Published in final edited form as:
J Immunol. 2014 November 1; 193(9): 4558–4567. doi:10.4049/jimmunol.1401372.

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

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Abstract

Bacterial infection and its associated sepsis are devastating clinical entities which 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 targeting 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 (ROS) in BMDMs. Supportively, over-expression of miR-15a/16 using miRNA mimics led to decreased phagocytosis and decreased generation of mitochondrial ROS. Mechanistically, deletion of miR-15a/16 up-regulated the expression of toll-like receptor 4 (TLR4) *via* targeting the principle transcriptional regulator PU.1 locating on the promoter region of TLR4, and further modulated TLR4's downstream signaling molecules, including Rho GTPase Cdc 42 and TRAF6. Additionally, deficiency of miR-15a/16 also facilitated TLR4-mediated pro-inflammatory 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.

Keywords

miR-15a/16; bacterial infection; macrophage; phagocytosis

Introduction

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

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Disclosures: The authors have no financial conflicts of interest.

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-bounded vesicle named phagosome (5). Next, the phagosome is generated *via* the fusion of phagosome and lysosomes to destroy the targeted pathogen (5). Additionally, the lysis of pathogenic microorganisms is facilitated by a variety of other toxic products including reactive oxygen species (ROS), such as the nitric oxide (NO), hydrogen peroxide (H_2O_2) and superoxide anion (O_2^-) (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 pro-inflammatory cytokines and chemokines that result in neutrophil infiltration and a state of inflammation in the tissue after infection (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, i.e., bactericidal functions and potential tissue damaging effects.

The toll-like receptors (TLRs) are a class of innate immune receptors which 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, interleukin-1 receptor-associated kinase (IRAK) has been implicated in the signal transduction of TLR/IL-1 receptor (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 nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling. Furthermore, IRAK-1 regulates tumor necrosis factor receptor (TNFR) super family-induced signaling pathways as well (10). For example, after TLR4 activation, the TLR4, MyD88, IRAK4 and IRAK1 form a complex, later phosphorylated, 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 up-regulation of scavenger receptors (SRs), leading to a robust increase in macrophage/monocyte phagocytosis of both gram negative and positive bacteria (11, 12). However, despite its important roles in mediating phagocytosis, TLR signaling components must be tightly controlled to avoid excessive inflammation which can potentially lead to tissue injury.

In recent years, MicroRNAs (miRNAs) have emerged as important regulator for many cellular pathways, including the TLR signaling. MiRNAs are single-stranded non-coding RNA molecules (containing roughly 22 nucleotides) that function in transcriptional and post-transcriptional gene regulation, by forming imperfect base pairs (13). MiRNAs have emerged as important controllers of many cellular events, including TLR signaling (14). MicroRNAs 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 elevated in septic patients (21).

In this current 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 together during the generation of knock-outs (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 due to its importance in pathogen clearance, neutrophil recruitment and inflammatory signal process, as well as its 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 directly addressing 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 Jackson Laboratory (Bar Harbor, Maine) and cross-bred to generate the myeloid specific miR-15a/16$^{-/-}$ Cre mice. TLR-4$^{-/-}$ mice were also obtained from 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 current study. 1) cecal ligation and puncture (CLP) model as described previously (23). Briefly, middle of cecum was ligated and was punctured with two holes using 21g needle and, later, resuscitated with 1 mL saline. 2) Live bacteria-induced sepsis model as described in detail previously (24): E.coli-DH5a (108 cfu) was dissolved in 200 μL of PBS per mouse and later injected intraperitoneally. 3) LPS induced sepsis model, as described previously (24). LPS (25 mg/Kg) were injected

intraperitoneally. For survival, mice were checked every 24h and samples were collected after 24h after surgery or injection.

Isolation and differentiation of bone marrow derived macrophage (BMDM)

L929 cells were purchased from ATCC (Manassas, VA). L929 cells were cultured in DMEM media with 10% FBS and 1% Penicillin/Streptomycin at 37°C in a 5% CO_2 incubator. Cell culture media was 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 days.

