IRAK-M Is a Negative Regulator of Toll-like Receptor Signaling

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Summary

Toll-like receptors (TLRs) detect microorganisms and protect multicellular organisms from infection. TLRs transduce their signals through MyD88 and the serine/threonine kinase IRAK. The IRAK family consists of two active kinases, IRAK and IRAK-4, and two inactive kinases, IRAK-2 and IRAK-M. IRAK-M expression is restricted to monocytes/macrophages, whereas other IRAKs are ubiquitous. We show here that IRAK-M is induced upon TLR stimulation and negatively regulates TLR signaling. IRAK-M prevented dissociation of IRAK and IRAK-4 from MyD88 and formation of IRAK-TRAF6 complexes. IRAK-M$^{-/-}$ cells exhibited increased cytokine production upon TLR/IL-1 stimulation and bacterial challenge, and IRAK-M$^{-/-}$ mice showed increased inflammatory responses to bacterial infection. Endotoxin tolerance, a protection mechanism against endotoxin shock, was significantly reduced in IRAK-M$^{-/-}$ cells. Thus, IRAK-M regulates TLR signaling and innate immune homeostasis.

Introduction

The innate immune system is a host defense mechanism that is conserved evolutionarily from plants to humans (Medzhitov and Janeway, 1997). Essential components of the innate immune system are Toll-like receptors (TLRs), which recognize microbial products termed PAMPs (pathogen-associated molecular patterns). PAMP recognition leads to activation of the innate immune system, which in turn activates adaptive immunity (Medzhitov and Janeway, 1997). TLRs recognize specific PAMPs through their LRR (leucine-rich repeat)-containing extracellular domains: TLR2, TLR3, TLR4, TLR5, TLR6, and TLR9 recognize peptidoglycan, double-stranded RNA, LPS, Flagellin, mycoplasmal macrophage-activating lipopeptide-2 kDa (MALP-2), and CpG bacterial DNA, respectively (Alexopoulou et al., 2001; Hayashi et al., 2001; Hemmi et al., 2000; Hoshino et al., 1999; Poltorak et al., 1998; Qureshi et al., 1999; Takeuchi et al., 1999). Several different signaling components are involved in TLR signaling. The adaptor molecule MyD88 has dual binding domains, a TIR domain (Toll and IL-1-

receptor homology domain) and death domain (DD), and binds to the intracellular TIR domain of TLRs (Medzhitov et al., 1998; Wesche et al., 1997). Upon TLR stimulation, the serine/threonine kinase IRAK is recruited to the TLR signaling complex via a DD-DD interaction (Medzhitov et al., 1998). IRAK is phosphorylated either by auto- or crossphosphorylation (Cao et al., 1996; Wesche et al., 1999), losing affinity for the TLR signaling complex. IRAK is thus released from the complex, permitting binding to downstream molecules such as TRAF6, causing activation of NF-κB, JNK, p38, and ERK1/2 (Kawai et al., 1999; Medzhitov et al., 1998; Wesche et al., 1997; Zhang et al., 1999). Like IRAK, IRAK-2 and IRAK-4 are expressed ubiquitously (Li et al., 2002; Muzio et al., 1997). However, IRAK-M is restricted to monocytes/macrophages (Wesche et al., 1999). IRAK-2 and IRAK-M have no kinase activity but activate NF-κB when overexpressed in 293T cells and partially restore IL-1 signaling in IRAK$^{-/-}$ cells (Muzio et al., 1997; Wesche et al., 1999). All IRAKs act downstream of MyD88 and can bind to TRAF6 (Li et al., 2002; Medzhitov et al., 1998; Wesche et al., 1997, 1999), suggesting that IRAK family proteins play a redundant role. Indeed, IRAK and IRAK-4$^{-/-}$ cells and mice still partially respond to IL-1β and TLR stimulation (Kanakaraj et al., 1998; Suzuki et al., 2002; Swantek et al., 2000; Thomas et al., 1999).

Although the inflammatory response is critical to control the growth of pathogenic microorganisms (Cross et al., 1995; Eden et al., 1988; Hagberg et al., 1984; Shahin et al., 1987), excessive cytokine production is harmful to the host and can even be fatal (Beutler et al., 1985; Danner et al., 1991). Chronic repeated exposed to endotoxin (or LPS) causes a transient increase in the threshold to endotoxin challenge (Beeson, 1947; Greisman et al., 1966; Ziegler-Heitbrock, 1995). This "endotoxin tolerance" is a negative feedback mechanism to protect from endotoxic shock (Gustafson et al., 1995; Henricson et al., 1990; Salkowski et al., 1998). Several factors are involved in this process, including TLR4 downregulation (Nomura et al., 2000) and decreased NF-κB activation (Goldring et al., 1998; Kastenbauer and Ziegler-Heitbrock, 1999; Ziegler-Heitbrock et al., 1994). However, the underlying mechanisms are largely unknown. To investigate the role of IRAK-M, we generated IRAK-M$^{-/-}$ mice using gene targeting. Surprisingly, innate immunity was strongly enhanced in IRAK-M$^{-/-}$ mice, and IRAK-M$^{-/-}$ cells have strikingly impaired endotoxin tolerance, showing that IRAK-M negatively regulates TLR signaling.

Results

Molecular Cloning of *irak-M* and Generation of IRAK-M$^{-/-}$ Mice

A homology search for IRAK homologs in the EST databases and extension of the coding sequence by 5'-RACE resulted in the molecular cloning of the full-length cDNA encoding a novel mouse kinase of 596 amino acids and a calculated molecular mass of 68.7 kDa.

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BLAST search revealed that this kinase is the murine ortholog of human IRAK-M, sharing 73% amino acid sequence identity. Mouse IRAK-M has 12 serine/threonine kinase subdomains and a conserved lysine in the ATP binding site in subdomain II. However, like human, mouse IRAK-M lacks the catalytically active aspartate in subdomain VIB (see Supplemental Figure S1A at http://www.cell.com/cgi/content/full/110/2/191/DC1), suggesting that mouse IRAK-M lacks kinase activity. To assess the role of IRAK-M, we generated IRAK-M$^{-/-}$ mice. Homologous recombination was confirmed by Southern blot analysis (see Supplemental Figures S1B and S1C), and the absence of IRAK-M expression was confirmed by Western blot using an antibody against the C terminus of IRAK-M (see Supplemental Figure S1D). To eliminate the possibility of functional truncated protein containing the N terminus of IRAK-M, we performed Northern blot analysis using a probe for the N terminus of the *irak-M* gene. A smaller band of reduced expression level was observed in heterozygous and homozygous cells (see Supplemental Figure S1E). The sequence of this transcript obtained by RT-PCR revealed the transcribed gene to contain fused *irak-M* and *neo* genes and an in-frame stop codon, predicting a mutant product lacking a functional kinase domain (see Supplemental Figure S1F). Although we do not know if this transcript is translated and stably expressed in cells, an N-terminal IRAK-M fragment could be inhibitory for signaling (Wesche et al., 1999). This molecule does not appear to be functional in IRAK-M$^{-/-}$ mice, however, since heterozygous IRAK-M mice have no "inhibited" phenotype and since the phenotype of IRAK-M$^{-/-}$ mice is increased (not decreased) inflammation. IRAK-M$^{-/-}$ mice were born at the expected Mendelian ratio and showed no gross developmental abnormalities (data not shown).

Enhanced Response in IRAK-M$^{-/-}$ Macrophages upon TLR Stimulation

To characterize the effect of IRAK-M deficiency on TLR signaling, IRAK-M$^{-/-}$ macrophages were prepared from bone marrow and stimulated with various PAMPs. Surprisingly, IRAK-M$^{-/-}$ macrophages revealed significantly *increased* production of IL-12 p40, IL-6, and TNFα when compared to wild-type macrophages at 24 hr (Figures 1A–1C) and 6 hr after stimulation (not shown). IL-6 production was greater in IRAK-M$^{-/-}$ cells throughout the entire dose range of LPS (100 pg/ml to 1 mg/ml) (Figure 1D). Interestingly, although IRAK-M deficiency affected signaling by all TLRs tested, it had the strongest effect on TLR9, which is a receptor for CpG DNA (Figures 1A–1C).

