miR-15a/16 Regulates Macrophage Phagocytosis after Bacterial Infection

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miR-15a/16 Regulates Macrophage Phagocytosis after Bacterial Infection

Hyung-Geun Moon,*, Jincheng Yang,*, Yijie Zheng,* and Yang Jin*

Bacterial infection and its associated sepsis are devastating clinical entities that lead to high mortality and morbidity in critically ill patients. Phagocytosis, along with other innate immune responses, exerts crucial impacts on the outcomes of these patients. MicroRNAs (miRNAs) are a novel class of regulatory noncoding RNAs that target specific mRNAs for modulation of translation and expression of a targeted protein. The roles of miRNAs in host defense against bacterial sepsis remain unclear. We found that bacterial infections and/or bacterial-derived LPS enhanced the level of miR-15a/16 in bone marrow–derived macrophages (BMDMs). Deletion of miR-15a/16 (miR-15a/16−/−) in myeloid cells significantly decreased the bacterial infection–associated mortality in sepsis mouse models. Moreover, miR-15a/16 deficiency (miR-15a/16−/−) resulted in augmented phagocytosis and generation of mitochondrial reactive oxygen species in BMDMs. Supportively, overexpression of miR-15a/16 using miRNA mimics led to decreased phagocytosis and decreased generation of mitochondrial reactive oxygen species. Mechanistically, deletion of miR-15a/16 upregulated the expression of TLR4 via targeting the principle transcriptional regulator PU.1 locating on the promoter region of TLR4, and further modulated the downstream signaling molecules of TLR4, including Rho GTPase Cdc 42 and TRAF6. In addition, deficiency of miR-15a/16 also facilitated TLR4-mediated proinflammatory cytokine/chemokine release from BMDMs at the initial phase of infections. Taken together, miR-15a/16 altered phagocytosis and bacterial clearance by targeting, at least partially, on the TLR4-associated pathways, subsequently affecting the survival of septic mice.

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Bacterial infection is a common cause of sepsis and sepsis-associated organ failure, causing high morbidity and mortality (1). The pathogenesis of this devastating clinical entity remains unclear, and options for treatment are limited. The innate immune system constitutes the first line of host defense against bacterial infection (2). One of the most important initial innate immune responses is the phagocytosis of pathogens by residing macrophages (2). Patients with defects in phagocytic function typically experience early dissemination of infection, leading to severe sepsis and increased mortality (3). In addition, a reduced phagocytic activity during the first 24 h after admission has been recognized as a negative predictor for survival in septic patients (4).

During the process of phagocytosis, the phagocyte-bound pathogen is surrounded by a phagocyte membrane and then encapsulated in a membrane-bound vesicle named phagosome (5). Next, the phagosome is generated via the fusion of phagosome and lysosomes to destroy the targeted pathogen (5). In addition, the lysis of pathogenic microorganisms is facilitated by a variety of other toxic products including reactive oxygen species (ROS), such as the NO, hydrogen peroxide, and superoxide anion (3, 5).

These bactericidal agents produced and released by macrophages and neutrophils are often harmful to the normal host tissue. Furthermore, the interaction between pathogens and macrophages activates macrophages and prompts them to release proinflammatory cytokines and chemokines that result in neutrophil infiltration and a state of inflammation in the tissue postinfection (6). Although the prompt initiation of phagocytic activity in response to invading pathogens is considered the central element of host defense against sepsis, the mechanisms underlying phagocytosis remain inadequately investigated. Evidently, the process of phagocytosis requires a tight regulation, given its dual roles, that is, bactericidal functions and potential tissue-damaging effects.

The TLRs are a class of innate immune receptors that are essential in the recognition of microbial pathogens by host cells and the initiation of phagocytosis (7). TLR4 has been shown to mediate phagocytosis and translocation of Gram-negative bacteria in vivo using a bacterial peritonitis model (8). The MyD88, an essential component of the TLR signaling is required for TLR-mediated phagocytosis (9). Mechanistically, IL-1R–associated kinase (IRAK) has been implicated in the signal transduction of TLR/IL-1R family (10). IRAK-1 and IRAK-4, along with their two inactive counterparts IRAK-2 and IRAK-M, play important roles in mediating NF-κB and MAPK signaling. Furthermore, IRAK-1 regulates TNFR superfamily-induced signaling pathways as well (10). For example, after TLR4 activation, the TLR4, MyD88, IRAK-4, and IRAK-1 form a complex, later phosphorylate, subsequently interact/activate TRAF6, and ultimately activate the NF-κB and JNK/p38 pathways (10). The negative regulators of TLR/IL-1R–mediated signaling include IRAK-2 and IRAK-M. IRAK-M inhibits IRAK-1 dissociation from the receptor complex and blocks LPS-induced IRAK-1–TRAF6 interaction and NF-κB activation (10). Also TLRs, along with the MyD88, IRAKs, and p38, facilitate the upregulation of scavenger receptors, leading to a robust increase in macrophage/monocyte phagocytosis of both Gram-negative and -positive bacteria (11, 12). However, despite

