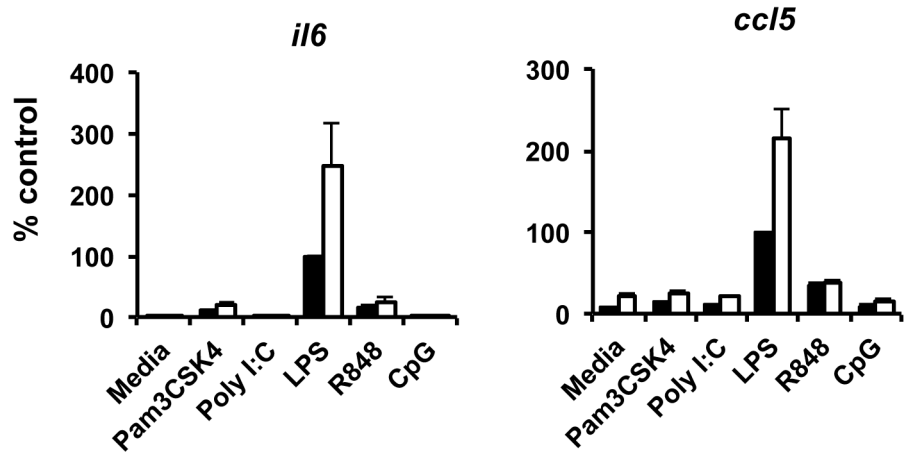
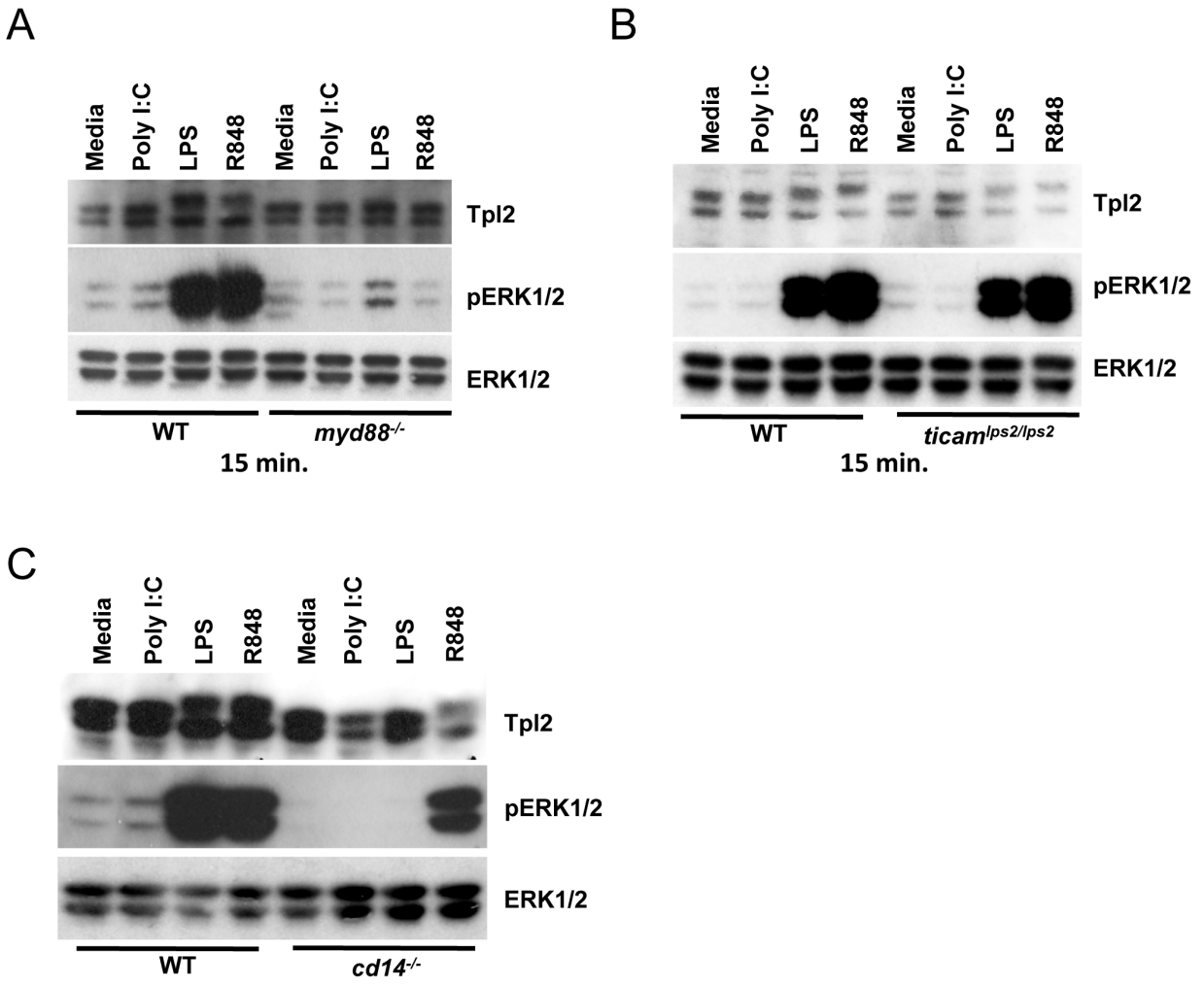


Figure 2.7: Model of Tpl2-ERK activation during TLR signaling. Stimulation of TLR2, 4 and 7 immediately activates the IKKβ-Tpl2-ERK inflammatory pathway. In addition to a TLR adaptor protein, activation of this pathway by TLR4 requires the TLR4 co-receptor CD14 and the tyrosine kinase Syk. Active IKKβ phosphorylates both NF-κB1 and Tpl2-p58 and leads to release of active Tpl2, which in turn phosphorylates ERK prior to Tpl2-p58 proteosomal degradation. ERK signaling facilitates processing and secretion of TNFα. TLR3 and 9 do not

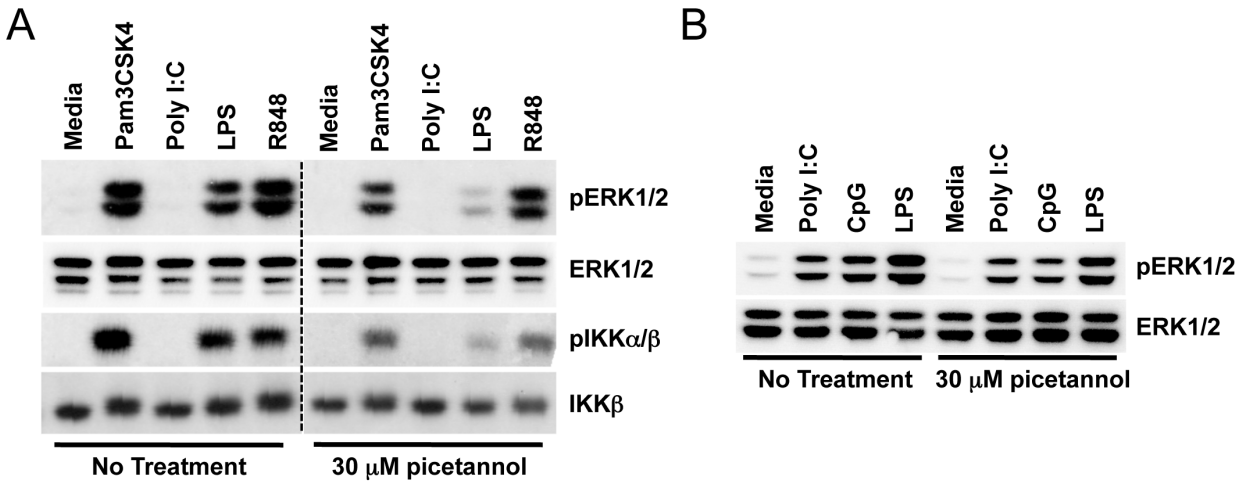
cause Tpl2-p58 phosphorylation-induced mobility shift, degradation or early ERK activation. Instead, TLR3 and 9 induce delayed ERK phosphorylation via autocrine signaling by ROS, which is generated in a Tpl2-dependent manner. Consequently, TLR3 and 9 induced delayed secretion of innate TNFα compared to other TLRs.



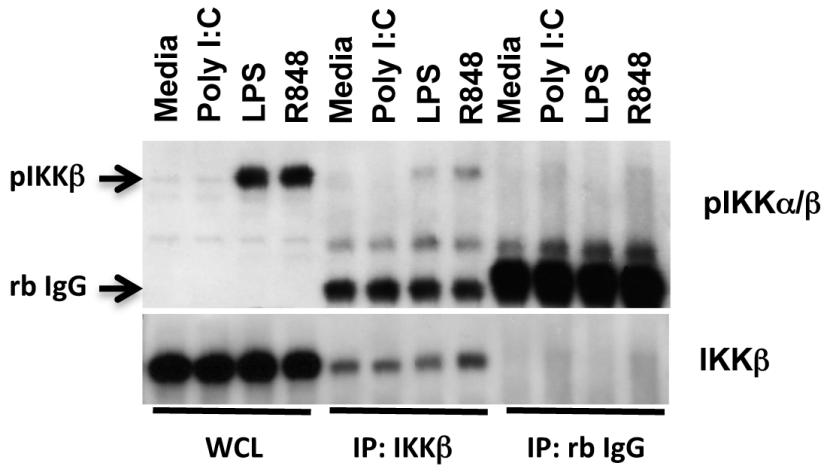
Supplementary Figure 2.8: Early expression of proinflammatory cytokines *il6* and *ccl5* are restricted to a subset of TLRs. (A) BMDMs from WT and *Tpl2*$^{-/-}$ mice were either left untreated or stimulated with Pam3CSK4, poly I:C, LPS, R848 or CpG for 1 h. Cells were lysed after collecting supernatants, and *il6* and *ccl5* mRNA expression were measured by real-time PCR relative to an actin control. Values are relative to WT cells treated with LPS, which was considered 100% in individual experiments. Bars indicate mean ± SEM from 3 - 4 independent experiments.



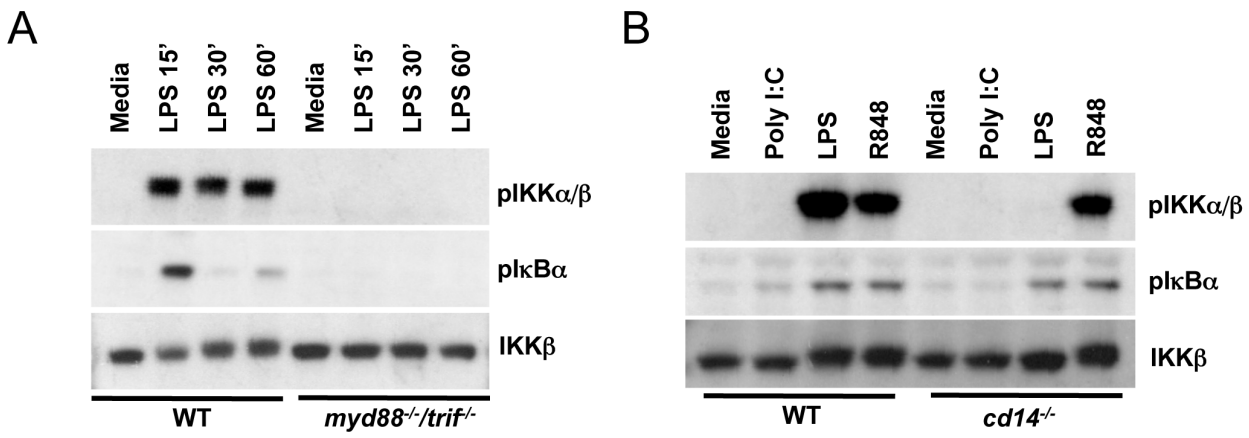
Supplementary Figure 2.9: Tpl2-p58 mobility shift and ERK phosphorylation by LPS occurs independent of either MyD88 or TRIF adaptor protein. (A-B) BMDMs from WT, *Myd88*$^{-/-}$ and *Ticam*$^{lps2/lps2}$ mice were stimulated with Poly I:C, LPS and R848 for 15 min. Note the delayed ERK activation in *Myd88*$^{-/-}$ BMDMs in response to LPS. **C)** BMDMs from WT and *Cd14*$^{-/-}$ mice were stimulated with poly I:C, LPS or R848 for 30 min. WCL were immunoblotted with antibodies recognizing Tpl2, phospho-ERK1/2 and total ERK1/2. Data are representative of at least 2 independent experiments.



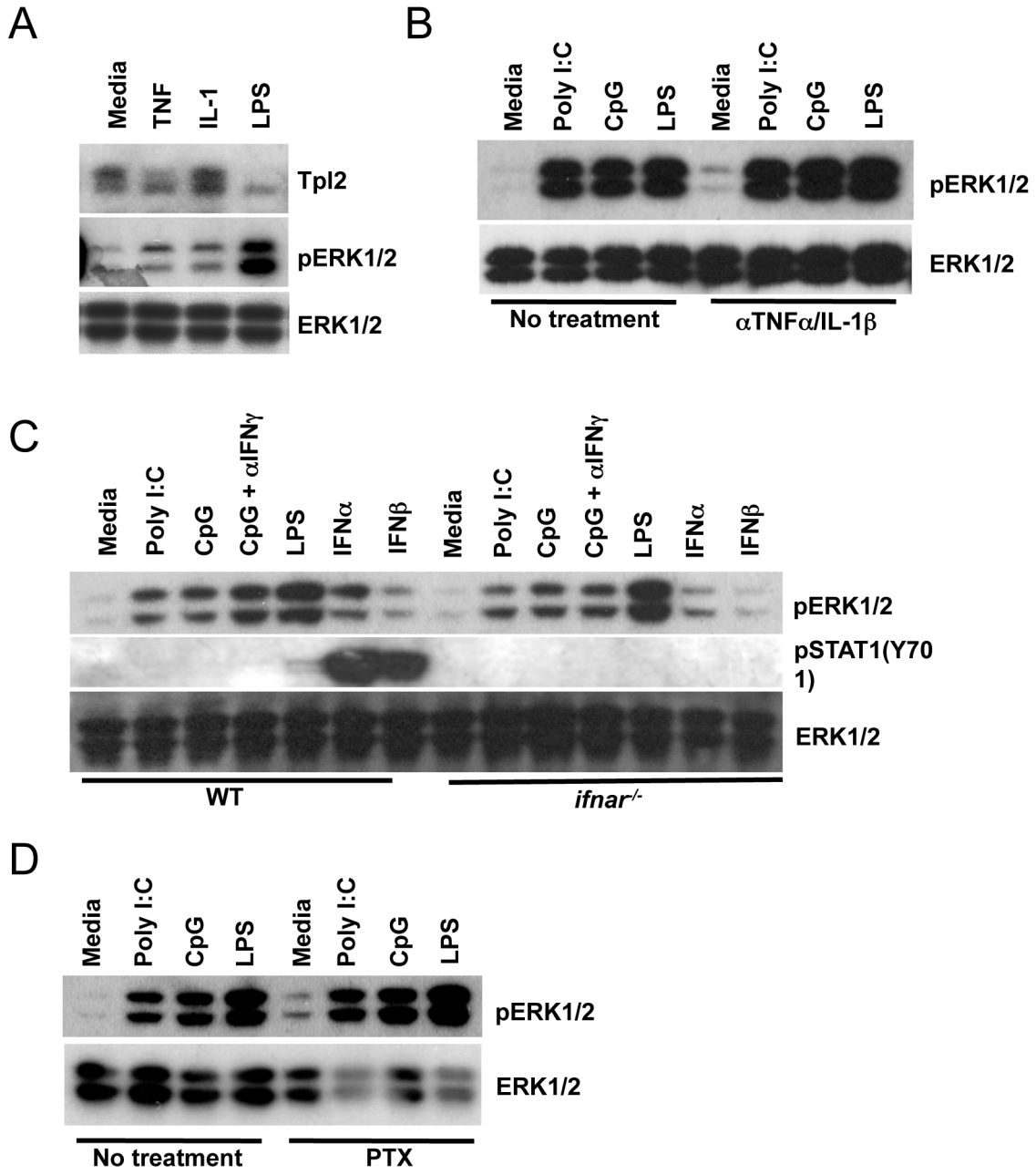
Supplementary Figure 2.10: Syk inhibition of pIKKα/β is selective for the TLR4 pathway. **(A)** BMDMs from WT mice were pretreated with piceatanol for 1hr before stimulating with Pam3CSK4, poly I:C, LPS or R848 for 15 min. **. (B)** BMDMs from WT mice were pretreated with piceatanol for 1hr before stimulating with poly I:C, LPS or CpG for 1 h. WCL were immunoblotted with antibodies recognizing phospho-ERK1/2, total ERK1/2, phospho-IKKα/β and total IKKβ. Data are representative of 3 independent experiments.



Supplementary Figure 2.11: LPS and R848, but not poly I:C, phosphorylates IKKβ. (A) BMDMs from WT mice were treated with poly I:C, LPS or R848 for 15 min. Whole cell lysates were subjected to immunoprecipitation with 1:100 IKKβ antibody or a control rabbit IgG antibody. Immunoprecipitates were washed, boiled in sample buffer and separated by SDS PAGE. WCL or immunoprecipitated proteins were immunoblotted with antibodies recognizing phospho-IKKα/β or total IKKβ. Data are representative of 2 independent experiments.



Supplementary Figure 2.12: Phosphorylation of IKKβ during TLR4 signaling requires either MyD88 or TRIF adaptor proteins and TLR4 co receptor CD14. (A) BMDMs from WT and *Myd88*$^{-/-}$*/Trif*$^{-/-}$ mice were left untreated or stimulated with LPS for 15, 30 or 60 min. **(B)** BMDMs from WT and *Cd14*$^{-/-}$ mice were stimulated with poly I:C, LPS or R848 for 30 min. WCL were immunoblotted with antibodies recognizing pIKKα/β, pIκBα, and IKKβ. Data are representative of 2-4 independent experiments.