Since IL-1 receptor (IL-1R) and TLR are members of an evolutionarily conserved receptor family and both require MyD88 for signaling, we tested whether IRAK-M deficiency affects IL-1R signaling. Bone marrow-derived macrophages were stimulated with IL-1β, and IL-6 production was measured. The IL-1β-induced production of IL-6 in IRAK-M$^{-/-}$ cells was higher in a time- and dose-dependent manner (Figure 1E).

Increased Inflammatory Responses of IRAK-M$^{-/-}$ Mice Challenged with Bacteria In Vitro and In Vivo

To investigate the roles of IRAK-M in host defense, we infected IRAK-M$^{-/-}$ macrophages with two gram-nega-
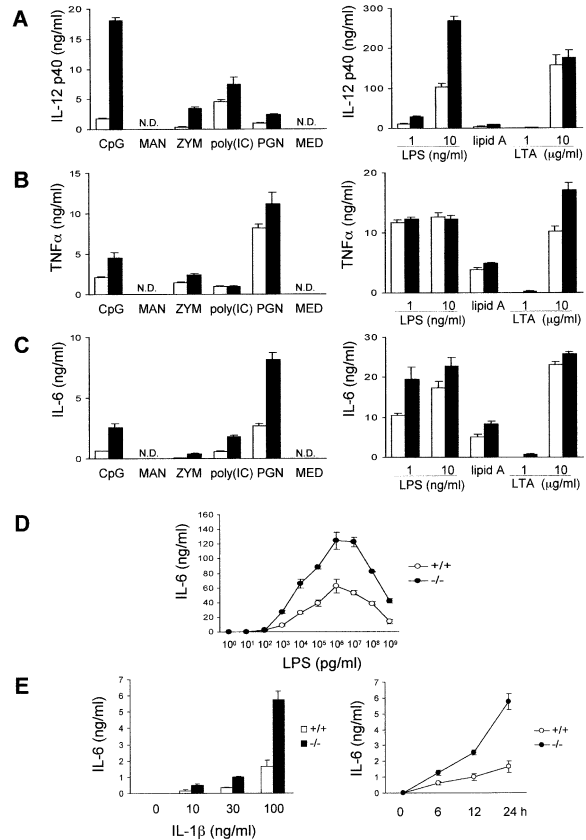

Figure 1. Increased Cytokine Production of IRAK-M$^{-/-}$ Macrophages upon PAMP Stimulation

(A) IL-12 p40: Bone marrow-derived macrophage from wild-type and IRAK-M$^{-/-}$ mice were plated in 24-well plates at a density of 2×10^5 cells/well. Cells were stimulated with 10 μM of CpG oligo DNA (CpG), 10 μg/ml of mannan (MAN), 10 μg/ml of zymosan (ZYM), 10 μg/ml of double-stranded RNA (poly(IC)), 10 μg/ml of peptidoglycan (PGN), 1 or 10 ng/ml of LPS, 10 μg/ml of Lipid A, 1 or 10 μg/ml of lipoteichoic acid (LTA), or medium alone (MED). After 24 hr, the concentration of IL-12 p40 in the supernatant was examined by ELISA. Experiments were repeated at least three times in triplicate with similar results. N.D. indicates not detected.

(B) TNFα: Bone marrow-derived macrophages from wild-type and IRAK-M$^{-/-}$ mice were stimulated as in (A). After 24 hr, TNFα in the supernatant was determined by ELISA. Experiments were repeated at least three times in triplicate with similar results. N.D. indicates not detected.

(C) IL-6: Bone marrow-derived macrophage from wild-type and IRAK-M$^{-/-}$ mice were stimulated as in (A). After 24 hr, IL-6 in the supernatant was determined by ELISA. Experiments were repeated at least three times in triplicate with similar results. N.D. indicates not detected.

(D) IL-6: Bone marrow-derived macrophage from wild-type and IRAK-M$^{-/-}$ mice were stimulated with LPS at the indicated concentration. After 24 hr, the concentration of IL-6 in the supernatant was examined by ELISA. Experiments were repeated twice in triplicate with similar results.

(E) Increased production of IL-6 by IRAK-M$^{-/-}$ macrophages on IL-1β stimulation. Bone marrow-derived macrophage were prepared from wild-type and IRAK-M$^{-/-}$ mice as (A) and stimulated with IL-1β as indicated for 24 hr (left) or with 100 ng/ml of IL-1β for indicated times (right). IL-6 in the supernatant was determined by ELISA. Experiments were repeated twice in triplicate with similar results.

tive bacteria, *Salmonella typhimurium* and *Escherichia coli,* and assessed cytokine production in the cell supernatants. Because wild-type *S. typhimurium* rapidly kills
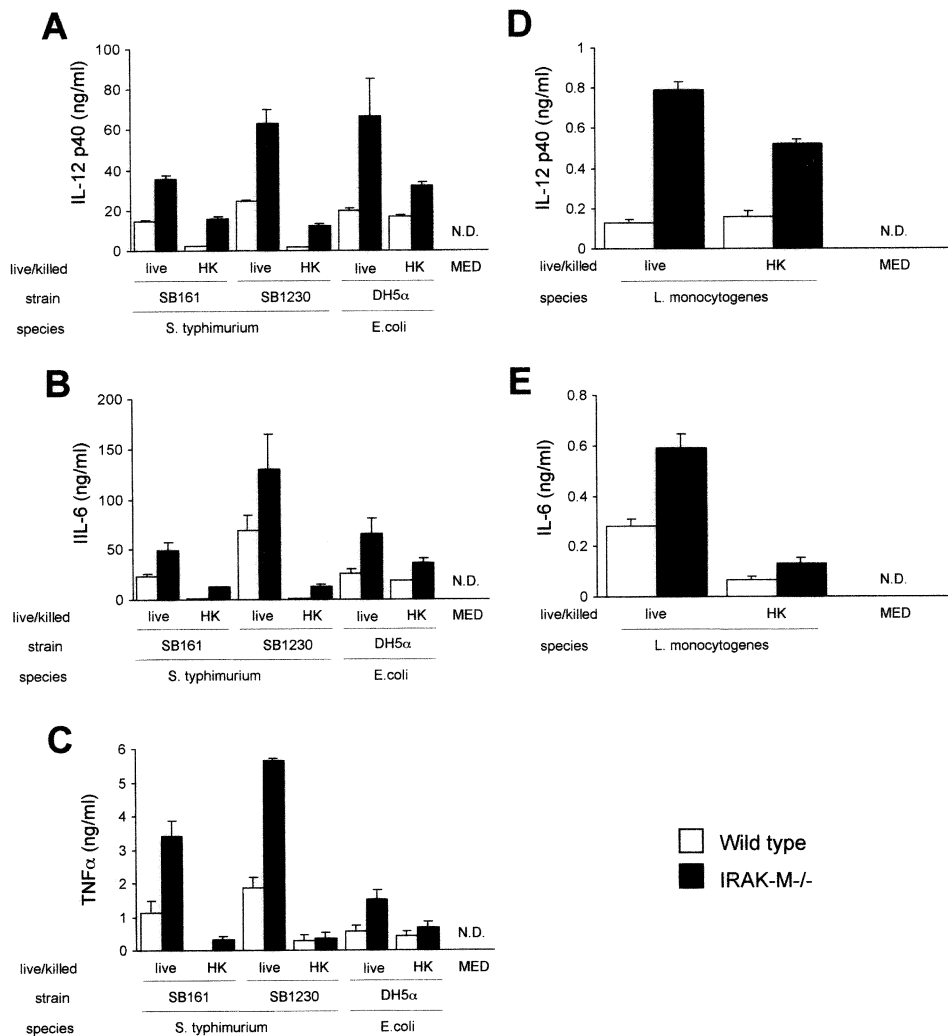
Figure 2. Increased Response of IRAK-M$^{-/-}$ Mice upon Bacterial Challenge In Vitro

(A) IL-12 p40 to gram-negative bacterial challenge. Bone marrow-derived macrophages were prepared from wild-type and IRAK-M$^{-/-}$ mice. Cells were infected with *Salmonella typhimurium* (strains SB161 and S1230) or *Escherichia coli* (strain DH5α) as described in Experimental Procedures. After infection (24 hr), the concentration of IL-12 p40 in the supernatant was examined by ELISA. Abbreviations: HK, heat-killed bacteria; N.D., not detected.