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Abbreviations used in this article: BMDM, bone marrow–derived macrophage; CLL, chronic lymphocytic lymphoma; CLP, cecal ligation and puncture; ESCIT, evolutionarily conserved signaling intermediate in the Toll pathways; HPRT, hypoxanthine-guanine phosphoribosyltransferase; IRAK, IL-1R–associated kinase; LB, lysogenic broth; miRNA, microRNA; ROS, reactive oxygen species; WT, wild type.

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its important roles in mediating phagocytosis, TLR signaling components must be tightly controlled to avoid excessive inflammation that can potentially lead to tissue injury.

In recent years, miRNAs (miRNAs) have emerged as important regulators for many cellular pathways, including the TLR signaling. miRNAs are single-stranded noncoding RNA molecules (containing roughly 22 nucleotides) that function in transcriptional and posttranscriptional gene regulation, by forming imperfect bp (13). miRNAs have emerged as important controllers of many cellular events, including TLR signaling (14). miRNAs have also been shown as an important link between the innate and adaptive immune systems, potentially playing a role in the pathogenesis of inflammatory diseases (15–19). The essential functions of miRNAs in many human disease processes have triggered robust interests and, as a result, the first miRNA mimic, miR-122 mimic, has entered into the hepatitis C virus phase 2 clinical trials (20). However, so far, the potential roles of miRNAs involved in bacterial infection–associated sepsis remain incompletely understood, despite scattered reports showing that the levels of certain miRNAs are increased in septic patients (21).

In this study, we sought to define whether miR-15a/16 could regulate phagocytosis by macrophages, in the settings of bacterial infection–associated sepsis. miR-15a and miR-16 both locate on the same chromosomal 13q14.3 region and, therefore, were collectively deleted during the generation of knockouts (22). We adopted a couple of well-established in vivo models, including the cecal ligation and puncture (CLP) model and LPS-induced sepsis model in mice. We chose macrophages as our cellular models because of their importance in pathogen clearance, neutrophil recruitment, and inflammatory signal process, as well as their convenience for in vitro studies. We further explored the underlying mechanisms by which miR-15a/16 regulates macrophage-mediated phagocytosis, and its interactions with TLR4 signaling. To the best of our knowledge, this is the first study to directly address the potential roles of miRNAs in bacterial sepsis. Our reports potentially provide a novel target for the development of the therapeutic and diagnostic strategies for polymicrobial sepsis.

Materials and Methods

Animals
Myeloid-specific Cre (004781) and miR-15a/16-null mice (miR-15a/16−/−) were purchased from The Jackson Laboratory (Bar Harbor, ME) and cross-bred to generate the myeloid-specific miR-15a/16−/− Cre mice. TLR4−/− mice were also obtained from The Jackson Laboratory. All animals were housed according to the guidelines of the American Association for Laboratory Animal Care, and the all protocols were approved by the Animal Research Committee of Brigham and Women’s Hospital.

Sepsis mouse models
We have used the following mouse sepsis models in this study: 1) CLP model described as previous (23); in brief, the middle of cecum was ligated and was punctured with two holes using 21-gauge needle and later resuscitated with 1 ml saline; 2) Live bacteria-induced sepsis model as described in detail previously (24): Escherichia coli–DH5α (108 cfu) was dissolved in 200 μl PBS per mouse and later injected i.p. 3) LPS-induced sepsis model, as described previously (24); LPS (25 mg/kg) was injected i.p. For survival, mice were checked every 24 h and samples were collected after 24 h after surgery or injection.

Isolation and differentiation of bone marrow–derived macrophage
L929 cells were purchased from American Type Culture Collection (Manassas, VA). L929 cells were cultured in DMEM media with 10% FBS and 1% penicillin/streptomycin at 37°C in a 5% CO2 incubator. Cell culture media were collected and filtered using 0.22-μm filters and kept at −20°C. Mouse bone marrow was isolated as described previously (25) and was cultured with 30% L929 media in DMEM complete media for 7 d.

Phagocytosis and mitochondrial ROS
E. coli–FITC (20 μg/ml; Invitrogen, Grand Island, NY) was added to the culture of bone marrow–derived macrophage (BMDM). After 6 h, cells were washed with PBS twice and incubated with mitoSOX (2.5 μM; Invitrogen, Grand Island, NY). After 10 min of 37°C incubation, cells were collected and fluorescence was measured using FLA–9600 (BD Bioscience, San Jose, CA). Analysis was performed using FlowJo software (BD Bioscience, San Jose, CA).