Supplementary Figure. 2.13: TLR3- and TLR9-mediated ERK activation occurs independently of autocrine signaling by TNFα, IL-1β, type I interferons and chemokines. **(A)** BMDMs from WT mice were either left untreated or treated with TNFα or IL-1β for 1 hr. **(B)** BMDMs from WT mice were either left untreated or treated with aTNF/IL-1β before stimulating with poly I:C, CpG or LPS for 1 hr. **(C)** BMDMs from WT and *Ifnar1*$^{-/-}$ mice were

stimulated with Poly I:C, CpG, CpG + anti-IFNγ, LPS, IFNα and IFNβ for 1 hr. **(D)** BMDMs from WT mice were pretreated with pertussis toxin for 30 min before stimulating with poly I:C, CpG or LPS for 1 hr. WCL were immunoblotted with antibodies recognizing Tpl2, phospho-ERK1/2, pSTAT1(Y701) and total ERK1/2. Data are representative of 2 independent experiments.

CHAPTER 3

TUMOR PROGRESSION LOCUS 2 PROMOTES ANTIVIRAL INTERFERON PRODUCTION, SIGNAL TRANSDUCTION AND $CD8^+$ T CELL RESPONSES TO PROTECT AGAINST INFLUENZA VIRUS

Teneema Kuriakose, Ralph A. Tripp and Wendy T. Watford. Submitted to *PLOS Pathogens* (In revision)

ABSTRACT

Mitogen-activated protein kinase (MAP) cascades are important in antiviral immunity through their regulation of interferon (IFN) production as well as virus replication. Although the serine-threonine MAP kinase tumor progression locus 2 (Tpl2/MAP3K8) has been implicated as a key regulator of Type I (IFNα/β) and Type II (IFNγ) IFNs, remarkably little is known about how Tpl2 might contribute to host defense against viruses. Herein, we investigated the role of Tpl2 in antiviral immune responses against influenza virus. We demonstrate that Tpl2 is an integral component of multiple virus sensing pathways, differentially regulating the induction of IFNα/β and IFNλ in a cell-type specific manner. We also identified both Tpl2/ERK and PI3K/mTOR/Akt pathways as positive regulators of TLR-mediated IFNλ induction in plasmacytoid dendritic cells. Although Tpl2 is important in the regulation of both IFNα/β and IFNλ, only IFNλ required Tpl2 for its induction during influenza virus infection both *in vitro* and *in vivo*. Further studies revealed an unanticipated function for Tpl2 in transducing Type I IFN signals that promote the expression of antiviral genes, including IFNλ and interferon-stimulated genes (ISGs), to limit virus replication. In addition to early innate alterations, impaired expansion of virus-specific $CD8^+$ T cells accompanied delayed viral clearance in *Tpl2*$^{-/-}$ mice. Consistent with its critical role in facilitating both innate and adaptive antiviral responses, Tpl2 was necessary for controlling virus replication and restricting morbidity and mortality associated with influenza virus infection. Collectively, these findings establish an essential role for Tpl2 in antiviral host defense mechanisms.

AUTHOR SUMMARY

Influenza virus infects millions of people annually causing significant morbidity, mortality and socio-economic burdens. Host immune responses against influenza virus are initiated upon virus recognition by specific intracellular receptors. Signals relayed from these receptors trigger various signaling cascades, which induce an antiviral immune response to control infection. Herein, we identified the serine-threonine kinase tumor progression locus 2 (Tpl2) as an essential component of virus sensing pathways, regulating induction of interferons (IFNs) and IFN-induced antiviral genes that restrict virus replication. We also demonstrate that Tpl2 is necessary for generation of effector $CD8^+$ T cells, which are required for viral clearance from infected lungs. Consistent with the impaired antiviral responses, Tpl2-deficient mice are defective in controlling virus replication and succumb to influenza virus infection with a normally low pathogenicity strain. Thus, our study identified Tpl2 as a host viral restriction factor that integrates antiviral innate and adaptive responses that restrict morbidity and mortality during influenza virus infection.

INTRODUCTION

Mitogen-activated protein kinase (MAP) cascades represent major intracellular signaling pathways activated in response to a variety of external stimuli. Their activation during infection leads to transcriptional induction of immune and inflammatory mediators. Although MAP kinase signaling is important in eliciting host protective responses, many viruses are known to utilize these pathways directly for their replication (1). Activation of MAP kinases occurs during virus recognition by pattern recognition receptors (PRRs) like toll-like receptors (TLRs) and RIG-I-like RNA helicases (RLH) (2). Virus sensing by these receptors activates multiple intracellular signaling cascades including NFκB, MAP kinase and IRF pathways that coordinately regulate induction of interferons (IFNs) which are important mediators of antiviral resistance (3). Among the MAP kinases, the MAP3 kinase tumor progression locus 2 (Tpl2/MAP3K8) plays an important role in regulating IFN production by promoting the ERK-dependent induction of *c-fos*, a component of AP-1 heterodimeric transcription factors (4). While Tpl2 is required for IFNα production by plasmacytoid dendritic cells (pDCs) and IFNγ secretion by $CD4^+$ T cells, it is a potent negative regulator of IFNβ in macrophages and DCs (4,5). Despite being identified as a major regulator of both Type I (IFNα/β) and Type II (IFNγ) IFNs, Tpl2 regulation of Type III IFNs (IFNλs) has not been investigated so far.

Tpl2 was initially identified as an oncogene that induces T cell lymphomas in rodents (6), but more recent studies have established its criticality in regulating both innate and adaptive immune responses via its cell type- and stimulus-specific activation of the ERK MAPK pathway. Tpl2 regulates signal transduction and cellular responses downstream of TLRs, cytokine receptors, antigen receptors and G protein-coupled receptors (4,7-9). In addition to IFNs, Tpl2 also regulates the production of other prominent immune mediators like TNFα, IL-1β, IL-10, IL-12

and COX-2 (4,10-12). Consequently, Tpl2 is essential for mounting effective immune responses during infections, and *Tpl2*$^{-/-}$ mice are more susceptible to *Toxoplasma gondii* (5), *Listeria monocytogenes* (11), *Mycobacterium tuberculosis* (13) and *Group B Streptococcus* (14). Surprisingly, there is still limited and contradictory information about how Tpl2 contributes to host defense against viruses. Early studies reported normal cytotoxic T cell responses against lymphocytic choriomeningitis virus (10) and resistance to mouse cytomegalovirus infection (14). However, another study delineating the signaling circuitry in virus sensing pathways implicated Tpl2 as a key regulator of both inflammatory and antiviral gene induction in response to model viral ligands (15). A recent study also reported increased replication of vesicular stomatitis virus in Tpl2-deficient mouse embryonic fibroblasts (MEFs) (16).

We recently demonstrated that among the TLRs implicated in virus sensing (TLRs 3, 7 and 9), Tpl2 plays a prominent role in TLR7 signaling (17). In this study, we investigated Tpl2's regulation of antiviral responses using a murine model of influenza virus infection, which relies upon TLR7 for virus sensing (18), ERK MAP kinase for virus replication (19) and where both IFNα/β and IFNλ are host protective (20). Our experiments demonstrate positive regulation of IFNλ and cell-type specific regulation of IFNα/β production in Tpl2-deficient cells following stimulation with model viral ligands that trigger influenza virus sensing receptors, TLR7 or RIG-I. However, during influenza virus infection IFNλ uniquely required Tpl2 for its induction. Moreover, Tpl2 directly transduced IFN signals, including ERK activation and STAT1^{ser727} phosphorylation, and was required for proper induction of antiviral ISGs. Impaired ISG induction coupled with reduced antigen-specific $CD8^+$ T cells resulted in failure to control virus replication and resulted in significant morbidity and mortality of *Tpl2*$^{-/-}$ mice to an otherwise low

pathogenicity strain of influenza. Collectively, this study establishes Tpl2 as a host-derived viral restriction factor that integrates antiviral responses to control influenza virus infection.

RESULTS

Tpl2 ablation enhances virus replication and inflammatory responses during influenza infection.

To determine whether Tpl2 regulates influenza virus replication, wild type (WT) and *Tpl2*$^{-/-}$ mice were infected with 10^4 plaque forming units (pfu) of mouse-adapted influenza virus A/HK-X31(H3N2) (X31), and viral titers in the lungs were evaluated on days 3, 5 and 7 post infection (pi). The lung viral titers were significantly higher in *Tpl2*$^{-/-}$ mice compared to WT mice at all time-points examined (Figure 3.1A). Notably, viral titers were more than ten-fold higher in *Tpl2*$^{-/-}$ lungs at day 7 pi. Consistent with increased virus replication, total cellular infiltration was significantly increased in the lungs of *Tpl2*$^{-/-}$ mice at day 7 pi (Figure 3.1B). This increase in virus replication was also observed in littermate control mice (Supplementary figure 3.9). In addition to viral titers, all proinflammatory cytokines, except TNFα, were significantly higher in the BALF of *Tpl2*$^{-/-}$ mice compared to WT mice (Figure 3.1C). The increased lung viral titers in *Tpl2*$^{-/-}$ mice early after infection on day 3 suggest a critical role for Tpl2 in limiting virus replication during influenza virus infection.

Tpl2 signaling in airway epithelial cells is necessary for limiting virus replication.

Airway epithelial cells are the primary targets for influenza virus infection. Early studies after the discovery of Tpl2 demonstrated high levels of Tpl2 expression in the lungs (21). Moreover, Tpl2 regulation of signal transduction and cytokine gene induction was also demonstrated in airway epithelial cells (22). To elucidate whether Tpl2 function in hematopoietic or stromal cell

compartment is important in limiting virus replication, we assessed lung viral titers in chimeric mice where WT bone marrow cells where grafted into either WT or *Tpl2*$^{-/-}$ recipients. At day 3 pi, lung viral titers were significantly high in *Tpl2*$^{-/-}$ mice reconstituted with WT hematopoietic cells (Figure 3.1D). This data demonstrate that Tpl2 signaling in the infected lung epithelia is necessary for limiting virus replication early after infection.

Tpl2 is required for optimal IFNλ production during influenza infection *in vivo* and *in vitro*.

Interferons are induced early during infection and are key factors initiating host protective antiviral responses (3). To determine whether the observed increase in viral titers in *Tpl2*$^{-/-}$ mice is due to defective induction of IFNs, WT and *Tpl2*$^{-/-}$ mice were infected with 10^6 pfu X31, and IFNα/β/λ levels in lung homogenate or BALF were measured at day 1 or day 3 pi. Induction of both IFNα and β were comparable between WT and *Tpl2*$^{-/-}$ lung homogenates and BALF (Figure 3.2A). Notably, IFNλ secretion was significantly reduced in *Tpl2*$^{-/-}$ mice following influenza virus infection (Figure 3.2B). Surprisingly, while IFNλ was induced to a much higher level compared to Type I IFNs in WT mice, there was minimal induction in *Tpl2*$^{-/-}$ mice in response to infection. Despite differences in IFN induction, total cellular infiltration and IFNγ levels in BALF were significantly elevated in *Tpl2*$^{-/-}$ mice compared to WT mice at day 3 pi (Supplementary Figure 3.10).

The observation that Tpl2 is uniquely required for IFNλ, but not IFNα or IFNβ, production in influenza-infected lungs is especially significant, because IFNλ is regarded as the principal IFN induced during influenza virus infection In addition to airway epithelium, pDCs are also considered to be a major source of IFNs during respiratory virus infections, including influenza, and the contribution of pDCs to IFN production in influenza virus-infected lungs is well

established (23). (20). Although we observed decrease in IFNλ levels at day 1 pi, a more consistent and significant reduction was observed at day 3 pi, which corresponds to the migration of pDCs to infected lungs (23). Since Tpl2 is required for macrophage and neutrophil migration during acute inflammation (9,24), we investigated whether Tpl2 similarly regulates the recruitment of pDCs to the infected lung. The reduction in IFNλ levels in influenza-infected *Tpl2*$^{-/-}$ mice was not due to impaired recruitment of pDCs, because the proportions and absolute numbers of pDCs recruited to the lungs were similar between WT and *Tpl2*$^{-/-}$ mice (Supplementary Figure 3.11). To investigate whether defective IFN induction in pDCs contributes to the reduced IFNλ in BALF from *Tpl2*$^{-/-}$ mice during influenza infection, bone marrow-derived pDCs (CD11c^{+}B220^{+}CD11b^{-}) from WT and *Tpl2*$^{-/-}$ mice were infected with influenza virus A/WSN/1933 (H1N1), and the production of IFNα, β and λ was assessed. Consistent with *in vivo* infections, the levels of both IFNα and IFNβ were comparable between WT and *Tpl2*$^{-/-}$ cells whereas IFNλ secretion was significantly less in *Tpl2*$^{-/-}$ pDCs infected with influenza virus (Figure 3.2C). A similar decrease in IFNλ production was also observed in Tpl2-deficient cells infected with X31 influenza virus (Supplementary Figure 3.12). Interestingly, no impairement in IFNλ production at day 3 pi was observed in *Tpl2*$^{-/-}$ mice reconstituted with WT hematopoietic cells (Figure 3.2 D-E). This suggests that Tpl2-dependent IFNλ induction in pDCs is important in maintaining high levels of IFNλ in influenza virus-infected lungs. Collectively, these data demonstrate the unique requirement of Tpl2 in IFNλ production during influenza infection *in vitro* and *in vivo*.

Tpl2 differentially regulates IFN production in response to model viral ligands in a cell type-specific manner.

During influenza virus infection, receptors of both TLR and RLR families recognize viral PAMPs and trigger rapid induction of IFNs. Recognition of viral components by PRRs typically occurs by respiratory epithelial cells, alveolar macrophages, DCs and plasmacytoid DCs (pDCs) in a cell type-specific manner (25). The major receptors involved in recognition of influenza virus are TLR7, which recognizes single-stranded viral RNA, and RIG-I, which recognizes the 5'-triphosphate of single-stranded RNA genomes (5'ppp RNA). The single-stranded RNA genome is recognized through endosomal TLR7 in pDCs (18) in contrast to epithelial cells and DCs where virus recognition is mediated primarily by the cytosolic sensor RIG-I (26). To investigate whether differential regulation of IFN production observed during infection is due to differences in Tpl2 regulation of PRRs involved in viral sensing, bone marrow-derived macrophages (BMDMs) and MEFs from WT and *Tpl2*$^{-/-}$ mice were either transfected with the RIG-I ligand 5'ppp RNA or stimulated with the TLR7 ligand R848, and IFN production was measured by ELISA. Consistent with previous studies using the TLR4 ligand LPS (4), IFNβ production was significantly increased in *Tpl2*$^{-/-}$ cells treated with both 5'ppp RNA and R848 (Figure 3.3A-C). This increase in IFNβ levels correlated with impaired ERK phosphorylation and *fos* induction in Tpl2-deficient cells in response to these ligands (Supplementary Figure 3.13). To determine whether Tpl2 regulates TLR7-mediated IFN production by pDCs, bone marrow-derived pDCs from WT and *Tpl2*$^{-/-}$ mice were treated with the TLR7 ligand, R848, and IFN levels were quantitated. Consistent with previous studies using the TLR9 ligand CpG (4), and in contrast to BMDMs, secretion of both IFNα and IFNβ (Figure 3.3D) were significantly decreased in culture supernatants from *Tpl2*$^{-/-}$ pDCs treated with R848. Notably, IFNλ secretion

was also significantly less in *Tpl2*$^{-/-}$ pDCs compared to WT cells in response to R848 (Figure 3.3D). Unlike *ifna* and similar to NFκB-regulated *il12p40* and *tnfa* (27), *ifnl3* transcription occurred early, by 2 hr of stimulation (Supplementary Figure 3.14). Collectively, these data demonstrate that Tpl2 regulates IFN production downstream of PRRs involved in influenza virus sensing in a cell type-specific manner.

Tpl2 positively regulates IFNλ production in pDCs in an ERK- and PI3K/mTOR/Akt-dependent manner.

The importance of IFNλs in host protection against many viruses is well established, however, the mechanisms that regulate their production are largely unexplored. Common mechanisms have been postulated to regulate Type I and III IFNs during viral infections (28,29). Despite their importance in mediating Type I IFN production in pDCs (4,30), the significance of MAP kinase and PI3 kinase cascades in murine IFNλ production has not been directly investigated. In order to elucidate the potential mechanism by which Tpl2 regulates IFNλ production in pDCs, we evaluated the involvement of ERK and PI3K-mTOR signaling in IFNλ induction. Tpl2 regulation of both ERK and mTOR-Akt signaling in different cell types has been reported before (8,31-33). In addition to the MEK/ERK pathway (4), Tpl2 also promotes mTOR/Akt signaling in pDCs as determined by a decrease in the proportion of phospho-Akt^{+} pDCs in the absence of Tpl2 signaling (Figure 3.4A-B). To confirm whether ERK, PI3K or mTOR signaling also contributes to IFNλ production in pDCs, cells were pre-treated with rapamycin (mTOR inhibitor), LY294002 (PI3K inhibitor) or U0126 (MEK inhibitor) 30 min prior to TLR stimulation, and CpG-induced IFNλ secretion was measured by ELSIA. CpG was used as the stimulant in these experiments because TLR9 ligation induced higher levels of IFNλ compared to TLR7 stimulation with R848. Phamacological inhibition of these signaling pathways

significantly reduced IFNλ secretion, and these levels were comparable to that observed in *Tpl2*$^{-/-}$ cells (Figure 3.4C). These results demonstrate the significance of both MAP kinase and PI3 kinase signaling cascades in regulating IFNλ production in pDCs.

Tpl2 promotes IFNAR-mediated IFNλ production in pDCs.

Robust production of Type I IFNs in pDCs is dependent upon IRF7 and autocrine IFN signaling, and consequently IFNα secretion is abrogated in both *irf7*$^{-/-}$ and *ifnar1*$^{-/-}$ pDCs (34). Similar to IFNα, and as reported previously (20), IFNλ production was abolished in *ifnar1*$^{-/-}$ pDCs infected with influenza virus (Figure 3.5A) demonstrating the absolute requirement for IFNAR signaling in IFNλ secretion by pDCs. Induction of IFNλ in response to direct IFN stimulation has been reported in hepatocyte carcinoma HepG2 cell lines (35). Although a high dose of IFNβ directly induced modest IFNλ secretion, the levels induced were lower than that induced by TLR-stimulation, demonstrating that IFN/IRF7 signaling alone is not sufficient for driving high levels of IFNλ secretion (Figure 3.5B). Nevertheless, Tpl2 contributed to IFNAR-induced IFNλ production, since significantly less IFNλ was secreted by *Tpl2*$^{-/-}$ pDCs directly treated with IFNβ (Figure 3.5B). In addition to demonstrating the role of Tpl2 in IFNAR-mediated IFNλ production, these data also suggest a role for Tpl2 in directly transducing Type I IFN signals.