Phagocytosis & mitochondrial ROS

E.coli-FITC (20 μg/ml; Invitrogen, Grand Island, NY) was added to the culture of BMDM. After 6 hours, cells were washed with PBS twice and incubated with mitoSOX (2.5μM; Invitrogen, Grand Island, NY). After 10 min of 37°C incubation, cells were collected and fluorescence was measured using FACS Canto II (BD Bioscience, San Jose, CA). Analysis was performed using FlowJo software (BD Bioscience, San Jose, CA)

In vitro bacterial clearance

E.coli-DH5α (10^7 cfu; New England Biolabs, Ipswich, MA) was added to BMDM (1×10^6 cells) for 6 & 24 h and 10 μL supernatant was taken from the cell culture media and was spread on a LB plates. Colony count was performed after 16h 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 4h and, then, cells were washed with cold PBS twice. Cells were harvested for transfer into WT mice intraperitoneally. Recipient peritoneal fluids were collected after 24h and cytokines were measured by ELISA.

RNA isolation and Real-time PCR

Total and miRNA were isolated using the miRNeasy®Mini Kit (Qiagen, 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′-agtgggtcaaggaacagaagca-3′, reverse:5′-ctttaccagctcatttctcacc-3′), CCL8 primers (forward:5′-tctacgcagtgcttctttgcc-3′, reverse:5′-aaggggatcttcagctttagta-3′), CCL20 (forward:5′-gcctctcgtacatacagacgc-3′, reverse:5′- ccatggctgctttggatcagc-3′), CCL22 (forward:5′-ctctgccatcacgtttagtgaa-3′, reverse:5′-gacggttatcaaaacaacgcc-3′), CXCL10 (forward:5′-ccaagtgctgccgtcattttc-3′, reverse:5′- ggctcgcagggatgatttcaa-3′) and PU.1(forward:5′-gcatctggtgggtggacaa-3′, reverse:5′-tcttgccgtagttgcgcag-3′). miR-15a and miR-16 Taqman assays were purchased from Applied Biosystems/Invitrogen (Grand Island, NY). Gene expression was normalized by HPRT or 18S, as previously described (26).

MicroRNA overexpression & inhibitor transfection

MicoRNA15a 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, 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 RIPPA buffer with protease inhibitors (Roche, Indianapolis, IN) following a previous described protocol (26). TLR4 antibody was purchased from Abcam (Cambridge, MA) and IRAK1, 2, M, RelA, RelA(p468); Rho GTPase Cdc42; TRAF6; PU.1 antibody were purchased from Cell Signaling Technology (Danvers, MA); and β-actin from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical Analysis (Anova)

The means of fold change in all figures were compared using two-way analysis of variance to test the differences among independent samples. With $p<.05$, the difference was considered statistically significant. Error bars were used to indicate the standard deviation.

Results

The level of miR-15a/16 increased robustly in BMDMs after bacterial infection and the 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 intraperitoneal injection (Fig. 1D). These results raised a question on how the bacterial infection affects the level of miR-15a and/or miR-16 in wild type (WT) mice. Notably, miR-15a and miR-16 share the same chromosomal region and cannot be separated during the generation of knock-out 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 (MOF) and in host-defense mechanisms. As shown in figure 2A-C, polymicrobial infection induced by either CLP, *E.coli* or gram-negative bacteria-derived LPS robustly up-regulated 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). In fact, LPS down regulated the level of miR-15a/16 in spleen (Fig. 2F). Macrophages are the important fist-line innate immune cells involved in sepsis (4). Next, we evaluated the level of miR-15a and miR-16 in bone marrow-derived

macrophages (BMDMs). There was a significant increase of miR-15a/16 level peaked around 4h 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). Additionally, 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 (6h or 24h post infection), the colony counts were evaluated in the supernatant of WT or miR-15a/16$^{-/-}$ BMDMs, as detailed in the material and methods. Less colonies of *E.coli* were found in the miR-15a/16$^{-/-}$BMDMs, starting at 6h post-infection and were dramatically obvious after 24h post-infection (Fig. 3D). Additionally, 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 figure 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 reactive oxygen species (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 to 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).

