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IRAK4 Activity Controls Immune Responses to Intracellular Bacteria *Listeria monocytogenes* and *Mycobacterium smegmatis*

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Abstract

Interleukin (IL)-1 receptor-associated kinase (IRAK) 4 is a central enzyme of the Toll-like receptor (TLR) pathways. This study tested the hypothesis that IRAK4 kinase activity is prerequisite for regulating innate immunity during infections with intracellular bacteria. To this end, we analyzed responses of macrophages obtained from mice expressing wild-type IRAK4 or its kinase-inactive K213M mutant (IRAK4^{KI}) upon infection with intracellular bacteria *Listeria monocytogenes* or *Mycobacterium smegmatis*. In contrast to robust induction of cytokines by macrophages expressing kinase-sufficient IRAK4, IRAK4^{KI} macrophages expressed decreased tumor necrosis factor- α , IL-6, IL-1 β , and C-C motif chemokine ligand 5 upon infection with *L. monocytogenes* or *M. smegmatis*. Bacterial infection of IRAK4^{KI} macrophages led to attenuated activation of IRAK1, mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)- κ B, impaired induction of inducible nitric oxide (NO) synthase mRNA and secretion of NO, but resulted in elevated microbial burdens. Compared to wild-type animals, systemic infection of IRAK4^{KI} mice with *M. smegmatis* or *L. monocytogenes* resulted in decreased levels of serum IL-6 and CXC motif ligand-1 but increased bacterial burdens in the spleen and liver. Thus, a loss of IRAK4 kinase activity underlies deficient cytokine and microbicidal responses during infection with intracellular bacteria *L. monocytogenes* or *M. smegmatis* via impaired activation of IRAK1, MAPKs, and NF- κ B but increases bacterial burdens, correlating with decreased induction of NO.

Keywords

Inflammation; Innate Immunity; Infection; Toll-like Receptor; Macrophage

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Authorship

GP conducted the experiments with *M. smegmatis* *in vitro* and *in vivo*, analyzed the results, and wrote the manuscript. MM performed macrophage *in vitro* experiments with *L. monocytogenes* and, in part, with *M. smegmatis*. AF and KK conducted experiments to analyze iNOS and cytokine mRNA expression in WT and IRAK4^{KI} BMDMs infected with bacteria and performed infection of mice with *L. monocytogenes*. KK determined levels of serum and organ cytokines by ELISA. AEM conceived the idea for the project, conceptualized the research, analyzed the data, edited and finalized the manuscript.

Conflict of interest. The authors declare that they have no conflicts of interest with the contents of this article

Introduction

Toll-like receptors (TLRs) sense microbes or host “alarmins” to activate innate immunity and orchestrate adaptive immune responses [1]. TLRs present on the plasma membrane (TLR1, TLR5, TLR6), endosomes (TLR3, TLR7, TLR9) or in both compartments (TLR2, TLR4) in myeloid cells express an ectodomain involved in ligand recognition, a transmembrane segment, and an intracellular Toll-IL-1R (TIR) signaling domain [1, 2]. Upon ligand recognition, the majority of TLRs recruit myeloid differentiation primary response protein (MyD) 88, followed by recruitment of interleukin (IL)-1R-associated kinase (IRAK) 4, IRAK1, IRAK2 and IRAK-M [2, 3]. Clustering of IRAK4 molecules induces IRAK4 kinase activity, leading to phosphorylation and activation of downstream IRAKs and engagement of tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6 and transforming growth factor- β -activated kinase (TAK) 1 [3–5]. TAK1 phosphorylates and activates MAPKs and inhibitor of nuclear factor (NF)- κ B kinase (IKK)- β to trigger activation and nuclear translocation of transcription factors, culminating in transcription of inflammatory cytokine genes [4]. TLR3 and TLR4 signal from endosomes via TIR domain-containing adapter inducing interferon (IFN)- β (TRIF) that recruits TRAF-associated NF- κ B activator-binding kinase-1 and IKK- ϵ . These kinases activate IFN regulatory factor (IRF)-3, leading to its nuclear translocation and transcription of type I IFNs [2]. The TRIF pathway also mediates delayed activation of MAPKs and NF- κ B via the receptor-interacting protein-1-TRAF6 signaling axis [2].

Studies with IL-1 β and defined TLR agonists have revealed that activated IRAK4 signals via the TAK1-IKK- β axis to mediate classical activation of NF- κ B, engages the MK2-dependent pathway to stabilize cytokine mRNAs [4, 6, 7], and elicits rapid activation of nucleotide-binding domain-like receptor containing pyrin domain 3 (NLRP3) inflammasome [8, 9]. Kinase-inactive (K213M) IRAK4 (IRAK4^{KI}) fails to mediate these responses [10], but signals via MAPK-extracellular regulated kinase (ERK) kinase 3 (MEKK3)-IKK- γ to trigger non-classical activation of NF- κ B. This pathway up-regulates cytokines (e.g. CC motif ligand (CCL) 2, CCL4, CCL7), signaling molecules (TLR2, IRAK2) and TLR inhibitors (A20, IRAK-M) whose expression is not regulated by mRNA stability [4, 6, 7, 10–13]. Macrophages and mice harboring IRAK4^{KI} are deficient in mounting responses to and containing infection with *Streptococcus pneumoniae* [13], a highly invasive extracellular Gram positive bacterial pathogen. However, the role of IRAK4 kinase activity in regulating innate immune responses during infections with intracellular bacteria, e. g. *Listeria monocytogenes* or mycobacteria, is largely unknown. Furthermore, while studies in MyD88- and IRAK4-deficient mice showed their involvement in nitric oxide (NO) production [14, 15], the role of IRAK4 kinase activity in inducing macrophage microbicidal activity and NO secretion has not been elucidated.