(B) IL-6 to gram-negative bacterial challenge. Wild-type and IRAK-M$^{-/-}$ macrophages were prepared and infected with *Salmonella typhimurium* (SB161 and SB1230) or *Escherichia coli* (DH5α) as described in (A). After infection (24 hr), the concentration of IL-6 in the supernatant was examined by ELISA. N.D. indicates not detected.

(C) TNFα to gram-negative bacterial challenge. Wild-type and IRAK-M$^{-/-}$ macrophages were prepared and infected with *Salmonella typhimurium* (S161 and S1230) or *Escherichia coli* (DH5α) as described in (A). After infection (24 hr), the concentration of TNFα in the supernatant was examined by ELISA. N.D. indicates not detected.

(D) IL-12 p40 to gram-positive bacterial challenge. Bone marrow-derived macrophages from wild-type and IRAK-M$^{-/-}$ mice were infected with *Listeria monocytogenes* as described in Experimental Procedure. After infection (24 hr), IL-12 p40 in the supernatant was determined by ELISA. Abbreviations: HK, heat-killed bacteria; N.D., not detected.

(E) IL-6 to gram-positive bacterial challenge. Wild-type and IRAK-M$^{-/-}$ macrophages were infected with *Listeria monocytogenes* as described in (D). After infection (24 hr), IL-6 in the supernatant was determined by ELISA. N.D. indicates not detected.

macrophages via the type III secretion system (Chen et al., 1996), we used two type III secretion-negative mutant strains. IRAK-M$^{-/-}$ macrophages challenged with live or heat-killed *S. typhimurium* and *E. coli* produced significantly increased amounts of IL-12p40, IL-6, and TNFα at 24 hr (Figures 2A–2C) and 6 hr (data not shown) after infection, compared to wild-type cells. IRAK-M$^{-/-}$ macrophages were also challenged with the gram-positive bacterium *Listeria monocytogenes*, and cytokine production was analyzed after infection. IRAK-M$^{-/-}$ macrophages produced increased IL-12p40 and IL-6, upon treatment with either live or heat-killed *L. monocytogenes* at 24 hr (Figures 2D and 2E) and 6 hr after infection (data not shown).

To investigate the role of IRAK-M in vivo, we infected IRAK-M$^{-/-}$ mice with a virulent strain of *S. typhimurium*. We again chose a type III secretion mutant of *S. typhimurium* that, although virulent in a mouse model of infection, gives significantly reduced intestinal pathology (Galan and Curtiss, 1989; Penheiter et al., 1997). IRAK-M$^{-/-}$ mice
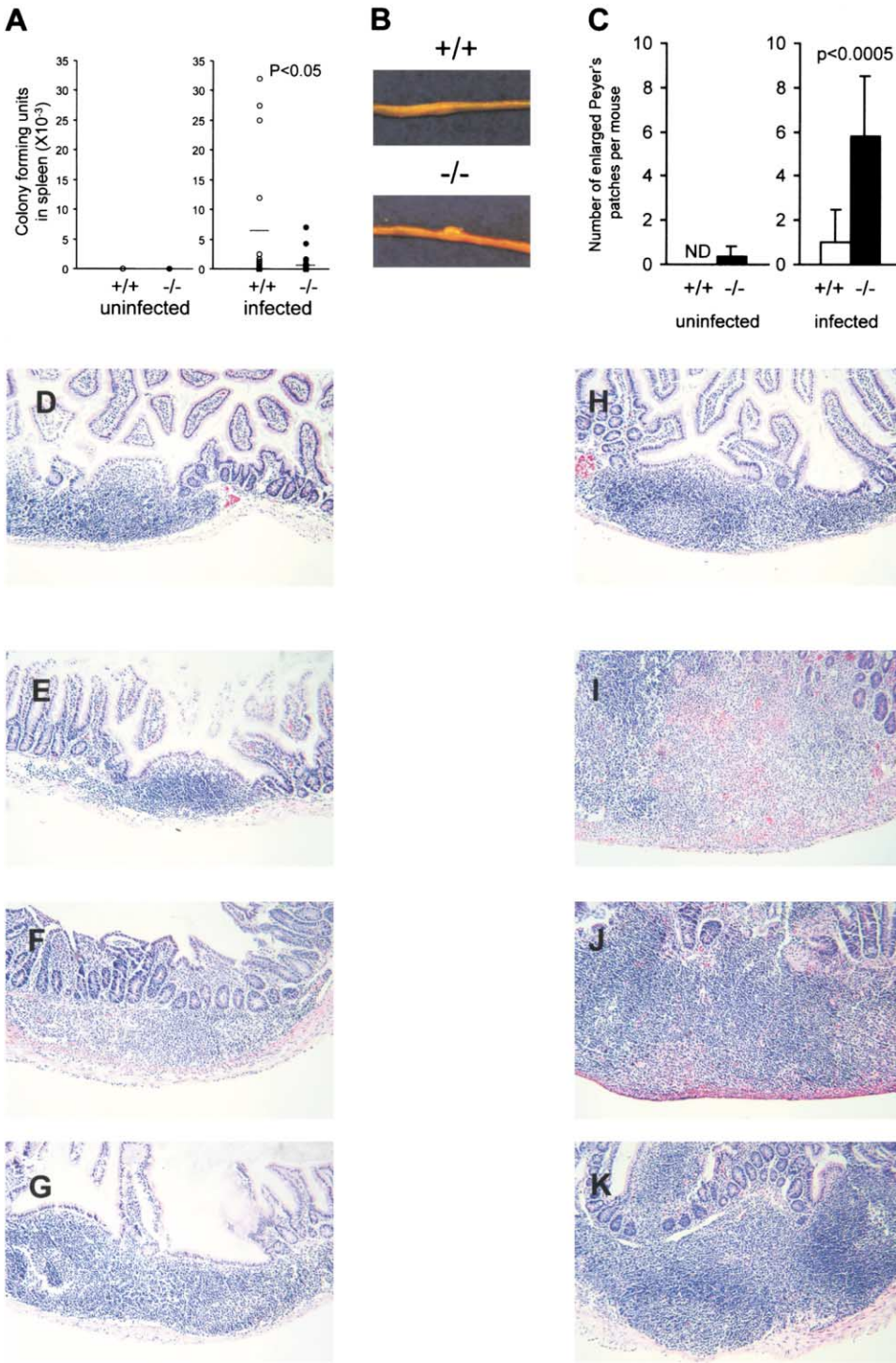
Figure 3. Increased Response of IRAK-M$^{-/-}$ Mice upon *Salmonella typhimurium* In Vivo

(A) No increased susceptibility to *Salmonella* infection in IRAK-M$^{-/-}$ mice. Wild-type and IRAK-M$^{-/-}$ mice were infected orally with *Salmonella typhimurium* SB161 at 10^9 bacteria per mouse. After infection (72 hr), mice were euthanized, and colony-forming units (CFU) of *Salmonella typhimurium* in spleen of wild-type and IRAK-M$^{-/-}$ mice were determined (right). Eighteen mice in each group were used for the experiments. The averages of *S. typhimurium* CFU in wild-type and IRAK-M$^{-/-}$ mice are indicated with bars. The p values were determined by the Mann-Whitney Test. Bacterial CFU of uninfected mice are also shown as control (n = 3 for each group, left).

(B) Enlarged Peyer's patch in small intestine of IRAK-M$^{-/-}$ mice upon *Salmonella* challenge in vivo. Wild-type and IRAK-M$^{-/-}$ mice were infected orally with 10^9 *Salmonella typhimurium* SB161 CFU per mouse. After infection (72 hr), mice were euthanized and the small intestines were examined. Typical appearance of the small intestine of wild-type and IRAK-M$^{-/-}$ mice are presented.

