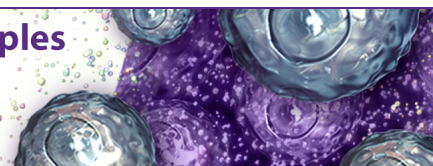


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MPYS Is Required for IFN Response Factor 3 Activation and Type I IFN Production in the Response of Cultured Phagocytes to Bacterial Second Messengers Cyclic-di-AMP and Cyclic-di-GMP

Lei Jin,¹ Krista K. Hill,¹ Holly Filak, Jennifer Mogan, Heather Knowles, Bicheng Zhang, Anne-Laure Perraud, John C. Cambier,² and Laurel L. Lenz²

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- κ B 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: 2595–2601.

Macrophages are among the first cells of the immune system to encounter invading pathogens. Hence, the ability of macrophages to sense pathogens and their products plays a crucial role in the initiation of inflammation and immune responses. A key mechanism by which macrophages mediate their function involves production of immune regulatory cytokines such as TNF, IL-6, and IFN- α/β . TNF and IL-6 play a largely protective role in the host response to bacterial infections (1–3). In contrast, the role of type I IFN in bacterial infection is more complex (4). Type I IFNs were suggested to protect mice against *Salmonella typhimurium* and group B streptococcal infections (5, 6) and suppress intracellular replication of *Legionella pneumophila* (7, 8). In contrast, type I IFNs increase host susceptibility to *Listeria monocytogenes* (9–12), *Mycobacterium tuberculosis* (13–15), and *Francisella tularensis* (16). The mechanisms by which type I IFNs promote susceptibility during these

infections are not currently clear. Nevertheless, it is important to understand how infection of host cells by intracellular bacteria elicits the production of type I IFNs.

A series of recent studies has established the critical role of the cytoplasmic DNA sensor absent in melanoma 2 (AIM2) in host defense against cytoplasmic bacteria, including *L. monocytogenes* and *F. tularensis* infections (17–23). Mice deficient in AIM2 are extremely susceptible to *F. tularensis*, suffering greater mortality and bacterial burden than wild-type mice. The increased susceptibility of AIM2 mutant mice is associated with defective caspase-1 activation and IL-1 β secretion by infected macrophages (18, 20, 21). However, AIM2 is not required for type I IFN production (24), indicating that sensing of bacterial DNA or DNA-like molecules by AIM2 contributes to caspase 1 activation but not type I IFN production.

Notably, type I IFN production in response to intracellular DNA was more recently shown to involve a distinct cytoplasmic DNA sensor, IFI16/p204 (25). This study suggested that IFI16 recruits STING/MPYS to activate the TBK1–IFN response factor 3 (IRF3) signaling pathway. Transfection of *L. monocytogenes* DNA into the cytosol of host cells also activates the TBK1–IRF3 pathway leading to IFN- β production (26), but the extent to which intact bacterial DNA accesses the cytosol of host cells during infection is not clear. Because bacterial multidrug efflux pumps enhance induction of IFN- α/β during *L. monocytogenes* infection, small-molecule substrates of these pumps also appear to elicit host cell production of type I IFN (27). Possible small-molecule substrates of such pumps include cyclic dinucleotide monophosphates, such as cyclic-di-AMP (c-di-AMP) and cyclic-di-GMP (c-di-GMP).

c-di-GMP influences bacterial cell survival, differentiation, colonization, biofilm formation, and bacteria–host interactions (28–31). Diverse immune cell populations have been shown to respond to c-di-GMP treatments both *in vivo* and *in vitro*. For

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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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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 μ g/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 JM8A3.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. From 52 clones, 6 positive clones were identified. 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/6J female mice for germline transmission. Both ES clones had successful germline transmission. The heterozygous mice were interbred to obtain wild-type, heterozygous, and homozygous littermates. 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 μ g/ml 2.4g2 Fc-receptor blocking Ab). Cells were then resuspended in BD Cytotfix/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 FACSCalibur. 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 μ g/ml streptomycin (Life Technologies), and 50 μ M

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 1×10^6 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 μ g/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 μ g/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 0.5×10^4 to 2×10^4 CFU log-phase *L. monocytogenes* (10403S) or i.p. with 1×10^4 to 5×10^4 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 1×10^6 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 μ l DMEM (Life Technologies) and increasing concentrations of c-di-GMP (2, 10, and 50 μ M final concentration; Biolog) or c-di-AMP (1, 3, and 9 μ M final concentration; Biolog). To a tube designated B, we added 50 μ l DMEM and 2 μ l 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 indicated 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.