In vitro bacterial clearance
E. coli–DH5α (107 cfu; New England Biolabs, Ipswich, MA) was added to BMDM (1 × 106 cells) for 6 and 24 h, and 10 μl supernatant was taken from the cell culture media and was spread on lysogeny broth (LB) plates. Colony count was performed after 16 h incubation in a 37°C incubator.

Primed macrophage transfer into peritoneal cavity
BMDM was primed with BSA or E. coli particle (20 μg/ml) for 4 h; then cells were washed with cold PBS twice. Cells were harvested for transfer into wild type (WT) mice i.p. Recipient peritoneal fluids were collected after 24 h, and cytokines were measured by ELISA.

RNA isolation and real-time PCR
Total and miRNA were isolated using the miRNeasyMini Kit (Qagen, Valencia, CA) following the manufacturer’s instructions. RNAs were converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Grand Island, NY). PCR amplifications were performed by CYBR mixture (Invitrogen, Grand Island, NY) with the following primers with 60°C annealing temperature: TLR4 primers (forward: 5′-agptggtggaacagcaagaca-3′, reverse: 5′-cttacacagcttcatc-3′), CCL8 primers (forward: 5′-ttctacagctttggctgc-3′, reverse: 5′-aaggctatgttcagtttagtgc-3′), CCL20 (forward: 5′-gcggctctgatatacag-3′, reverse: 5′-acgccagcctgatggac-3′), CCL22 (forward: 5′-ctgctcagcatctgaggttac-3′, reverse: 5′-gaagggctgatcagcgtac-3′), CXCL10 (forward: 5′-ccccatgggagctttgct-3′, reverse: 5′-ttgtgatgctgctca-3′), miR-15a and miR-16 TaqMan assays were purchased from Applied Biosystems/Invitrogen (Grand Island, NY). Gene expression was normalized by hypoxanthine-guanine phosphoribosyltransferase (HPRT) or 18S, as previously described (26).

miRNA overexpression and inhibitor transfection
miR-15a-1a and -16 overexpression mimics were purchased from Ambion (Grand Island, NY), and inhibitors were purchased from Sigma (St. Louis, MO). Transfection of mimics and inhibitors was followed by manufacturer’s instruction; jetPEI–Macrophage DNA transfection reagent (Polyplus, Illkirch, France) was used for transfection reagent.

ELISA
Mouse IL-1β, IL-6, IL-21, and TNF-α were purchased from R&D systems (Minneapolis, MN) and were used according to manufacturer’s instructions.

Western blot
Cells were harvested after twice washed with PBS and later suspended in radioimmunoprecipitation assay buffer with protease inhibitors (Roche, Indianapolis, IN) following a previous described protocol (26). TLR4 Ab was purchased from Abcam (Cambridge, MA), and IRAK1, IRAK2, M, RelA, RelA/p65, Rho GTPase Cdc42, TRAF6, PU.1 Ab were purchased from Cell Signaling Technology (Danvers, MA), and β-actin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical analysis (ANOVA)
The means of fold change in all figures were compared using two-way ANOVA to test the differences among independent samples. With p < 0.05, the difference was considered statistically significant. Error bars are used to indicate the SD.

Results
Level of miR-15a/16 increased robustly in BMDMs after bacterial infection and deletion of miR-15a/16 improved the survival rate in sepsis mouse models
Initially, we observed that myeloid-specific Cre-miR-15a/16−/− mice showed improved survival in all three different mouse models
used for sepsis. After demonstrating the successful deletion of miR-15a/16 (Fig. 1A), we observed a significantly higher survival rate and length in Cre-miR-15a/16−/− mice after CLP (Fig. 1B), live E. coli (Fig. 1C), and LPS i.p. injection (Fig. 1D). These results raised a question on how the bacterial infection affects the level of miR-15a and/or miR-16 in WT mice. Notably, miR-15a and miR-16 share the same chromosomal region and cannot be separated during the generation of knockout mice (22). We first determined the level of miR-15a and miR-16 in two vital organs using real-time PCR. Lung and spleen both play crucial roles in sepsis-associated multiple organ failure and in host-defense mechanisms. As shown in Fig. 2A–C, polymicrobial infection induced by either CLP, E. coli, or Gram-negative bacteria-derived LPS robustly upregulated the level of miR-15a in lungs; miR-16 expression was increased in lung tissue after live E. coli infection (Fig. 2B). Interestingly, in the spleen, neither miR-15a nor miR-16 showed significant increase after polymicrobial, E. coli, or LPS infection (Fig. 2D–F). Macrophages are the important first-line innate immune cells involved in sepsis (4). Next, we evaluated the level of miR-15a and miR-16 in BMDMs. There was a significant increase of miR-15a/16 level peaked around 4 h by LPS stimulation as a result of an early response of host defense (Fig. 3A). Furthermore, LPS augmented miR-15a/16 in BMDMs in a dose-dependent manner (Fig. 3B). In addition, this LPS-induced miR-15a/16 expression seemed to be NF-κB and JNK pathway dependent but p38/PI3K independent (Fig. 3C). These findings indicated a critical function of miR-15a/16 involved in the initial innate immunity and host defense after bacterial infection.