(C) Increased number of enlarged Peyer's patches in IRAK-M$^{-/-}$ mice upon *Salmonella* challenge in vivo. Wild-type and IRAK-M$^{-/-}$ mice were infected with *Salmonella typhimurium* as described in (B). After infection (72 hr), mice were euthanized, and enlarged Peyer's patches in the

were infected with *S. typhimurium* orally and sacrificed 72 hr later to assess the intestinal inflammation and splenic bacterial load. IRAK-M$^{-/-}$ mice challenged with *S. typhimurium* showed grossly enlarged Peyer's patches (Figure 3B). Further, the actual number of enlarged Peyer's patches was significantly increased in infected IRAK-M$^{-/-}$ mice compared to wild-types (Figure 3C). Histological examination of Peyer's patches in IRAK-M$^{-/-}$ mice infected with S. *typhimurium* revealed severe inflammatory infiltrates in Peyer's patches with numerous polymorphonuclear cells (Figures 3I–3K) and accompanying hemorrhage (Figures 3I and 3J), in significant contrast to wild-type mice, which showed only mild Peyer's patch inflammation (Figures 3E–3G). The bacterial organ load was examined using spleens of infected mice. In spite of the increased inflammatory response in the gut, the number of bacterial colony-forming units (CFU) in spleens of infected IRAK-M$^{-/-}$ mice were not increased compared to wild-type mice (Figure 3A), suggesting that the increased inflammatory response in IRAK-M$^{-/-}$ mice was due to enhanced innate immunity rather than enhanced susceptibility to infection.

Enhanced TLR Signaling in IRAK-M$^{-/-}$ Cells

TLR stimulation activates NF-κB, JNK, p38, and ERK1/2 through the signaling molecules MyD88 and IRAK (Kawai et al., 1999; Medzhitov et al., 1998). We therefore examined the activation of these downstream effectors of TLR signaling in IRAK-M$^{-/-}$ cells. IRAK-M$^{-/-}$ macrophages were stimulated with CpG DNA or LPS, and the activation of NF-κB, JNK, p38, and ERK1/2 was analyzed. CpG DNA stimulation of IRAK-M$^{-/-}$ macrophages showed more rapid phosphorylation and degradation of IκBα as compared to wild-type cells (Figure 4A). Phosphorylation of JNK, p38, and ERK1/2 was faster and stronger in IRAK-M$^{-/-}$ macrophages than in wild-type cells (Figure 4A), indicating enhanced signaling in CpG DNA-stimulated IRAK-M$^{-/-}$ macrophages and suggesting that IRAK-M negatively regulates these signaling pathways. LPS-stimulated IRAK-M$^{-/-}$ macrophages also showed enhanced signaling to NF-κB, JNK, p38, and ERK, although the augmentation was not as great as for CpG (Figure 4B). To eliminate the possibility that IRAK-M$^{-/-}$ cells are somehow primed and sensitive to *any* stimuli, we stimulated IRAK-M$^{-/-}$ macrophages with TNFα. There was no increased NF-κB, JNK, p38, and ERK activation upon TNFα stimulation, indicating that the increased IRAK-M$^{-/-}$ signaling is specific to TLR stimulation (Figure 4C).

IRAK-M Is Required for Endotoxin Tolerance

Our results showing that IRAK-M is a negative regulator of TLR signaling suggested that IRAK-M might be in-

volved in the induction of endotoxin tolerance. If so, then IRAK-M might be expected to be inducible by TLR signaling. To examine this, wild-type macrophages were stimulated with LPS, and the levels of *irak-M* and *irak* mRNA were assessed by Northern blotting. *irak-M* mRNA was significantly induced by LPS stimulation, whereas *irak* mRNA was not induced (Figure 5A). The protein levels of IRAK-M, IRAK, MyD88, and TRAF6 were also examined by Western blotting. The expression of IRAK-M was induced by LPS, whereas the expression of IRAK, MyD88, and TRAF6 were not (Figure 5B). We next determined the ability of IRAK-M$^{-/-}$ macrophages to develop endotoxin tolerance. IRAK-M$^{-/-}$ macrophages were first stimulated with 10 or 100 ng/ml of LPS (primary LPS stimulation). After incubation for the indicated periods, cells were restimulated with 10 ng/ml of LPS (second LPS stimulation), and cytokine production was examined after secondary LPS stimulation. At each time point, cytokine levels were compared to the cytokine levels of macrophages that received only the second LPS stimulation. As shown by previous studies (Nomura et al., 2000), wild-type macrophages showed reduced cytokine production in accordance with a longer incubation time and a higher dose of LPS (Figure 5C), indicating that endotoxin tolerance is dependent on the duration and dose of the primary LPS treatment. IRAK-M$^{-/-}$ macrophages showed a lack of endotoxin tolerance, and the cytokine levels produced upon LPS restimulation were not decreased as much as in restimulated wild-type macrophages (Figure 5C). IL-6 and TNFα production after short incubation times was even increased compared to that of nonpretreated macrophages, indicating that IRAK-M is essential for endotoxin tolerance and that the absence of this negative regulator causes abnormal enhancement of inflammatory cytokine production. After 24 hr of incubation, however, IRAK-M$^{-/-}$ macrophages showed reduced IL-6 and TNFα production and almost no IL-12p40 production, suggesting a possible second mechanism to mediate endotoxin tolerance, which operates at later time points in IRAK-M$^{-/-}$ cells.

Inhibition of TLR Signaling by IRAK-M

IRAK and IRAK-4 are positive signal transducers, whereas our data show IRAK-M to be a negative regulator. IRAK activation leads to the phosphorylation of IRAK and subsequent formation of IRAK and TRAF6 complexes (Kojima et al., 1998). To test whether IRAK-M inhibits the ligand-stimulated association of IRAK and TRAF6, we transfected 293T cells with either control or IRAK-M expression vectors. Cells were stimulated with IL-1β, and the association of endogenous IRAK and TRAF6 was examined. In mock-transfected cells, the

small intestine of wild-type and IRAK-M$^{-/-}$ mice were enumerated (right). Enlarged Peyer's patches were readily apparent and up to ~3 mm in diameter. A total of ten mice in each group were used in the experiments. The p values were determined by the Student's t test. The number of enlarged Peyer's patches in uninfected mice is also shown as control (n = 7 for wild-type and n = 6 for mutant mice, left). N.D. indicates not detected.
(D–K) Increased inflammatory response in Peyer's patches of IRAK-M$^{-/-}$ mice upon *Salmonella* challenge. Wild-type and IRAK-M$^{-/-}$ mice were infected with *Salmonella typhimurium* as described in (B). After infection (72 hr), mice were euthanized, and Peyer's patches were removed, fixed with 10% formalin, and stained by hematoxylin and eosin (H&E). Three typical Peyer's patches of wild-type mice (E, F, and G) and IRAK-M$^{-/-}$ mice (I, J, and K) after infection are presented. Typical Peyer's patches of uninfected wild-type mice (D) and IRAK-M$^{-/-}$ mice (H) are presented as controls. All figures are shown at the same magnification.
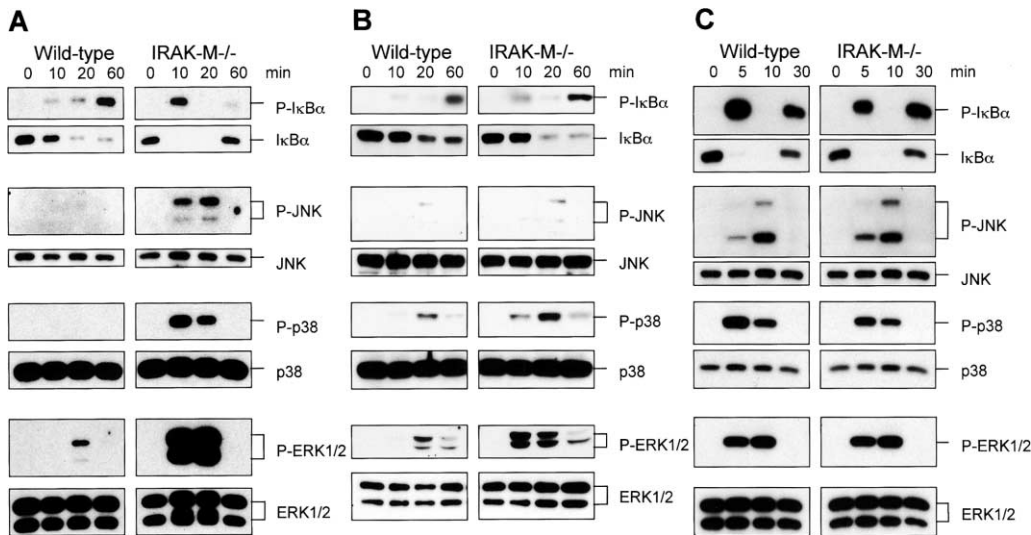
A   **B**   **C**