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

To further confirm the observations found above, we over-expressed miR-15a/16 in BMDMs using miR-15a/16 mimics. As shown in figure 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 in cells over-expressing miR-15a/16 (Fig. 4B). Again, directly visualized FITC-labeled *E.coli* particles were less in the cells over-expressing miR-15a/16 compared to WT cells (Fig. 4C). Similarly, the generation of mitochondrial ROS decreased in miR-15a/16 over-expressing BMDMs compared to 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 down-stream pathway components, including the IRAK-1/2 and RelA/p65 (p468), all increased in miR-15a/16$^{-/-}$ cells (Fig. 5B). On the other hand, 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 above 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 over-expressed miR-15a/16 using the miR-15a or miR-16 mimics. Over-expression of miR-15a/16 significantly decreased both expression of TLR4 (Fig. 6A) and 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 over-expressing 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 pro-inflammatory cytokine releases after bacterial infection, given that miR-15a/16 modulated TLR4 expression as illustrated above. After bacterial infections, IL-1β, IL-6 and IL-21 are all critical cytokines that promotes innate immunity and regulates 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). Additionally, mRNA expression level of CCL8, CCL20, CCL22 and CXCL10 were robustly elevated in the miR-15a/16$^{-/-}$ cells compared to 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 4h. We next transferred these primed BMDMs into the WT mice peritoneally as described in methods. Peritoneal fluids were collected after 24h and cytokines were measured by ELISA. Consistent with our above *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, while the deletion of TLR4 (TLR4$^{-/-}$) partially reversed the effects of miR-15a/16 inhibitors (Fig 7D). On the other hand, over-expression of miR-15a/16 using miR-15a/16 mimics suppressed all the above 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 after infection or LPS stimulation in BMDMs.

Discussion

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* up-regulation TLR4 and its transcriptional regulator PU.1 at both basal level and after bacterial infection (Fig 9). Furthermore, miR-15a/16 regulated the level of Rho GTPase Cdc42, a key participant in phagocytosis which 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 pro-inflammatory cytokines and chemokines were also augmented in miR-15a/16$^{-/-}$ cells in the early phase of infection, *via* regulating TLR4-associated TRAF6 (27). These increased pro-inflammatory cytokines/chemokines potentially facilitate the recruitment of neutrophils and bacterial clearance. As a result, deletion of miR-15a/16 improved the survival rate of septic mice, compared to WT mice using sepsis mouse models. Mechanistically, engagement of TLR4 results in the recruitment of mitochondria to macrophage phagosomes and augments mROS production (27). This response requires translocation of the TRAF6 to mitochondria and the interactions between TRAF6 and the protein 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$^{-/-}$) resulted in the up-regulation of TRAF6 at the acute phase of LPS stimulation, therefore, potentially facilitated ROS generation (Fig 9). Further experiments to explore the above potential mechanisms are needed in future studies.

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.

MicroRNAs are a novel class of regulatory noncoding RNAs, which function primarily by targeting specific mRNAs 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 (CLL), 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. On the other hand, other miRNAs (eg, 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 which directly demonstrated that microRNA modulates the sepsis associated innate immunity, i.e., 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 current 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 which 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, while 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 antigen 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 antigen presentations. In contrast, neutrophils are short-lived cells that functions 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 pro-apoptotic effects of miR-15a/16 on CLL 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 are not only resulted from the failure of appropriate innate immunity mediated by myeloid

originated cells, but also a significant contribution of solid organ failure, i.e., 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 organ and tissue express 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 up-regulation 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 tissue/organ/cells. In the setting of devastating bacterial infections, the natural host responses would be to avoid inducing the factors which can be harmful for hosts. Interestingly, in BMDMs, significant and consistent induction of miR-15a/16 was observed. Additionally, our data suggested that the elevated level 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 (Fig. 3-4). Next question arisen here is that if these miRNAs are harmful to the hosts after bacterial infections, how and why these endogenously expressed miR-15a/16s were up-regulated? 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 up-regulated 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. Although the up-regulation 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 after infection. 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/SIRS) and

cause negative outcomes. Supportively, *Xie et al* had shown a positive correlation between circulating miR-15a/16 in serum and mortality of patients with sepsis (21).

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 a novel therapeutic/diagnostic tools for both infectious sepsis and non-infectious SIRS.

Acknowledgments

This work was supported by NIH R01 HL102076 (Y.J.)