In this paper, we demonstrate that infection of IRAK4^{KI} macrophages with intracellular bacteria *Listeria monocytogenes* or *Mycobacterium smegmatis* led to deficient induction of TNF- α , IL-6, IL-1 β , CCL5 and NO, inhibited activation of IRAK1, MAPKs and NF- κ B, comparable phosphorylation of IRF3, but increased bacterial burdens. Furthermore, mice harboring kinase-dead IRAK4 exhibited decreased levels of serum IL-6 and CXCL1 but increased splenic and liver microbial burdens after systemic infection with *M. smegmatis* or

L. monocytogenes. Thus, IRAK4 kinase activity is prerequisite for mounting innate immune responses and restraining intracellular bacterial survival during infection with *L. monocytogenes* or *M. smegmatis*.

Materials and Methods

Antibodies (Abs) and Reagents

The following Abs were used: anti-phospho (p)-IRAK1, anti-IRAK1, anti-p65 NF- κ B (Santa Cruz Biotechnology, Santa Cruz, CA); anti-p-IRAK-4, anti-p-p38, anti-p-ERK1/2, anti-ERK1/2, anti-p38, anti-I κ B- α , anti-p-p65, anti-p-IRF3, and anti- β -tubulin (Cell Signaling Technology, Inc., Danvers, MA). Pam3Cys, S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-Ser-Lys4-OH], was purchased from InvivoGen (San Diego, CA).

Mice, Infection and Macrophage Isolation

C57BL/6J mice were from the Jackson Laboratory (Bar Harbor, Maine, USA). IRAK4^{KI} mice [7, 8, 10, 12, 13] were provided by Drs. Kirk Staschke (Eli Lilly, Indianapolis, IN) and Stefanie Vogel (University of Maryland School of Medicine, Baltimore, MD). Mice were housed in the University of Connecticut Health Center animal facility, and 6-week-old male and female mice were used. All animal procedures were approved by the Institutional Animal Care and Use Committee. For infections, wild-type (WT) or IRAK4^{KI} mice were i.p. administered *M. smegmatis* (5×10^6 CFUs) or *L. monocytogenes* (2×10^4 CFU), similar to doses used in previous reports [16, 17]. Six hours post infection (hpi), blood samples were obtained by retroorbital punctures and serum was prepared to analyze cytokines. At 48 hpi, spleens and livers were homogenized in PBS containing protease and phosphatase inhibitors (ThermoFisher Scientific, Waltham, MA) and bacterial colony forming units (CFUs) were enumerated by plating organ homogenates on the respective agars [16, 18]. To generate bone marrow-derived macrophages (BMDMs), bone marrow cells from femurs and tibias were differentiated for 7 days in DMEM (Mediatech, Herndon, VA, USA), supplemented with 10% FBS (Atlanta Biologicals, Flowery Branch, GA), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin (complete (c) RPMI) and 15% L929 cell-conditioned, macrophage-colony-stimulating factor-containing supernatant [6].

Bacterial cultures and infection

Green fluorescent protein (GFP)-expressing *M. smegmatis* and *L. monocytogenes* were provided by Dr. Volker Briken (University of Maryland College Park, College Park, MD) and Dr. Kamal Khanna (New York University School of Medicine, New York, NY), respectively. *M. smegmatis* were cultured in 7H9 broth containing 0.5% glycerol, 0.05% Tween-80, 10% oleic albumin dextrose complex enrichment (OADC) and 40 μ g/ml kanamycin. *L. monocytogenes* was cultured in BHI media containing 10 μ g/ml chloramphenicol. Bacteria were grown to mid-log phase, washed in PBS, and added to cells cultured in antibiotic-free DMEM at the respective multiplicity of infections (MOI) for 2 h. Cells were washed in PBS and incubated in complete (c) DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin and 100 μ g/ml gentamycin.

Assessment of bacterial CFUs

BMDMs were grown overnight in 24-well plates (10^6 cells/well), infected for 1 h with *L. monocytogenes* (MOI 1) or 2 h with *M. smegmatis* (MOI 10) in antibiotic-free DMEM, washed with PBS, incubated in cDMEM containing 100 µg/ml gentamycin for the indicated times, and lysed in PBS containing 0.05% Tween-80 [19]. Spleens and livers from *M. smegmatis*- or *L. monocytogenes*-infected mice were homogenized in PBS containing protease and phosphatase inhibitors (ThermoFisher Scientific). The number of CFUs per 10^6 cells or in organ homogenates was determined by plating 10-fold serial dilutions of lysates on Middlebrook 7H10 plates complemented with 0.5% glycerol, 10% OADC enrichment, 40 µg/ml kanamycin (*M. smegmatis*) or blood heart infusion agar plates containing 10 µg/ml chloramphenicol (*L. monocytogenes*) [19]. Plates were incubated at 37°C and CFUs were counted after 24–48 h.

Analyses of bacterial uptake by flow cytometry

BMDMs (10^6 cells) were treated for 1 h with GFP-expressing *L. monocytogenes* (multiplicity of infection (MOI) 1) or for 2 h with *M. smegmatis* (MOI 10) in antibiotic-free medium, washed, and incubated in cDMEM containing 100 µg/ml gentamycin on ice or at 37°C for the indicated times. Cells were detached, washed in ice-cold PBS containing 3% FBS, stained with Zombie® Aqua cell viability dye (Biolegend), fixed for 15 min in 3.7% formaldehyde, and analyzed by FACS on a LSRII Flow Cytometer (BD Biosciences) to measure GFP fluorescence. The data were analyzed using the FlowJo software (Tree Star). BMDMs were gated using forward vs side scatters, live cells were gated as Zombie® Aqua-negative events, and GFP mean fluorescence intensity (MFI) values were analyzed.

