MPYS Is Required for IFN Response Factor 3 Activation and Type I IFN Production in the Response of Cultured Phagocytes to Bacterial Second Messengers Cyclic-di-AMP and Cyclic-di-GMP


_J Immunol_ published online 3 August 2011
http://www.jimmunol.org/content/early/2011/08/03/jimmunol.1100088

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2011/08/04/jimmunol.1100088.DC1

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Cyclic-di-GMP and cyclic-di-AMP are second messengers produced by bacteria and influence bacterial cell survival, differentiation, colonization, biofilm formation, virulence, and bacteria–host interactions. In this study, we show that in both RAW264.7 macrophage cells and primary bone marrow-derived macrophages, the production of IFN-β and IL-6, but not TNF, in response to cyclic-di-AMP and cyclic-di-GMP requires MPYS (also known as STING, MITA, and TMEM173). Furthermore, expression of MPYS was required for IFN response factor 3 but not NF-κB activation in response to these bacterial metabolites. We also confirm that MPYS is required for type I IFN production by cultured macrophages infected with the intracellular pathogens Listeria monocytogenes and Francisella tularensis. However, during systemic infection with either pathogen, MPYS deficiency did not impact bacterial burdens in infected spleens. Serum IFN-β and IL-6 concentrations in the infected control and MPYS−/− mice were also similar at 24 h postinfection, suggesting that these pathogens stimulate MPYS-independent cytokine production during in vivo infection. Our findings indicate that bifurcating MPYS-dependent and -independent pathways mediate sensing of cytosolic bacterial infections. The Journal of Immunology, 2011, 187: 000–000.

## References

Abbreviations used in this article: AIM2, absent in melanoma 2; BMM, bone marrow-derived macrophages; c-di-AMP, cyclic-di-AMP; c-di-GMP, cyclic-di-GMP; DC, dendritic cell; ES, embryonic stem; hpi, hours postinfection; IRF3, IFN response factor 3; KO, knockout; MOI, multiplicity of infection; WT, wild-type.

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Received for publication January 11, 2011. Accepted for publication June 28, 2011.

This work was supported in part by National Institutes of Health Grants R01AI055701 (to L.L.L.) and R01AI062739-05S1, R01AI062739-05S2, R01AI062739-05S3, and P01AI022950-22 (to I.C.C.). I.C.C. is an Ida and Cecil Green Professor of Immunology.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AIM2, absent in melanoma 2; BMM, bone marrow-derived macrophages; c-di-AMP, cyclic-di-AMP; c-di-GMP, cyclic-di-GMP; DC, dendritic cell; ES, embryonic stem; hpi, hours postinfection; IRF3, IFN response factor 3; KO, knockout; MOI, multiplicity of infection; WT, wild-type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100088
example, c-di-GMP induces dendritic cell (DC) maturation and triggers the production of IFN-β and a number of other cytokines and chemokines by DCs and macrophages (28, 29). It has also been shown that the amount of c-di-AMP secreted by L. monocytogenes strains correlates linearly with their IFN-β-inducing activity (32). Additionally, cytosolic delivery of c-di-AMP induces production of type I IFNs (32). IFN-β production in response to cytosolic c-di-AMP or c-di-GMP is dependent on TBK1 and IRF3 but independent of MyD88/TIR-domain-containing adapter-inducing IFN-β and MAVS (29, 32).

MPYS has been shown to play an essential role in the induction of IFN-β by intracellular dsDNA and by L. monocytogenes (33). However, it is not known whether MPYS acts as a general sensor of cytosolic bacterial infection in macrophages or contributes to IFN-β production in response to c-di-AMP or c-di-GMP. In this report, we address these questions and show that MPYS is essential for macrophage IL-6 and IFN-β production in response to cytosolic delivery of c-di-AMP and c-di-GMP, as well as for infections by the cytosolic bacterial pathogens L. monocytogenes and F. tularensis. These data reveal an important role for MPYS in the detection of cytosolic bacterial pathogens and their cyclic dinucleotide metabolites.