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Abbreviations

BMDM	bone marrow-derived macrophage
miRNA	microRNA
TLRs	toll-like receptors
LPS	lipopolysaccharide
IRAK	interleukin-1 receptor-associated kinase

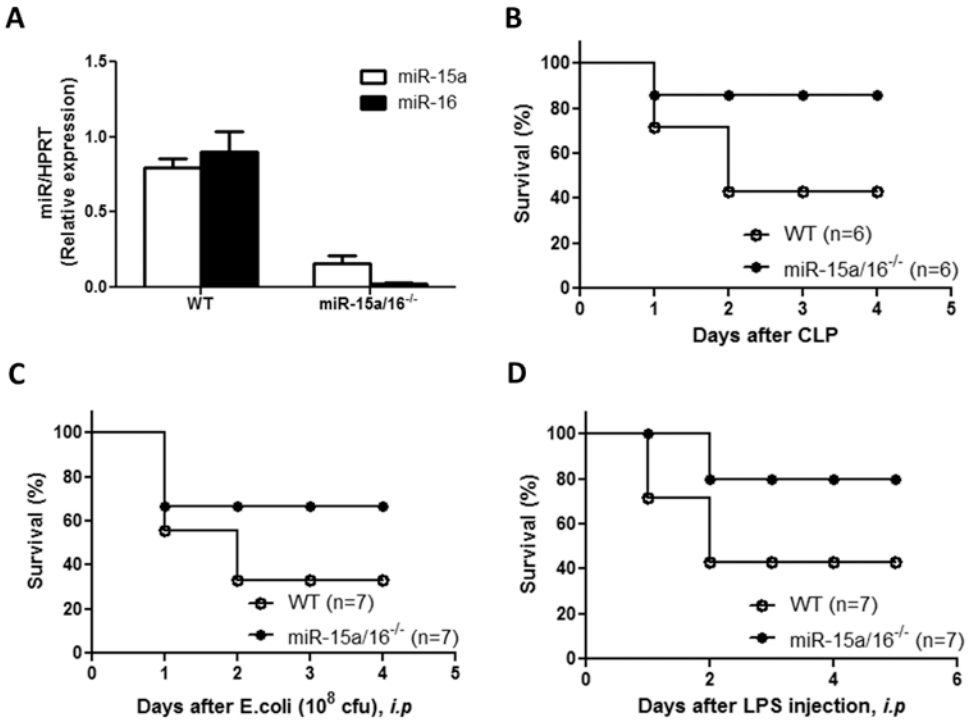

Figure 1. Deletion of miR-15a/16 conferred protective effects on sepsis using mouse models

(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* (10^8 cfu/mouse) injection intraperitoneally (C) and LPS (25 mg/kg) injection intraperitoneally (D)

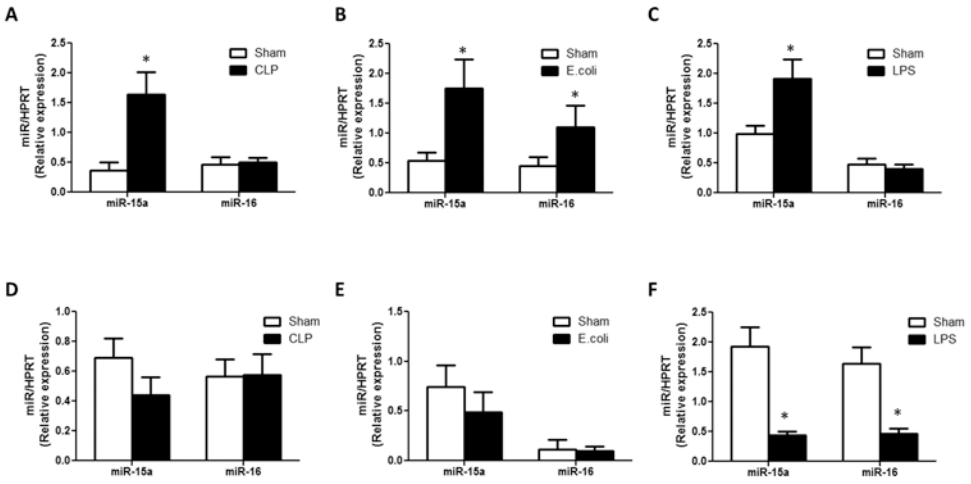

Figure 2. Expression of miR-15a and miR-16 in mouse tissue and cells after bacterial infection

(A)-(C): Expression of miR-15a and miR-16 in mouse lung tissue after CLP (A), *E.coli* injection *i.p.* (B) and LPS injection i.p. (C)

(D)-(F): Expression of miR-15a and miR-16 in mouse spleen tissue after CLP (D), *E.coli* injection *i.p.* (E), and LPS injection i.p. (F)