Figure 4. Increased Signaling in IRAK-M$^{-/-}$ Macrophages upon CpG Oligo DNA and LPS Stimulation

(A) Strongly increased signaling in IRAK-M$^{-/-}$ macrophages upon CpG oligo DNA stimulation. Bone marrow-derived macrophages from wild-type and IRAK-M$^{-/-}$ mice were stimulated with 10 μM of CpG oligo DNA for the indicated periods. Cell lysates were prepared and blotted with anti-phospho-IκBα, anti-IκBα, anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-ERK1/2, and anti-ERK1/2 antibodies.
(B) Increased signaling in IRAK-M$^{-/-}$ macrophages upon LPS stimulation. Bone marrow-derived macrophages were stimulated with 10 ng/ml of LPS for the indicated times. Cell lysates were blotted with anti-phospho-IκBα, anti-IκBα, anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-ERK1/2, and anti-ERK1/2 antibodies.
(C) No enhancement of signaling in IRAK-M$^{-/-}$ macrophages upon TNFα stimulation. Bone marrow-derived macrophages were stimulated with 10 ng/ml of TNFα for the indicated times. Cell lysates were blotted with anti-phospho-IκBα, anti-IκBα, anti-phospho-JNK, anti-JNK, anti-phospho-p38, anti-p38, anti-phospho-ERK1/2, and anti-ERK1/2 antibodies.

IRAK/TRAF6 complex was induced 3 min after stimulation and disappeared by 30 min, suggesting that an IRAK/TRAF6 complex is formed transiently (Figure 6A, left). In contrast to the sharp band of IRAK in the total lysate, TRAF6-associated IRAK migrated diffusely, suggesting that activated phospho-IRAK was associated with TRAF6. In transfected IRAK-M cells, the IRAK/TRAF6 complex was not induced (Figure 6A, right). We next tested whether IRAK/TRAF6 complex formation by TLR stimulation is also inhibited by IRAK-M expression. We transfected TLR2-expressing 293T cells with either empty vector or IRAK-M expression vectors. Cells were stimulated with peptidoglycan, and the association of IRAK and TRAF6s was examined. Again, IRAK/TRAF6 association was observed in mock-transfected cells but not in IRAK-M-transfected cells (Figure 6B).

These results suggested two ways to explain IRAK-M's inhibitory role. First, IRAK-M might inhibit the association of IRAK and TRAF6 directly. Second, IRAK-M could affect the activation of IRAK by TLR/IL-1R stimulation. To test the first possibility, we cotransfected 293T cells with HA-tagged TRAF6 and FLAG-tagged IRAK together with IRAK-M expression vectors. Lysates were prepared and immunoprecipitated by anti-HA antibody, and the association of IRAK and TRAF6 was examined. Overexpression of wild-type IRAK causes the appearance of slowly migrating bands that reflect IRAK autophosphorylation (Cao et al., 1996; Wesche et al., 1999; Yamin and Miller, 1997). Coexpression of IRAK and TRAF6 caused their association, and IRAK-M expression did not inhibit this association (Figure 6C, left). We also tested the association of TRAF6 and kinase inactive IRAK (IRAKKD, K206A mutant). Again, expres-

sion of IRAK-M did not affect association of IRAKKD and MyD88. Thus, IRAK-M does not affect the association of IRAK with TRAF6. Next, we tested whether IRAK-M could inhibit recruitment of IRAK to the TLR signaling complex. We cotransfected HA-tagged MyD88, Flag-tagged IRAKKD, and Flag-tagged IRAK-M into 293T cells. After immunoprecipitation using an anti-HA antibody, MyD88-associated molecules were analyzed by Western blotting with an anti-Flag antibody. Cotransfection of MyD88 and IRAK resulted in the association of these two molecules (Figure 6D, left). Surprisingly, cotransfection of IRAK-M together with MyD88 and IRAK resulted in enhanced, not decreased, association of IRAK with MyD88, suggesting that IRAK-M does not inhibit the recruitment of IRAK to MyD88. Further, we tested whether phosphorylated IRAK associates with MyD88. Wild-type IRAK and MyD88 were cotransfected, and their association was examined. Transfection of wild-type IRAK and MyD88 resulted in readily detectable slowly migrating bands and only a low level of the faster migrating band, suggesting that most IRAK was phosphorylated under these conditions. (Figure 6D, right gel, first lane). Coimmunoprecipitation studies showed that phosphorylated IRAK did not associate with MyD88 (Figure 6C, top). In contrast, cotransfection of IRAK-M together with MyD88 and wild-type IRAK resulted in increased relative levels of the faster migrating (unphosphorylated) band (Figure 6C, middle), suggesting that IRAK-M inhibits IRAK phosphorylation. These immunoprecipitation studies also detected an enhanced association of IRAK and MyD88. Notably, even phosphorylated IRAK, which has little affinity for MyD88, also remained associated with MyD88 in the presence of IRAK-M, indi-
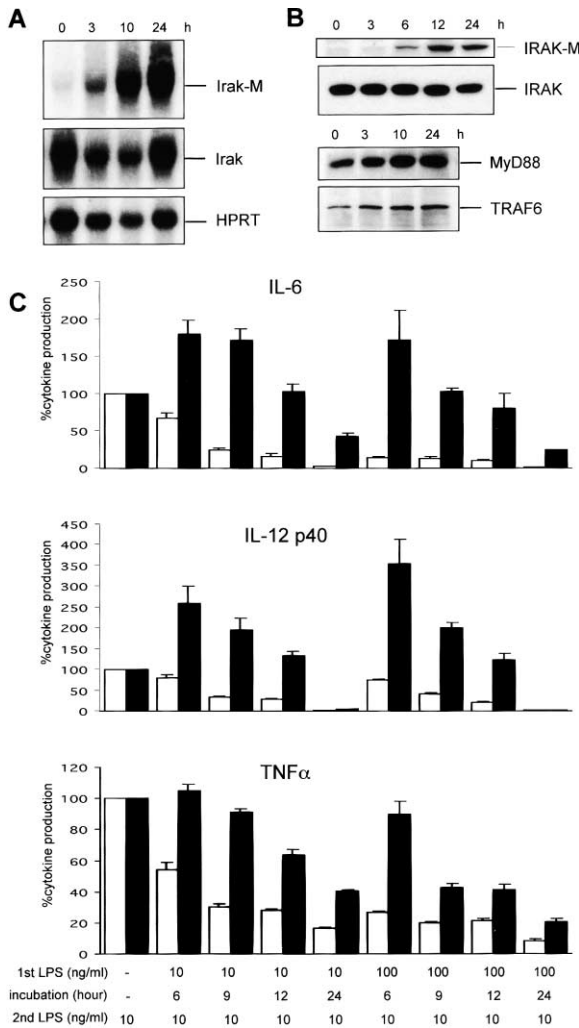
Figure 5. IRAK-M Expression Is Induced by Endotoxin and Is Required for Endotoxin Tolerance

(A) Induction of *irak-M* mRNA in macrophages after LPS stimulation. Bone marrow-derived macrophages were stimulated with 10 ng/ml of LPS for the indicated times. The level of *irak-M*, *irak*, and *HPRT* mRNA were determined by Northern blot analysis using total RNA and *irak*-, *irak-M*-, and *HPRT*-specific ^{32}P-labeled DNA probes. Hypoxanthine phosphoribosyltransferase (HRPT) was used as control.
(B) Induction of the expression of IRAK-M in macrophages after LPS stimulation. Bone marrow-derived macrophages were stimulated with 10 ng/ml of LPS for the indicated times. The levels of IRAK-M, IRAK, MyD88, and TRAF6 were examined in cell lysates by Western blot analysis using anti-IRAK-M, anti-IRAK, anti-MyD88, and anti-TRAF6 antibodies.
(C) Perturbed endotoxin tolerance in IRAK-M$^{-/-}$ macrophages. Endotoxin tolerance was induced in bone marrow-derived macrophages from wild-type or IRAK-M$^{-/-}$ mice by preactivation with 10 or 100 ng/ml of LPS (1st LPS). After the indicated incubation period, cells were washed and restimulated with 10 ng/ml of LPS (2nd LPS). After 24 hr, IL-6, IL-12 p40, and TNFα was determined by ELISA. The concentrations of cytokines in each sample were normalized to the concentration of the sample that received the second stimulation alone, and values are represented as percentages of the cytokine levels in samples receiving second stimulation alone. White bar indicates wild-type macrophages; black bar indicates IRAK-M$^{-/-}$ macrophages.

cating that IRAK-M increases the affinity of both phosphorylated and unphosphorylated forms of IRAK for MyD88. These data suggest that IRAK-M does not inhibit the recruitment of IRAK to the TLR signaling complex, but rather that IRAK-M inhibits the dissociation of IRAK from TLR signaling complex after IRAK activation.