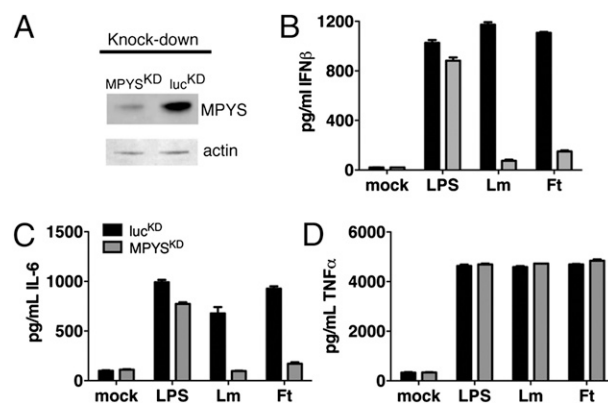


FIGURE 1. MPYS is required for IFN- β production in response to infection with *L. monocytogenes* and *F. tularensis*. **A**, Whole-cell lysate from RAW264.7 cells expressing luciferase-knockdown (*lucKD*) or MPYS-knockdown (*MPYSKD*) construct were fractionated by SDS-PAGE (10% NuPAGE), transferred 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*.

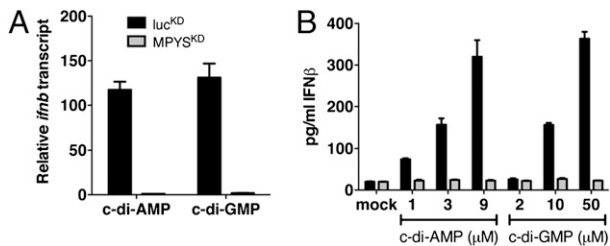


FIGURE 2. MPYS is required for IFN- β production in response to treatment with cyclic dinucleotides. *A*, RAW264.7 *luc*^{KD} or *MPYS*^{KD} macrophages were treated with 9 μ M c-di-AMP or 50 μ M c-di-GMP and harvested for RNA at 12 h posttreatment. Quantitative PCR (TaqMan method) was performed for IFN- β gene expression, and results were normalized to those of GAPDH. *B*, RAW264.7 *luc*^{KD} or *MPYS*^{KD} 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 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'-CTCATCATTGGAAGACTTACCAGAAAC-3'); IFN- β antisense (5'-CAGAGTCCGCTCTGATGCT-3'); IFN- β probe (5'-ATGCCTCAGATGAGTGGTGGTTGCA-3'); GAPDH sense (5'-GGGAAGCCCATCA-CCATCTT-3'); GAPDH antisense (5'-ACATACTCAGCACCGGCCTC-3'); GAPDH probe (5'-AGCGAGACCCCACTAACATCAAATGGG-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 (*luc*^{KD}) or exon 5 of MPYS (*MPYS*^{KD}) and that has been reported before (36). Immunoblotting of cell lysates with anti-MPYS Ab confirmed the efficiency of MPYS knockdown in the *MPYS*^{KD}, but not *luc*^{KD}, cells (Fig. 1*A*). We next investigated the effects of MPYS knockdown on cytokine production after infection of the *luc*^{KD} and *MPYS*^{KD} cells with *L. monocytogenes* or *F. tularensis*. Both cells responded to LPS treatment with robust secretion of IFN- β protein (Fig. 1*B*). 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. 1*C*, 1*D*). 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 *luc*^{KD} and *MPYS*^{KD} 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