Deletion of miR-15a/16 facilitated the phagocytic and bactericidal functions of BMDMs

Next, we focused on the function of miR-15a/16 in BMDMs after bacterial infection. One of the most important innate immune responses is to clear the invading bacteria via phagocytosis (5). Thus, we evaluated the potential effects of miR-15a/16 on the capability of BMDM-mediated phagocytosis using live E. coli as previously described (24). We first isolated BMDMs from WT and Cre-miR-15a/16−/− mice. After the designated time of E. coli infection (6 or 24 h postinfection), the colony counts were evaluated in the supernatant of WT or miR-15a/16−/− BMDMs, as detailed in Materials and Methods. Fewer colonies of E. coli were found in the miR-15a/16−/− BMDMs, starting at 6 h postinfection, and were dramatically obvious after 24 h postinfection (Fig. 3D). In addition, consistent results were obtained using the miR-15a or miR-16 inhibitor-transfected BMDMs (Fig. 3E). To directly evaluate the effects of miR-15a/16 on BMDM-mediated phagocytosis, we applied the E. coli–FITC particles into either WT or miR-15a/16−/− BMDMs and assayed the results using flow cytometer. As shown in Fig. 3F, significantly more FITC-labeled E. coli particles were found in miR-15a/16−/− BMDMs (Fig. 3F). Mechanistically, deletion of miR-15a/16 augmented the Rho GTPase Cdc42 (Fig. 3G), suggesting a potential role in actin polymerization. Mitochondrial ROS plays vital roles in mediating the bactericidal effects after bacterial infections (27). We found that the generation of mitochondrial ROS was significantly increased in the miR-15a/16−/− BMDMs, compared with WTs (Fig. 3H). After immediate exposure to LPS, the level of TRAF6, a key regulator of ROS generation, was also elevated in miR-15a/16−/− BMDMs (Fig. 3I).

Overexpression of miR-15a/16 suppressed the phagocytic and bactericidal functions of BMDMs

To further confirm the observations found earlier, we overexpressed miR-15a/16 in BMDMs using miR-15a/16 mimics. As shown in Fig. 4A, transfection of miR-15a/16 mimics into the BMDMs resulted in a rapid but transient increase on miR-15a/16 level in these cells. Colony counts of E. coli were significantly increased

![FIGURE 1](http://www.jimmunol.org/) Deletion of miR-15a/16 conferred protective effects on sepsis using mouse models. (A) Basal level of miR-15a/16 expression in BMDMs isolated from WT and miR-15a/16−/− mice. miR-15a/16 was normalized using HPRT. (B–D) Survival of WT mice or mononuclear cell–specific Cre-miR-15a/16−/− mice after CLP (B) E. coli (10⁸ cfu/mouse) injection i.p. (C) and LPS (25 mg/kg) injection i.p. (D).
in cells overexpressing miR-15a/16 (Fig. 4B). Again, directly visualized, FITC-labeled E. coli particles were less in the cells overexpressing miR-15a/16 compared with WT cells (Fig. 4C). Similarly, the generation of mitochondrial ROS decreased in miR-15a/16 overexpressing BMDMs compared with WTs (Fig. 4D). Taken altogether, miR-15a/16 is critical for altering bacterial clearance in BMDMs.

**MiR-15a/16 modulated the phagocytic and bactericidal functions of the BMDMs via regulating the TLR4-associated signaling pathways**

We next explored the underlying mechanisms by which miR-15a/16 modulated BMDM-associated phagocytosis and bactericidal effects. Initially, we evaluated the expression of TLR4 in the absence of miR-15a/16 in BMDMs. Deletion of miR-15a/16 significantly increased the TLR4 expression at the basal level, and this expression became much more robustly elevated after LPS treatment (Fig. 5A). We next confirmed this observation at the protein level using WB analysis (Fig. 5B). TLR4 protein level and its downstream pathway components, including the IRAK-1/2 and RelA/p65 (p468), all increased in miR-15a/16 overexpressing BMDMs compared with WTs (Fig. 5D). In contrast, the IRAK-M, which is the negative regulator of IRAK-1/2, significantly decreased in the miR-15a/16−/− cells (Fig. 5B).