Tpl2 mediates IFN signaling and induction of IFN-stimulated genes (ISGs).

Both IFNα/β and IFNλ are known to induce expression of ISGs that establish an antiviral state in infected tissue to prevent virus replication and spread (36). Because of the involvement of Tpl2 in IFNAR signaling (Figure 3.5B) and because of the observed increase in early virus replication in *Tpl2*$^{-/-}$ mice (Figure 3.1A), we questioned whether Tpl2 regulates IFN signaling and induction of ISGs. To test whether Tpl2 regulates IFN signaling, BMDMs from WT and *Tpl2*$^{-/-}$ mice were stimulated with IFNα or IFNβ, and activation of downstream cascades, especially STAT1, which

is the principle regulator of IFN responses, were evaluated by immunoblotting. BMDMs were used in these experiments due to limited availability of pDCs. The phosphorylation of STAT1 Tyr701 and Ser727 residues, which is necessary for maximal STAT1 transcriptional activation, were examined (37). While phosphorylation of Tyr701 was increased in *Tpl2*$^{-/-}$ compared to WT cells in response to stimulation with Type I IFNs, a reproducible and statistically significant reduction in Ser727 phosphorylation was observed in *Tpl2*$^{-/-}$ cells (Figure 3.6A-B). In addition to the classical JAK-STAT pathway, signaling via the Type I IFN receptor also activates other downstream cascades including MAP kinases (38). Despite the existence of multiple MAP3 kinases, Tpl2 has an essential, non-redundant role in transducing ERK activation signals during TLR, TNF- and IL-1-receptor signaling (7,8). We therefore investigated whether Tpl2 is similarly required for ERK activation during Type I IFN signaling, or whether other MAP3Ks could fulfill this role. ERK phosphorylation was strongly induced by both IFNα and IFNβ. Importantly, ERK phosphorylation was absent in *Tpl2*$^{-/-}$ BMDMs stimulated with IFNα/β demonstrating an absolute requirement for Tpl2 in transducing ERK activation signals in response to Type I IFNs (Figure 3.6A). Of note, unlike LPS- and TNFα-treated BMDMs and similar to poly I:C-, CpG-, and IL-1β-treated BMDMs (17,39), no mobility shift (indicative of phosphorylation) or degradation of the p58 isoform of Tpl2 was detected following stimulation with Type I IFNs (Figure 3.6A). Consistent with our previous studies (40), both Tpl2 protein and mRNA expression were induced upon either IFNβ stimulation or influenza virus infection (Figure 3.6A and Supplementary Figure 3.15). To investigate whether the impairment in IFN signaling in *Tpl2*$^{-/-}$ cells influenced the induction of ISGs, the expression of several ISGs known to be important in limiting influenza virus infection was determined (25). BMDMs were treated with IFNβ, and the expression of *ifitm3*, *isg15* and *oasl2* were measured over a period of 4 hrs.

While the induction of *ifitm3* and *oasl2* gene expression was significantly less in *Tpl2*$^{-/-}$ cells, the expression of *isg15* was unaffected by Tpl2 ablation (Figure 3.6C). Collectively, these data demonstrate that Tpl2 promotes ERK and STAT1^{Ser727} phosphorylation and the transcriptional induction of ISGs during Type I IFN signaling.

Since ISGs are major contributors to antiviral resistance, the induction of ISGs in lungs was investigated to determine if this was similarly impaired in *Tpl2*$^{-/-}$ mice following influenza virus infection. Consistent with impaired expression in IFNβ-treated Tpl2-deficient BMDMs, *ifitm3* and *oasl2* were expressed at significantly reduced levels in *Tpl2*$^{-/-}$ mice compared to WT, while *isg15* was expressed at similar levels between the groups (Figure 3.6D). These data demonstrate that Tpl2 signaling promotes the induction of ISGs to limit virus replication during influenza infection.

Tpl2 ablation limits induction of antigen specific CD8$^+$ T cells and enhances susceptibility to influenza infection.

Even though the observed reduction in ISGs could explain the early increase in viral titers, a more pronounced and biologically significant difference in the increase in viral titers was observed at day 7 pi which correlated with the recruitment of influenza-specific CD8$^+$ T cells to the lungs (41). Since many seminal studies have identified CD8$^+$ T cells as the major mediators of influenza virus clearance from infected lungs (42,43), we investigated whether virus-specific CD8$^+$ T cell responses are impaired in *Tpl2*$^{-/-}$ mice. Consistent with defective viral clearance observed in *Tpl2*$^{-/-}$ mice, induction of protective nucleoprotein (NP)-specific CD8$^+$ T cells (44) was significantly reduced in BAL cells from *Tpl2*$^{-/-}$ mice compared to WT animals (Figure 3.7A-B). In addition, secretion of antigen-specific IFNγ was also decreased in BAL cells from *Tpl2*$^{-/-}$ mice (Figure 3.7C). During the course of this experiment, we unexpectedly observed severe

clinical signs in *Tpl2*$^{-/-}$ mice although the mice were infected with the low pathogenicity A/HK-X31(H3N2) (X31) influenza virus. To confirm whether Tpl2 ablation alters the susceptibility to influenza virus infection, WT and *Tpl2*$^{-/-}$ mice were infected with 10^4 pfu of X31 virus, and weight loss and clinical symptoms were monitored over a period of 14 days. All *Tpl2*$^{-/-}$ mice exhibited severe clinical signs and succumbed to infection by day 10 post-infection (pi), whereas all WT animals survived and returned to pre-infection body weights by day 14 pi (Figure 3.7D-E). A similar increase in susceptibility to infection and decrease in antigen specific $CD8^+$ T cell response were also observed in *Tpl2*$^{-/-}$ mice infected with the virulent PR8 (A/Puerto Rico/8/34 (PR8; H1N1)) strain (Supplementary Figure 3.16). Collectively, these data demonstrate the critical role of Tpl2 in promoting viral clearance and restricting morbidity and mortality associated with influenza virus.

DISCUSSION

Tpl2 is now appreciated to regulate the induction of Type I and Type II IFNs as well as other cytokines that may contribute to antiviral responses. However, there is very limited information on how Tpl2 coordinates antiviral immune responses *in vivo*. In this study, we demonstrate Tpl2's obligate role in promoting antiviral responses and viral clearance during influenza virus infection. These findings are important because influenza virus is a ubiquitous seasonal virus that afflicts millions of people annually, causing significant morbidity, mortality and socio-economic burdens (45). Therefore, understanding the role of host factors like Tpl2 in restricting morbidity and mortality associated with influenza virus infection is critical for developing disease intervention strategies. Mechanistically, Tpl2 exerts its effects by promoting induction of ISGs and virus-specific $CD8^+$ T cells that facilitate viral clearance as shown in the proposed model

(Figure 3.8). Thus, the findings reported here establish an essential role for Tpl2 in host protective innate and adaptive antiviral responses.

The increased mortality observed in *Tpl2*$^{-/-}$ mice infected with X31 virus was surprising because infection with this low pathogenicity virus does not typically cause severe clinical signs or mortality in mice. Even though IFNλ production was impaired in *Tpl2*$^{-/-}$ mice, this defect is not sufficient to explain their increased morbidity and mortality, because several studies have shown that *either* Type I or Type III IFN alone is sufficient to limit influenza virus infection (20,46,47). Early increases in virus replication and defective viral clearance likely potentiate the inflammatory response, which is considered a major factor contributing to morbidity and mortality during pathogenic influenza infection (48). In addition to impaired $CD8^+$ T cell responses (43), the reduction in expression of some ISGs may also contribute to the enhanced pathogenesis, since defective expression of individual antiviral factors, like IFITM3, can alter the course of infection (49). Although MAP kinase pathways are known to be activated in response to IFNs, the importance of Tpl2 in regulating IFN-inducible effectors has not yet been described. Interestingly, not all ISGs were similarly affected by Tpl2 ablation. The complexity of the IFN response is not completely understood, since multiple signaling cascades and transcription factors activated during IFN signaling can independently or cooperatively regulate transcriptional response to IFNs (38). Importantly, our data demonstrate that Tpl2 directly transduces IFN signals leading to the phosphorylation of ERK and STAT1^{Ser727}.

Previous studies have demonstrated the significance of STAT1^{Ser727} phosphorylation for full transcriptional activation and induction of ISGs (37,50). Conflicting reports exist regarding the identity of the serine kinase responsible for STAT1^{Ser727} phosphorylation; different kinases including p38, ERK and PKC-δ have been implicated (51-53). Importantly, an association of

ERK with STAT1 and a requirement of ERK activity for expression of ISGs have been demonstrated (54). The contribution of Tpl2 in promoting IRF7-dependent transcription has also been demonstrated recently (16). However, normal induction of IFNα/β during influenza virus infection argue against a major role for IRF7 in the observed phenotype since IRF7 is regarded as the 'master regulator' of Type I IFN induction (34). In addition to regulating ISG transcription, Tpl2-ERK signaling also regulates the phosphorylation of the translation initiation factor eIF4E, which is involved in cap-dependent translation of many genes, including ISG15 (33,55). Thus, the Tpl2-ERK pathway regulates biological effects of IFNs at both transcriptional and posttranscriptional levels.

Induction of IFNα, like IFNλ, is dependent on IFN feedback signaling (34), however, Tpl2 did not have a detectable role in IFNα production during influenza virus infection, either *in vitro* or *in vivo*. This suggests that the reduction in IFNλ levels observed in *Tpl2*$^{-/-}$ mice or cells is not simply a secondary effect of decreased secretion of IFNα/β, but rather suggests that unique mechanisms selectively drive IFNλ induction. Although Type I and Type III IFNs have common regulatory elements in their promoters and are usually co-expressed in response to viruses and TLR ligands (35), selective induction of IFNλ by transcription factors like NFκB and IRF1 independently of Type I IFNs has been reported (56,57). The distinct requirement for Tpl2 in IFNλ induction likely represents the unique requirement of the IFNλ promoter for an early NFκB-dependent priming event. In support of this, previous studies have reported differential regulation of *ifna* and *ifnl* by IRFs and NFκB, respectively (57,58).

Even though IFNAR signaling is necessary for IFNλ secretion, Type I IFN stimulation alone is not sufficient to induce high levels of IFNλ. This suggest that synergism between TLR and IFNAR signaling is critical for IFNλ production in pDCs. Importantly, we show that Tpl2 is a

component of both pathways regulating IFNλ production, since Tpl2 ablation reduced IFNλ secretion in response to either TLR ligands R848 and CpG or direct stimulation with IFNβ. With the exception of a very recent study reporting that p38, but not ERK, is required for *ifnl1* expression in human cells (56), the roles of MAPK or PI3K pathways in the regulation of IFNλs have not been evaluated. Although the regulation of IFNλ1 by PI3K-mTOR is still unexplored, our data demonstrate a different mechanism of IFNλ3 regulation that relies on the ERK pathway compared to the p38-dependent regulation described for IFNλ1. Therefore, in addition to transcription factors (29), diverse signaling cascades also specify induction of different IFNλs.

In addition to antiviral innate responses, we also identified a critical role for Tpl2 in induction of antigen-specific $CD8^+$ T cell responses. This is in contrast to a recent study reporting a major role for Tpl2 in human, but not murine, $CD8^+$ T cell responses (59). The impaired induction of virus-specific $CD8^+$ T cells resulting in defective viral clearance and increased mortality in *Tpl2*$^{-/-}$ mice clearly warrants detailed studies on Tpl2 regulation of effector $CD8^+$ T cell responses.

Overall, our study establishes Tpl2 as a host factor with intrinsic ability to restrict influenza virus replication and also demonstrates immune regulatory functions of Tpl2 within the lungs. The involvement of Tpl2 in major virus sensing pathways as well as antiviral signaling cascades suggests a key role for Tpl2 in integrating antiviral responses. Whether Tpl2 similarly restricts the replication of other classes of viruses requires further investigation. The findings reported here also suggest that the proposed therapeutic inhibition of Tpl2 during chronic inflammatory diseases might predispose patients to viral infections.

MATERIALS AND METHODS

Ethics Statement

All animal experiments were performed in accordance to the national guidelines provided by the "The Guide for Care and Use of Laboratory Animals" and The University of Georgia Institutional Animal Care and Use Committee (IACUC). The Institutional Animal Care and Use Committee (IACUC) of the University of Georgia approved all animal experiments under animal use protocol A2011 06-019. Embryonated specific pathogen free (SPF) chicken eggs were purchased from Sunrise Farms, New York and virus stocks were generated by inoculating the allantoic cavity of 9- to 11-day-old SPF eggs as per the animal use protocol A2010 07-135 approved by the University of Georgia IACUC.

Mice and viruses

Wild type (WT) C57BL/6J ($CD45.2^+$) mice and *Ifnar1*$^{-/-}$ mice were purchased from The Jackson Laboratory. *Tpl2*$^{-/-}$ mice backcrossed to C57B6/J were kindly provided by Dr. Philip Tsichlis (Tufts University) and Thomas Jefferson University. To generate chimeric mice, WT ($CD45.2^+$) or *Tpl2*$^{-/-}$ recipient mice were lethally irradiated with 1000 rad and reconstituted with donor B6.SJL-*Ptprc*^{a}*Pepc*^{b}/BoyJ ($CD45.1^+$) or *Tpl2*$^{-/-}$ bone marrow cells. Chimeric mice were maintained for 8 weeks. Animals were housed in sterile microisolator cages in the Central Animal Facility of the College of Veterinary Medicine. Influenza viruses A/HKX31 (H3N2), A/Puerto Rico/8/34 (PR8; H1N1) and A/WSN/1933 (H1N1) stocks were propagated in the allantoic cavity of 9- to 11-day-old embroynated SPF chicken eggs at 37 °C for 72 hr and viral titers were enumerated by plaque assays (60).

***In vivo* infections**

Age-matched, 6- to 8-week-old WT, *Tpl2*$^{-/-}$, *Ifnar1*$^{-/-}$, *Ifnar1*$^{-/-}$ /*Tpl2*$^{-/-}$ or chimeric mice were anesthetized with 250 mg/kg Avertin (2,2,2-tribromoethanol) followed by intranasal infection with influenza A/HK-X31 (H3N2) in 50 μl PBS. Control mice were mock-infected with a similar dilution of allantoic fluid. To determine lung viral titers, whole lungs from WT and *Tpl2*$^{-/-}$ mice infected with 10^4 pfu of X31 virus were harvested on days 3, 5 and 7 pi. Lungs were placed in 1 ml PBS and dissociated with a bead mill homogenizer (Qiagen), and virus titers were enumerated by plaque assays. To assess susceptibility to influenza infection, WT and *Tpl2*$^{-/-}$ mice infected with 10^4 pfu of X31 virus were observed over a period of 14 days. Body weighs were recorded daily, and mice exhibiting severe signs of disease or more than 30% weight loss were euthanized. To measure IFN and cytokine secretion, mice infected with 10^6 or 10^4 pfu of X31 virus were euthanized 3 or 7 days pi, and bronchoalveolar lavage fluid (BALF) or BAL cells were obtained by washing the lungs twice with 1 mL PBS. Cells were recovered by centrifugation of the lavage fluid for 10 min at 250xg. BALF from the first wash was used for quantitation of cytokine secretion. Cellular recruitment was assessed by quantifying total leukocyte recovery from both washes.

Measurement of antigen specific CD8$^+$ T cell responses

Mice infected with 10^4 pfu of X31 virus were euthanized on day 10 pi, and cells were obtained by washing the lungs twice with 1 mL PBS. BAL cells were stained with anti-CD4, anti-CD8 (eBiosciences), and H2D^{b}NP$_{366–374}$ tetramer (NIH Tetramer Core Facility, Emory University, Atlanta, GA) for 30 min at 4^0C and fixed in 1% formaldehyde. Cells were acquired on a BD LSRII flow cytometer and analyzed using FlowJo software (Tree Star, Inc.). For IFNγ measurement, BAL cells were stimulated with a cocktail of influenza immunodominant peptides

($NP_{366–374}$, $PA_{224–233}$, $PB1_{703–711}$) (1 μg/mL) for 24 hr at 37^0C, and IFNγ levels in culture supernatant was measured by ELISA (eBiosciences).

Cell culture

Bone marrow derived macrophages (BMDMs), pDCs and mouse embryonic fibroblasts (MEFs) were generated from age- and sex-matched mice as described previously (17,61). $CD11c^+CD11b^-B220^+$ pDCs were sorted using a Beckman Coulter MoFlo XDP cell sorter. In some experiments, cells were used on day 10 without sorting (referred as Flt3 ligand-derived DCs). Triggering of RIG-I was accomplished by directly delivering 5'-triphosphate RNA (5'ppp RNA; 0.5 μg/mL) or a control RNA to the cytosol of BMDMs or MEFs using LyoVec transfection reagent (InvivoGen). 20 μL 5'ppp RNA or control RNA (100 μg/mL) was incubated with 200 μL LyoVec (62.5 μg/mL) at room temperature for 15 min to form complexes. Twenty-five microliters of the complexes were used to stimulate $2.5x10^5$ BMDMs or $0.5x10^5$ MEFs per well for 24 hr. BMDMs at $1x10^6$/mL were also treated with R848 (InvivoGen) (1 μg/mL) for 24 hr. To investigate IFN signaling, BMDMs at $1x10^6$/mL were treated with rmIFNα (2000 IU/mL; R&D Systems), or rhIFNβ (10 ng/mL; Peprotech) for 1-4 hr.

Plasmacytoid DCs at 0.5-$1x10^6$/mL concentrations were left untreated or stimulated with R848 (1 μg/mL) or CpG ODN2395 (10 μg/mL) (InvivoGen) or 50 ng/mL rhIFNβ (PeproTech) or infected with WSN virus at a MOI of 0.2 for 24 hr. In some experiments, cells were pretreated with LY294002 hydrochloride (20 μM), rapamycin (30 nM) or U0126 (20 μM) (Sigma) for 30 min before stimulating with CpG.