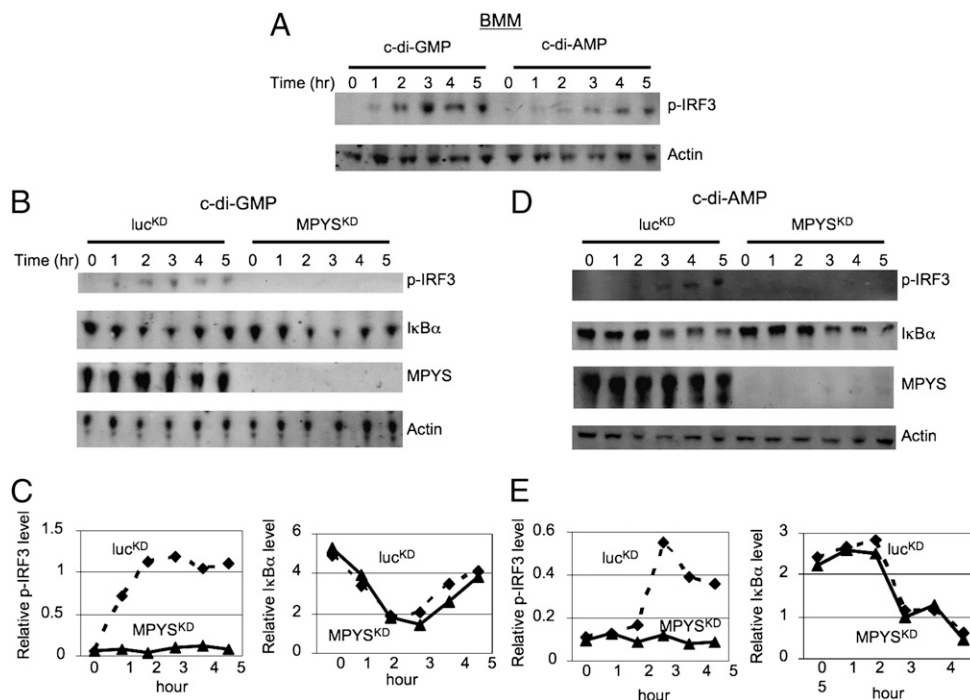


FIGURE 3. MPYS is selectively required for IRF3 activation in response to treatment with cyclic dinucleotides. *A*, BMM were treated with 20 μ M synthetic c-di-GMP or c-di-AMP as described in *Materials and Methods*. Whole-cell lysates were separated by SDS-PAGE (10% NuPAGE), transferred to polyvinylidene fluoride, and probed with Abs against p-IRF3 (Cell Signaling) and actin (Santa Cruz). *B*, RAW264.7 *luc*^{KD} or *MPYS*^{KD} macrophages were treated with c-di-GMP (20 μ M) as before and harvested at the indicated times. Whole-cell lysates were separated and blotted as in *A*, then probed with Abs to p-IRF3, I κ B α (Cell Signaling), actin, or MPYS (34). *C*, The intensities of p-IRF3 (left panel) and I κ B α (right panel) bands in *B* were quantified using Odyssey 2.1 software and normalized to actin. The relative intensities of p-IRF3 and I κ B α are plotted against time. *D*, RAW264.7 *luc*^{KD} cells and *MPYS*^{KD} cells were treated with c-di-AMP (20 μ M) as before. Activation of IRF3 and degradation of I κ B α were evaluated as in *B*. *E*, The relative intensities of p-IRF3 and I κ B α were determined as in *C*. Each experiment of this figure was performed at least three times.

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 luc^{KD} and MPYS^{KD} cells with increasing concentrations of cytosolic synthetic c-di-AMP and c-di-GMP (Fig. 2). The results clearly showed that the MPYS^{KD} 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. 3A). Likewise, this prolonged increase in absolute and relative p-IRF3 was seen in control RAW264.7 luc^{KD} cells receiving either c-di-GMP or c-di-AMP (Fig. 3B–E). Unlike control cells, MPYS^{KD} 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 I κ B α (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- κ B activation.

We also evaluated IRF3 activation and I κ B α degradation after *L. monocytogenes* infection. In both BMM and RAW264.7 luc^{KD} cells, we observed MPYS-dependent p-IRF3 at 3 h postinfection and MPYS-independent I κ B α degradation within 4 h of infection (Supplemental Fig. 1).

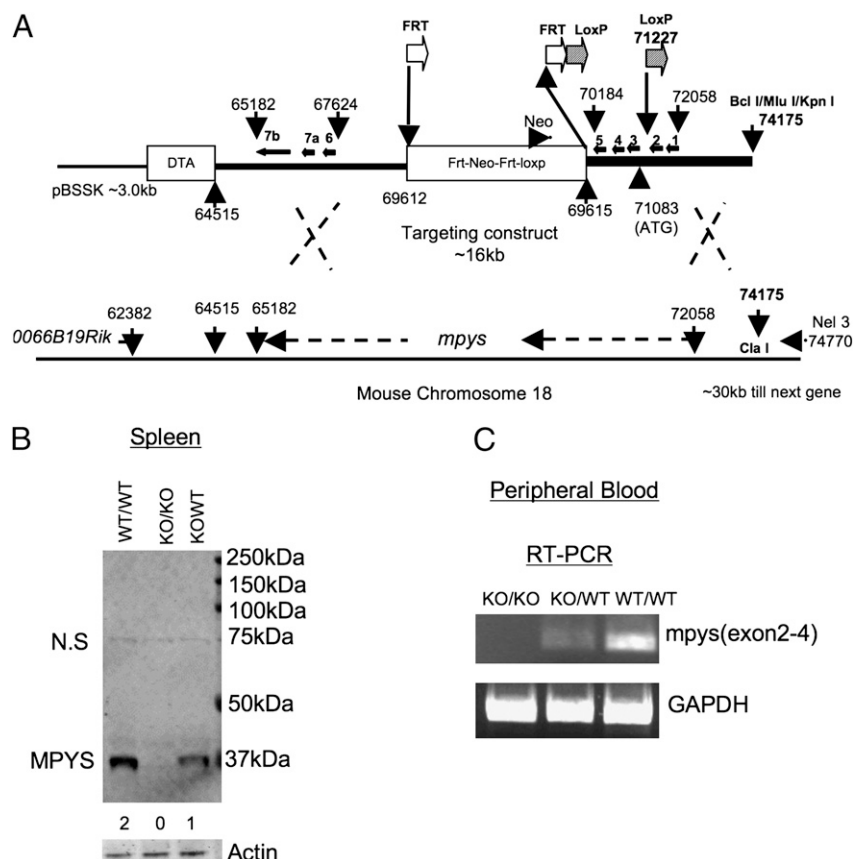
MPYS-deficient BMM do not make IFN- β in response to cyclic dinucleotide monophosphates