Materials and Methods

Knockdown of MPYS in RAW264.7 macrophages

RAW264.7 cells were transduced as previously described with retroviruses expressing either MPYS-knockdown or luciferase-knockdown constructs (34). Transduced cells were selected using medium containing 8 µg ml puromycin. The efficiency of MPYS knockdown was confirmed by immunoblotting using anti-MPYS Ab (34).

Generation of MPYS-knockout mice

Linearized targeting vector, which covers ~10 kb of the genomic region in MPYS locus on mouse chromosome 18, was transfected into JMS3.N1 embryonic stem (ES) cells originated from the C57BL/6 strain, followed by the selection for neomycin positive and diphtheria toxin (DTA) negative clones. Targeted clones were screened by PCR. Two of these ES clones were subjected to the generation of chimera mice by injection using C57BL/6J blastocysts as the ES clones were identified. Two of these ES clones were subjected to the targeted changes of the genomic region in MPYS locus on mouse chromosome 18, was transfected into JMS3.N1 embryonic stem (ES) cells originated from the C57BL/6J strain, followed by the selection for neomycin positive and diphtheria toxin (DTA) negative clones. Targeted clones were screened by PCR. Two of these ES clones were subjected to the generation of chimera mice by injection using C57BL/6J blastocysts as the host. The male chimeras (chimerism – 95% determined by coat color) were mated with C57BL/6 female mice for germline transmission. Both ES clones had successful germline transmission. The heterozygous mice were interbred to obtain wild-type, heterozygous, and homozygous litters. The genotypes of the mice were determined by genomic PCR and intracellular MPYS staining in mouse peripheral blood. Animals were generated at the National Jewish Health Mouse Genetics Core Facility. Animal care and handling was performed according to institutional animal care and use committee guidelines.

Intracellular MPYS staining

Mouse blood was collected by cheek bleeding. RBCs were lysed, and white cells were harvested and washed in FACS buffer (PBS with 2% FBS, 0.05% sodium azide, and 0.2 µg/ml 2.4G2 Fc-receptor blocking Ab). Cells were then resuspended in BD Cytofix/Cytoperm buffer (BD Biosciences) for 20 min at room temperature. BD Perm/Wash buffer (BD Biosciences) was added into the cell suspension. Cells were collected and washed with BD Perm/Wash buffer again. Cells were suspended in BD Perm/Wash buffer containing rabbit anti-MPYS Ab for 20 min at room temperature. Cells were collected and washed with BD Perm/Wash buffer twice and incubated with goat anti-rabbit Alexa-647 for 20 min at room temperature. Cells were collected and washed with BD Perm/Wash buffer, then analyzed using a FACS Calibur. Data were analyzed by FlowJo software (Tree Star, San Carlos, CA).

Bone marrow-derived macrophage culture

Bone marrow-derived macrophages were generated as described (35). Briefly, bone marrow cells harvested from mouse femurs were cultured in DMEM (Life Technologies) containing 20% FBS (Biosource), 10% L cell conditioned media as a source of CSF-1, 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Life Technologies), 100 units/ml penicillin plus 100 µg/ml streptomycin (Life Technologies), and 50 µM 2-mercaptoethanol. The medium was exchanged after 3 d, and cells were stained for the macrophage marker F4/80 at day 7.

L. monocytogenes and F. tularensis infections

Macrophages were seeded in 12-well plates at 1 × 10⁵ cells/well. The following day, the medium was exchanged with fresh antibiotic-free medium. L. monocytogenes (strain 10403S) were grown to log phase and added to the cell cultures at a multiplicity of 10 infectious bacterial cells per macrophage cell (multiplicity of infection; MOI). After 30 min, gentamicin (50 µg/ml; Life Technologies) was added to the medium to kill all extracellular bacteria. At 1 h postinfection, the medium was exchanged. F. tularensis (strain LVS) were grown to log phase and added to cell cultures at an MOI of 100. Two hours later, gentamicin (50 µg/ml) was added to the medium to kill all extracellular bacteria, and 1 h later the medium was exchanged. At time points indicated, supernatants were collected and analyzed for cytokines by ELISA. Cells were harvested, washed in PBS, and then lysed in 0.02% Nonidet P-40 buffer. Serial dilutions of lysate were plated onto TSB agar plates.