To further determine whether TLR4 is the target of miR-15a/16 in macrophages, we evaluated whether miR-15a/16 alters the level of transcription regulators located on the promoter region of TLR4. PU.1 has been a well-known principle transcriptional regulator on the TLR4 promoters (28). Interestingly, at the basal level, the deletion of miR-15a/16 markedly stimulated the expression of PU.1 (Fig. 5C), although this effect subsided after LPS stimulation (Fig. 5C). We also confirmed the observation found earlier at the protein level using the WB analysis. Deletion of miR-15a/16 increased the PU.1 protein expression (Fig. 5D).

We next confirmed this result using the gain-of-function approach. We overexpressed miR-15a/16 using the miR-15a or miR-16 mimics. Overexpression of miR-15a/16 significantly decreased both expression of TLR4 (Fig. 6A) and of IRAK-1 (Fig. 6B), but augmented IRAK-M (Fig. 6B). As expected, PU.1 transcription (Fig. 6C) and protein level (Fig. 6D) were markedly decreased after overexpressing miR-15a/16. These results indicated miR-15a/16 suppresses TLR4 expression by regulating PU.1 transcription after LPS stimulation or bacterial infection.

**MiR-15a/16 modulated the BMDM-derived cytokines and chemokines immediately after bacterial infections**

Activation of TLR4 not only participates in phagocytosis, but also leads to downstream release of inflammatory modulators, including a variety of cytokines and/or chemokines (29). We further evaluated the role of miR-15a/16 on proinflammatory cytokine releases after bacterial infection, given that miR-15a/16 modulated TLR4 expression as illustrated earlier. After bacterial infections, IL-1β, IL-6, and IL-21 are all critical cytokines that promote innate immunity and regulate inflammatory response to fulfill the bactericidal effects (30). We found that deletion of miR-15a/16 significantly increased the level of IL-1β, IL-6, and IL-21, but not TNF-α (Fig. 7A). In addition, mRNA expression levels of CCL8, CCL20, CCL22, and CXCL10 were robustly elevated in the miR-15a/16−/− cells compared with WT cells (Fig. 7B). To test whether the deletion of miR-15a/16 in BMDMs truly has effects on the secretion of cytokines in vivo, we first isolated the BMDMs from WT and miR-15a/16−/− mice and primed them using E. coli or BSA (control) for 4 h. We next transferred these primed BMDMs into the WT mice peritoneally as described in Materials and Methods. Peritoneal fluids were collected after 24 h, and cytokines were measured by ELISA. Consistent with our earlier in vitro data, we found that deletion of miR-15a/16 in BMDMs augmented the secretion of IL-1β, IL-6, and IL-21 (Fig. 7C). Furthermore, we transfected the miR-15a/16 inhibitors into BMDMs obtained from both WT and TLR-4−/− mice. After exposure to LPS, WT cells transfected with miR-15a/16 inhibitors released a higher amount of IL-1β and IL-6, whereas the deletion of TLR4 (TLR4−/−) partially reversed the effects of miR-15a/16 inhibitors (Fig. 7D). In contrast, overexpression of miR-15a/16 using miR-15a/16 mimics suppressed all the earlier cytokines including IL-1β, IL-6, and IL-21 (Fig. 8A), as well as the expression of chemokines in BMDMs (Fig. 8B). Taken together, miR-15a/16 modulated production of cytokines and expression of chemokines postinfection or LPS stimulation in BMDMs.
Our studies demonstrated that miR-15a/16 carries an essential role in mediating the bacterial sepsis, particularly in the immediate/early stage after bacterial invasion. Deletion of miR-15a/16 in the myeloid cells resulted in an increased phagocytic and bactericidal capability of BMDMs, via upregulation of TLR4 and its transcriptional regulator PU.1 both at basal level and after bacterial infection (Fig. 9). Furthermore, miR-15a/16 regulated the level of Rho GTPase Cdc42, a key participant in phagocytosis that spurs actin polymerization and subsequently enables the plasma membrane to encircle its target (31). GTPase Cdc42 has previously been reported to participate in the intermediate pathway of TLR4-JAK2 signaling on macrophage-mediated phagocytosis (32). The TLR4-mediated proinflammatory cytokines and chemokines were also augmented in miR-15a/16<sup>−/−</sup> cells in the early phase of infection, via regulating TLR4-associated TRAF6 (27). As a result, deletion of miR-15a/16 improved the survival rate of septic mice, compared with WT mice using sepsis mouse models. Mechanistically, engagement of TLR4 results in the recruitment of mitochondria to macrophage phagosomes and augments mitochondrial ROS production (27). This response requires translocation of the TRAF6 to mitochondria and the interactions between TRAF6 and ECSIT (evolutionarily conserved signaling intermediate in the Toll pathways) in the mitochondria. ECSIT plays a crucial role in mitochondrial respiratory chain assembly. Interactions between TRAF6 and ECSIT lead to ECSIT ubiquitination and enrichment at the mitochondrial periphery, ultimately resulting in an increased mitochondrial and cellular ROS generation (27). Our results indicated that deletion of miR-15a/16 (miR-15a/16<sup>−/−</sup>) resulted in the upregulation of TRAF6 at the acute phase of LPS stimulation, therefore potentially facilitating ROS generation (Fig. 9). Further experiments to explore the earlier potential mechanisms are needed in future studies.