Isolation of RNA and Real-Time PCR

RNA was extracted with TRIzol (ThermoFisher Scientific, Waltham, MA), cDNA was prepared from 1 µg of total RNA using the reverse transcription system (Promega) and analyzed by real-time PCR on a MyIQ real-time PCR machine (Bio-Rad). The following primers were used - glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*): 5'-GCTGACCTGCTGGATTACATT-3' (forward), 5'-GTTGAGAGATCATCTCCACCA-3' (reverse); *Tnf-α*: 5'-CCCAGGCAGTCAGATCATCTTC-3' (forward), 5'-GCTTGAGGGTTTGCTACAACATG-3' (reverse); pro-*Il-1β*: 5'-CTGGTGTGTGACGTTCCCAT-3' (forward), 5'-GATTCTTTCCTTTGAGGCCCA-3' (reverse); inducible NO synthase (*iNos*): 5'-CGCCTTCAACACCAAGGTTG-3' (forward), 5'-TGGGGACAGTCTCCATTCCCA-3'; *Il-6*: 5'-TCAGGAAATTTGCCTATTGAAAATTT-3' (forward), 5'-GCTTTGTCTTTCTTGTATCTTTTAAGTTGT-3' (reverse). The data were calculated as a ratio of expression of gene of interest normalized to that of *Gapdh*, normalized to medium treatments and expressed as folds induction [12].

Western blot analyses

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 5 mM NaF, 2 mM sodium orthovanadate, 1 mM PMSF, 1 × complete protease inhibitors), and resuspended in Laemmli buffer (50 mM Tris-Cl, pH 6.8, 10% glycerol, 2%

SDS, 0.1% bromophenol blue, 5% 2-mercaptoethanol), boiled for 5 min, separated on 4–20% polyacrylamide gels (Fisher Scientific, Waltham, MA), transferred to polyvinylidene fluoride membranes (Bio-Rad), blocked, and probed with the respective Abs, as described [12].

Analyses of cytokine secretion and NO release

Organ homogenates and supernatants from cell stimulated with TLR agonists or infected with bacteria were cleared by centrifugation (400 g, 10 min), and cytokine levels were determined by ELISA using commercial kits (BioLegend, San Diego, CA) per manufacturer's recommendations. The ELISA kits had the following limits of detection: 16 pg/ml (IL-1 β), 4 pg/ml (TNF- α , IL-6 and CXCL1) and 31.20 pg/ml (CCL5). Nitrite (NO $_2^-$; product of NO breakdown) concentrations in the supernatants were examined by the Griess method [20]. NO $_2^-$ was quantified using sodium nitrite (NaNO $_2$) as a standard.

Statistical Analysis

Data were processed by the GraphPad Prism software package (GraphPad Software, San Diego, CA), using one-way ANOVA with the Tukey's post-hoc test to compare multiple experimental groups, or using Student's t-test to perform pair-way comparisons, and the data were expressed as mean \pm standard deviation (SD).

Results

Macrophages expressing IRAK4^{KI} show reduced expression of cytokines upon infection with *L. monocytogenes* or *M. smegmatis*

In response to TLR/IL-1R agonists, IRAK4 kinase activity is prerequisite for induction of inflammatory mediators whose expression is regulated by mRNA stability [4, 6, 7, 10–13] and for host resistance against *S. pneumoniae* [13], an extracellular bacterial pathogen. To explore the role of IRAK4 kinase activity in mounting macrophage cytokine responses during infection with intracellular bacteria, we used mice expressing WT or kinase-deficient (K213M) IRAK4. Infection of BMDMs from WT mice with *L. monocytogenes* up-regulated levels of TNF- α , IL-6 and pro-IL-1 β mRNA by 25–43, 10–20 and 58–62 folds (Fig. 1A–C) and led to robust secretion of TNF- α , IL-6, IL-1 β and CCL5 (Fig. 1E–H). In contrast, IRAK4^{KI} BMDMs exhibited 62–91% inhibition of these responses (Fig. 1). Likewise, IRAK4^{KI} BMDMs infected with *M. smegmatis* secreted markedly decreased (67–95% inhibition) levels of TNF- α , IL-1 β , IL-6 and CCL5 compared to robust cytokine responses of macrophages expressing WT IRAK4 (Fig. 2). IRAK4^{KI} BMDMs infected with GFP-expressing *L. monocytogenes* or *M. smegmatis* showed similar or higher levels of MFI for GFP compared to WT BMDMs (Fig. 1D, Fig. 2E), and exhibited similar bacterial CFUs immediately after infection (Fig. 5A, B). These data indicate that the differences in cytokine induction between WT and IRAK4^{KI} BMDMs are not due to alterations in the bacterial uptake. Thus, a loss of IRAK4 kinase activity confers deficient expression of inflammatory cytokines by BMDMs infected with *L. monocytogenes* or *M. smegmatis*.

A loss of IRAK4 kinase activity impairs activation of IRAK1, MAPK and NF- κ B in macrophages infected with *L. monocytogenes* or *M. smegmatis*

Next, we addressed the biochemical basis for attenuated responses of IRAK4^{KI} macrophages during bacterial infection. WT BMDMs infected with *L. monocytogenes* showed robust phosphorylation of IRAK4, decreased levels of unmodified IRAK1, degradation of I κ B- α , and phosphorylation of p38 MAPK and p65 NF- κ B, whereas these responses were markedly attenuated in IRAK4^{KI} BMDMs (Fig. 3A). During infection with *M. smegmatis*, WT, but not IRAK4^{KI}, BMDMs exhibited efficient phosphorylation of IRAK1, ERK and p38 MAPKs and p65 NF- κ B (Fig. 3B). Notably, IRAK4-independent, TRIF-driven [21] phosphorylation of IRF3 was induced comparably in WT and IRAK4^{KI} BMDMs (Fig. 3C). These results indicate that deficiency in IRAK4 kinase activity underlies impaired phosphorylation and modifications of IRAK1, activation of ERK and p38 MAPK, degradation of I κ B- α and phosphorylation of p65 NF- κ B in BMDMs infected with *L. monocytogenes* or *M. smegmatis* but does not inhibit IRF3 activation.