IRAK-4 is the newest member of the IRAK protein family; it acts as a signal transducer upon TLR and IL-1 stimulation downstream of MyD88 (Li et al., 2002; Suzuki et al., 2002). We tested whether IRAK-M also inhibits the release of IRAK-4 from MyD88. The vectors expressing HA-tagged MyD88, V5-tagged IRAK-4, and FLAG-tagged IRAK-M were transfected into 293T cells, and the association of MyD88 and IRAK-4 was examined. Like IRAK, the overexpression of IRAK-4 led to its autophosphorylation, and this phosphorylated form failed to associate stably with MyD88 (Figure 6E, first and third lanes; Li et al., 2002). IRAK-M coexpression caused not only the association of IRAK-M with MyD88 but also the induced association of IRAK-4 with MyD88 (Figure 6E, second and fourth lanes), suggesting that IRAK-M may inhibit dissociation of IRAK-4 from MyD88.

Discussion

Innate immunity is a first line of defense against pathogenic microorganisms (Medzhitov and Janeway, 1997). The innate immune system stimulated via TLRs activates the adaptive immune system by the production of proinflammatory cytokines such as IL-1β, IL-6, TNFα, or IL-12 and the induction of key surface molecules, which drive T cell activation, including MHC, CD40, CD80, and CD86 (Akira et al., 2001; Medzhitov and Janeway, 1997; Schnare et al., 2001). Cytokines, however, induce pronounced positive feedback in the immune system, which, if left unchecked, can cause severe immunopathology. Indeed, a number of pathologies such as Crohn's and inflammatory bowel disease are postulated to result from disregulated innate immunity (Van Heel et al., 2001). However, the mechanisms by which innate immunity is held in check are largely unknown.

We show here that the kinase IRAK-M exerts this critical negative regulatory role. Consistent with this function, macrophages from IRAK-M$^{-/-}$ mice showed enhanced proinflammatory cytokine production when infected with either live or dead bacteria (Figures 2A–2E). Further, IRAK-M$^{-/-}$ mice had greatly exacerbated intestinal inflammatory responses to challenge with the enteric pathogenic bacteria *Salmonella typhimurium* (Figures 3D–3K). Compared to wild-type mice, infected IRAK-M$^{-/-}$ mice exhibited enlarged and inflamed Peyer's patches, the site of *Salmonella* colonization of the intestinal tract. The exacerbated response of IRAK-M$^{-/-}$ mice is likely the result of enhanced TLR signaling. Consistent with this, IRAK-M$^{-/-}$ macrophages stimulated with known TLR agonists such as LPS or CpG DNA displayed increased NF-κB and MAP kinase activation (Figures 4A and 4B), both well-characterized outputs of TLR stimulation (Kawai et al., 1999; Medzhitov et al., 1998; Zhang et al., 1999).

Persistent stimulation with LPS results in endotoxin tolerance, where responses are dampened by hitherto poorly understood negative regulatory mechanisms. We
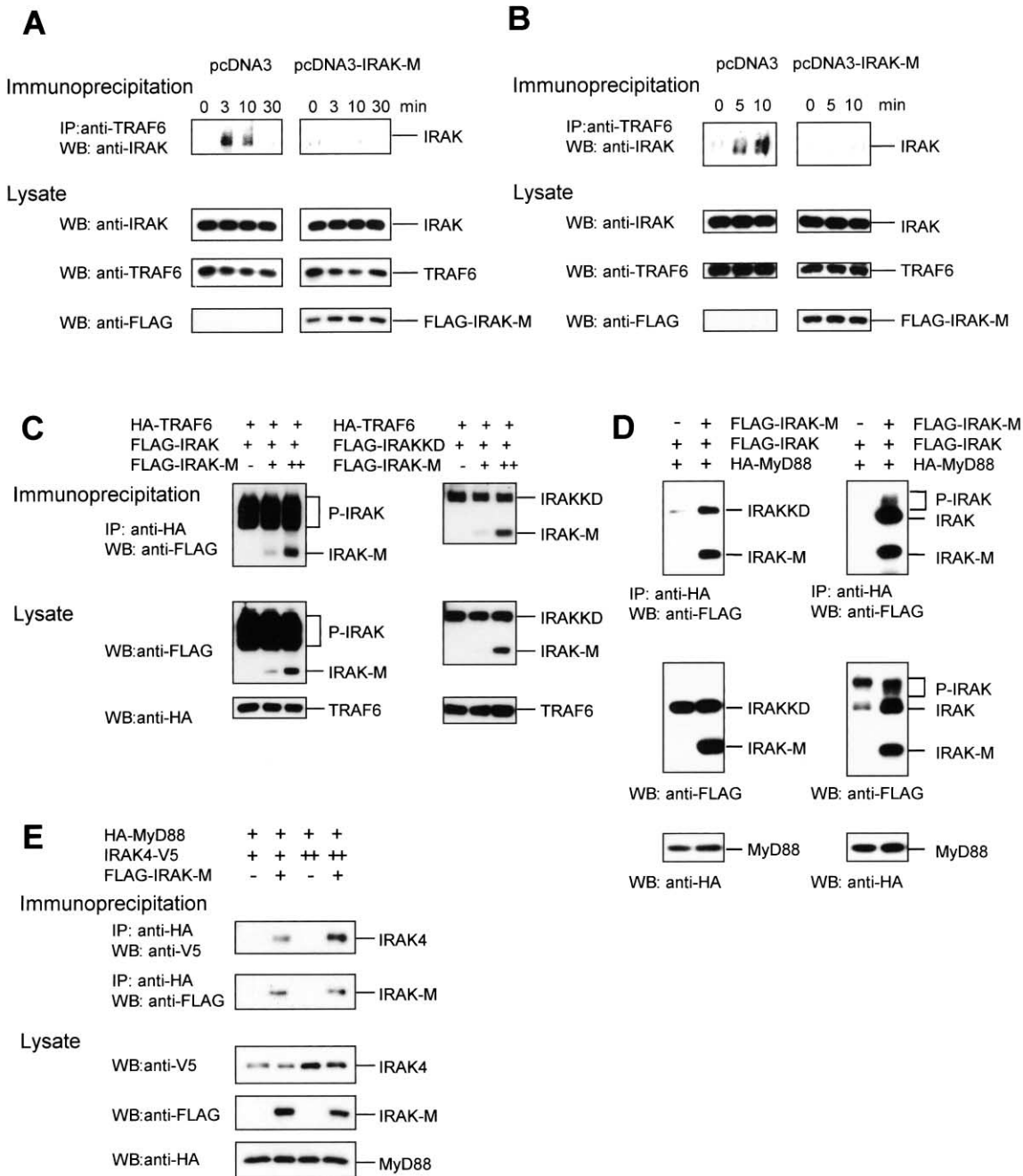
Figure 6. A Molecular Mechanism of Inhibition of TLR Signaling by IRAK-M

(A) Inhibition of IRAK/TRAF6 complex formation on IL-1β stimulation by IRAK-M overexpression. 293T cells were transfected with either pcDNA3 or pcDNA3-IRAK-M vectors. After 24 hr, cells were stimulated with 10 ng/ml of IL-1β for the indicated periods. Lysates were immunoprecipitated with an anti-TRAF6 antibody, and the association with IRAK and TRAF6 was examined by Western blotting with an anti-IRAK antibody. The expression levels of IRAK, TRAF6, and IRAK-M in cell lysates was examined by Western blotting using anti-IRAK, anti-TRAF6, and anti-FLAG antibodies, respectively.