Cytokine measurements

Cytokine levels were measured by ELISA (IFNλ and IFNγ, eBioscience; IFNβ, PBL Interferon Source) or bead-based detection assays (Mouse IFNα Flowcytomix simplex, eBioscience; Mouse inflammation cytokine bead array, BD Biosciences).

Analysis of mRNA expression

Cells stimulated with R848 or IFNs were lysed using TRK lysis buffer (Omega Bio-Tek). For *in vivo* infections, RNA lysates were prepared from tissue after homogenizing whole lungs. RNA was extracted using a Total RNA Kit (Omega Bio-Tek). Real-time PCR was performed after synthesizing cDNA using a High capacity cDNA Reverse Transcription kit (Applied Biosystems). The expression of *irf7* (Mm00516791_g1), *il28b (ifnl3)* (Mm00663660_g1), *ifitm3* (Mm00847057_s1), *isg15* (Mm01705338_s1), *oasl2* (Mm00496187-m1), *il12b* (Mm00434174_m1), *il6* (Mm00446190_m1), *tnfα* (Mm00443258_m1), *ifna (Mm03030145-gH), ccl5 (Mm01302427-m1)* and *actinb* (4352341E-1112017) were determined by RT-PCR (Applied Biosystems). RT-PCR reactions were performed in microAmp Fast plates (Applied Biosystems) using SensiFAST Probe Hi-ROX kit (Bioline) and a StepOnePlus RT-PCR machine (Applied Biosystems). Relative gene expression levels were calculated by normalizing the Ct levels of the target gene to both endogenous actin levels and an unstimulated WT control using the ΔΔCt method.

Protein Analysis

Cell lysates were separated on 4-12% gradient gels (Invitrogen) and were transferred to PVDF membranes using the iBlot Gel Transfer system (Invitrogen). Membranes were probed with various antibodies followed by horseradish peroxidase (HRP)-labeled secondary antibodies. Protein bands were visualized by enhanced chemiluminescent reagent (Lumigen) and Amersham

Hyperfilm ECL (GE Healthcare). The following antibodies were used for immunoblotting: Tpl2 (Cot M-20), ERK1 and ERK2 (Santa Cruz Biotechnology), p-ERK1/2 (Thr202/Tyr204), p-STAT1 (Tyr701), p-STAT1 (Ser727) and STAT1 (Cell Signaling Technology).

Intracellular staining

Cells harvested after overnight stimulation were fixed, permeabilized with triton buffer (PBS+0.5%triton+0.1%BSA) and stained for p-Akt (Ser473) according to manufacturers' protocol (Cell Signaling Technology). Samples were acquired on a BD LSRII flow cytometer and analyzed using FlowJo software (Tree Star, Inc.).

Statistical analysis

Data represent means ± SEM, except where indicated. P-values were determined by two-tailed Students *t*-test, and significance was assigned for p-values <0.05. Kaplan-Meier analysis using PRISM software was performed to estimate percentage survival of WT and *Tpl2*$^{-/-}$ groups infected with influenza virus, and *p* value was determined using a Mantel-Cox test.

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FIGURES

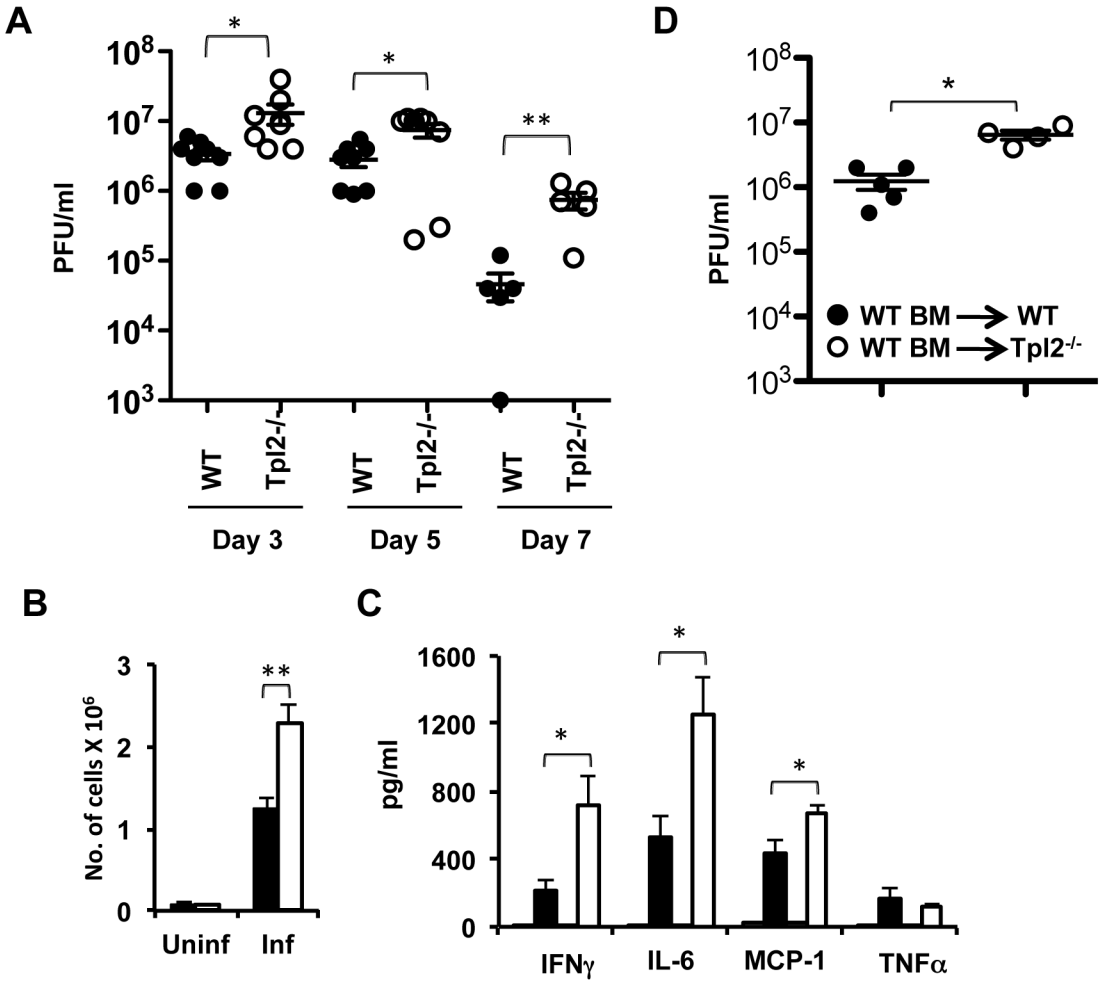
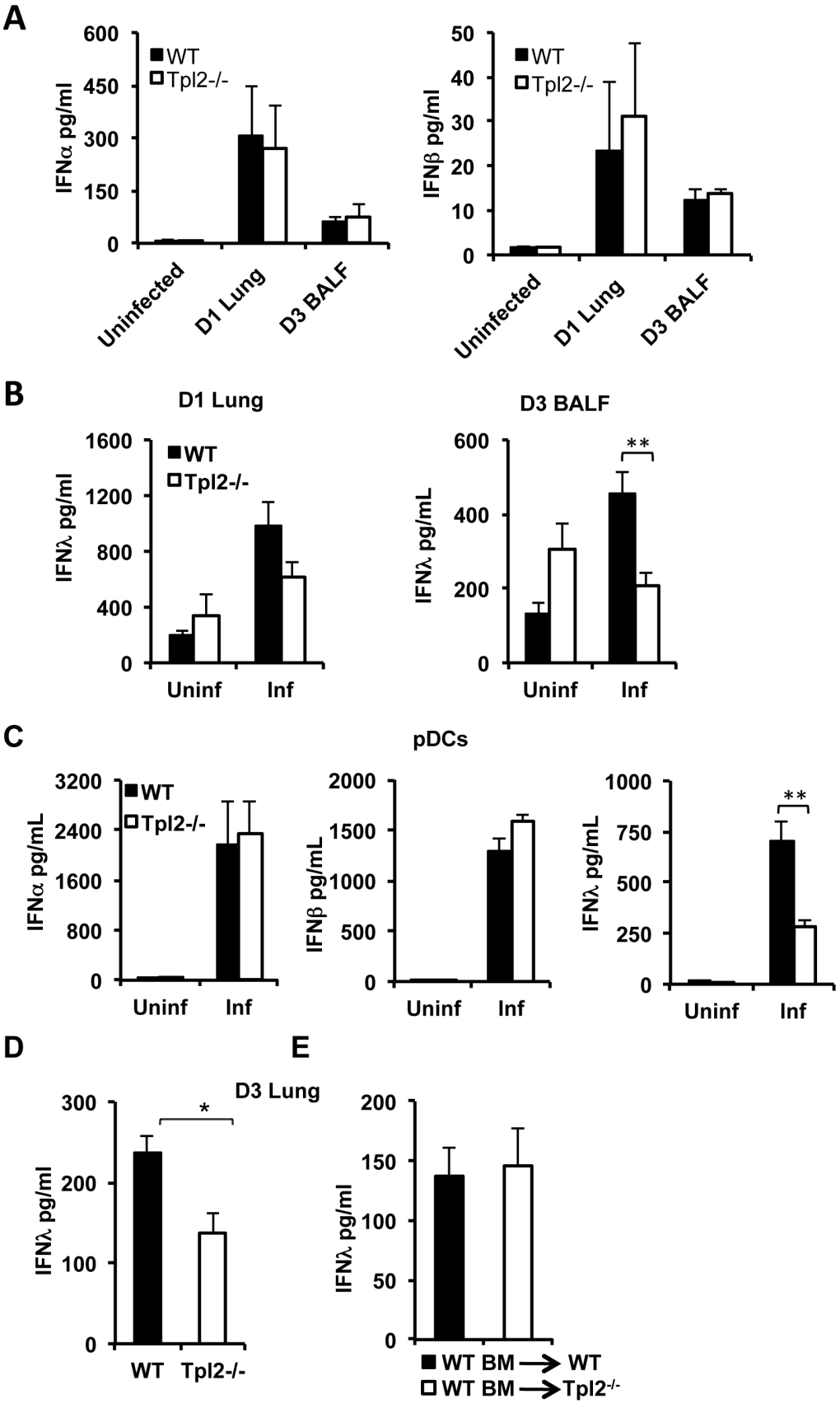


Figure 3.1: Tpl2 ablation enhances virus replication and inflammatory responses during influenza infection. (A) WT and *Tpl2*$^{-/-}$ mice were intranasally infected with 10^4 pfu of X31 virus and lung viral titers were enumerated by plaque assays; n=8 (D3 and D5) or n=5 (D7). WT and *Tpl2*$^{-/-}$ mice were infected with 10^4 pfu of X31 virus, and the number of cells recovered **(B)** and cytokine levels **(C)** in BALF were measured on D7 pi; n=6 uninfected and 10 (WT) and 8

(*Tpl2*$^{-/-}$) infected. **(D)** Chimeric mice were intranasally infected with 10^4 pfu of X31 virus and lung viral titers D3 pi were enumerated by plaque assays . * indicates $p<0.05$, ** indicates $p<0.01$.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600
450
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IFNα pg/ml
WT
Tpl2-/-
Uninfected
D1 Lung
D3 BALF
50
40
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20
10
0
IFNβ pg/ml
WT
Tpl2-/-
Uninfected
D1 Lung
D3 BALF
B
D1 Lung
1600
1200
800
400
0
IFNλ pg/ml
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200
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100
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IFNλ pg/ml
WT BM → WT
WT BM → Tpl2-/-

Figure 3.2: Tpl2 is required for optimal IFNλ production during influenza virus infection *in vitro* and *in vivo*. (A) WT and *Tpl2*$^{-/-}$ mice were infected with 10^6 pfu of X31 virus, and IFNα and β levels in D1 lung homogenate or D3 BALF were measured (For IFNα, n=4 uninfected and 7 infected mice per group; for IFNβ, n=2 uninfected and 3 infected per group). **(B)** WT and *Tpl2*$^{-/-}$ mice were infected with 10^6 pfu of X31 virus, and IFNλ levels in D1 lung homogenate or D3 BALF were measured (n=5 uninfected and 14 infected (WT) and 13 (*Tpl2*$^{-/-}$) infected). **(C)** pDCs from WT and *Tpl2*$^{-/-}$ mice were infected with WSN virus at an MOI of 0.2 for 24 hr, and IFN α, β and λ levels were measured. WT and *Tpl2*$^{-/-}$ mice **(D)** or chimeric mice **(E)** were infected with 10^4 pfu of X31 virus, and IFNλ levels in lung homogenates D3 pi was measured by ELISA. Data are representative of 3-4 independent experiments. ** indicates $p<0.01$

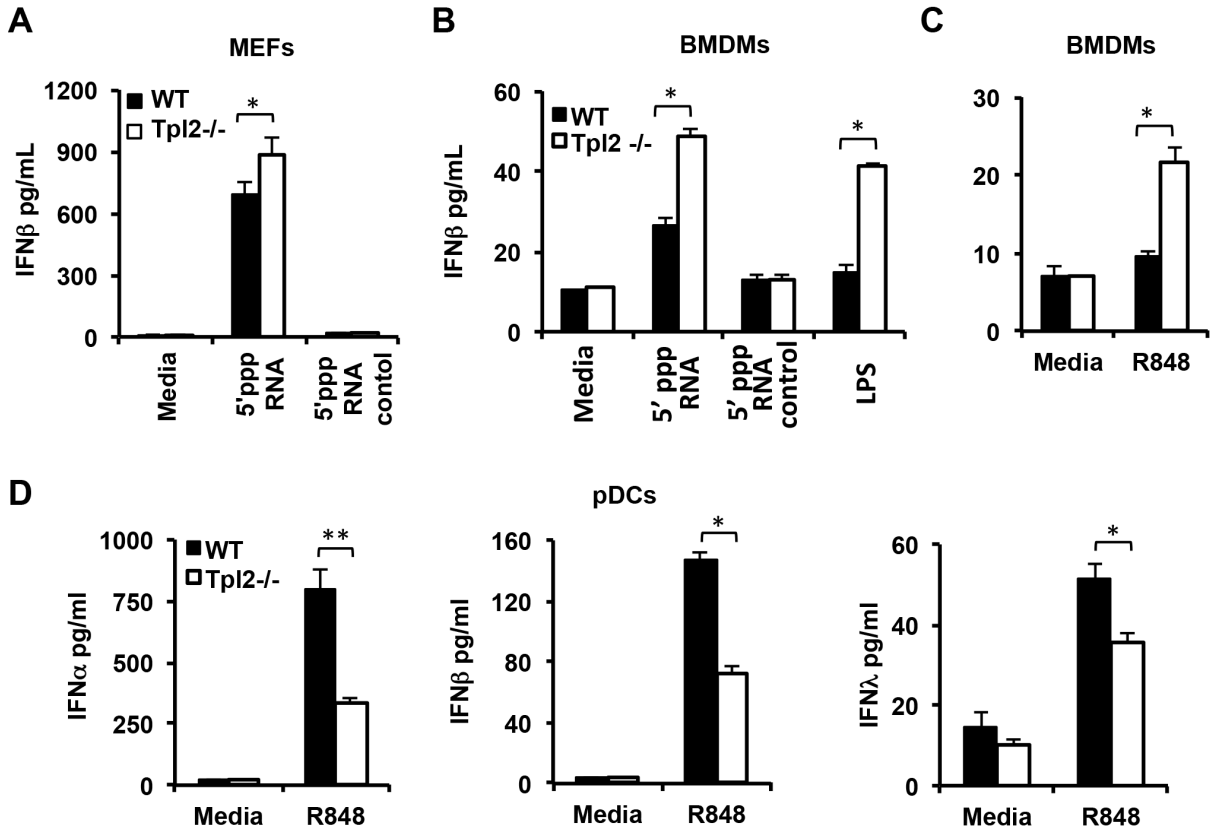


Figure 3.3: Tpl2 differentially regulates IFN production in response to model viral ligands in a cell type specific manner. MEFs **(A)** or BMDMs **(B-C)** from WT and *Tpl2*$^{-/-}$ mice were transfected with 5'ppp RNA or stimulated R848 or LPS, and IFNβ levels were measured by ELISA. **(D)** Plasmacytoid DCs were stimulated with R848 for 24 hr, and IFN α and β and λ levels were measured. Data are representative of 3-4 independent experiments. Graphs show means±SD. * indicates $p<0.05$, ** indicates $p<0.01$