Mouse infections

Mice between 6 and 8 wk of age were used for all in vivo experiments. Mice were infected (tail vein) with 0.5 × 10⁶ to 2 × 10⁶ CFU log-phase L. monocytogenes (10403S) or i.p. with 1 × 10⁵ to 5 × 10⁵ CFU F. tularensis (strain LVS). After 48–72 h, spleens were harvested. Bacterial CFUs were determined by dilution plating as previously described (12).

Cyclic-di-GMP and cyclic-di-AMP activation

Macrophages were seeded in a 12-well plate at 1 × 10⁶ cells/well overnight before medium was exchanged. Cells were treated with Lipofectamine according to the manufacturer’s instructions. Briefly, in a tube designated A, we added 50 µl DMEM (Life Technologies) and increasing concentrations of c-di-GMP (2, 10, and 50 µM final concentration; Biolog) or c-di-AMP (1, 3, and 9 µM final concentration; Biolog). To a tube designated B, we added 50 µl DMEM and 2 µl Lipofectamine 2000 (Invitrogen). Each tube was then vortexed for 1 s and incubated at room temperature for 5 min before the solution from tube A was transferred to tube B, followed by vortexing for 2 s and incubation at room temperature for 20 min. The mixture was then added dropwise into the macrophage culture followed by incubation at 37˚C. At designated time points, supernatants were collected for cytokine ELISAs, and cells were washed in PBS and harvested for total RNA.

Cytokine ELISAs

Supernatants from treated and control macrophages were collected at designated time points to measure concentrations of IL-6 and TNF (BD Pharmingen) or IFN-β (IFNSource) using commercial ELISAs. Supernatants were stored at −20˚C before assay.

FIGURE 1. MPYS is required for IFN-β production in response to infection with L. monocytogenes and F. tularensis. A, Whole-cell lysate from RAW264.7 cells expressing luciferase-knockdown (lucKD) or MPYS-knockdown (MPYSKD) construct were fractionated by SDS-PAGE (10%) and transferred to nitrocellulose, and stained with anti-MPYS or anti-actin Ab, respectively. B–D, lucKD or MPYSKD macrophages were infected with L. monocytogenes (MOI 10) or F. tularensis LVS (MOI 100). Supernatants were collected at 12 hpi, and ELISAs were performed for IFN-β (B), IL-6 (C), and TNF (D). Lm, L. monocytogenes; Ft, F. tularensis.
transcript abundance was normalized to ifnb

CAGAGTCCGCCTCTGATGCT

Odyssey 2.1 software and normalized to actin. The relative intensities of p-IRF3 and I

to p-IRF3, I

polyvinylidene fluoride, and probed with Abs against p-IRF3 (Cell Signaling) and actin (Santa Cruz).

FIGURE 3. MPYS is selectively required for IRF3 activation in response to treatment with cyclic dinucleotides. A, BMM were treated with 20 μM synthetic c-di-AMP or c-di-GMP as described in Materials and Methods. Whole-cell lysates were separated by SDS-PAGE (10% NuPAGE), transferred to polyvinylidene fluoride, and probed with Abs against p-IRF3 (Cell Signaling) and actin (Santa Cruz). B, RAW264.7 lucKD or MPYSKD macrophages were treated with 9 μM c-di-AMP or 50 μM c-di-GMP and harvested for RNA at 12 h posttreatment. Quantitative PCR (TaqMan method) was performed for IFN-β gene expression, and results were normalized to those of GAPDH. B, RAW264.7 lucKD or MPYSKD cells were treated with increasing concentrations of c-di-AMP or c-di-GMP as before. At 12 h posttreatment, supernatants were collected and analyzed by ELISA for IFN-β production.