***L. monocytogenes* or *M. smegmatis* infection of macrophages harboring IRAK4^{KI} leads to impaired NO production**

NO is critical for host defense against *L. monocytogenes* and mycobacteria [18, 20, 22–26], as evidenced by increased susceptibility to infections in mice subjected to pharmacological inhibition of inducible NO synthase or in carriers of polymorphisms rendering the enzyme dysfunctional [24, 27, 28]. While MyD88 is required for NO production [29], the involvement of IRAK4 in triggering NO secretion in response to bacterial infection is unknown. To delineate the role of IRAK4 kinase activity, we examined induction of iNOS mRNA and production of NO by macrophages derived from WT or IRAK4^{KI}-expressing mice during infection with live intracellular bacteria. Figure 4 demonstrates that infection of WT BMDMs with *L. monocytogenes* (A, B) or *M. smegmatis* (C, D) markedly increased expression of iNOS mRNA (A, C) and induced NO-dependent nitrite production (B, D), while IRAK4^{KI} macrophages had impaired iNOS mRNA induction and secreted significantly lower levels of NO. Thus, a loss of IRAK4 kinase activity impairs the capacity of macrophages to respond to infection with *L. monocytogenes* or *M. smegmatis* by production of NO, suggesting their deficient microbicidal functions.

Increased microbial loads in IRAK4^{KI} BMDMs infected with *L. monocytogenes* or *M. smegmatis*

In the next series of experiments, we examined whether deficient NO production by *L. monocytogenes*- or *M. smegmatis*-infected IRAK4^{KI} macrophages affects intracellular bacterial replication. Infection of WT and IRAK4^{KI} BMDMs with *L. monocytogenes* or *M. smegmatis* resulted in comparable bacterial CFUs immediately after infection and within the initial period of 3 hpi (*L. monocytogenes*) or 6 hpi (*M. smegmatis*) (Fig. 5A, B). However, compared to WT macrophages, significantly higher CFUs were recovered from IRAK4^{KI} BMDMs at 6 and 24 hpi with *L. monocytogenes* (Fig. 5A) and at 24–72 hpi with *M. smegmatis* (Fig. 5B). These data indicate that deficiency of kinase activity of IRAK4 promotes bacterial replication and survival in macrophages.

Mice harboring IRAK4^{KI} exhibit reduced serum IL-6 and CXCL1 but increased splenic and liver CFUs following systemic infection with *M. smegmatis* or *L. monocytogenes*

IRAK4 expression is prerequisite for immune defense against Gram-positive and some Gram-negative bacterial pathogens [15], and IRAK4 activity controls innate immune responses and susceptibility to infection with an extracellular bacterial pathogen *S. pneumoniae* in mice [13]. However, the role of IRAK4 kinase activity for host resistance against intracellular bacteria *in vivo* remains elusive. To address this question, we performed i.p. infection of WT and IRAK4^{KI} mice with *M. smegmatis* or *L. monocytogenes* and determined levels of serum cytokines and microbial burdens in the spleen and liver. Figure 6 demonstrates that IRAK4^{KI} mice had significantly lower expression of serum IL-6 and CXCL1 (66% and 77% reduction, respectively) compared to their levels observed in WT animals at 6 hpi with *M. smegmatis* (A). Notably, significantly higher CFUs were detected 48 hpi in the spleen and liver of *M. smegmatis*-infected IRAK4^{KI} mice compared to WT mice (Fig. 6B). Compared to WT animals, i.p. infection of IRAK4^{KI} mice with *L. monocytogenes* led to lower levels of CXCL1 (Fig. 6C) and IL6 (data not shown) in the serum, spleen and liver but yielded higher splenic and liver CFUs (Fig. 6D). Thus, during systemic infection with *M. smegmatis* or *L. monocytogenes*, a loss of IRAK4 kinase activity curtails induction of IL-6 and CXCL1 while increasing splenic and liver bacterial burdens, indicating a critical role for IRAK4 activity for host defense against intracellular bacteria.

Discussion

IRAK4 integrates signaling emanating from the majority of TLRs, except TLR3, to trigger induction of inflammatory cytokine genes, and mediates rapid, transcription-independent activation of NLRP 3 inflammasome [2, 4, 6–9]. Human IRAK4-deficient patients have recurrent pyogenic infection [30, 31], and IRAK4^{-/-} mice succumb to infections with *S. aureus* or *S. pneumoniae* [13, 15, 32]. In response to defined IL-1R/TLR ligands, IRAK4 activity is required to induce cytokines whose expression is regulated by mRNA stability, and to optimally activate MAPKs and NF- κ B via the TAK1-IKK β pathway [4, 6, 7, 10–13]. Kinase-inactive IRAK4 adapter triggers MEKK3-dependent activation of IKK- α and IKK- γ , which initiates I κ B- α phosphorylation without degradation and induce a subset of cytokines (CCL2, CCL4, CXCL10) and negative regulators (IRAK-M, A20) [4, 6, 7, 10–13]. While IRAK4 kinase deficiency renders mouse macrophages hyporesponsive to extracellular bacteria *S. aureus* or *S. pneumoniae* and conferred increased susceptibility to *S. pneumoniae* infection in mice [13, 15], the role of IRAK4 activity in host responses to intracellular bacteria *L. monocytogenes* and mycobacteria has been unknown.

This paper shows that macrophages harboring kinase-inactive IRAK4 exhibited deficient activation of IRAK1, MAPKs and NF- κ B, and reduced expression of TNF- α , IL-6, IL-1 β and, to a lesser extent, CCL5 upon infection with *L. monocytogenes* or *M. smegmatis*. Compared to WT cells, IRAK4^{KI}-expressing BMDMs showed equal infectivity at the start of infection and even higher bacterial uptake and survival at later times post infections, indicating that deficient cytokine responses cannot be explained by lower microbial burdens. *L. monocytogenes* and *M. smegmatis* express ligands activating TLR2 (mycobacterial lipoarabinomannan, listeria-derived lipoproteins), TLR7 (bacterial ssRNA) or TLR9

(bacterial DNA) [33–37]. These TLRs activate IRAK4 enzymatic activity to induce IRAK4-dependent phosphorylation and activation of IRAK1 [3–5]. In line with the requirement for IRAK4 to activate IRAK1 [3–5], we observed deficient IRAK4 and IRAK1 phosphorylation and reduced IRAK1 modifications (reflected in decreased levels of unmodified IRAK1) in IRAK4^{KI} BMDMs infected with bacteria. Phosphorylated, activated IRAK1 engages TRAF6-TAK1 to interact and activate the IKK complex, mediating activation of MAPKs and NF- κ B [4, 6, 7, 10–13]. We show that impaired auto-phosphorylation of IRAK4 and activation of IRAK1 in IRAK4^{KI} BMDMs infected with *L. monocytogenes* or *M. smegmatis* was accompanied by deficient phosphorylation of ERK and p38 MAPK, p65 NF- κ B and reduced I κ B- α degradation.