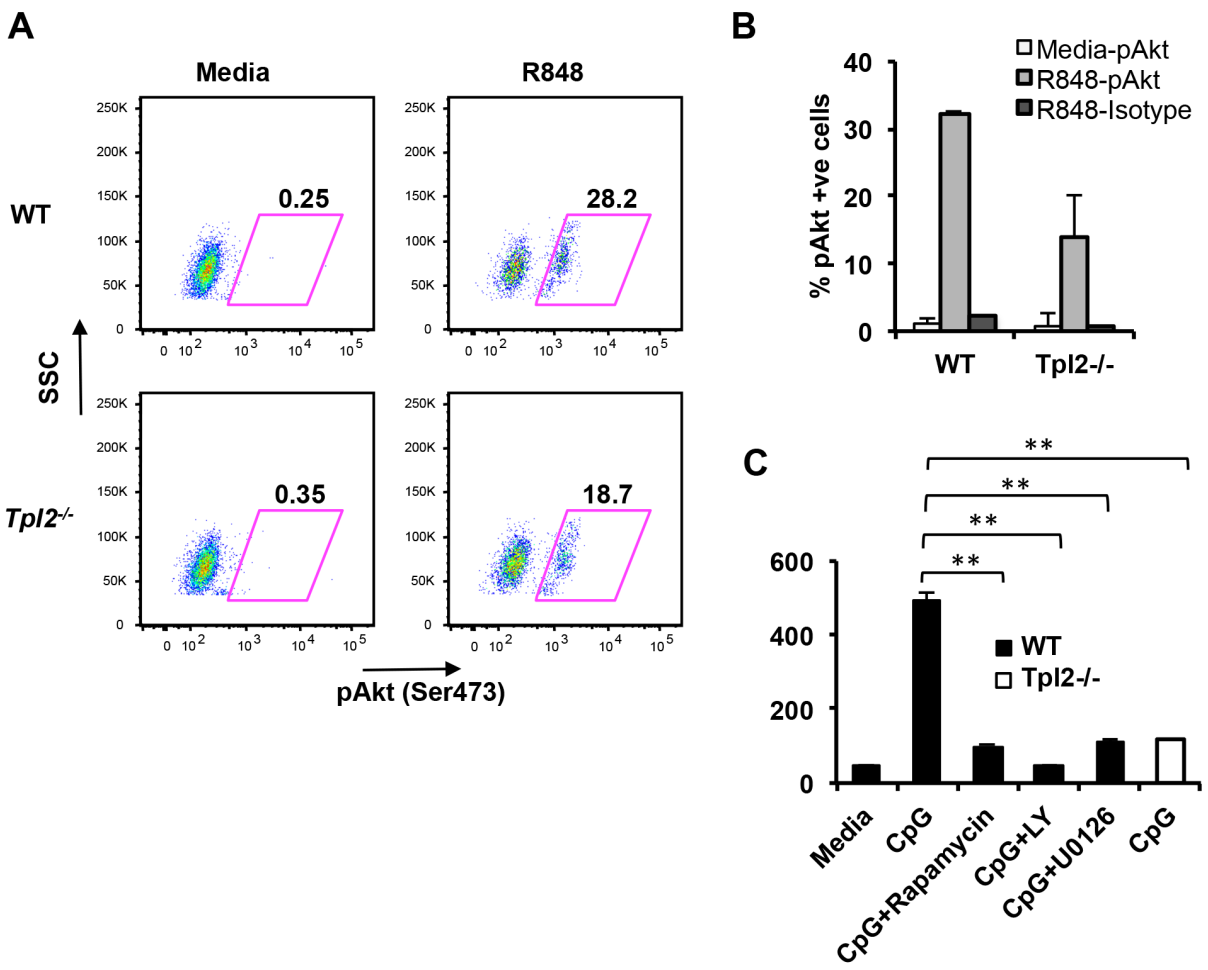


Figure 3.4: Tpl2 promotes IFNλ production in pDCs in an ERK and PI3K/mTOR/Akt-dependent manner. pDCs from WT and *Tpl2*$^{-/-}$ mice were stimulated with R848 overnight, and analyzed by intracellular staining for pAkt^{Ser473}. **(A)** Representative flow cytometry plots **(B)** Proportion of pAkt positive cells from 2 independent experiments **(C)** pDCs were pretreated with inhibitors for 30 min before stimulation with CpG, and IFNλ levels were measured by ELISA. Data are representative of 2 (A-B) or 3 (C) independent experiments. Graphs show means±SD. ** indicates $p<0.01$

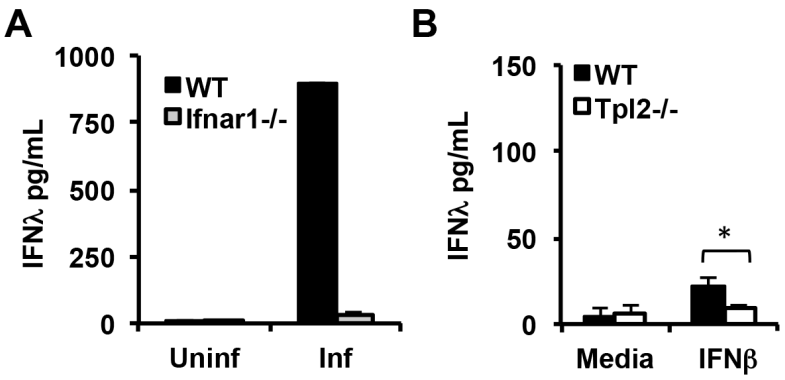


Figure 3.5: Tpl2 promotes IFNAR-mediated IFNλ production in pDCs. (A) Flt3 ligand-derived DCs from WT and *ifnar1*$^{-/-}$ mice infected with WSN virus for 24 hr, and IFNλ secretion was measured by ELISA. **(B)** Flt3 ligand-derived DCs from WT and *Tpl2*$^{-/-}$ mice were treated with IFNβ for 24 hr, and and IFNλ secretion was quantitated by ELISA. Data are representative of 2 (A) or 3 (B) independent experiments. Graphs show means±SD. * indicates $p<0.05$

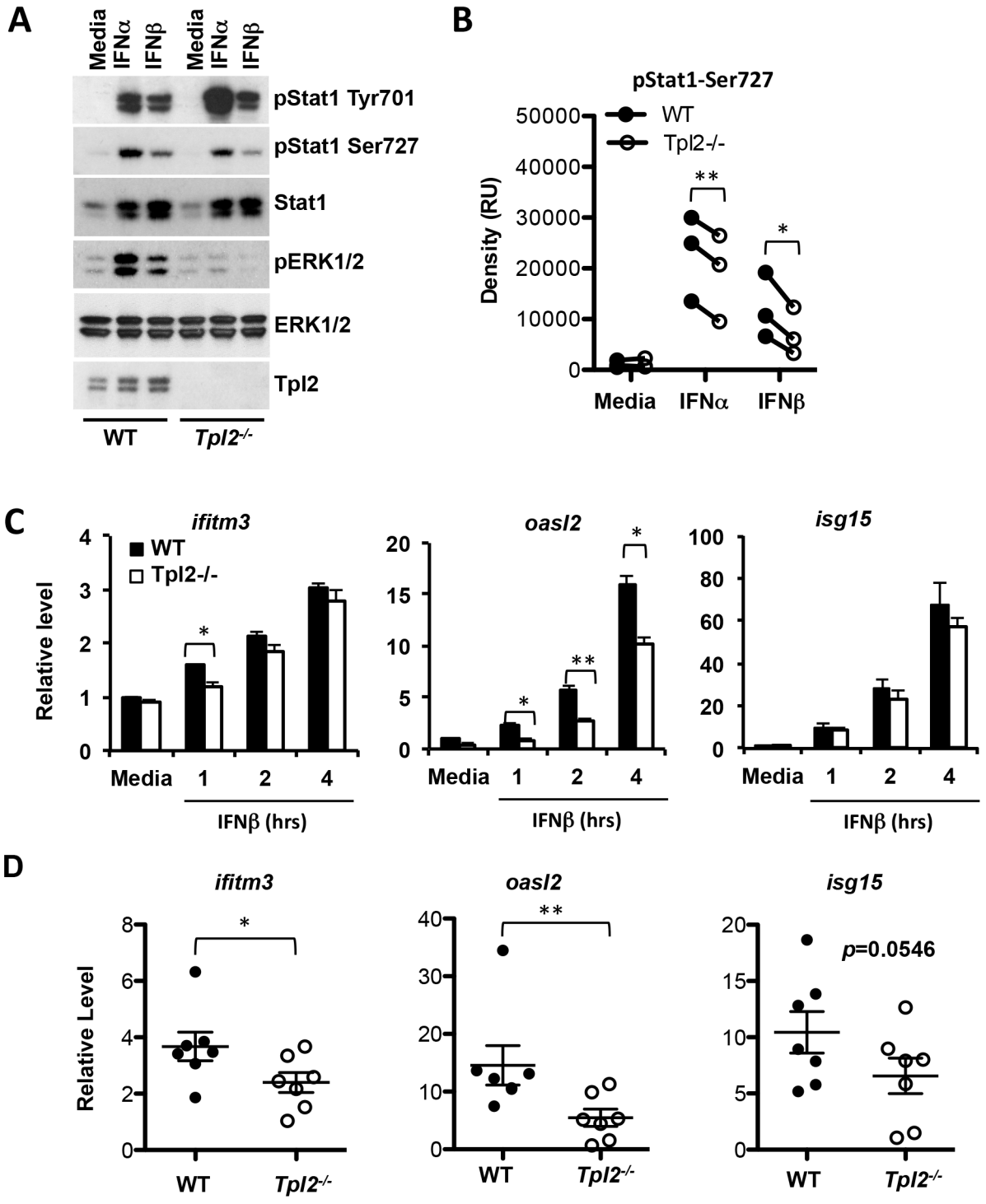


Figure 3.6: Tpl2 mediates IFN signaling and induction of ISGs. (A) BMDMs from WT and *Tpl2*$^{-/-}$ mice were stimulated with IFNα or IFNβ for 1 hr, and STAT1 and ERK phosphorylation were assessed by immunoblotting. Data are representative of 3 independent experiments. **(B)**

Average intensities of pSTAT1^{Ser727} bands by densitometric analysis. Data collected on the same day are connected by lines. **(C)** BMDMs were treated with IFNβ, and expression of *ifitm3*, *isg15* and *oasl2* were measured by RT-PCR with normalization to actin mRNA and WT untreated control. Data are pooled from 3 independent experiments. **(D)** WT and *Tpl2*$^{-/-}$ mice were infected with 10^6 pfu of X31 virus, and the expression of *ifitm3*, *isg15* and *oasl2* in lung tissue D1 pi was measured by RT-PCR with normalization to actin mRNA and WT uninfected sample (n=7). * indicates $p<0.05$, ** indicates $p<0.01$

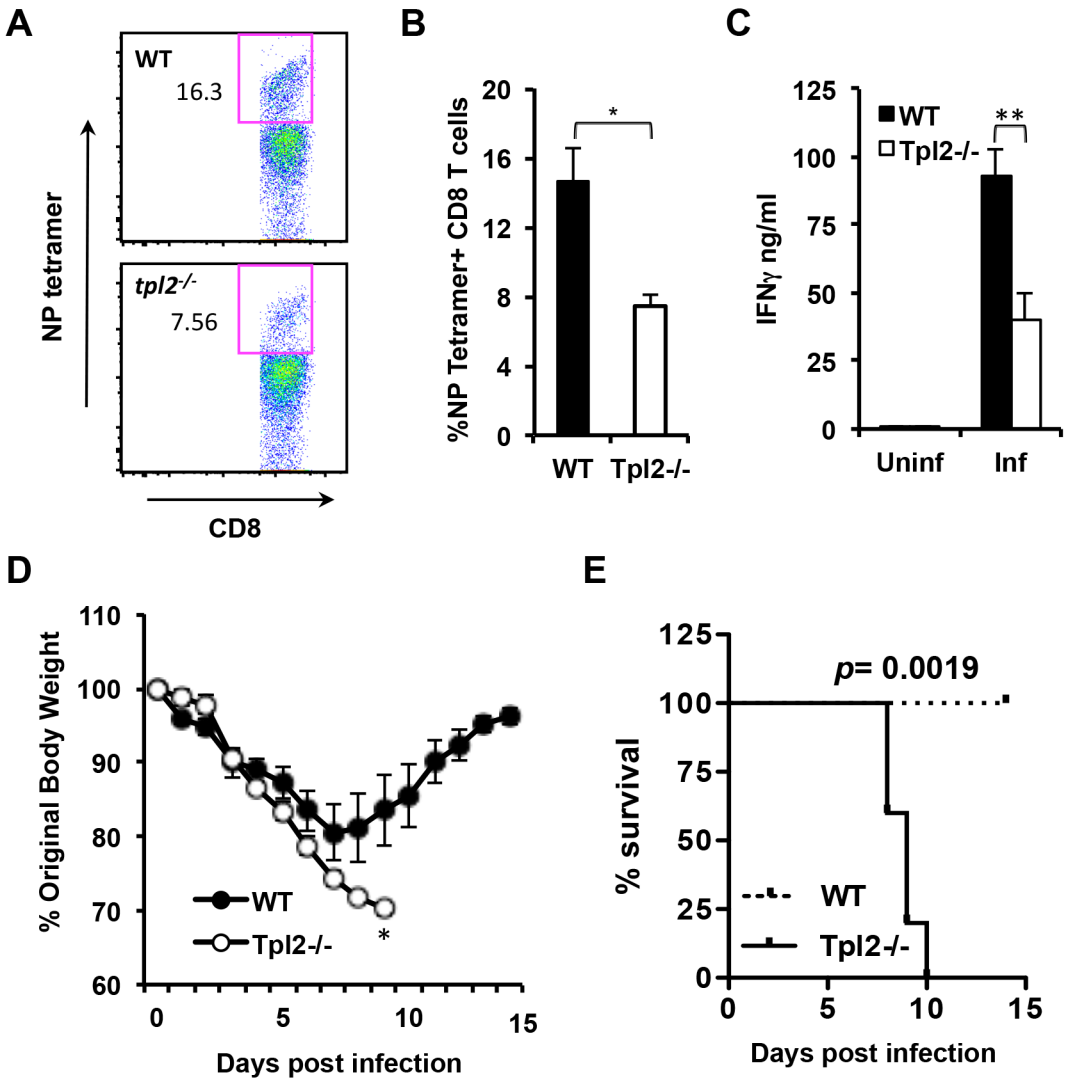


Figure 3.7: Tpl2 ablation limits antigen-specific CD8$^+$ T cell responses and enhances susceptibility to influenza infection. (A-B) WT and *Tpl2*$^{-/-}$ mice were infected with 10^4 pfu of X31 virus, and the proportion of $NP_{366–374}$ tetramer positive CD8$^+$ T cells in BAL were assessed. **(C)** BAL cells were stimulated with a cocktail of influenza immunodominant peptides for 24 hr and secretion of IFNγ was measured by ELISA. n=5. **(D-E)** WT and *Tpl2*$^{-/-}$ mice were infected with 10^4 pfu of X31 virus, body weights were recorded daily for 14 days, and mice exhibiting

severe signs of disease, including more than 30% weight loss were euthanized. Data are representative of 3 independent experiments; n=5. * indicates $p<0.05$, ** indicates $p<0.01$.

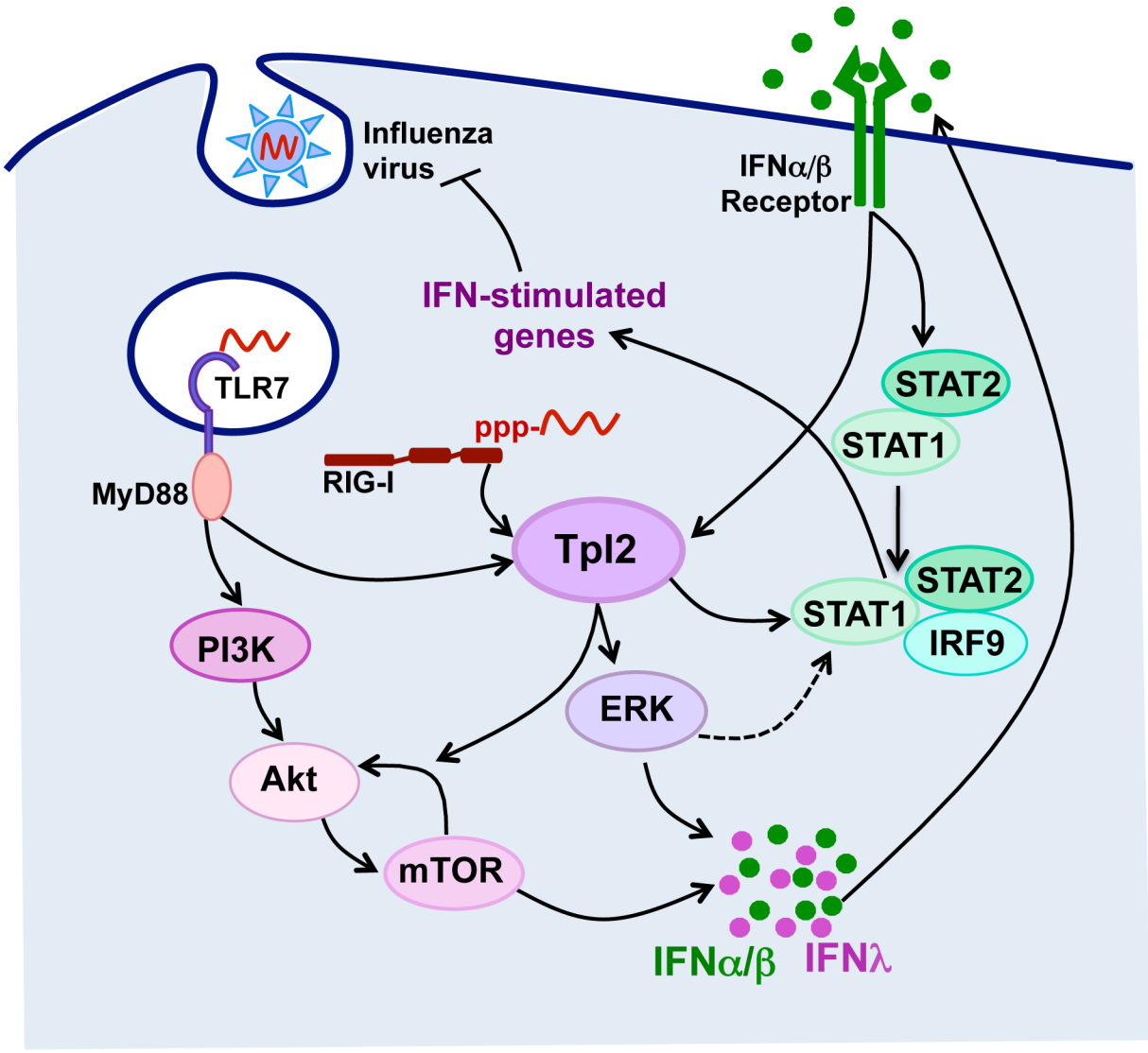
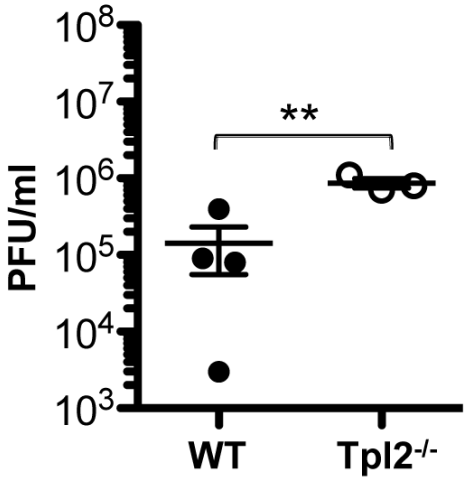
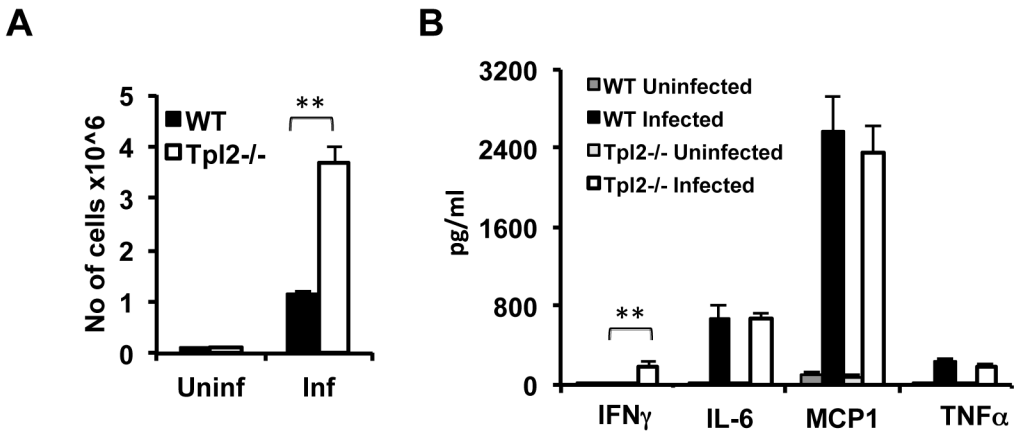