RT-PCR

Macrophages were cultured and treated as described. Total RNA was isolated from triplicate wells of macrophages at the indicated times using the Qiagen RNeasy Plus mini kit (Qiagen) and reverse-transcribed using the Qiagen RNeasy Plus mini kit (Qiagen) and reverse-transcribed using oligodeoxythymidylate primers and the IMPROM II FT system (Promega) as previously described (12). cDNAs were used as template for 29–32 cycles of PCR with Taq polymerase (Invitrogen). Primers and probes used for quantitative PCR (TaqMan method) were as follows: IFN-β sense (5'-CTCATCATTCAAGACTTACCAGAAAC-3'); IFN-β antisense (5'-CAGAGTCCGCCTCTGATGCT-3'); IFN-β probe (5'-ATGCCCTAGATGAATGCAGTGTTGCGCA-3'); GAPDH sense (5'-GGGAAGCCCATCA-CCATCT-3'); GAPDH antisense (5'-ACATACTCAGACCCGGCTC-3'); GAPDH probe (5'-AGGGAGACCCCACTAACATCAAATGGG-3'). ifnb transcript abundance was normalized to gapdh.

Results

MPYS knockdown selectively impairs IL-6 and IFN-β production in response to L. monocytogenes and F. tularensis infections in RAW264.7 cells

To investigate the requirement for MPYS in macrophage sensing of cytosolic bacterial pathogens, we used the RAW264.7 macrophage cell line that stably expresses short hairpin RNAs targeting either luciferase (lucKD) or exon 5 of MPYS (MPYSKD) and that has been reported before (36). Immunoblotting of cell lysates with anti-MPYS Ab confirmed the efficiency of MPYS knockdown in the MPYSKD, but not lucKD, cells (Fig. 1A). We next investigated the effects of MPYS knockdown on cytokine production after infection of the lucKD and MPYSKD cells with L. monocytogenes or F. tularensis. Both cells responded to LPS treatment with robust secretion of IFN-β protein (Fig. 1B). However, only the control cells efficiently secreted IFN-β when infected with L. monocytogenes (MOI of 10) or F. tularensis (MOI of 100). Likewise, the ability of macrophages to respond to infection by secretion of IL-6, but not TNF, required MPYS (Fig. 1C, 1D). These effects were not due to any discernible effects of MPYS expression on the ability of bacteria to infect the macrophages (data not shown); the doubling times of L. monocytogenes in lucKD and MPYSKD cells were 52.6 and 50 min. Thus, our data reveal that MPYS is essential for production of IL-6 and IFN-β, but not TNF, in response to cytosolic bacterial infection by both Gram-positive (L. monocytogenes) and Gram-negative (F. tularensis) bacteria.

MPYS knockdown impairs the recognition of cyclic dinucleotide monophosphates in RAW264.7 cells

It has been shown that secreted levels of the second messenger c-di-AMP correlate with the degree of type I IFN production in response...
to L. monocytogenes infection (32). Cytosolic delivery of the bacterial metabolites c-di-AMP or c-di-GMP to macrophage elicits IFN-β production (29, 32). To evaluate whether MPYS participates in sensing of these bacterial metabolites, we compared ifnb transcription and IFN-β secretion by lucKΔD and MPYSKΔD cells with increasing concentrations of cytosolic synthetic c-di-AMP and c-di-GMP (Fig. 2). The results clearly showed that the MPYSKΔD macrophages failed to produce IFN-β in response to either cyclic dinucleotide monophosphate.