Of note, WT and IRAK4^{KI}-expressing BMDMs exhibited comparable phosphorylation of TRIF-dependent IRF3, indicating a tailored impact of IRAK4 kinase activity primarily on MyD88-dependent signaling cascades, in line with previous findings [12, 13]. We also demonstrate herein different dependence on IRAK4 kinase activity for the induction of various cytokines in infected macrophages, with strict dependence for TNF- α , IL-6, and IL-1 β and lower dependency for CCL5. Sensing of mycobacterial species and *L. monocytogenes* involves several sensors, e.g. TLR2, TLR3, TLR4, TLR7 and TLR9 that engage MyD88-IRAK4-driven and the TRIF-dependent pathways [20, 22, 33, 37–39]. Since CCL5 can be induced via the TRIF pathway [21] that does not utilize IRAK4, while TNF- α , IL-6 and IL-1 β rely primarily on the MyD88-IRAK4 module [40], this could explain the observed differences. Furthermore, IRAK4 kinase activity is required to stabilize mRNA for several cytokines, e.g. TNF- α , IL-6, IL-1 β , while CCL5 induction could be less dependent on mRNA stabilization, analogous to other cytokines (CCL2, CCL4, CCL7) [6]. We also do not exclude that IRAK4 kinase activity could uniquely influence epigenetic regulation of various cytokine genes. Studies are ongoing to delineate the mechanisms responsible for differential dependence of various cytokines on IRAK4 kinase activity.

Our data obtained during *in vitro* infection of BMDMs with *L. monocytogenes* and *M. smegmatis* extend observations on the prerequisite role of IRAK4 activity for optimal activation of IRAK1, MAPKs and NF- κ B in cells stimulated with TLR/IL-1R agonists [6, 7, 10–13]. However, they are in contrast to a report employing IRAK4 chemical inhibitor in human monocytes that showed a redundant role for IRAK4 kinase activity to mediate phosphorylation of IRAK1, ERK MAPK and p65 NF- κ B [41]. It is plausible that differences in cell types (mouse vs human) and/or different inhibitory modes imparted by IRAK4 genetic mutations (K213M) vs. chemical inhibitors could be responsible for the observed differences. In addition, different engagement of TLRs and TLR cooperation with other receptors occurring during stimulation with defined ligands vs infection with bacteria could differentially affect signaling strengths. It is possible that strong TLR signaling could efficiently activate MEKK3 or engage additional pathways (e.g. phosphoinositide-3 kinase) by kinase-inactive IRAK4 adapter, accounting for MAPK and NF- κ B activation. Under lower signaling strengths, MEKK3 engagement is impaired or does not occur and activation of the IRAK-1-TRAF6-TAK1 axis is required for optimal responses. Experiments are in progress to address this hypothesis.

Transcription of *Tnf- α* , *Il-6* and *Il-1 β* genes is governed by coordinated activation of MAPK-driven transcription factors (e.g. activator protein-1) and IKK- β -mediated activation of NF- κ B and recruitment of transcription factors to the respective cytokine promoters [2]. In line with this notion, we demonstrate that a loss of IRAK4 kinase activity led to impaired activation of MAPK and NF- κ B in *L. monocytogenes*- and *M. smegmatis*-infected BMDMs and significantly lowered expression of TNF- α , IL-6 and IL-1 β . It is likely that during infection with *L. monocytogenes* or *M. smegmatis*, the presence of IRAK4^{KI} in macrophages impairs both recruitment of transcription factors to promoters of the respective cytokine genes and activation of the MK2-dependent pathway of stabilization of cytokine mRNAs, as was shown for defined TLR agonists [4]. Reduced production of IL-1 β by IRAK4^{KI}-expressing macrophages upon infection with *L. monocytogenes* or *M. smegmatis* is likely to result from decreased transcription of pro-IL-1 β mRNA. Notably, IRAK4 kinase activity is indispensable for rapid activation of NLRP3 inflammasome that produces active caspase-1 to generate mature IL-1 β for secretion [8, 9]. Therefore, we cannot also exclude that impaired activation of NLRP3-driven caspase-1 in IRAK4^{KI} macrophages contributes to reduced IL-1 β secretion in response to bacterial infection. Experiments are ongoing to dissect the impact of deficiency in IRAK4 kinase activity on activation of NLRP3 and other inflammasomes during macrophage infection with *L. monocytogenes* or *M. smegmatis*.

NO is one of the major bactericidal mediators involved in host defense against *L. monocytogenes* and mycobacterial species [18, 20, 22-26], and genetic mutations in inducible NO synthase or its pharmacological inhibition have been linked to listeriosis and mycobacterial infections [24, 27, 28]. NO production requires MyD88 [29], but the role of IRAK4 kinase activity in inducing NO during bacterial infections has not been explored. We show significantly impaired capacities of macrophages harboring IRAK4^{KI} to up-regulate iNOS mRNA and to induce NO upon infection with *L. monocytogenes* or *M. smegmatis* compared to robust responses exhibited by WT macrophages. To the best of our knowledge, our study is the first showing that IRAK4 kinase activity is prerequisite to NO production during macrophage sensing of bacterial pathogens. Deficient activation of NO production in IRAK4^{KI}-expressing macrophages could be accounted by cell-intrinsic mechanisms. In this scenario, the alternative MEKK3-IKK- γ pathway induced by inactive IRAK4 adapter [4, 10, 11] cannot compensate for the loss of activity of the TAK1-IKK- β signaling axis that requires IRAK4 kinase activity [4, 6, 7, 10-13, 42]. Alternatively, mechanisms of indirect regulation could also play a role, as macrophages devoid of IRAK4 kinase activity show deficient induction of a variety of cytokines, including those implicated in up-regulation of NO production (IL-1 β) via NF- κ B- and STAT-1-dependent induction of iNOS transcription [43]. Additional studies are required to elucidate the exact molecular basis of impaired NO production in macrophages harboring kinase-inactive IRAK4.