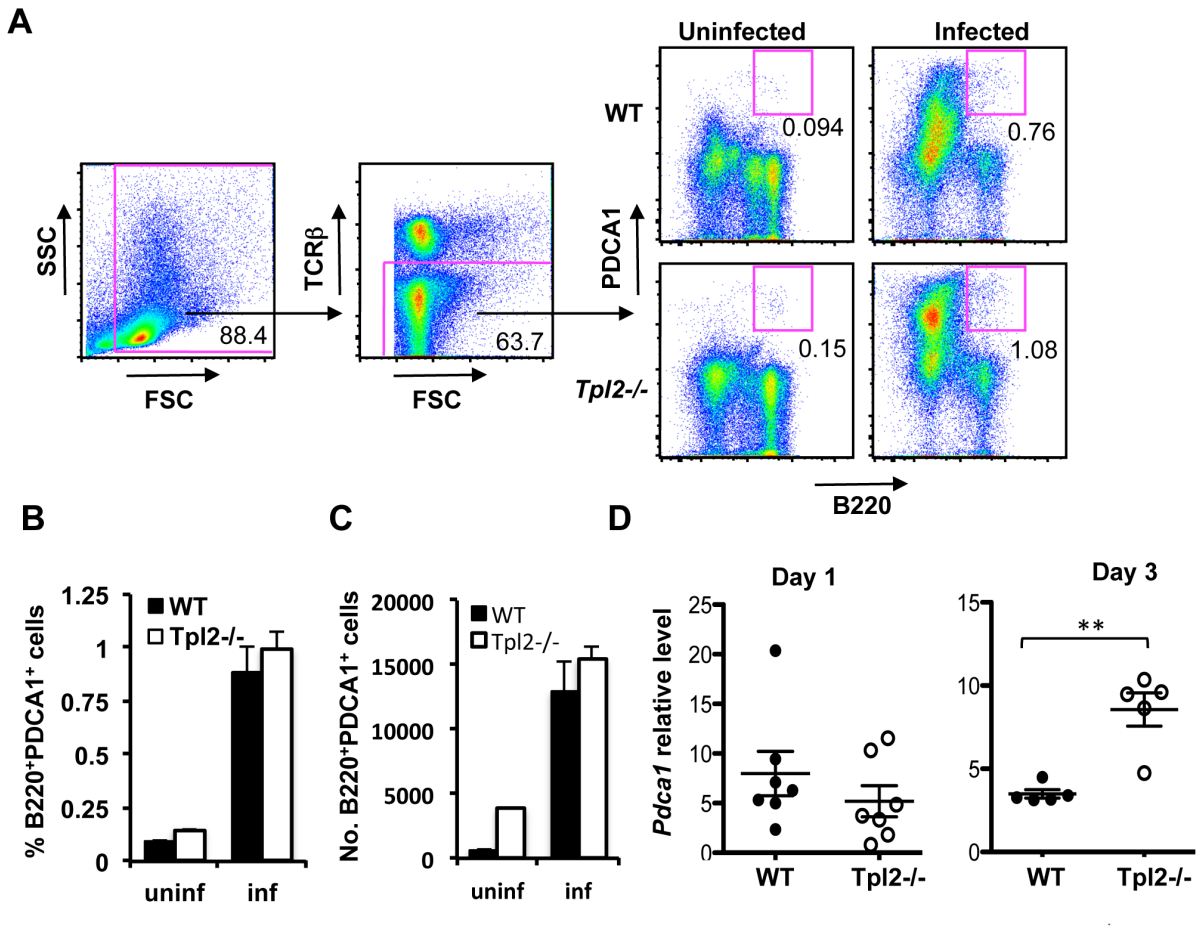
Figure 3.8: Model of Tpl2 regulation of antiviral immune responses. Recognition of influenza virus by TLR7 (in pDCs) or RIG-I (in other cell types) induces secretion of IFNα/β or IFNλ in a Tpl2-dependent manner. In addition to ERK, Tpl2 regulates Akt^{ser473} phosphorylation and PI3K/mTOR-mediated IFNλ production. Tpl2 directly transduces Type I IFN signals leading to the phosphorylation of ERK and STAT1^{Ser727} thereby regulating induction of ISGs, which are important mediators of antiviral resistance. In addition to early innate responses, Tpl2 promotes expansion of virus-specific CD8^{+} T cells that facilitate viral clearance from infected lungs (not pictured).



Supplementary Figure 3.9: Virus replication was increased in *Tpl2*$^{-/-}$ littermate control mice (A) WT and *Tpl2*$^{-/-}$ littermate mice were intranasally infected with 10^4 pfu of X31 virus and lung viral titers were enumerated by plaque assays on D7 pi; n=4 WT and 3 *Tpl2*$^{-/-}$. ** indicates $p<0.01$.

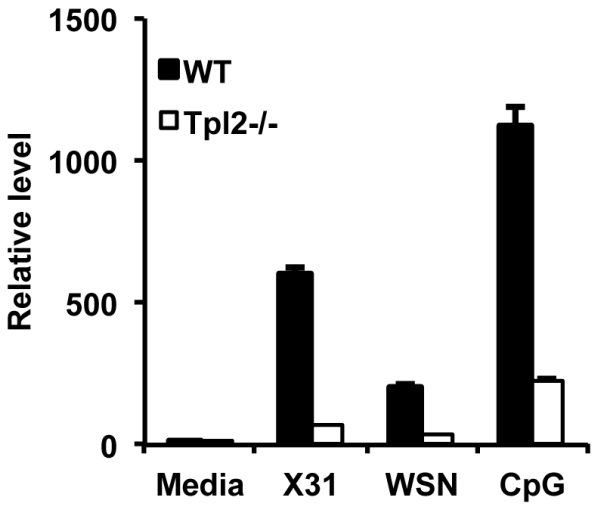


Supplementary Figure 3.10: Inflammatory responses are increased in the lungs of *Tpl2*$^{-/-}$ mice early after infection. WT and *Tpl2*$^{-/-}$ mice were infected with 10^6 pfu of X31 virus, and the number of cells recovered **(A)** and cytokine levels **(B)** in BALF were assessed on D3 pi; n=6 uninfected and 12 (WT) and 10 (*Tpl2*$^{-/-}$) infected. ** indicates $p<0.01$.

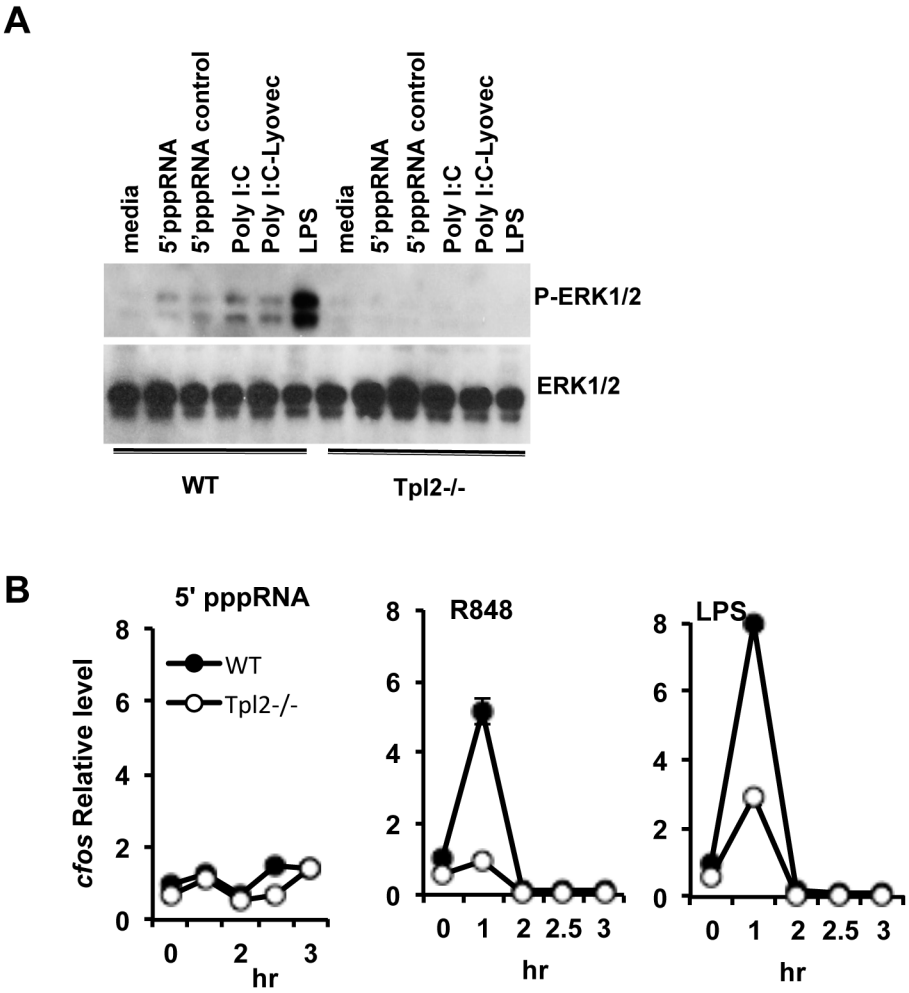


Supplementary Figure 3.11: Recruitment of pDCs to lungs during influenza infection occurs independently of Tpl2. Animals infected with 10^6 pfu of X31 virus were anesthetized with a lethal dose of avertin and lungs were perfused with 25 mL PBS/heparin sodium solution *in vivo*. Harvested lungs were minced and incubated in 1.25 mM EDTA for 30 min at 37°C. The tissue was further incubated in collagenase diluted in RPMI (6 mg/mL) at 37°C for 30 min. Supernatants from digestions were passed through a 70 µm cell strainer. Cells were enriched by Percoll (GE Healthcare) gradient purification using a 47/67% gradient. Cells at the interface were collected and stained with antibody cocktail containing anti- CD16/32, CD11c-PE, PDCA1-APC, CD8-eFlour, B220-FITC and TCRb-Percp Cy5.5 for 15 min at 4^0C and fixed in 1% formaldehyde. Samples were run on a BD LSRII flow cytometer and analyzed using FlowJo

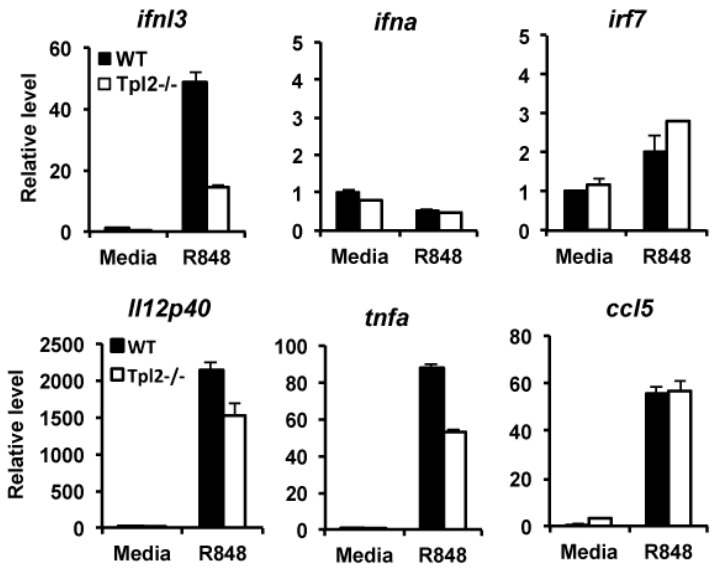
software (Tree Star, Inc.). **(A)** Representative flow plots Proportion **(B)** and absolute numbers **(C)** of $B220^{+}PDCA1^{+}$ cells; n= 1 uninfected and 3 infected WT and *Tpl2*$^{-/-}$ mice. **(D)** WT and *Tpl2*$^{-/-}$ mice were infected with 10^{6} pfu of X31 virus, and the expression of *pdca1* in lung tissue D1 or D3 pi was measured by RT-PCR with normalization to actin mRNA and WT uninfected sample (D1, n=7; D3, n=5).



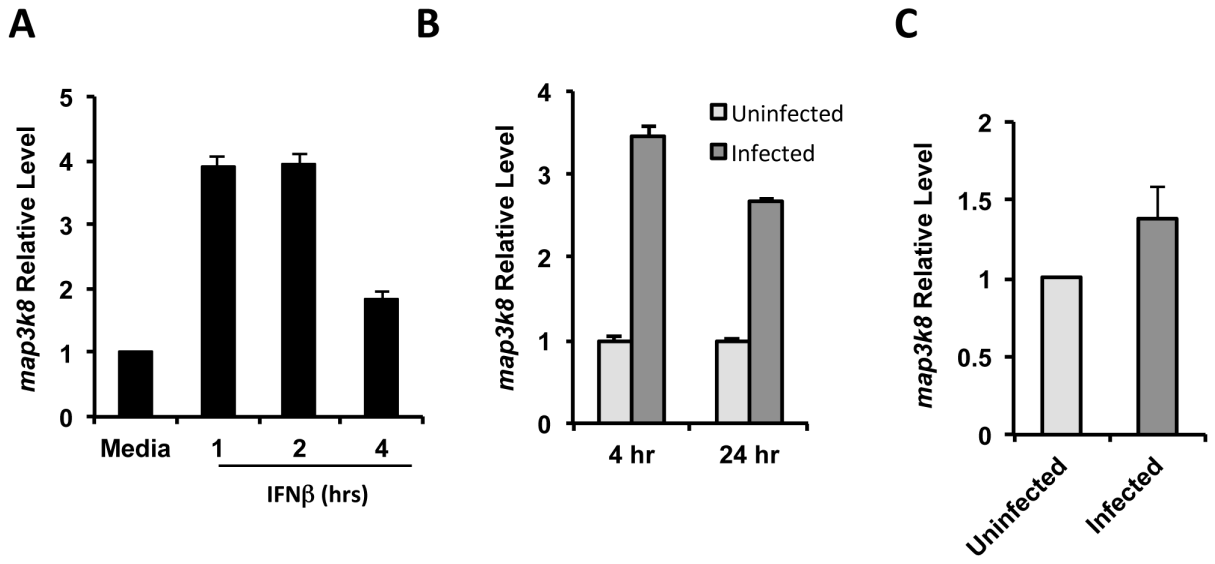
Supplementary Figure 3.12: Tpl2 is required for IFNλ induction in pDCs infected with X31 influenza virus. (A) Flt3 ligand derived DCs from WT and *Tpl2*$^{-/-}$ mice were infected with X31 or WSN virus at an MOI of 10 or stimulated with CpG for 24 hr and IFNλ expression was measured by RT-PCR with normalization to actin mRNA and WT uninfected sample.



Supplementary Figure 3.13: ERK phosphorylation and *fos* induction were impaired in Tpl2-deficient BMDMs stimulated with model viral ligands. (A) BMDMs from WT and *Tpl2*$^{-/-}$ mice were left untreated or stimulated with TLR or RLR ligands for 2 hr, and ERK phosphorylation was assessed. **(B)** BMDMs were stimulated with 5'ppp RNA, R848 or LPS for 0-3 hr, and *c-fos* was measured by Real-time PCR relative to an actin control and WT untreated samples. Data are representative of 3 independent experiments.



Supplementary Figure 3.14: Tpl2-dependent induction of IFNλ occurs early after stimulation like other NFκB-regulated proinflammatory cytokines. Plasmacytoid DCs from WT and *Tpl2*$^{-/-}$ mice were left untreated or stimulated with R848 for 2 hr, and expression of *ifnl3, ifna, irf7, il-12p40*, *tnfa*, and *ccl5* were measured by Real-time PCR relative to an actin control and WT untreated samples. Data are representative of 2 independent experiments.



Supplementary Figure 3.15: Tpl2 is induced in response to type I IFNs and influenza infection. (A) BMDMs from WT mice were stimulated with IFNb, and Tpl2 gene expression (*map3k8)* was measured by RT-PCR with normalization to endogenous actin mRNA and the WT untreated control. Data are pooled from 3 independent experiments. **(B)** pDCs from WT mice were infected with WSN virus at an MOI of 0.2 for 4 or 24 hr, and *map3k8* expression was measured. **(C)** WT mice were infected with 10^6 pfu of X31 virus, and the expression of *map3k8* in lung tissue D1 p.i. was measured by RT-PCR with normalization to actin mRNA and WT uninfected sample; n=2 uninfected and 7 infected mice.