All figures represent two independent experiments with identical results. * $p<0.05$

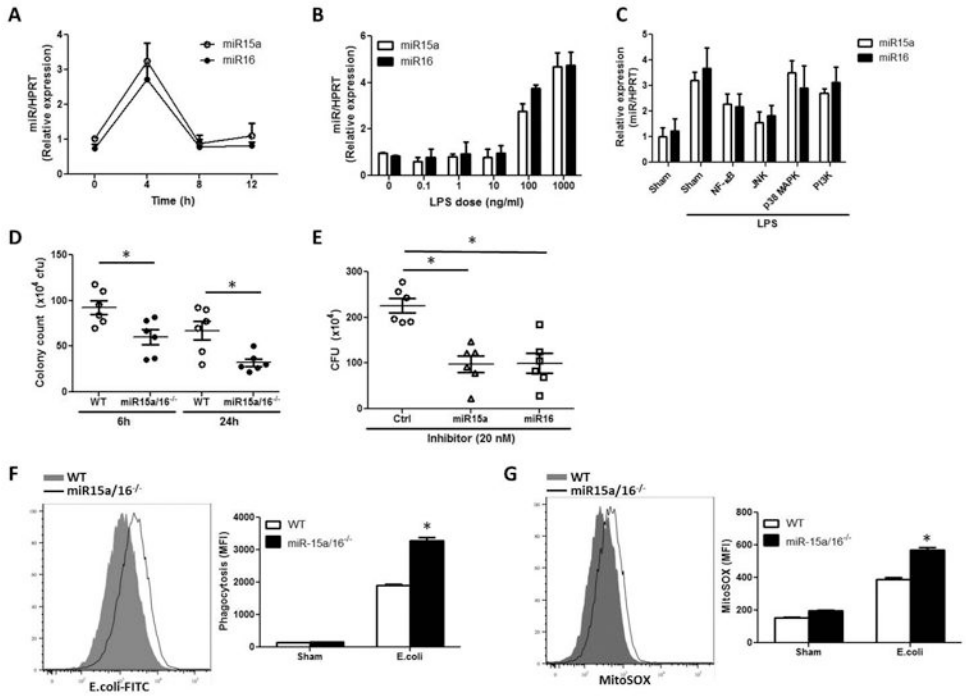

Figure 3. Deletion of miR-15a/16 promoted BMDM-mediated phagocytosis

(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 pre-treated with designated pathway inhibitors, followed by LPS. Expression of miR-15a or miR-16 were determined (D) *E.coli*-DH5a (10^7 cfu) was added to the WT and miR-15a/16$^{-/-}$ BMDM (1×10^6). After mixing, the cell culture supernatant (10 μl) was taken at 6h & 24h and sub-cultured in LB plates. Colony counts were determined.(E) Inhibitors of miR-15a or miR-16 were transfected into mouse BMDMs. After 24h, *E.coli*-DH5a (10^7 cfu) was added to the BMDMs. 10 μl mixture was taken at 6h 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$^{-/-}$ BMDMs. FITC was assayed using flow cytometer(G) Expression of Rho GTPase Cdc42 after LPS (time course) in WT and miR-15a/16$^{-/-}$ BMDMs (H) Mitochondrial ROS (MitoSOX) was measured 6h after *E.coli*-FITC stimulation (I) Expression of TRAF6 after LPS (time course) in WT and miR-15a/16$^{-/-}$ BMDMs
All figures represent 3 independent experiments with identical results. * $p<0.05$