(B) Inhibition of IRAK/TRAF6 complex formation on PGN stimulation by IRAK-M overexpression. TLR2-transfected 293T cells (FLAG-TLR2/CD14/293T cells) were transfected with either pcDNA3 or pcDNA3-IRAK-M expression vectors. After 24 hr, cells were stimulated with 10 μg/ml of PGN for the indicated periods. Lysates were prepared and immunoprecipitated with anti-TRAF6 antibody, and association with IRAK and TRAF6 was examined by Western blotting and anti-IRAK antibody. The expression levels of IRAK, TRAF6, and IRAK-M in cell lysates were examined by Western blotting using anti-IRAK, anti-TRAF6, and anti-FLAG antibodies, respectively.

(C) IRAK-M does not inhibit the association of IRAK (left) or IRAKKD (right) to TRAF6. HA-tagged TRAF6, Flag-tagged IRAK, or IRAKKD expression vectors with or without Flag-tagged IRAK-M expression vectors were cotransfected into 293T cells. Lysates were prepared and immunoprecipitated with anti-HA antibody, and the association between TRAF6 and IRAK (left) or between TRAF6 and IRAKKD (right) were examined by Western blotting with an anti-Flag antibody. The expression levels of IRAK, IRAKKD, and IRAK-M, or MyD88 in lysates were examined by Western blotting using anti-FLAG or anti-HA antibodies, respectively.

show that IRAK-M is a key component of this important control system. Thus, IRAK-M$^{-/-}$ macrophages were significantly impaired in the development of tolerance upon repeated stimulation with LPS (Figure 5C). IRAK-M$^{-/-}$ macrophages, however, retained some capacity to develop LPS tolerance at late time points, suggesting the existence of additional regulatory mechanisms to control the response to LPS. The recently reported downregulation of TLR4 may be one such mechanism (Nomura et al., 2000).

How does IRAK-M exert its function? A notable feature of IRAK-M is that it lacks kinase activity (Wesche et al., 1999) and has a weak capacity to be phosphorylated (Wesche et al., 1999). It is therefore likely that these features are important for its negative regulatory role. However, the role of the kinase activity of IRAK in TLR signaling is controversial. Indeed, kinase-inactive mutants of IRAK, as well as kinase-inactive IRAK-M and IRAK-2, can still activate NF-κB when overexpressed in cultured cells (Knop and Martin, 1999; Maschera et al., 1999; Muzio et al., 1997; Wesche et al., 1999). Further, overexpression of kinase-deficient IRAK mutants can restore NF-κB activation in IRAK$^{-/-}$ cells upon IL-1β stimulation (Knop and Martin, 1999; Li et al., 1999). We show that kinase-inactive IRAK-M exerts a negative regulatory role in TLR/IL-1R signaling, suggesting autophosphorylation as important for signaling by this kinase family. Indeed, a kinase-inactive mutant of IRAK-4 has a dominant-negative effect on IL-1R signaling (Li et al., 2002).

We propose the following model for IRAK-M function. Activation of TLRs by PAMPs dimerizes these receptors, following which IRAK, IRAK-4, and the adaptor protein MyD88 are recruited, resulting in IRAK and IRAK-4 activation and their subsequent phosphorylation (Figure 7A). Phosphorylation of IRAK or IRAK-4 results in a conformational change, causing loss of affinity for the TLR signaling complex and allowing the stimulation of downstream signaling through association with signaling molecules such as TRAF6. IRAK-M inhibits this process by inhibiting dissociation of IRAK and IRAK-4 from the TLR signaling complex by either inhibiting the phosphorylation of IRAK and IRAK-4 or stabilizing the TLR/MyD88/IRAK(-4) complex (Figure 7B). This inhibitory mechanism has greater effect after cells are exposed to LPS because IRAK-M levels are increased when cells are stimulated with LPS; in this way, IRAK-M induces LPS tolerance (Figure 5). Despite their lack of kinase activity, IRAK-M and IRAK-2 are able to complement NF-κB activation in IRAK$^{-/-}$ cells to some degree, albeit less effectively than wild-type IRAK (Wesche et al., 1999). We propose that this may occur upon their phosphorylation by another kinase(s), such as IRAK-4, that may be pres-
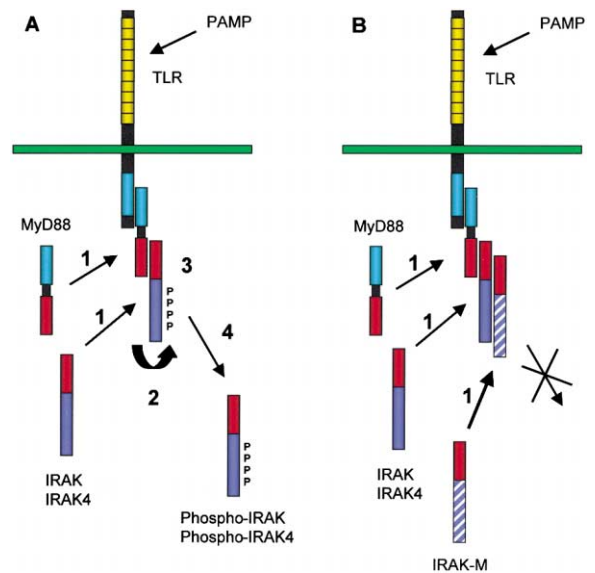

Figure 7. Model for the Regulation of TLR Signaling by IRAK-M

(A) Activation of IRAK/IRAK-4 upon TLR stimulation in the absence of IRAK-M. PAMP stimulation of TLR induces multimerization of these receptors, which in turn causes recruitment of MyD88 and IRAK to TLRs (1). Proximity of IRAK/IRAK-4 causes auto- or cross-phosphorylation (2). The phosphorylation of IRAK/IRAK-4 then causes a conformational change (3). The conformational change of IRAK/IRAK-4 results in reduced affinity for the TLR signaling complex, and IRAK/IRAK-4 are released to make possible the activation of downstream molecules. For simplicity, other adaptor molecules in TLRs, such as Tollip (Burns et al., 2000) and Tirap/Mal (Fitzgerald et al., 2001; Horng et al., 2001), are not included in the figure.
(B) Inhibition of TLR signaling by IRAK-M. In the presence of IRAK-M, TLR stimulation by PAMPs results in the recruitment of not only IRAK/IRAK-4 but also IRAK-M to the signaling complex. This association inhibits the release of IRAK/IRAK-4 from the TLR signaling complex by either inhibiting the phosphorylation of IRAK/IRAK-4 or stabilizing the TLR/MyD88/IRAK(-4) complex, resulting in the interruption of downstream signaling.

ent in the TLR signaling complex or, alternatively, may be an artifact created by overexpression of these inactive kinases.

Like IRAK-M, IRAK-2 may also function as a negative regulator of TLR/IL-1R signaling. Indeed, both proteins lack kinase activity (see Supplemental Figure S1A at http://www.cell.com/cgi/content/full/110/2/191/DC1; Muzio et al., 1997; Wesche et al., 1999) and increase expression upon stimulation (Figure 5A; Wesche et al., 1999). However, these related proteins are expressed differently; while IRAK-M is preferentially expressed in monocytes/macrophages cells, IRAK-2 is ubiquitous

(D) Expression of IRAK-M increases the association of MyD88 with IRAKKD (left) and IRAK (right). HA-tagged MyD88, Flag-tagged IRAKKD, or IRAK expression vectors with or without Flag-tagged IRAK-M expression vectors were cotransfected into 293T cells. Lysates were prepared and immunoprecipitated with anti-HA antibody, and the association of MyD88 with IRAKKD (left), or IRAK (right) was examined by Western blotting using an anti-Flag antibody. The expression levels of IRAKKD, IRAK, and IRAK-M or of MyD88 in lysates was examined by Western blotting using anti-FLAG or anti-HA antibodies, respectively.
(E) Expression of IRAK-M induces the association of MyD88 with IRAK-4. HA-tagged MyD88 and V5-tagged IRAK-4 expression vectors with or without Flag-tagged IRAK-M expression vectors were cotransfected into 293T cells. Lysates were prepared and immunoprecipitated with anti-HA antibody, and the association of MyD88 with IRAK-4 or IRAK-M was examined by Western blotting with anti-V5 or anti-FLAG antibodies. The expression levels of IRAK-4, IRAK-M, or MyD88 in lysates were examined by Western blotting using anti-V5, anti-FLAG, or anti-HA antibodies, respectively.