Previous reports demonstrated the requirement of TLR2 and MyD88 for restricting intracellular replication of *Coxiella burnetii* [44, 45], and enhanced growth of *Legionella pneumophilla* in the lung of MyD88^{-/-} mice [46]. Furthermore, downregulation of IRAK1 expression via micro-RNA 142-3p was shown to facilitate intracellular replication of *M. bovis* Bacillus Calmette-Guérin [47]. These findings highlight the importance of the MyD88-IRAK-1 axis in regulating survival of intracellular bacteria in the macrophage. However, the role of IRAK4 kinase activity in restraining intracellular bacteria is poorly

understood. This study presents an important observation that deficiency in IRAK4 kinase activity leads to increased survival of intracellular *L. monocytogenes* and *M. smegmatis* in the macrophage. Our findings extend a previous publication that revealed higher lung microbial burdens in IRAK4^{KI} mice infected with extracellular bacterial pathogen *S. aureus* [13]. Collectively, they show that IRAK4 kinase activity is prerequisite to restrict survival of intracellular bacteria *L. monocytogenes* and *M. smegmatis* in the macrophage, possibly, due to its requirement for the induction of NO, one of the important microbicidal mediators. In addition, impaired production of IL-1 β by macrophages harboring IRAK4^{KI} could “report” impaired inflammasome activation, which could create an intracellular niche favoring bacterial replication. Finally, impaired MAPK and NF- κ B activation by IRAK4^{KI} macrophages could impair activation of autophagy, in turn leading to increased intracellular bacterial survival. Studies are in progress to address the role of these mechanisms in promoting intracellular growth of bacteria in IRAK4^{KI} macrophages.

Pennini et al [13] revealed that IRAK4 kinase activity is prerequisite for innate immune defense against *S. pneumoniae* and clearance of infection. To uncover the role of IRAK4 kinase activity for infections with intracellular bacteria *in vivo*, we analyzed serum and organ cytokines and microbial burdens in WT and IRAK4^{KI} mice after systemic infection with *M. smegmatis* or *L. monocytogenes*. This paper demonstrates for the first time that a loss of IRAK4 kinase activity underlies deficient induction of serum IL-6 and CXCL1 but increases bacterial CFUs in the spleen and liver following i.p. infection with *M. smegmatis* or *L. monocytogenes*.

Taken collectively, our paper shows that a loss of IRAK4 kinase activity leads to impaired production of TNF- α , IL-6, IL-1 β , and CCL5 in macrophages infected with *L. monocytogenes* and *M. smegmatis*, under conditions of similar bacterial uptake immediately and at early times after infection. We demonstrate for the first time that in response to infection with *L. monocytogenes* or *M. smegmatis*, IRAK4^{KI} macrophages exhibited low induction of NO, while showing increased intracellular bacterial survival. These results suggest that triggering of the non-classical pathway of NF- κ B activation by kinase-inactive IRAK4 adapter in the macrophage is insufficient for mounting efficient cytokine responses and antibacterial resistance. Finally, we demonstrate deficient induction of serum cytokines and increased bacterial burdens in the spleen and liver in *M. smegmatis*- or *L. monocytogenes*-infected mice harboring kinase-dead IRAK4, indicating a critical role for IRAK4 activity for host defense against systemic infection with *M. smegmatis* or *L. monocytogenes in vivo*.

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Abbreviations

IL	interleukin
TLR	Toll-like receptor
IRAK	IL-1R-associated kinase
MAPK	mitogen-activated protein kinase
NF-κB	nuclear factor- κ B
NO	nitric oxide
TIR	Toll-IL-1 receptor domain
MyD88	myeloid differentiation primary response protein 88
IRAK4^{KI}	K213M kinase-inactive IRAK4
TNF	tumor necrosis factor
TRAF	TNFR-associated factor
TAK	transforming growth factor- β -activated kinase
IKK	inhibitor of NF- κ B kinase
TRIF	TIR domain-containing adapter inducing interferon- β
IFN	interferon
IRF	IFN regulatory factor
ERK	extracellular regulated kinase
MEKK	MAPK-ERK kinase
CXCL	CXC motif ligand
CCL	CC motif ligand
NLRP	nucleotide-binding domain-like receptor containing pyrin domain
Pam3Cys	S-[2,3-bis(palmitoyloxy)-(2-RS)-propyl]-N-palmitoyl-(R)-Cys-Ser-Lys4-OH
GFP	green fluorescent protein
BMDMs	bone marrow-derived macrophages
c	complete
MFI	mean fluorescent intensity
MOI	multiplicity of infections

hpi	hours post infection
iNOS	inducible NO synthase
CFUs	colony-forming units
hpi	hours post infection
WT	wild-type
Abs	antibodies
LPS	lipopolysaccharide
MOI	multiplicity of infection
MFI	mean fluorescence intensity
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
SD	standard deviation