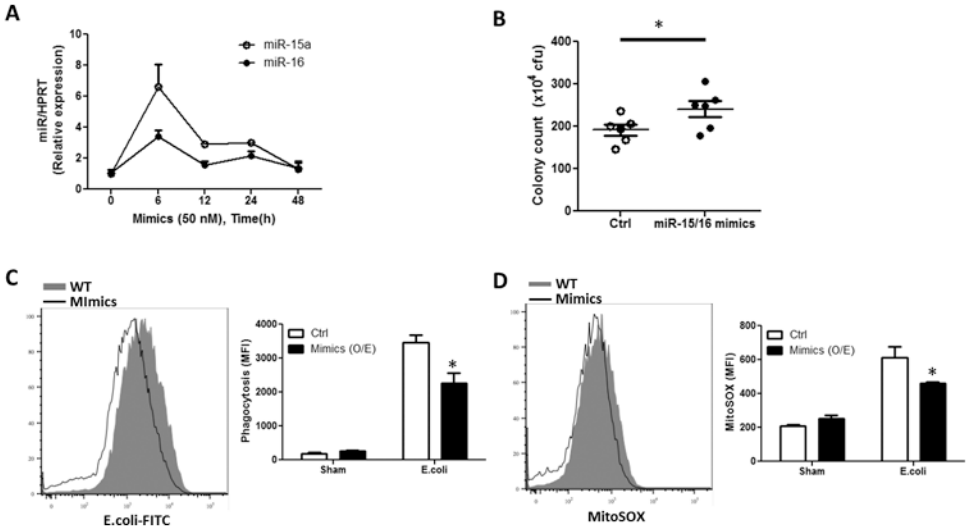

Figure 4. Overexpression of miR-15a/16 inhibited BMDM-mediated phagocytosis

Mimics of miR-15a and miR-16 (50 nM each) were co-transfected into the BMDMs.

(A) Expression of miR-15a and miR-16 in the BMDMs after mimics transfection.

(B) *E.coli*-DH5a (10^7 cfu) was added to the WT and miR-15a/16 over-expressing BMDMs (1×10^6) and colony counts was determined after 6h

(C). Phagocytosis of FITC-labeled *E.coli* particle (20 μg/ml) in WT and miR-15a/16 over-expressing BMDMs. FITC was assayed using flow cytometer.

(D) Mitochondrial ROS (MitoSOX) was measured 6h after *E.coli*-FITC stimulation in WT and miR-15a/16 over-expressing BMDMs.

All figures represent 2 independent experiments with identical results. * $p<0.05$

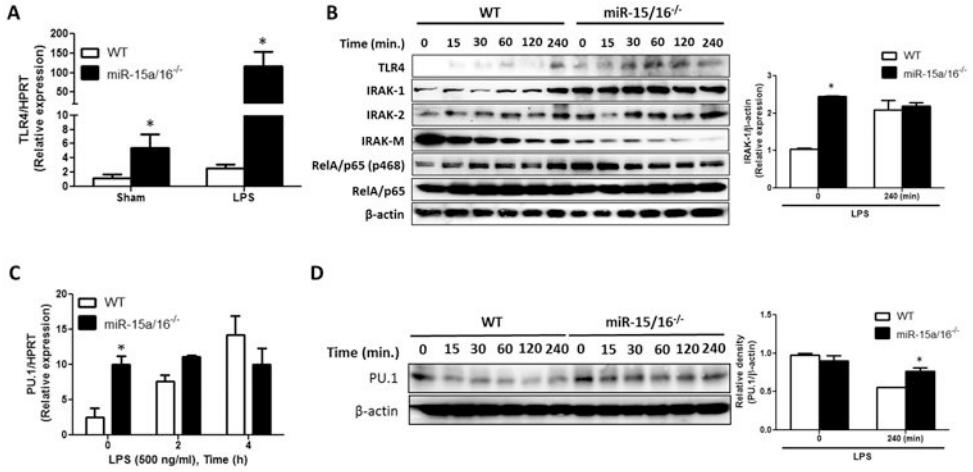

Figure 5. Deletion of miR-15a/16 up-regulated the TLR4 signaling in BMDMs

(A) TLR4 mRNA level after 4h 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 4h, 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 2 independent experiments with identical results. * $p<0.05$

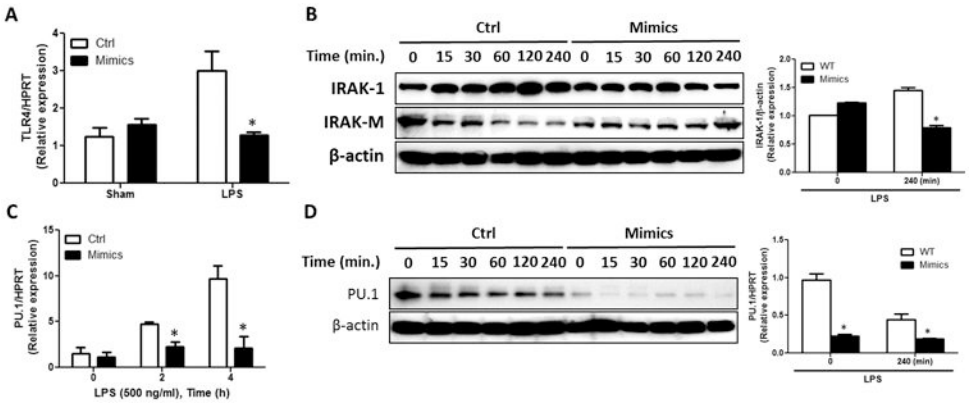

Figure 6. Over-expression of miR-15a/16 down-regulated the TLR4 signaling in BMDMs