**MPYS is required for IRF3 activation in response to cyclic dinucleotide monophosphates in RAW264.7 cells**

Expression of TBK1 and IRF3 is required for production of IFN-α/β by mouse bone marrow-derived macrophages (BMM) infected with L. monocytogenes or cytosolic delivery of c-di-AMP or c-di-GMP (29, 32). We further observed that introduction of either cyclic dinucleotide monophosphate caused a prolonged increase in phosphorylation of IRF3 at Ser96 (p-IRF3) in the BMM (Fig. 3). Similarly, this prolonged increase in absolute and relative p-IRF3 was seen in control RAW264.7 lucKΔD cells receiving either c-di-AMP or c-di-GMP (29, 32). We further observed that introduction of either cyclic dinucleotide monophosphate caused a prolonged increase in phosphorylation of IRF3 at Ser96 (p-IRF3) in the BMM (Fig. 3). Likewise, this prolonged increase in absolute and relative p-IRF3 was seen in control RAW264.7 lucKΔD cells receiving either c-di-GMP or c-di-AMP (Fig. 3B–E). Unlike control cells, MPYSKΔD macrophages failed to accumulate p-IRF3 in response to either c-di-AMP or c-di-GMP (Fig. 3B–E). Yet, MPYS was not required for the induced degradation of IκBα (Fig. 3B–E). These data indicate that MPYS lies upstream of IRF3 activation in the pathway leading from cyclic dinucleotide monophosphate sensing to ifnb transcription, but this requirement for MPYS does not extend to NF-κB activation.

We also evaluated IRF3 activation and IκBα degradation after L. monocytogenes infection. In both BMM and RAW264.7 lucKΔD cells, we observed MPYS-dependent p-IRF3 at 3 h postinfection and MPYS-independent IκBα degradation within 4 h of infection (Supplemental Fig. 1).

**FIGURE 4.** Generation of MPYS-KO mice by homologous recombination. A, Strategy to generate MPYS-KO mice. The genomic sequence of mouse MPYS gene is derived from a BAC clone RP24-490M12 (~140 kb). MPYS gene consists of 8 exons and spans from 65,182 to 72,058 bp in the BAC. Protein translation starts from exon 3. A hypothetical gene, 1700066B19Rik, is ~2.8 kb downstream of MPYS, and there is no protein-coding gene in the ~30-kb region upstream of MPYS. The targeting construct covers ~10-kb genomic region in the MPYS locus (64,515~74,175). The targeting construct has a neo gene inserted in intron 5 and a diphtheria toxin gene at the 3′-end of the MPYS gene. The neo gene is flanked by Frt elements and one LoxP site. Another LoxP site is inserted in intron 2. Thus, using tissue-specific Flp or Cre transgenic mice, we can also generate conditional KO or conditional WT MPYS mice. B, Western blot analysis of MPYS from WT, KO, and heterozygous MPYS littermates. Spleenocytes were lysed in RIPA buffer containing 0.1% SDS and run on a reducing SDS-PAGE gel. The blot was probed with rabbit α-MPYS Ab. This experiment was repeated more than three times. N.S., nonspecific. C, RT-PCR analysis of MPYS transcript from peripheral blood. RT-PCR was performed using cDNA from peripheral blood cells of WT, KO, and heterozygous MPYS littermates with primers for exon 2, 3, and 4 of the MPYS transcript. This experiment was repeated twice.
is required for c-di-NMP–induced IRF3 activation, but not NF-κB activation, in RAW264.7 macrophage cell line and in primary BMM.

The apparent mass of MPYS increases in response to L. monocytogenes and c-di-GMP activation

To confirm further the involvement of MPYS in L. monocytogenes and c-di-NMP–induced type I IFN response, we investigated whether MPYS is activated by these stimuli. Previous studies indicated that MPYS forms a transient homodimer in response to Sendai virus infection (36, 37) and intracellular dsDNA in 293T cells (38). We found that a high-mass MPYS form corresponding with the homodimer size was also elicited in response to L. monocytogenes infection of 293T cells (Fig. 7A). Furthermore, cytosolic c-di-GMP induced transient formation of these putative MPYS homodimers in BMM (Fig. 7B). These data suggest that homodimer formation is a hallmark of MPYS activation in response to diverse infectious and microbial stimuli.