**FIGURE 3.** Deletion of miR-15a/16 promoted BMDM-mediated phagocytosis. (A) Expression of miR-15a and miR-16 after LPS (500 ng/ml) in mouse BMDM. (B) Dose-dependent responses of miR-15a/16 expression in mouse BMDMs after LPS treatment (C) BMDMs were pretreated with designated pathway inhibitors, followed by LPS. Expression of miR-15a or miR-16 was determined. (D) E. coli-DH5a (10<sup>7</sup> cfu) was added to the WT and miR-15a/16<sup>−/−</sup> BMDMs (1 × 10<sup>8</sup>). After mixing, the cell culture supernatant (10 μl) was taken at 6 and 24 h and subcultured in LB plates. Colony counts were determined. (E) Inhibitors of miR-15a or miR-16 were transfected into mouse BMDMs. After 24 h, E. coli-DH5a (10<sup>7</sup> cfu) was added to the BMDMs. A 10-μl mixture was taken at 6 h, and colony counts were performed on LB plate. (F) Phagocytosis of FITC-labeled E. coli particle (20 μg/ml) in WT and miR-15a/16<sup>−/−</sup> BMDMs. FITC was assayed using flow cytometer. (G) Expression of Rho GTPase Cdc42 after LPS (time course) in WT and miR-15a/16<sup>−/−</sup> BMDMs. (H) Mitochondrial ROS (MitoSOX) was measured 6 h after E. coli-FITC stimulation. (I) Expression of TRAF6 after LPS (time course) in WT and miR-15a/16<sup>−/−</sup> BMDMs. All figures represent three independent experiments with identical results. *<i>p</i> < 0.05.
Although we showed that TLR4-related pathways were targeted by miR-15a/16 to regulate macrophage phagocytosis and cytokine release, TLR4 is clearly not the only target of miR-15a/16. Given that TLR4 plays essential roles in sepsis/inflammation/innate immunity, we focused our current reports on miR-15a/16–regulated TLR4 pathways. Further exploration on other potential targets of miR-15a/16 and more detailed mechanistic analyses are required in future studies.

**FIGURE 4.** Overexpression of miR-15a/16 inhibited BMDM-mediated phagocytosis. Mimics of miR-15a and miR-16 (50 nM each) were cotransfected into the BMDMs. (A) Expression of miR-15a and miR-16 in the BMDMs after mimics transfection. (B) *E. coli*–DH5a (10⁷ cfu) was added to the WT and miR-15a/16–overexpressing BMDMs (1 × 10⁶), and colony counts was determined after 6 h. (C) Phagocytosis of FITC-labeled *E. coli* particle (20 μg/ml) in WT and miR-15a/16–overexpressing BMDMs. FITC was assayed using flow cytometer. (D) Mitochondrial ROS (MitoSOX) was measured 6 h after *E. coli*–FITC stimulation in WT and miR-15a/16 overexpressing BMDMs. All figures represent two independent experiments with identical results. *p < 0.05.