To extend our observation that MPYS is required for sensing intracellular cyclic dinucleotide monophosphates to the primary cells, we used BMM generated from MPYS-knockout mice recently developed in Dr. Cambier's laboratory (L. Jin and J.C. Cambier, full characterization of the knockout mice will be described elsewhere). The knockout (KO) mice were generated through homologous recombination (Fig. 4A), and the homologous integration event was confirmed by genomic PCR and sequencing (Supplemental Fig. 2A–C). The endogenous *MPYS* gene was replaced by the targeting construct that has a neo gene inserted into intron 5 of the *MPYS* gene (Fig. 4A). Western blot in splenocytes (Fig. 4B) and intracellular staining in peripheral blood (Supplemental Fig. 2D) confirmed the lack of MPYS protein expression. RT-PCR in cells from peripheral blood, using primers that amplify exons 2, 3, and 4 of the *MPYS* gene, also confirmed an absence of MPYS transcripts (Fig. 4C). We next confirmed the requirement for MPYS expression in the response to c-di-GMP using BMM cultured from wild-type (WT), MPYS^{+/-}, and MPYS^{-/-} mice (Fig. 5). Cytosolic delivery of c-di-GMP resulted in significant IFN- β secretion from the BMM only when MPYS was expressed. These results indicated that MPYS is required for the IFN- β response of BMM to cyclic di-nucleotide monophosphate.

Cyclic dinucleotide monophosphates fail to activate IRF3 in MPYS-deficient BMM

We next evaluated c-di-NMP-induced IRF3 and NF- κ B activation in BMM from MPYS^{-/-} and WT mice. Consistent with our observations in RAW264.7 MPYS^{KD} cells (Fig. 3), MPYS^{-/-} BMM did not have detectable phosphorylation of IRF3 after intracellular introduction of c-di-GMP and c-di-AMP, though the degradation of I κ B α was still observed (Fig. 6A, 6B). Thus, MPYS

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. Splenocytes 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.



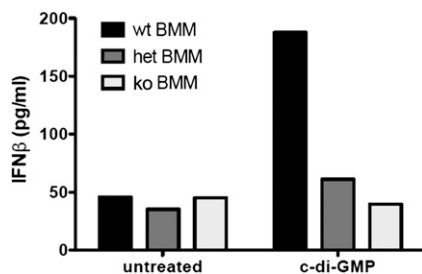


FIGURE 5. MPYS-deficient BMM are defective for IFN- β production in response to treatment with cyclic dinucleotide monophosphates. BMM from indicated mice were treated with 50 μ M synthetic c-di-GMP as before. At 12 h posttreatment, supernatants were collected and analyzed by ELISA for IFN- β production. Experiments were repeated twice with similar results.

is required for c-di-NMP-induced IRF3 activation, but not NF- κ B 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 IFN- α/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.