(A) TLR4 mRNA level 4h after LPS (500 ng/ml) stimulation in WT and miR-15a/16 over-expressing BMDMs. TLR4 expression level was normalized by HPRT.

(B) Protein expression of IRAK-1 and IRAK-M in WT and miR-15a/16 over-expressing BMDMs. Cells were treated with LPS (500 ng/ml). After the designated time points, protein levels were analyzed using the Western Blot analysis (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 4h respectively. PU.1 expression level was normalized using HPRT.

(D) PU.1 protein level in WT and miR-15a/16 over-expressing BMDMs after LPS stimulation (left panel), analyzed using Western Blot analysis. PU.1 expression was normalized by β-actin (right panel).

All figures represent 2 independent experiments with identical results. * $p<0.05$

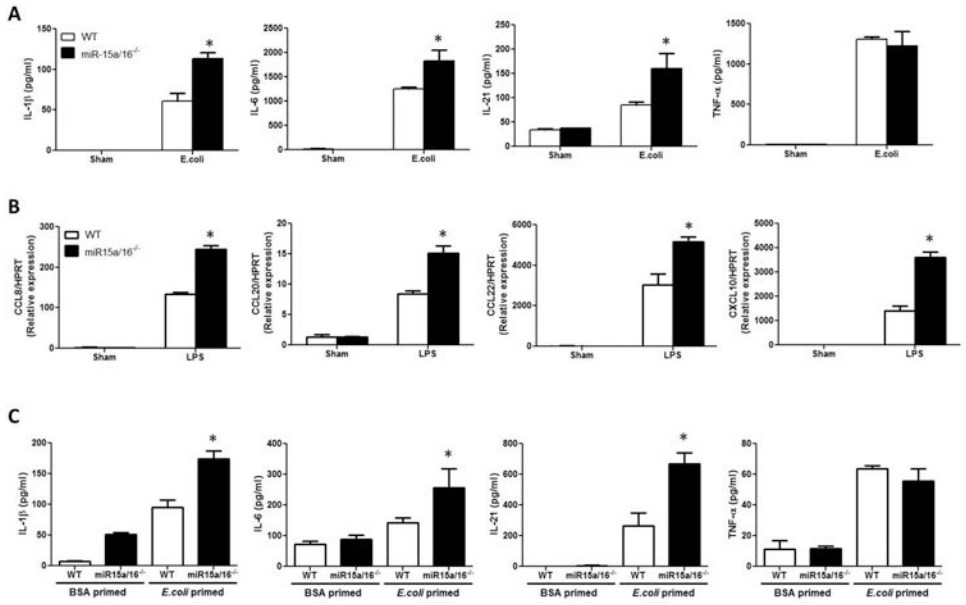

Figure 7. Cytokine and chemokine levels in WT or miR-15a/16$^{-/-}$ BMDMs after bacterial infections

(A) WT and miR-15a/16$^{-/-}$ BMDMs were treated with *E.coli*-FITC particles (20 μg/ml). After 6h, cytokines were measured using ELISA.

(B) WT and miR-15a/16$^{-/-}$ BMDMs were treated with LPS (500 ng/ml). After 4h, 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 4h, cells were harvested and injected into WT mice intra-peritoneally. Peritoneal fluids were collected after additional 24h 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, 6h). IL-1β and IL-6 were measured using ELISA

All figures represent 3 independent experiments with identical results. * $p<0.05$ copmared to WT-LPS, **$p<0.05$ compared to LPS-Ctrl