MPYS deficiency does not dramatically impact bacterial burdens during L. monocytogenes and F. tularensis infection

As mentioned earlier, mice deficient for expression of the receptor for IFNα/β demonstrate increased resistance to L. monocytogenes and F. tularensis. To determine whether MPYS deficiency might likewise reduce host susceptibility, we compared bacterial burdens from control B6, MPYS+/−, and MPYS−/− mice infected with L. monocytogenes. We found that bacterial burdens in spleens were comparable in infected WT and MPYS−/− mice at 72 h postinfection (hpi) (Fig. 8A). This was a surprising observation because under similar conditions, IFNAR1−/− mice have significantly reduced burdens of L. monocytogenes (Ref. 12 and data not shown). We also found that F. tularensis burdens were similar at 48 hpi in WT, MPYS+/−, and MPYS−/− mice (Fig. 8B). Consistent with their similar susceptibilities, we found that MPYS deficiency significantly reduced production of IFN-β and IL-6 at early times (8 h) after L. monocytogenes and F. tularensis infections (Fig. 8C, 8D, Supplemental Fig. 3), but not at later (24 h) times (Fig. 8E, 8F). These data suggest MPYS is required only for the initial IFN-β and IL-6 production in response to infections by these cytosolic bacterial pathogens.

Discussion

In this report, we established that the multitransmembrane protein MPYS plays an essential role in the sensing of cytosolic cyclic dinucleotide monophosphate in macrophages. MPYS mediated the activation of IRF3, but not NF-κB, in response to the bacterial metabolites c-di-AMP and c-di-GMP. Thus, our findings are consistent with the notion that macrophages sense both Gram-positive and Gram-negative cytosolic bacteria via ligation of a receptor or receptors for cyclic dinucleotide metabolites that are released from these bacteria. While this article was under review, Dr. Vance’s group also published evidence that implicates MPYS/STING in type I IFN production in response to c-di-NMPs (39).

Bacterial second messengers c-di-AMP and c-di-GMP are ubiquitously expressed in bacterial species but absent in higher eukaryotes (39). It was previously suggested that c-di-GMP acts as a danger signal when sensed by eukaryotic cells (40). Indeed, several studies have shown that c-di-GMP has strong immunostimulatory properties (28, 41). More recent studies demonstrated that introduction of c-di-GMP or c-di-AMP into macrophages also activated type I IFN production independent of TLRs and intracellular RNA sensing pathways (29). Our studies thus provide mechanistic insight into the host response to these ubiquitously expressed bacterial second messengers; specifically, by demonstrating an essential role of MPYS in the activation of IRF3 (but not NF-κB) and the production of type I IFNs by macrophages treated with c-di-AMP, c-di-GMP, or during infections with L. monocytogenes and F. tularensis. These studies do not necessarily indicate that MPYS is itself a receptor for c-di-AMP or c-di-GMP. Rather, the differences in kinetics of IRF3 activation in response to c-di-GMP and c-di-AMP and the lack of a requirement for MPYS in the activation of NF-κB by these ligands suggest that a proximal receptor or receptors is responsible for binding these cyclic dinucleotides prior to independent activation of both the MPYS–TBK1–IRF3 and the NF-κB pathways.

MPYS is a potent type I IFN stimulator (33). It mediates type I IFN responses to intracellular dsDNA of bacterial or mammalian origin (42). This intracellular dsDNA response is motif-independent but length-dependent (26). Recent studies identified the proteins AIM2 and IFI16 as sensors of intact cytosolic DNA. AIM2 activates caspase-1 and subsequent release of IL-1β and IL-18 during L. monocytogenes and F. tularensis infections (17–22, 43). It is not known whether AIM2 also senses the presence of bacterial cyclic dinucleotides; however, AIM2 does not mediate the activation of IRF3 and thus does not lead to production of type I IFNs (24, 25). IFI16 interacts with MPYS and activates IRF3 in response to dsDNA of >25 nt (∼13 kDa) (25). Thus, whereas IFI16 may activate MPYS–IRF3 in response to large dsDNA fragments released from dying bacteria, we speculate that another receptor may sense the small-molecule cyclic dinucleotide mono-
in vivo infections. Consistent with our observation that WT and (18, 33) but indicate that the situation is more complex during
F. tularensis burdens at 24 hpi. At this early time, bacterial burdens in tissues of
mutations in MPYS did not impact splenic
matitis virus infection. Likewise, Sauer et al. (39) published that (12 hpi) IFN-
that, in vivo, MPYS was required only for early (6 hpi) but not late
reminiscent of results from Dr. Barber’s group (33, 42) showing
quired only for the early (8 hpi) but not late (24 hpi). The type I IFN production by
these cells, or perhaps other cell types that are infected in vivo, is
presumably independent of MPYS and may instead use other
stimulators. It will be of interest to investigate the nature of this
MPYS-independent pathway or pathways.