(Muzio et al., 1997; Wesche et al., 1999). Because TLR expression is high in myeloid lineage cells and IL-1 receptors are ubiquitous (McMahan et al., 1991; Muzio et al., 2000), IRAK-M may be the main regulator of TLR signaling, whereas IRAK-2 is a regulator of IL-1R signaling. Studies using IRAK-2$^{-/-}$ mice should elucidate the role of IRAK-2.

We have identified IRAK-M as a negative regulator of TLR signaling. IRAK-M is induced by TLR stimulation and required for endotoxin tolerance, indicating that IRAK-M is a key component of a feedback regulatory system of innate immunity. IRAK-M therefore plays a critical role in the maintenance of homeostasis of the innate immune system.

Experimental Procedures

Molecular Cloning and Expression Vectors
Full-length mouse IRAK-M cDNA was obtained by an EST clone (GenBank accession number AA930623) and 5′-RACE (rapid amplification of cDNA ends) using mouse cDNA of LPS-activated macrophages and primer 5′-cct ata tga gca acg gga cgc tt. Mammalian expression vectors encoding NH2-terminal Flag-tagged mouse IRAK and IRAKKD were a kind gift of Sankar Ghosh (Yale). A construct encoding Flag-tagged human IRAK-M was a kind gift of Zaodan Cao, Tularik, Inc. (Wesche et al., 1999). Human IRAK-4 cDNA was obtained by RT-PCR using human liver cDNA (CLONTECH) and primers 5′-cac cat gaa caa acc cat aac acc atc aac and 5′-aga agc tgt cat ctc ttg cag cag, cloned into a pcDNA3.1 expression vector (Invitrogen) and verified by sequencing.

Generation of IRAK-M$^{-/-}$ Mice
A 129SV/J genomic library (Stratagene) was screened with the murine irak-M cDNA to obtain six phage-carrying overlapping genomic clones encompassing irak-M. The targeting vector replaced a 1.2 kb genomic fragment containing three exons encoding two-thirds of the kinase domain with the loxP-flanked neomycin resistance (neo) gene expression cassette. The targeting vector was linearized with Not I and electroporated into W9.5 ES cells. Clones resistant to G418 and gancyclovir were selected, and homologous recombination was confirmed by Southern blotting. Eight out of 70 clones screened were positive for homologous recombination. Three targeted clones were injected into C57BL/6 blastocysts and transferred into pseudopregnant foster mothers, and the resulting male chimeric mice were bred to C57BL/6 females to obtain +/− mice. Germline transmission was verified for all three ES clones by Southern blot analysis of tail DNA from F1 offspring. Interbreeding of +/− mice was performed to generate −/− IRAK-M mice. The phenotypes of all three lines were identical.

Reagents
Lipopolysaccharide (LPS) from Salmonella abortus equi, Lipid A from Escherichia coli, lipoteichoic acid (LTA) from Staphylococcus aureus, Mannan from Saccharomyces cerevisiae, and Zymosan A from Saccharomyces cerevisiae were from Sigma. Peptidoglycan (PGN) from Staphylococcus aureus was from Fluka. Poly (I-C) was from Amersham Pharmacia Biotech. Phosphorothioate-modified CpG oligo DNA (tccatgacgttcctgacgtt) was synthesized in the HHMI Biopolymer & W.M. Keck Biotechnology Resource Laboratory in Yale University. Recombinant human IL-1β and murine TNFα were purchased from R&D. The anti-Flag M2 monoclonal antibody, anti-HA antibody, and rabbit anti-IRAK-M antibody were from Sigma, BabCO, and Chemicon International, respectively.

Bone Marrow-Derived Macrophages
Bone marrow-derived macrophages were prepared as described (Celada et al., 1984). Briefly, bone marrow from tibia and femur was obtained by flushing with DMEM (Invitrogen). The medium was DMEM supplemented with 20% heat-inactivated fetal calf serum (FBS), glutamine (both from Invitrogen), and 30% L929 supernatant containing macrophage-stimulating factor. Bone marrow cells were

cultured in 10 ml at an initial density of 4×10^5 cells/ml in 100 mm petri dish (Becton Dickinson) at 37°C in humidified 10% CO_2 for 5 days. At day 3, 5 ml of medium was added. Cells were harvested with cold DPBS (Invitrogen), washed, resuspended in DMEM supplemented with 10% FBS, and used at a density of 2×10^5/ml for experiments unless mentioned otherwise. Cells were left untreated for at least 4 hr at 37°C in 10% CO_2 prior to further handling.

Listeria Infection of Macrophages
The cells were cultured without antibiotics, and L. monocytogenes (ATCC strain 43251) was added at an MOI of 50 bacteria per macrophage. After incubation for 30 min, extracellular bacteria were removed by washing the cells three times with DPBS. To prevent reinfection, the cells were cultured in medium containing gentamicin sulfate (50 μg/ml, Invitrogen).

Salmonella typhimurium and E. coli Infection of Macrophages In Vitro
The S. typhimurium strain SB161, which carries a nonpolar mutation in the invG gene, has been previously described (Kaniga et al., 1994). In vitro infection of macrophages with S. typhimurium was performed as described (Chen et al., 1996). Briefly, macrophages were seeded without antibiotics in 24-well dishes at 2×10^5 cells/ well. Eighteen hours later, macrophages were infected with SB161 or the E. coli strain DH5-α at an MOI of 50 bacteria per macrophage at 37°C in DMEM + 10% FBS. After 25 min, macrophages were washed 3 times with HBSS, and 100 μg/ml gentamicin was added to kill any extracellular bacteria. Culture media was collected at 6 and 24 hr postinfection for cytokine measurements.

Salmonella Challenge of Mice In Vivo
Age- and sex-matched groups of mice were infected orally with Salmonella typhimurium strain SB161 at 10^9 bacteria per mouse. Mice were euthanized 72 hr after infection. Enlarged Peyer's patches were fixed with 10% formalin and stained by Hematoxylin and eosin (H&E). Each spleen mouse was homogenized in 10 ml of BSG buffer, and serial dilutions of the homogenate were plated on LB/Strep agar plates and incubated at 37°C for 18 hr, and colony-forming units (CFU) were counted.

Cytokine Production from Macrophages
Bone marrow-derived macrophages were cultured with LPS, Lipid A, LTA, PGN, Mannan, Zymosan, poly(I-C), CpG DNA, or media alone as indicated for 6 and 24 hr. Macrophages were infected with Salmonella typhimurium or Listeria monocytogenes and cultured for 6 and 24 hr. IL-12 p40, IL-6, and TNF-α were measured by ELISA.

Northern Blot Analysis
Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instruction. Total RNA (20 μg) was then separated by electrophoresis, blotted onto nitrocellulose (Amersham), and probed with ^{32}P-labeled DNA probes. The irak-, irak-M-, and HPRT-specific probes were generated by PCR using forward primer 5′-gccagtggaaagtgatgagagtg and reverse primer 5′-gaaaaagcct gatgacagcagttg for murine irak, forward primer 5′-tccttcaggtgtcctt ctccactg and reverse primer 5′-cctcttctccattggcttgctc for murine irak-M, and forward primer 5′-gttggatacaggccagactttgttg and reverse primer 5′-gagggtaggctggcctataggct for HPRT.

Western Blot Analysis and Immunoprecipitation
Cell lysis, immunoprecipitation, and blotting were as described (Kobayashi et al., 1999). The membrane was blotted with an antibody to phosphorylated-IκBα, IκBα, phosphorylated-JNK, JNK, phosphorylated-p38, p38, phosphorylated-ERK1/2, ERK1/2 (Cell Signaling), IRAK-1 (Santa Cruz and Upstate), TRAF6 (Santa Cruz), MyD88 (StressGen), IRAK-M (Chemicon International), FLAG-tag (Sigma), HA-tag (BabCO), and V5-tag (Invitrogen).

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Accession Numbers

The GenBank accession number of mouse IRAK-M is AF461763.