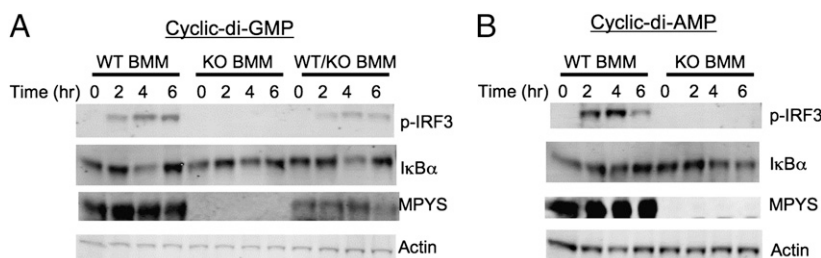
Discussion

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- κ B, 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- κ B) 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- κ B 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- κ B 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-1 β 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-

FIGURE 6. MPYS-deficient BMM fail to activate IRF3 in response to treatment with cyclic dinucleotide monophosphates. A and B, BMM from indicated mice were treated with 20 μ M synthetic c-di-GMP (A) or c-di-AMP (B) for indicated times as before. Whole-cell lysates were separated by SDS-PAGE and probed with indicated Abs as for Fig. 3. These experiments were repeated twice.



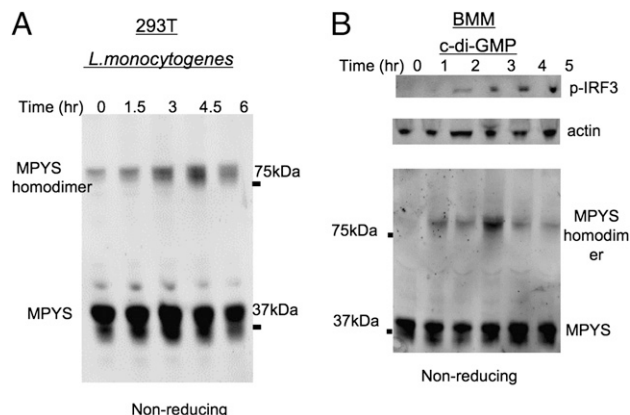


FIGURE 7. MPYS forms homodimers in response to *L. monocytogenes* infection and cytosolic c-di-GMP activation. **A**, HEK-293T cells were infected with *L. monocytogenes* as in Fig. 1 for the indicated time. Cells were then lysed in RIPA buffer containing 0.1% SDS and fractionated using nonreducing SDS-PAGE gels. Blots were probed with the anti-MPYS Ab. **B**, BMM were treated with c-di-GMP (20 μM) as for Fig. 3. Cells were then lysed and fractionated on a nonreducing gel. Blots were probed with indicated Abs. High-m.w. MPYS that previous studies have reported to be homodimers are indicated. These experiments were repeated more than three times.

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 required only 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 stomatitis 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

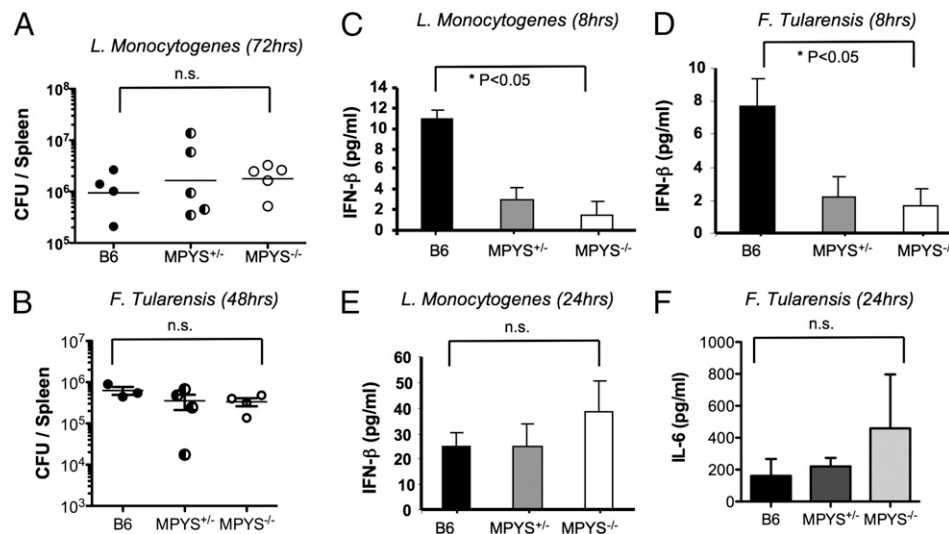
WT and IFNAR1^{-/-} mice are similar, but they diverge by 72 hpi with *L. monocytogenes*. We thus evaluated *L. monocytogenes* burdens at this later time (Fig. 8) and found that MPYS^{-/-} mice also harbored similar bacterial loads in the spleens at this time. Together, these data demonstrate that alternate, 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.

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 down-regulate 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

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 dilution 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. Experiments were performed twice.



MPYS^{-/-} mice reveal there are additional mechanisms for 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 infectious and other diseases.

Disclosures

The authors have no financial conflicts of interest.

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