Although MPYS was clearly required for production of type I
IFN in response to L. monocytogenes infection of BMM and bone
marrow-derived DCs [Supplemental Fig. 4 (42)], our data from
infected mice revealed additional MPYS-independent production of
type I IFN in vivo. A subpopulation of macrophages or TNF
and inducible NO synthase-producing DCs are thought to be the
main source for in vivo production of IFN-β during in vivo L.
monocytogenes infection (44–46). The type I IFN production by
these cells, or perhaps other cell types that are infected in vivo, is
presumably independent of MPYS and may instead use other
stimulators. It will be of interest to investigate the nature of this
MPYS-independent pathway or pathways.

The detection of bacterial components in the cytosol of macrophages appears to be the major mechanism responsible for type I IFN production by cultured phagocytes. It was originally thought that such detection enabled the host to stimulate an appropriate response to the presence of intracellular bacteria (47). Yet, many of the intracellular bacteria that elicit production of type I IFNs appear to replicate better in mice that are capable of responding to these cytokines. It is unclear why the host produces type I IFNs during bacterial infections in which the production of such IFNs is deleterious to host resistance. It is also unclear why type I IFN production (often counterprotective) and IL-6 production (often protective) are linked. We and others have speculated that IFN production may be a consequence of the strong evolutionary pressure to respond against aggressive viral infections, where type I IFNs are protective. Alternatively, type I IFN production may limit damage from unchecked inflammatory responses (48, 49). Alternatively, or additionally, certain pathogens may have evolved strategies to avoid the antibacterial consequences of type I IFNs while benefiting from the ability of these cytokines to downregulate inflammatory responses.

In summary, the results of this study revealed that MPYS is required for type I IFN production by cultured macrophages and
bone marrow-derived DCs in response to the bacterial second
messengers c-di-AMP and c-di-NMP. However, studies with

phosphates (~600 Da). Regardless of the receptors involved in
cyclic dinucleotide sensing by phagocytes, it will be important to
better understand how MPYS interacts with such receptors and
activates the TBK1–IRF3 pathway.

Our data in this study have confirmed previous reports that
MPYS is important for type I IFN production in response to in vitro
infection of macrophages by L. monocytogenes and F. tularensis
(18, 33) but indicate that the situation is more complex during
in vivo infections. Consistent with our observation that WT and
MPYS−/− mice have comparable bacterial loads during systemic
L. monocytogenes and F. tularensis infections, MPYS was re-
quired for the early (8 hpi) but not late (24 hpi) IFN-β and
IL-6 production in response to these bacteria. These data are
reminiscent of results from Dr. Barber’s group (33, 42) showing
that, in vivo, MPYS was required only for early (6 hpi) but not late
(12 hpi) IFN-α and -β production in response to vesicular stoma-
matitis virus infection. Likewise, Sauer et al. (39) published that
mutations in MPYS did not impact splenic L. monocytogenes
burdens at 24 hpi. At this early time, bacterial burdens in tissues of
WT and IFNAR1−/− mice are similar, but they diverge by 72 hpi
with L. monocytogenes. We thus evaluated L. monocytogenes
burdens at this later time (Fig. 8) and found that MPYS−/− mice
also harbored similar bacterial loads in the spleens at this time.
Together, these data demonstrate that alternate, MPYS-independent
mechanisms exist to elicit production of type I IFNs and the ensuing suppression of host resistance during in vivo
L. monocytogenes and F. tularensis infections.