**FIGURE 5.** Deletion of miR-15a/16 upregulated the TLR4 signaling in BMDMs. (A) TLR4 mRNA level after 4-h LPS (500 ng/ml) stimulation in WT and miR-15a/16−/− BMDMs. TLR4 mRNA level was normalized by HPRT. (B) Protein level of TLR4 signaling components after LPS (500 ng/ml) in miR-15a/16−/− BMDMs detected using the Western blot analysis. WT and miR-15a/16−/− BMDMs were treated with LPS (500 ng/ml) for the designated time points (left panel). IRAK-1 expression was normalized by β-actin (right panel). (C) PU.1 mRNA level after LPS (500 ng/ml) for 2 and 4 h, respectively. PU.1 expression level was normalized using HPRT. (D) PU.1 protein level in WT and miR-15a/16−/− BMDMs after LPS stimulation (left panel), analyzed using Western blot analysis. PU.1 expression was normalized by β-actin (right panel). All figures represent two independent experiments with identical results. *p < 0.05.
miRNAs are a novel class of regulatory noncoding RNAs, which function primarily by targeting specific miRNAs for modulation of translation and subsequently altering the expression of the targeted protein (33). Both miR-15a and miR-16 are located on the same chromosomal 13q14.3 region and share common functions in cancer biology (34). Previous reports have shown that miR-15a/16 primarily act as tumor suppressors (35). Expression of these miRNAs inhibits cell proliferation and promotes apoptosis of chronic lymphocytic lymphoma, pituitary adenomas, and prostate carcinoma cells, via targeting multiple oncogenes, including BCL2, MCL1, CCND1, and WNT3A (35). Modulation of phagocytosis via TLR4 pathway can potentially be a novel function identified for miR-15a/16, suggesting that these miRNAs not only promote apoptosis, but also regulate inflammation and innate immune responses. In contrast, other miRNAs (e.g., miR-27b, miR-101, miR-147, miR-155) also participate in LPS-associated signaling in macrophages (36–40). Several other miRNAs including miR150, miR-181b, and miR-223 have been linked to sepsis (41–43). However, our report, to the best of our knowledge, is the first one that directly demonstrates that miRNA modulates the sepsis-associated innate immunity, that is, phagocytosis and bacteria clearance, using CLP mouse models. Instead of observational data, we used the cell-specific Cre-miR-15a/16 knockout mice and performed both in vitro and in vivo assays to support our findings. The cross talks among miR-15a/16 and other miRNAs in the development of sepsis will be investigated in the near future.

Our study focuses on the immediate/early responses of the host in the presence of invading bacteria. We did not address whether the deletion of miR-15a/16 prolongs the survival of macrophages or neutrophils, nor explain how the half-lives of these phagocytic cells change in the presence or absence of miR-15a/16. Phagocytosis and killing of invading pathogens are two vital tasks that monocytes/macrophages and neutrophil have to fulfill in the presence of bacterial infection (4). Previous reports have demonstrated that phagocytosis has differential effects on the apoptosis of macrophages or neutrophils (44). Macrophages become apoptotic after phagocytosis of extracellular bacteria, whereas the phagocytosis of the same bacteria by neutrophils slows down the spontaneously occurring apoptosis of these cells (45). Elevated apoptosis in macrophages is restricted mainly to the cells that contain bacteria (44). In fact, apoptosis of macrophages sometimes acts as a prerequisite for killing off the invading bacteria (46). Furthermore, the phagocytosis and subsequent apoptosis of macrophages correlate with the decreased capacity for Ag process and presentation by these macrophages (47). Therefore, potentially, the deletion of miR-15a/16 augmented phagocytosis, increased the subsequent apoptosis of macrophages, and ultimately decreased Ag presentations. In contrast, neutrophils are short-lived cells that function mainly to eliminate pathogens, and their prolonged survival in the setting of bacterial infection benefits the host (48). Macrophage apoptosis or inhibition of neutrophil apoptosis is generally considered to diminish the innate immune response during infection (49). Given the differential responses on apoptosis between macrophages and neutrophils after bacterial phagocytosis, we hypothesize that miR-15a/16 may also carry differential effects on cell survival and apoptosis between macrophages and neutrophils. Therefore, the proapoptotic effects of miR-15a/16 on chronic lymphocytic lymphoma cells cannot be readily extrapolated to either macrophages or neutrophils. However, we speculated that miR-15a/16 decreases neutrophil survival, but not macrophages.

The high mortality and morbidity of severe septic patients after bacterial infection have not only resulted from the failure of appropriate innate immunity mediated by myeloid originated cells, but also a significant contribution of solid organ failure, that is, the malfunctions involved in the vital organs, such as lung, liver, and kidney. Although not included in our studies, we found that almost all the vital organs and tissues express a significant amount of miR-15a/16. It is quite likely that miR-15a/16 expressed in these solid organs also play crucial roles related to cell death and tissue damage. To focus on the innate immunity mediated by inflammatory cells, we chose the myeloid-specific Cre-miR-15a/16−/− mice to perform in vivo studies (Fig. 1). Therefore, one drawback of our report is that the functions of other solid-organ/tissue-
derived miR-15a/16 were not investigated. However, given its known function as a tumor-suppressor gene, we hypothesize that the bacterial infection–induced miR-15a/16 upregulation can potentially cause detrimental cell death and organ damage. This will also be investigated in the subsequent studies in the near future.

Mir-15a/16 is endogenously expressed universally in many tissues/organs/cells. In the setting of devastating bacterial infections, the natural host responses would be to avoid inducing the factors that can be harmful for hosts. Interestingly, in BMDMs, significant and consistent induction of miR-15a/16 was observed. In addition, our data suggested that the elevated levels of miR-15a/16 were from host cells, but not the microbes (data not shown). The Gram-negative, bacteria-derived LPS induced miR-15a/16 expression in BMDMs promptly, as short as 4–6 h after the infection (Fig. 2). We have shown that elevated miR-15a/16 aggravated bacterial infection via decreased phagocytosis (Figs. 3 and 4). The next question that arose in this study is that if these miRNAs are harmful to the hosts after bacterial infections, how and why were these endogenously expressed miR-15a/16s upregulated?