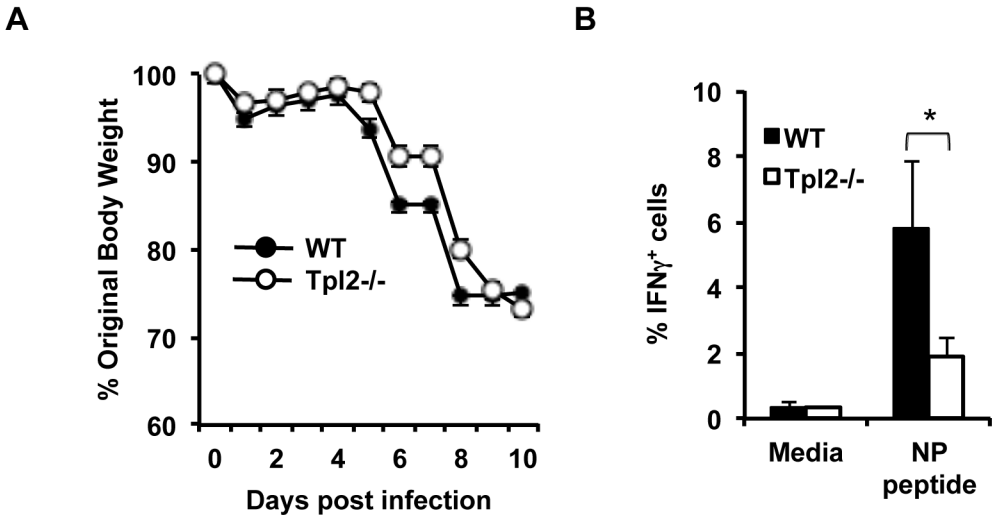


Figure 3.16: Increased susceptibility to infection and decreased antigen-specific CD8$^+$ T cell responses in *Tpl2*$^{-/-}$ mice infected with A/Puerto Rico/8/34 (PR8; H1N1). (A) WT and *Tpl2*$^{-/-}$ mice were infected with 30 pfu of PR8 virus, and body weights were recorded daily for 10 days. Mice were euthanized on D10 pi, BAL cells were collected by lavaging the lungs. Cells were enumerated and stimulated with nucleoprotein (NP) specific peptide. Cells were fixed, permeabilized, and stained using anti-mouse CD8 and IFNγ monoclonal antibodies. Samples were acquired on a BD LSRII flow cytometer and analyzed using FlowJo software (Tree Star, Inc.). **(B)** The proportions of IFNγ$^+$ CD8 T cells in WT and *Tpl2*$^{-/-}$ samples. * indicates $p<0.05$.

CHAPTER 4

TUMOR PROGRESSION LOCUS 2 PROMOTES AKT-FOXO1 SIGNALING AND IFNγ PRODUCTION IN CD8$^+$ T CELLS

Teneema Kuriakose and Wendy T. Watford. To be submitted to *Journal of Interferon and Cytokine Research*

ABSTRACT

Secretion of IFNγ is a common effector function of both $CD4^+$ and $CD8^+$ T cells (CTLs); however, distinct mechanisms regulate IFNγ induction in these two cell types. Our previous studies have identified the serine-threonine kinase tumor progression locus 2 (Tpl2/Cot/MAP3K8) as a critical regulator IFNγ production in $CD4^+$ T cells. Herein, we demonstrate that Tpl2 is also necessary for proper induction of IFNγ in CTLs. Unlike $CD4^+$ T cells, induction of transcription factors STAT4, T-bet and Eomes were unaffected by Tpl2 ablation. Tpl2 was also dispensable for STAT4 phosphorylation. In contrast, activation of Akt-FOXO1 signaling, which is known to promote CTL effector functions including IFNγ production, was impaired in Tpl2-deficient CTLs. Collectively, these data demonstrate differences in Tpl2-dependent regulation of IFNγ in T cells and establish the significance of Tpl2 in regulating CTL effector functions.

INTRODUCTION

$CD8^+$ cytotoxic T lymphocytes (CTLs) are key mediators of host immune responses against intracellular pathogens and tumors. Naïve $CD8^+$ T cells undergo rapid expansion upon stimulation and differentiate into effector and memory CTLs that control infection and provide long-term protective immunity. In addition to the lysis of infected cells, secretion of IFNγ is another major effector function of CTLs (1). The crucial role for IFNγ in cell-mediated immunity is confirmed by increased susceptibility to infections observed in IFNγ-receptor knockout mice despite normal cytolytic functions (2,3).

Although both $CD4^+$ and $CD8^+$ T cells secrete IFNγ, the regulation of IFNγ production varies considerably between these two cell types. STAT4 and T-bet transcription factors are critical for antigen receptor- and IL-12-mediated IFNγ production in $CD4^+$ T cells, but T cell receptor (TCR)-mediated IFNγ production is STAT4-independent in CTLs (4). Moreover, unlike $CD4^+$ T cells, CTLs demonstrate significant IFNγ secretion independent of IL-12 (4). However, IFNγ production via IL-12/IL-18 stimulation is STAT4-dependent in both $CD4^+$ and $CD8^+$ T cells (4). Although T-bet is important in antigen-specific induction of IFNγ in CTLs (5), another T-box transcription factor eomesodermin (Eomes) is also critical for CTL effector functions, including T-bet–independent IFNγ induction (6).

We have previously reported the critical role of the serine/threonine kinase, tumor progression locus 2 (Tpl2) in promoting IFNγ production in $CD4^+$ T cells (7). Even though Tpl2 is known to regulate IFNs and other inflammatory cytokines (8,9), limited and contradictory studies have addressed the role of Tpl2 in regulating CTL effector functions. Initial studies using *Tpl2*$^{-/-}$ mice reported normal cytotoxic responses against LCMV-infected cells (9). Another recent study demonstrated decreased effector functions in human, but not murine, CTLs in the absence of

Tpl2 signaling (10). Finally, our recent investigation of the role of Tpl2 in antiviral responses showed defective induction of antigen-specific CTLs in influenza virus-infected *Tpl2*$^{-/-}$ mice (Kuriakose, T. *et al*., manuscript submitted). In this study, we investigated whether Tpl2 regulates TCR- and cytokine-mediated IFNγ production in CTLs in a cell intrinsic manner using *in vitro* expanded CD8^{+} T cells from wild type (WT) and Tpl2-deficient mice.

RESULTS AND DISCUSSION

Tpl2 promotes IFNγ production in CTLs

As previously been reported in CD4^{+} T cells and in IFNα-treated CD8^{+} T cells (7,11), induction of Tpl2 in murine CD8^{+} T cells was observed in response to both TCR and cytokine stimulation (Figure 4.1A). To investigate whether Tpl2 regulates IFNγ production in CTLs, sorted CD44^{lo}CD62L^{hi} CD8^{+} T cells cultured for 5 days were stimulated with plate-bound anti-CD3 and -CD28 or cytokines, and IFNγ production was quantitated. Similar to CD4^{+} T cells, *Tpl2*$^{-/-}$ CTLs showed impaired induction of IFNγ in response to TCR signals as well as IL-12/IL-18 stimulation as measured by intracellular cytokine staining or ELISA (Figure 4.1B-E). Moreover, expression of *Ifng* mRNA was also significantly less in Tpl2-deficient cells compared to WT cells (Figure 4.1F). Consistent with normal cytotoxic responses reported previously (9), *Tpl2*$^{-/-}$ cells expressed similar levels of both granzyme and perforin as WT cells (Supplementary Figure 4.3). Collectively, these data demonstrate that Tpl2 is necessary for IFNγ production in CTLs.

Although CD4^{+} and CD8^{+} T cells show distinct regulation of IFNγ production, our data identified Tpl2 as a common signaling component required for proper induction of IFNγ in T cells. These results are in contrast to the findings of Chowdhury *et al*. who reported a major role for Tpl2 in human, but not murine, CTL effector functions (10). This discrepancy could be due to differences in the nature of the CD8^{+} T cell population (sorted naïve cells in the present study

versus total enriched $CD8^+$ T cells in the former study) or due to differences in culture conditions. In this regard, previous studies have demonstrated profound influence of culture conditions on CTL effector functions (12). Impaired secretion of IFNγ observed in our experiments correlated with reduced levels of *Ifng* mRNA demonstrating that Tpl2 signaling is required for transcriptional induction of IFNγ.

Decreased induction of IFNγ in Tpl2-deficient CTLs correlates with impaired activation of Akt-FOXO1 signaling.

Tpl2 is necessary for proper expression of key transcription factors, STAT4 and T-bet in $CD4^+$ T cells (7). In order to identify the potential mechanism by which Tpl2 regulates IFNγ production in CTLs, we investigated whether induction of transcription factors was also impaired in *Tpl2*$^{-/-}$ CTLs. However, unlike Tpl2-deficient $CD4^+$ T cells, STAT4 mRNA and protein levels as well as STAT4 phosphorylation were comparable between WT and *Tpl2*$^{-/-}$ CTLs (Figure 4.2A-B). Moreover, both *Tbx21* and *Eomes* were similarly induced in WT and *Tpl2*$^{-/-}$ cells demonstrating that Tpl2 does not have a role in regulating expression of transcription factors in CTLs (Figure 4.2A). Although expression of *Tbx21* was very similar between WT and *Tpl2*$^{-/-}$ cells, we observed a modest, but consistent increase in *Eomes* levels in *Tpl2*$^{-/-}$ CTLs in response to both cytokine and TCR signals. One signaling cascade known to negatively regulate *Eomes* expression is the mTOR-Akt-FOXO1 pathway [13,14]. Importantly, Akt activity is also required for inactivation of FOXO transcription factors and proper recruitment of RNA polymerase II to initiate *Ifng* transcription (14,15). Association of Tpl2 with Akt and Tpl2 regulation of Akt phosphorylation has been previously demonstrated (16,17). Therefore, we examined whether Tpl2 regulates Akt-FOXO1 signaling in CTLs. Consistent with the defective induction of *Ifng*,

phosphorylation of both Akt and FOXO1 were impaired in Tpl2-deficient CTLs compared to WT CTLs (Figure 4.2C). These data suggest that Tpl2 promotes *Ifng* expression via regulation of Akt-FOXO1 signaling.

Consistent with cell-type specific differences in Tpl2-dependent responses reported previously (18), our study demonstrates differences between $CD4^+$ and $CD8^+$ T cells in transcriptional regulation of major transcription factors, STAT4 and T-bet. We also demonstrate Tpl2 regulation of Akt signaling in CTLs as previously reported in other cell types (16,17). Since Akt-FOXO1 signaling is important in regulation of memory responses, further studies are warranted to investigate a role for Tpl2 in regulation of transcriptional programs that control $CD8^+$ T cell fate. Overall, our study establishes the significance of Tpl2 in IFNγ production during cell-mediated immune responses.

MATERIALS AND METHODS

Mice

Wild type (WT) C57BL/6J mice were purchased from The Jackson Laboratory. *Tpl2*$^{-/-}$ mice backcrossed to C57B6/J were kindly provided by Dr. Philip Tsichlis (Tufts University) and Thomas Jefferson University. Breeding colonies were maintained in the Central Animal Facility of the College of Veterinary Medicine. All animal experiments were performed in accordance to the national guidelines provided by the "The Guide for Care and Use of Laboratory Animals" and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Georgia.

Isolation and stimulation of CTLs

Naive $CD44^{lo}CD62L^{hi}$ $CD8^{+}$ T cells were purified from single-cell suspensions of mouse splenocytes and lymph nodes using a Beckman Coulter MoFlo XDP cell sorter. Cells were cultured in complete RPMI on plates precoated with 5 μg/ml each of anti-CD3 and anti-CD28 for 3 days followed by 2 days culture in medium supplemented with 40 U/ml IL-2. Cells were then washed and rested in RPMI medium for 3-4 h before restimulation with anti-CD3/CD28 or cytokines.

For cytokine secretion, 2×10^5 cells/200 μl were stimulated with varying doses of IL-12 alone or in combination with IL-18 (10ng/ml) or stimulated with plate-bound anti-CD3/CD28 (5 μg/ml) 24 h, and IFN-γ in cell culture supernatants was quantified by ELISA. For intracellular cytokine staining, 1×10^6 cells/ml were stimulated under the same conditions for 4 h with Golgi transport inhibitor. Cells were fixed, permeabilized, and stained using anti-mouse CD8 and IFNγ monoclonal antibodies. Samples were acquired on a BD LSRII flow cytometer and analyzed using FlowJo software (Tree Star, Inc.).

For mRNA analysis, 1×10^6 cells/ml were stimulated under the same conditions for 4 h, and *Map3k8 (Tpl2)*, *Ifng*, *Stat4*, *Tbx21*, *Eomes, Granzyme* and *Perforin* mRNA levels were measured by real-time PCR. Relative gene expression levels were calculated by normalizing the Ct levels of the target gene to both endogenous actin levels and an unstimulated WT control using the ΔΔCt method.

Protein Analysis

Cell lysates were separated on 4-12% gradient gels (Invitrogen) and were transferred to PVDF membranes using the iBlot Gel Transfer system (Invitrogen). Membranes were probed with various antibodies followed by horseradish peroxidase-labeled secondary antibodies. Protein

bands were visualized by enhanced chemiluminescent reagent (Lumigen) and Amersham Hyperfilm ECL (GE Healthcare). The following antibodies were used for immunoblotting: p-STAT4 (Tyr693), p-STAT1 (Tyr701), STAT4, pAkt (Ser473), Akt, p-FOXO1 (Ser256) and FOXO1 (Cell Signaling Technology).

Statistical analysis

Data represent means ± SEM. P-values were determined by paired Students *t*-test, and significance was assigned for p-values <0.05.

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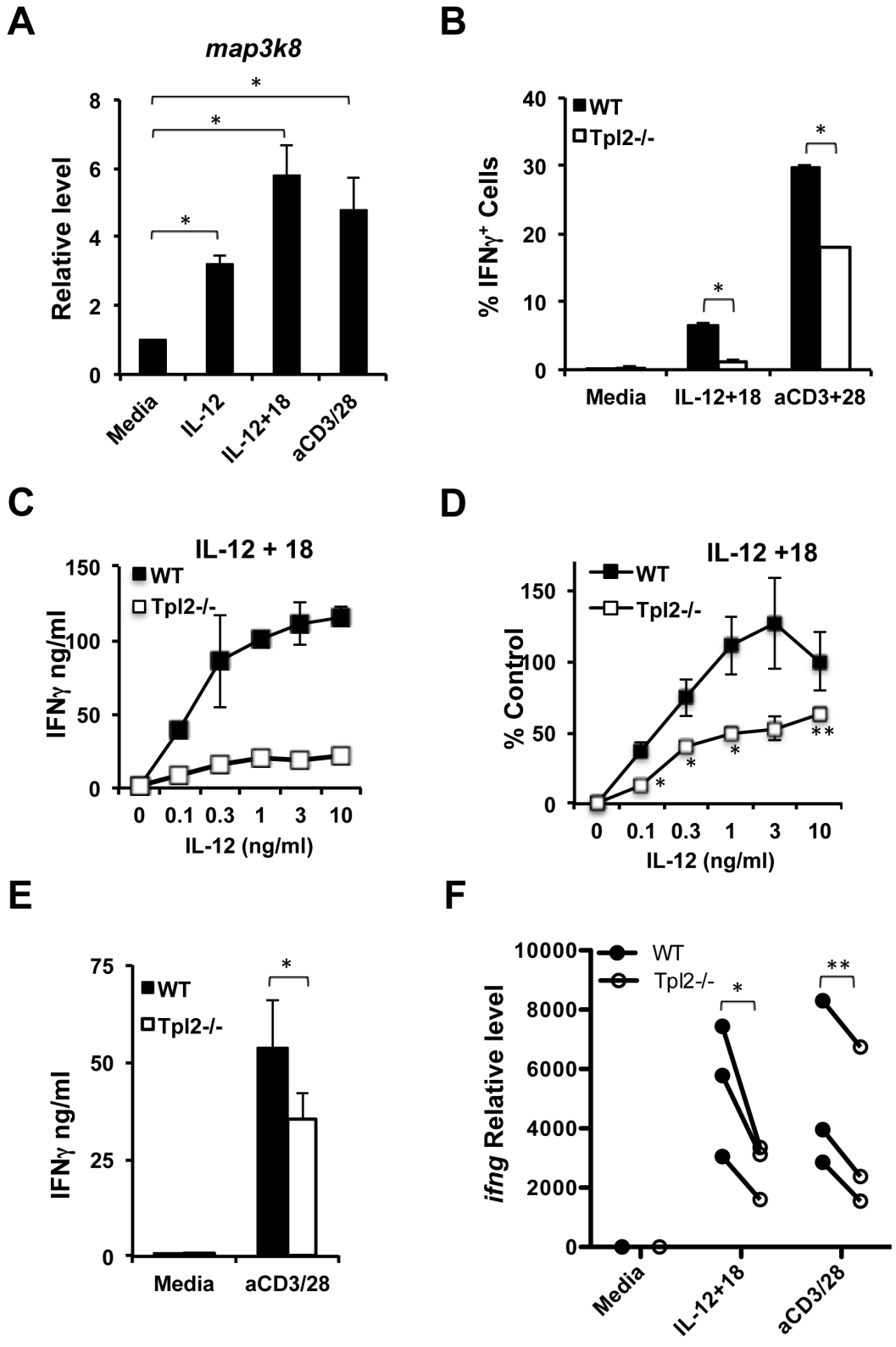
FIGURES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map3k8
Relative level
Media
IL-12
IL-12+18
aCD3/28
B
WT
Tpl2-/-
% IFNγ+ Cells
Media
IL-12+18
aCD3+28
C
IL-12 + 18
WT
Tpl2-/-
IFNγ ng/ml
IL-12 (ng/ml)
D
IL-12 +18
WT
Tpl2-/-
% Control
IL-12 (ng/ml)
E
WT
Tpl2-/-
IFNγ ng/ml
Media
aCD3/28
F
WT
Tpl2-/-
ifng Relative level
Media
IL-12+18
aCD3/28