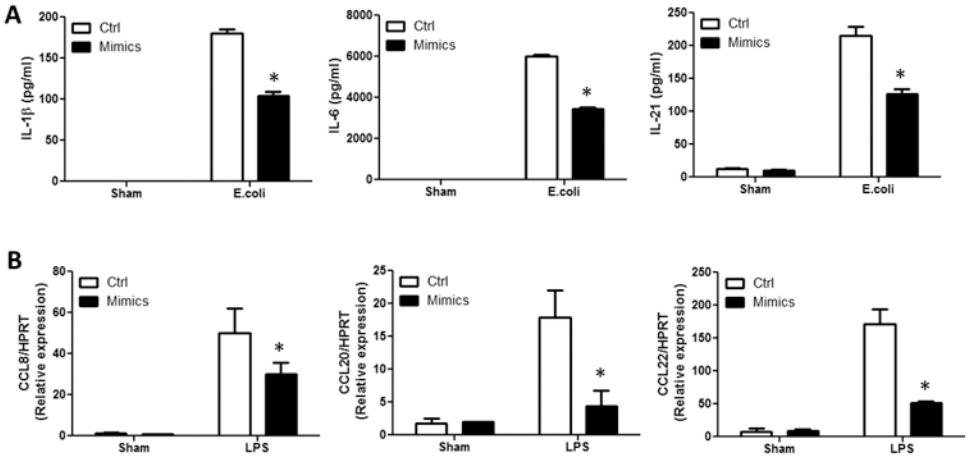

Figure 8. Cytokine and chemokine levels in WT or miR-15a/16 over-expressing BMDMs after bacterial infections

(A) WT and miR-15a/16 over-expressing BMDMs were treated with *E.coli*-FITC particles (20 μg/ml). After 6h, cytokines were measured using ELISA.

(B) WT and miR-15a/16 over-expressing BMDMs were treated with LPS (500 ng/ml). After 4h, RNA was isolated. Chemokine gene expressions were determined using real-time PCR and were normalized by HPRT.

All figures represent 3 independent experiments with identical results. * $p<0.05$

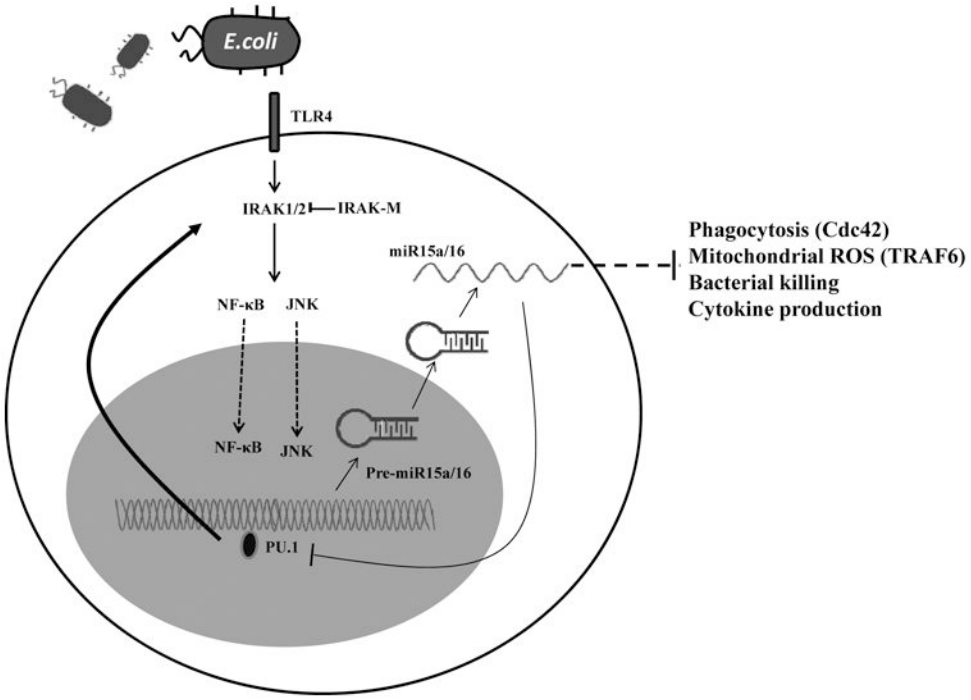

Figure 9. Proposed mechanisms involved in the functions of miR-15a/16 in macrophages

miR-15a/16 down-regulated the expression of TLR4 *via* targeting PU.1 locating on the promoter region of TLR4 and modulated TLR4 down-stream 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.