We believe that the miR-15a/16 is induced by bacterial infection more specifically in BMDMs, but not universally in all other organs. In fact, our data supported this hypothesis (Fig. 2). We found that bacterial infections, in fact, differentially induced the miR-15a/16 in different tissue/cells. MiR-15a was highly upregulated in lung tissue, but no significant changes were found in the spleen. Next, although increased miR-15a/16 significantly decreased the phagocytosis of myeloids, they also blunted the release of a group of inflammatory cytokines/chemokines, including IL-1β, IL-6, and IL-21, which are essential in the activation of systemic immune responses and runaway inflammation (30). The short-lasting burst of miR-15a/16 may be crucial in priming myeloids and modulating the overall immune responses to avoid uncontrolled systemic inflammation. However, there is a balance between the beneficial and detrimental effects of miR-15a/16. If the burst of miR-15a/16 is too strong and long-lasting, apparently, decreased phagocytosis will result in uncontrolled propagation of bacteria and devastating outcomes. We have also started to investigate on how the burst of miR-15a/16 in BMDMs occurs.

![FIGURE 7. Cytokine and chemokine levels in WT or miR-15a/16^{-/-} BMDMs after bacterial infections. (A) WT and miR-15a/16^{-/-} BMDMs were treated with E. coli–FITC particles (20 μg/ml). After 6 h, cytokines were measured using ELISA. (B) WT and miR-15a/16^{-/-} BMDMs were treated with LPS (500 ng/ml). After 4 h, RNA was isolated. Chemokine gene expressions were determined using real-time PCR and normalized using HPRT. (C) WT and miR-15a/16^{-/-} BMDMs were primed with BSA-FITC or E. coli–FITC; after 4 h, cells were harvested and injected into WT mice intraperitoneally. Peritoneal fluids were collected after an additional 24 h, and the cytokines secreted into the peritoneal fluids were measured using ELISA. (D) WT and TLR4^{-/-} BMDMs were transfected with miR-15a/16 inhibitors, followed by stimulation with LPS (500 ng/ml, 6 h). IL-1β and IL-6 were measured using ELISA. All figures represent three independent experiments with identical results. *p < 0.05 compared with WT-LPS, **p < 0.05 compared with LPS-Ctrl.](http://www.jimmunol.org/)}
Although the upregulation of miR-15a/16 may be associated with NF-κB or JNK pathways (Fig. 3), the overall systemic effects of miR-15a/16 might be associated with the release of stored miR-15a/16, but not the de novo generation of these miRNAs postinfection. These detailed mechanisms are currently under investigation, and our current reports primarily focus on the functional roles of miR-15/16.

Besides the direct clearance of invading bacteria, phagocytosis is also crucial for clearing the dying neutrophils and solid tissue cells. If the process of phagocytosis is defected, these damaged neutrophils and structural cells will accumulate and generate robust amount of inflammatory factors, which subsequently trigger systemic inflammation (sepsis/systemic inflammatory response syndrome) and cause negative outcomes. Supportively, Xie et al. (21) had shown a positive correlation between circulating miR-15a/16 in serum and mortality of patients with sepsis.

In summary, miR-15a/16 derived from BMDMs played a crucial role in altering phagocytosis and inflammatory responses after bacterial infection. Targeting of these miRNAs, particularly after the very initial phase of infection, may provide novel therapeutic/diagnostic tools for both infectious sepsis and noninfectious systemic inflammatory response syndrome.

**FIGURE 8.** Cytokine and chemokine levels in WT or miR-15a/16-overexpressing BMDMs after bacterial infections. (A) WT and miR-15a/16-overexpressing BMDMs were treated with *E. coli*-FITC particles (20 μg/ml). After 6 h, cytokines were measured using ELISA. (B) WT and miR-15a/16-overexpressing BMDMs were treated with LPS (500 ng/ml). After 4 h, RNA was isolated. Chemokine gene expressions were determined using real-time PCR and were normalized by HPRT. All figures represent three independent experiments with identical results. *p < 0.05.

**FIGURE 9.** Proposed mechanisms involved in the functions of miR-15a/16 in macrophages. miR-15a/16 downregulated the expression of TLR4 via targeting PU.1 locating on the promoter region of TLR4 and modulated TLR4 downstream signaling molecules, including the Rho GTPase Cdc 42 and TRAF6. Subsequently, miR-15a/16 altered phagocytosis and bacterial clearance, as well as cytokine releases in macrophages, via targeting at least partially, on the TLR4-associated pathways.