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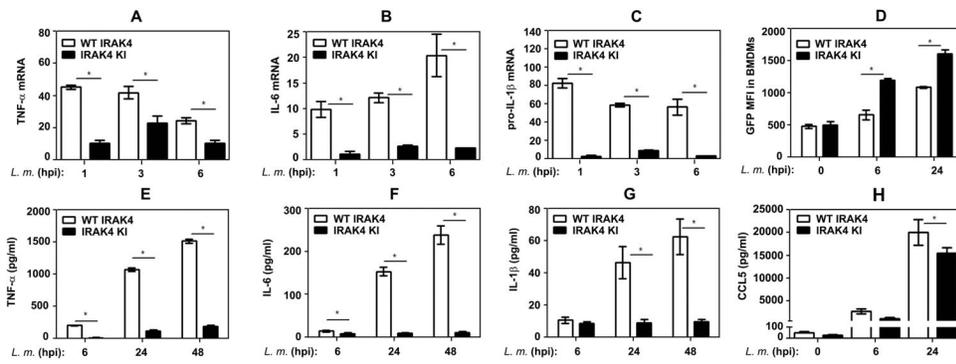


Figure 1. Macrophages expressing kinase-inactive IRAK4 exhibit deficient cytokine responses upon infection with *L. monocytogenes*

BMDMs from mice expressing WT or IRAK4^{KI} were infected with GFP-expressing *L. monocytogenes* (MOI 1), washed and incubated for the indicated times. A–C: RNA was isolated, reverse-transcribed and subjected to real-time qPCR with gene-specific primers. D: BMDMs were fixed and subjected to FACS analyses to determine MFI of GFP-positive cells. E–H: Cytokines levels in cell-free supernatants were examined by ELISA. The results (mean \pm SD) of a representative experiment (n=3) are depicted. *p<0.05.

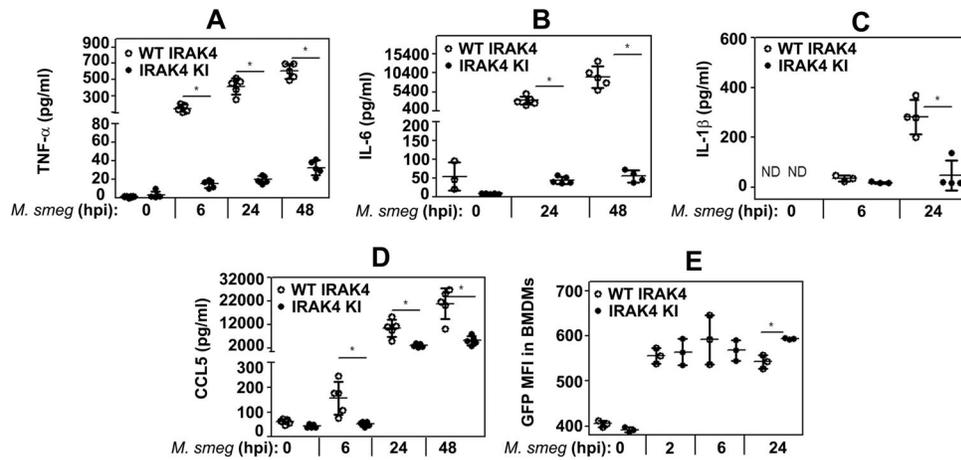


Figure 2. Infection with *M. smegmatis* results in attenuated cytokine responses in macrophages harboring kinase-inactive IRAK4

After infection with GFP-expressing *M. smegmatis* (MOI 10), BMDMs were washed and incubated for the indicated times. A–D: Cytokines levels were analyzed in cell-free supernatants by ELISA. E: BMDMs were fixed and analyzed by FACS to determine MFI of GFP-positive cells. The summary data (mean \pm SD) of 3 experiments (individual mice indicated as dots) are shown. * $p < 0.05$.

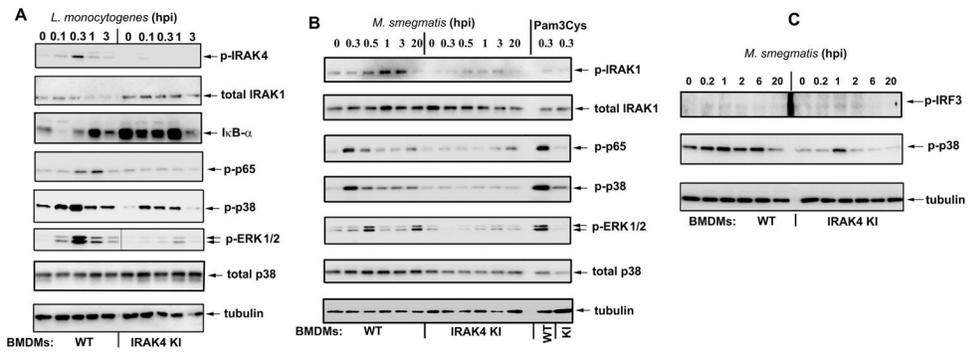


Figure 3. IRAK-4 kinase activity is required for activation of IRAK1, MAPKs and NF- κ B, but not IRF3, in BMDMs infected with *L. monocytogenes* or *M. smegmatis*
 BMDMs from WT or IRAK4^{KI} mice were infected with *L. monocytogenes* (A) or *M. smegmatis* (B, C) (MOI 1 and 10, respectively). After washing, cells were incubated as shown, cell extracts were prepared and examined by Western blot analyses with the indicated Abs. The data of a representative (n=3) experiments are depicted.