FIGURE 8. MPYS is required for early but not late IFN-β or IL-6 production in vivo. A and B, MPYS−/−, MPYS+/−, and their WT littermates (B6) were infected with 10,000 CFU L. monocytogenes (i.v.) (A) or 5000 CFU F. tularensis (i.p.) (B). Spleens were harvested, homogenized, and di-
I
lution plated to determine bacterial burdens 72 h (A) or 48 h (B) later. Each point indicates an individual mouse. Bars indicate the mean values.

C–F, MPYS−/−, MPYS+/−, and their WT littermates (B6) (n = 3) were infected as above with L. monocytogenes (C, E) or F. tularensis (D, F). Sera were collected at indicated
times. IFN-β and IL-6 concentrations were measured by ELISA. Experi-
ments were performed twice.
Disclosures

The authors have no financial conflicts of interest.

References


19. Sauer, J. D., C. E. Witte, J. Zemansky, B. Hanson, P. Lauer, and D. A. Portnoy. 2004. Mice lacking the type I interferon receptor are resistant to Type I IFN production during in vivo bacterial infections. Further study is needed to characterize the nature of these mechanisms and their contributions to host defense or susceptibility to infections and other diseases.


Figure S1. MPYS is required for IRF3 activation, but not IκBα degradation, in *L. monocytogenes* infected macrophages. **A.** BMM (1×10^6 cells) were infected with *L. monocytogenes* and at the indicated times after infection, WCL were prepared and probed as in Fig. 3 with Abs to p-IRF3, IκBα, and actin. **B.** The intensities of p-IRF3 (left panel) and IκBα (right panel) in the WCL from *L. monocytogenes* infected BMM were quantified and normalized to actin. Relative intensity values are plotted against time. **C.** RAW264.7 luc^KD^ or MPYS^KD^ cells were infected with *L. monocytogenes* as in A. WCL were prepared at the indicated times after infection and evaluated for p-IRF3, IκBα, MPYS, and actin. **D.** The normalized intensities of p-IRF3 (left panel) and IκBα (right panel) were quantified and plotted as in B. These experiments have been repeated more than three times.
Figure S2. MPYS-KO mice Screening. A. PCR screening strategy for MPYS KO mice. In a homologous recombination event, the targeting construct will replace the endogenous MPYS locus. Thus, the two primers nel3 at 74770 (outside of the target region) and neo will be on the
same mouse chromosome 18. PCR using these primers will yield a 5.7kb fragment in the positive clone. The same 5.7kb PCR fragment should also contain the Cla I site at 74175 and the LoxP site at 71227. B. Genomic PCR by nel3-neo primers in the positive ES clone JM5 and offspring from chimera mice injected with the JM5 ES clone, E4, E5 and E6 mice. C. PCR sequencing in the nel3-neo fragment. The Cla I site at 74175 and Lox P site at 71227 were highlighted. D. Intracellular staining of MPYS in peripheral blood from indicated mice. The intracellular staining was done as described in Material and Methods. Rabbit anti-MPYS polyclonal Ab was used followed by goat anti-rabbit Alexa 647. All the genomice PCR and intracellular staining were repeated twice.

Figure S3. MPYS is required for early IL-6 production in response to *F. tularensis* infection *in vivo*. MPYS**, MPYS**/* and their WT littermates (B6) (n=3) were infected with *F. tularensis* as in Fig.8 and serum IL-6 was measured by ELISA. Experiments were done twice.
Figure S4. MPYS is required for *L. monocytogenes* induced IFNβ production in BMDC.

Colony-stimulated factor (GM-CSF)-dependent DC (GM-DC) were generated by culturing BM cells in the presence of murine recombinant GM-CSF (R&D Systems). GM-DC from MPYS<sup>−/−</sup> or WT littermates (B6) were infected with *L. monocytogenes* (MOI of 10). Medium supernatants were collected after 21hrs and IFNβ was measured as before. Experiments were done twice.