Figure 4.1: Tpl2 promotes IFNγ production in CTLs (A) WT CTLs were stimulated with IL-12, IL-12/IL-18 or anti-CD3/28 for 4h and *Map3k8 (Tpl2)* expression was measured by RT-PCR. WT and *Tpl2*$^{-/-}$ CTLs were stimulated with anti-CD3/28 or cytokines for 4h **(B)** or 24h **(C, D, E),** and IFNγ production was measured by intracellular staining **(B)** or ELISA **(C, D, E)**. **(C)** Representative data **(D)** Data pooled from three independent experiments. For pool data, values are relative to WT cells treated with IL-12/IL-18 (10 ng/ml), which was considered 100% in individual experiments. **(F)** WT and *Tpl2*$^{-/-}$ CTLs were stimulated with anti-CD3/28 or cytokines for 4h and *Ifng* expression was measured by RT-PCR. Data collected on the same day are connected by lines. **(A, B, D, E, F)** Data are from three independent experiments. Data represent means ± SEM. * indicates $p<0.05$

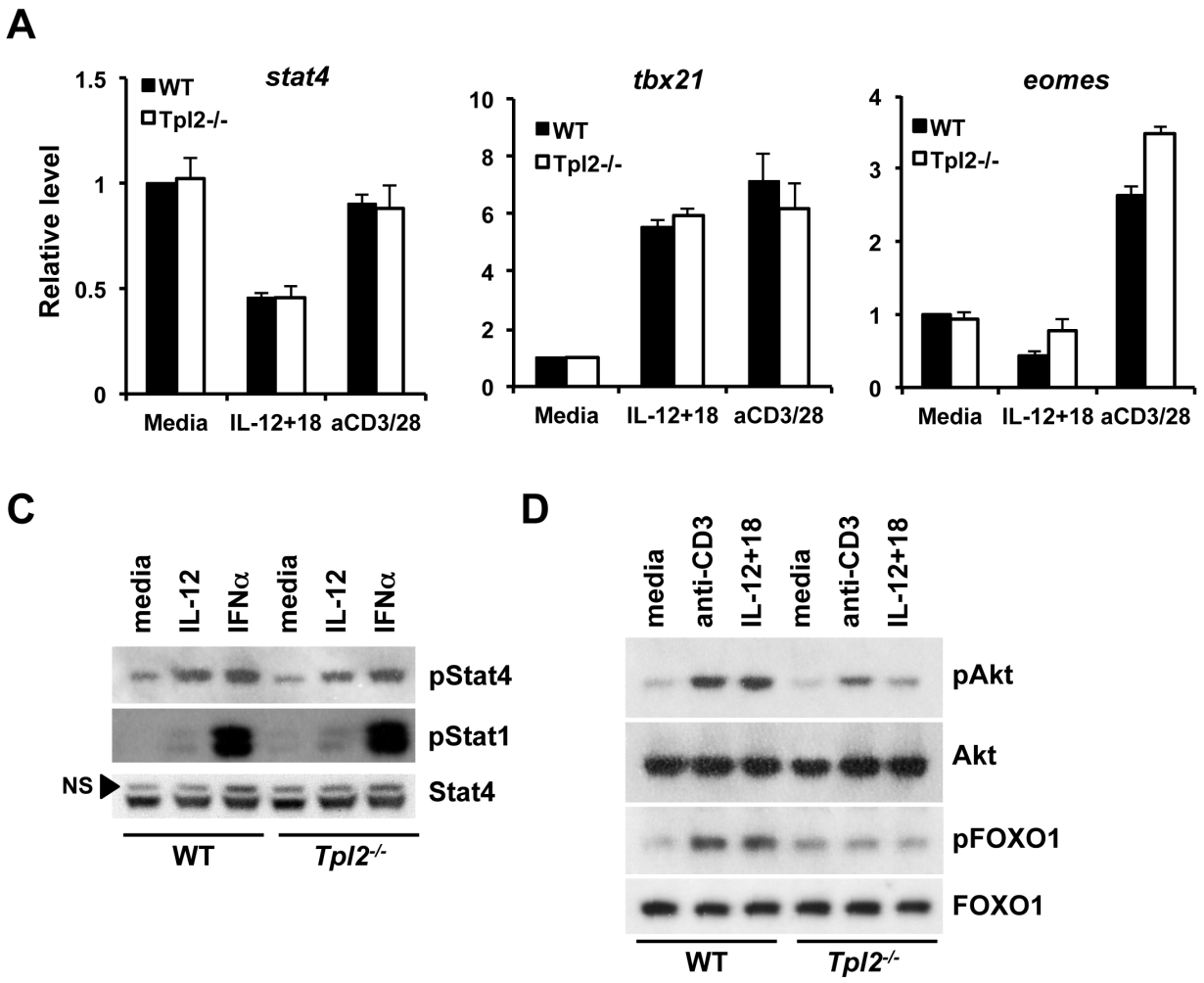
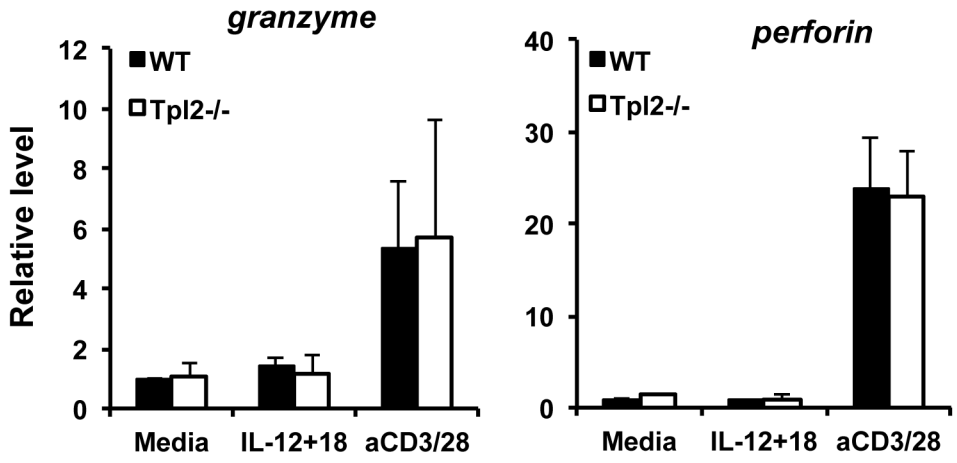


Figure 4.2: Tpl2 is dispensable for STAT4 phosphorylation, but is necessary for activation of Akt-FOXO1 signaling. (A) WT and *Tpl2*$^{-/-}$ CTLs were stimulated with anti-CD3/28 or cytokines for 4h and expression of *Stat4, Tbx21* and *Eomes* were measured by RT-PCR. **(B)** WT and *Tpl2*$^{-/-}$ CTLs were stimulated with IL-12 or IFNα for 20 min, and STAT4 and STAT1 phosphorylation was assessed by immunoblotting. (NS – non-specific) **(C)** WT and *Tpl2*$^{-/-}$ CTLs were stimulated with IL-12/IL-18 or anti-CD3 for 20 min, and Akt and FOXO1 phosphorylation was assessed by immunoblotting. Data are from **(A)** or representative **(B, C)** of 3 independent experiments. Data represent means ± SEM.



Supplementary Figure 4.3: Tpl2 is dispensable for *granzyme* and *perforin* expression in CTLs. (A) WT and *Tpl2*$^{-/-}$ CTLs were stimulated with plate-bound anti-CD3/28 or cytokines for 4 hrs and expression of granzyme and perforin were measured by RT-PCR. Data are 3 independentexperiments. Data represent means ± SEM.

CHAPTER 5

CONCLUSION

Tpl2 is now appreciated to regulate the induction of IFNs and other early, innate cytokines that may contribute to antiviral responses (1-4); however, no studies have directly addressed the role of Tpl2 in immune regulation during viral infections. Therefore the main aim of this study was to determine how Tpl2 is involved in virus sensing and antiviral innate and adaptive immune responses. The major findings are summarized below and in Figure 5.1:

1. Although Tpl2 regulates immune and inflammatory responses downstream of all toll-like receptors (TLRs), only a subset of TLRs tested, specifically TLR2, 4 and 7, directly initiate IKKβ-Tpl2-ERK signaling that dictates expression of proinflammatory cytokines and TNFα secretion. TLR3 and 9 do not directly couple to this pathway but induce ERK phosphorylation via NADPH-oxidase-dependent autocrine signaling. Activation of the IKKβ-Tpl2-ERK pathway by TLR4 requires the TLR4 co-receptor, CD14, and the tyrosine kinase, Syk, in addition to either the MyD88 or TRIF adaptor protein.
2. Tpl2 is essential for generation of antimicrobial reactive oxygen species (ROS) during TLR signaling.
3. Tpl2 differentially regulates the induction of Type I (IFNα/β) and Type III (IFNλ) IFNs in a cell-type- and stimulus-specific manner. In plasmacytoid DCs, in addition to ERK, Tpl2 regulates Akt phosphorylation and both Tpl2-ERK and PI3K-mTOR pathways are positive regulators of TLR-mediated IFNλ production.

4. Tpl2 is required for IFNλ secretion during influenza virus infection *in vitro* and *in vivo*.
5. Tpl2 transduces Type I IFN signals promoting the induction of interferon-stimulated genes (ISGs) that limit virus replication.
6. Tpl2 signaling is necessary for proper expansion of virus-specific $CD8^+$ T cells. Tpl2 also promotes antigen- and cytokine-induced IFNγ production in $CD8^+$ T cells in a cell intrinsic manner. Tpl2 regulation of effector $CD8^+$ T cell responses correlated with Tpl2-dependent activation of Akt-FOXO1 signaling.
7. Tpl2 limits virus replication, facilitates viral clearance and restricts morbidity and mortality during influenza virus infection.

Overall, this study demonstrates the critical role of Tpl2 in pathogen sensing as well as in the regulation of antiviral innate and adaptive immune responses.

In addition to providing a better understanding about key events that distinguish early innate responses by diverse TLRs, the observed differences in Tpl2-ERK activation have many implications regarding the role of Tpl2 in immune and inflammatory responses. The exclusion of IKKβ-Tpl2-ERK signaling by TLR9 may be a regulatory mechanism to limit autoimmune diseases initiated by 'self' DNA. Furthermore, direct activation of Tpl2 during TLR7 signaling suggests that Tpl2 is likely to play a preferential role in host defense against RNA viruses that trigger TLR7. The increased replication of influenza virus in *Tpl2*$^{-/-}$ mice is consistent with previous *in vitro* studies reporting increased replication of vesicular stomatitis virus in Tpl2-deficient cells (5). The essential role of Tpl2 in ROS production helps to explain the increased susceptibility to intracellular bacterial infections in *Tpl2*$^{-/-}$ mice (3,6). Since ROS is also important in antiviral responses (7), defective induction of ROS may also enhance susceptibility

to viral infections. The molecular events linking Tpl2 to ROS production are currently unknown, and further studies are warranted to identify the signaling events that link Tpl2 to NOX enzymes. Even though IFNλs are secreted in response to many viruses and are important for antiviral resistance (8,9), the mechanisms controlling induction of these IFNs are less well understood (10). In this study, Tpl2 was identified as a major regulator of IFNλ in pDCs. Tpl2 is a component of both TLR and IFNAR pathways regulating IFNλ production, since Tpl2 ablation reduced IFNλ secretion in response to either TLR ligands R848 and CpG or direct stimulation with IFNβ. Although Tpl2 regulates TLR-mediated Type I IFN production in pDCs, Tpl2 signaling did not have a detectable role in IFNα/β production during *in vitro* and *in vivo* influenza virus infection. Notably, while Tpl2 is critical for ERK activation during TLR signaling, influenza virus is known to activate ERK via the Raf/MEK pathway (11). Thus virus-induced activation of divergent kinases and transcription factors could differentially regulate induction of Type I versus Type III IFNs. Selective induction of Type III IFNs by distinct transcription factors is further supported by the unique role for IRF1 in regulating the expression of IFNλ, but not IFNβ (10). Importantly, our data revealed selective, Tpl2-dependent regulation of IFNλ during influenza virus infection. Although both Tpl2-ERK and PI3K-mTOR pathways are positive regulators of IFNλ, the precise mechanism by which Tpl2 regulates IFNλ induction is unknown. Further studies are needed to determine whether Tpl2 mediates IFNλ induction via regulation of transcription factors involved in IFN gene induction like NFκB, IRF7, AP-1 and STAT1.

The findings from this study also establish an essential role for Tpl2 in the phosphorylation of ERK and STAT1^{ser727} during IFN signaling. Although MAP kinase pathways are known to be activated in response to IFNs, the importance of Tpl2 in transducing IFN signals and regulating

IFN-inducible effectors has not been described before. Interestingly, not all ISGs were similarly affected by Tpl2 ablation at the mRNA level. However, since Tpl2 signaling also regulates cap-dependent translation of many genes, Tpl2 may also regulate biological effects of IFNs at posttranscriptional levels (12,13). The essential role of Tpl2 in IFN signaling and induction of ISGs also helps to explain the enhanced pathogenesis of *Tpl2*$^{-/-}$ mice during influenza infection, since defective expression of individual ISGs, like IFITM3, are sufficient to alter the course of infection (14). In addition to demonstrating the involvement of Tpl2 in virus sensing pathways, this study establishes Tpl2 as a host factor with intrinsic ability to restrict influenza virus replication and demonstrates immune regulatory functions of Tpl2 within the lungs during influenza virus infection. To our knowledge, this is the first report demonstrating Tpl2's obligate role in regulating antiviral IFN responses and promoting viral clearance *in vivo*. These findings clearly necessitate the evaluation of Tpl2 functions in airway epithelial cells, the primary target of respiratory viral infections.

Similar to antiviral innate responses, Tpl2 is also important in adaptive $CD8^+$ T cell responses during influenza virus infection. Tpl2 promotes both the expansion of virus-specific $CD8^+$ T cells as well as IFNγ secretion during recall response. Further experiments demonstrated a T cell intrinsic role for Tpl2 signaling in induction of IFNγ, which correlated with Tpl2-dependent activation of Akt-FOXO1 cascade. Tpl2 regulation of Akt-FOXO1 signaling in $CD8^+$ T cells also suggests a role for Tpl2 in memory responses as well as in antiviral responses during chronic viral infections (15,16).

The findings from this study are important, especially because influenza virus is a ubiquitous seasonal virus that afflicts millions of people annually, causing significant morbidity, mortality and socio-economic burdens (17). Whether Tpl2 similarly restricts the replication of other

classes of viruses requires further investigation. Overall, this study demonstrates the critical role for Tpl2 in integrating antiviral innate and adaptive immune responses and also establishes the significance of Tpl2 in host defense mechanisms during viral infections. The identification of Tpl2 as a host viral restriction factor provides further insights for developing disease intervention strategies and antiviral therapies aimed at controlling virus replication and virus-induced inflammatory responses.

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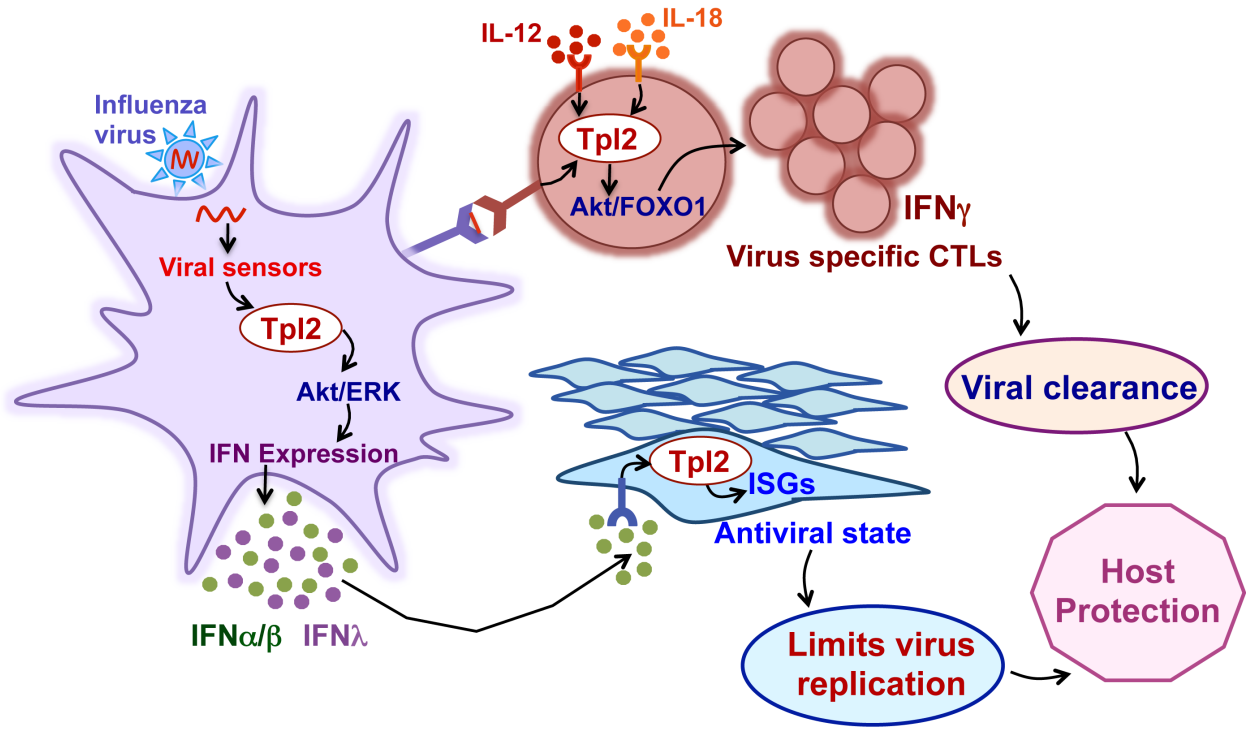

Figure 5.1: Schematic representation of Tpl2 regulation of antiviral responses. During influenza virus infection, the viral RNA is sensed by various viral sensors that activate downstream signaling cascades leading to induction of Type I and Type III IFNs in a Tpl2-dependent manner. Tpl2 also transduces IFN signals regulating the expression of IFN-stimulated genes, which induce an antiviral state that limits virus replication and cell-to-cell spread. In addition to innate responses, Tpl2 mediates antigen receptor and cytokine receptor signaling in $CD8^+$ T cells. Consistent with this, Tpl2 promotes secretion of IFNγ and expansion of virus-specific $CD8^+$ T cells that are important in facilitating viral clearance from infected lungs. Therefore, by integrating both innate and adaptive antiviral responses, Tpl2 enhances host protection and restricts morbidity and mortality during influenza virus infection.