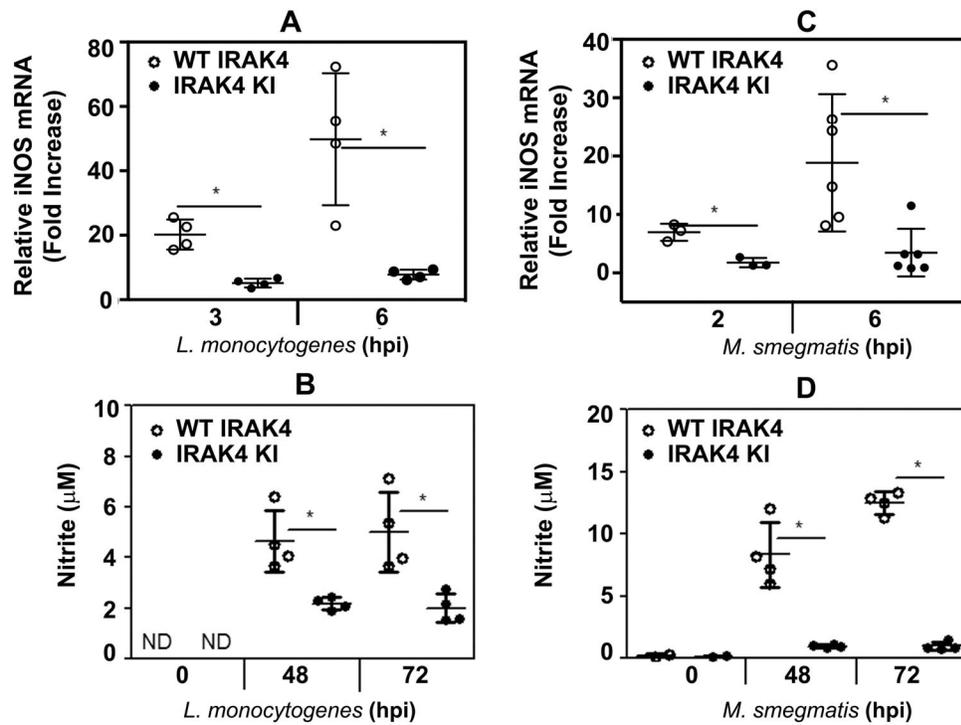


Figure 4. Deficient induction of iNOS mRNA and secretion of NO in IRAK4^{KI}-expressing macrophages infected with *L. monocytogenes* or *M. smegmatis*
 BMDMs obtained from WT or IRAK4^{KI} mice were infected with *L. monocytogenes* (MOI 1, A, C) or *M. smegmatis* (MOI 10, B, D), washed and incubated for the indicated times. RNA samples were reverse-transcribed and subjected to real-time PCR analyses of iNOS mRNA expression (A, B). Cell-free supernatants were examined by the Griess assay to determine the levels of nitrite (B, D). The results (mean \pm SD) or a representative experiment (n=3) are depicted. *p<0.05.

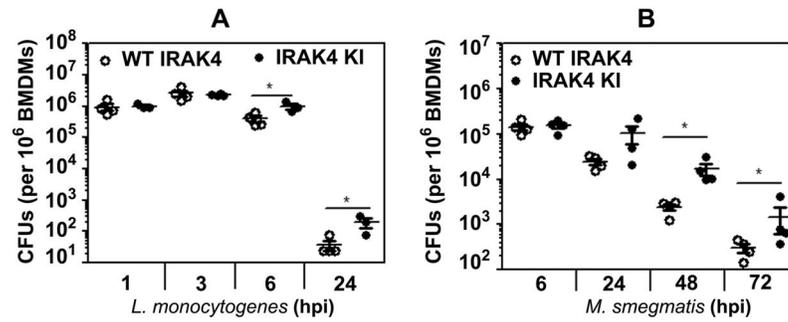


Figure 5. Macrophages harboring IRAK4^{KI} exhibit increased microbial CFUs after infection with *L. monocytogenes* or *M. smegmatis*

BMDMs expressing kinase-sufficient or kinase-deficient IRAK4 were infected with *L. monocytogenes* (MOI 1) or *M. smegmatis* (MOI 10). After washing, cells were incubated in gentamycin-containing medium for the indicated times, followed by lysing and plating cell lysates on agar plates to determine bacterial CFUs. The data (mean ± SD) represent a summary of three experiments, with individual mice shown in dots. *p<0.05.

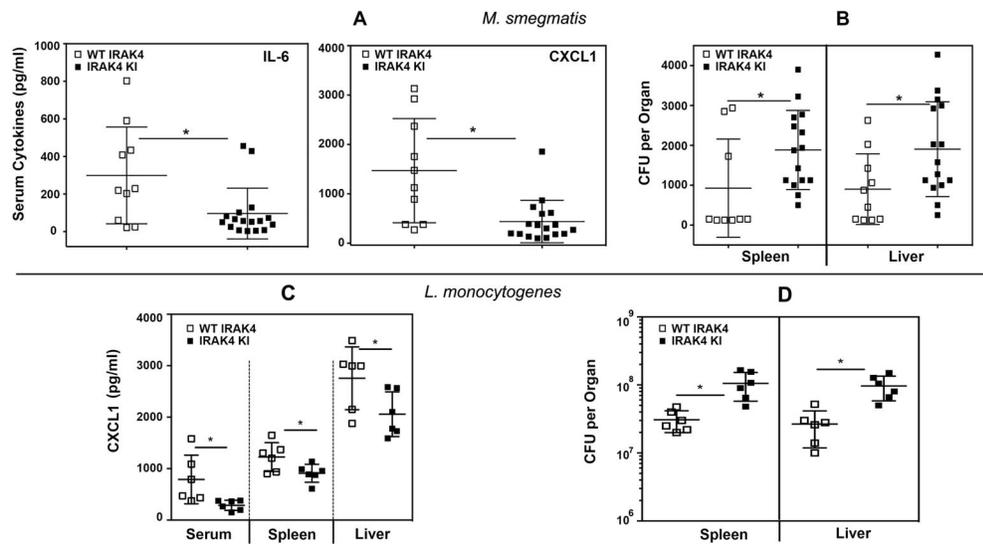


Figure 6. Mice harboring kinase-dead IRAK4 exhibit deficient levels of IL-6 and CXCL1 but increased splenic and liver CFUs following systemic infection with *M. smegmatis* or *L. monocytogenes*

WT or IRAK4^{KI} mice were infected i.p. with *M. smegmatis* (5×10^6 CFUs, A and B) or *L. monocytogenes* (2×10^4 CFUs, C and D). A: Serum was obtained 6 hpi and analyzed by ELISA for IL-6 and CXCL1 levels. B and D: 48 hpi, spleens and livers were homogenized and serial dilutions of organ homogenates were plated on the respective agar plates to determine CFUs. C: Organ homogenates were analyzed by ELISA to determine levels of CXCL1. Shown are the results (mean \pm SD) of 3 experiments with dots corresponding to individual mice. * $p < 0.05$