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MicroRNA-149 Negatively Regulates TLR-Triggered Inflammatory Response in Macrophages by Targeting MyD88

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ABSTRACT

MicroRNAs (miRNAs) have been shown to be important regulators of TLR signaling pathway at the post-transcriptional level. In this study, the potential role of miR-149 was explored in murine alveolar macrophage RAW264.7 cells. Our results demonstrated a dynamic change of the expressions of miR-149 expression and MyD88 in macrophage RAW264.7 upon *Mycobacterium bovis* Bacillus Calmette-Guerlin (BCG) infection or lipopolysaccharide (LPS) stimulation. The presence of BCG or LPS dynamically reduced the miR-149 expression, along with a substantially striking increase of MyD88 expression in these cells. More importantly, overexpression of miR-149 in RAW264.7 cells was associated with a significant decrease of MyD88 protein expression, as well as a reduced production of inflammatory mediator NF-κB 1, TNF-α and IL-6 in response to BCG infection or LPS stimulation. Further studies using immunoblotting assay against anti-MyD88 antibody and microRNA targeting luciferase reporter assay revealed that miR-149 was able to directly target the 3′-UTR of MyD88 mRNA and post-transcriptionally regulated MyD88 protein expression. These data suggested that miR-149 might be a key player of immune modulator for TLR/MyD88 signaling pathway in macrophages, which may through a mechanism of negatively regulating MyD88-dependent Toll-like receptors signaling pathway. J. Cell. Biochem. 115: 919–927, 2014.

KEY WORDS: microRNA-149; TLR; MyD88; INFLAMMATORY RESPONSE

Toll-like receptors (TLRs) are a family of pattern recognition receptors that detect conserved microbial components during infection and initiate an inflammatory response, which are commonly expressed by cells of the immune system including macrophages and dendritic cells, as well as other sentinel cells such as epithelial cells. Up to date, 13 TLRs (namely TLR1–TLR13) have been identified in mice and humans, and TLR4 and TLR2 are two of the most studied members [Medvedev, 2013]. The activation of TLRs is one of the most common defense mechanisms in host innate immunity against the invasion of pathogenic microorganisms, in which the TLRs recognize specific pathogen-associated molecular patterns (PAMP) that are present in microbial components, and

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sequentially trigger the cascade of inflammatory responses [Banerjee and Gerondakis, 2007]. In this context, different pathogens may activate distinct TLRs and induce distinct gene expression profiles, which not only activates the host innate immunity but also triggers an antigen-specific acquired immune response. Following the recognition of a PAMP, TLR binds its adaptor proteins, such as the MyD88, which in turn, recruits the interleukin-1 receptor-associated kinase (IRAK) complex. The IRAK complex includes four subunits: two active kinases (IRAK-1 and IRAK-4) and two noncatalytic subunits (IRAK-2 and IRAKM) [Takeda et al., 2003; O′Neill and Bowie, 2007], in which the IRAK-1 is phosphorylated by IRAK-4, the phosphorylated IRAK-1 then associates with TNF receptor-associated factor 6 (TRAF6) to activate the signaling of the NF-κB and Mitogen-activated protein kinases (MAPKs). The IRAK-TRAF6 complex is able to induce the phosphorylation and degradation of IκB via the IKK complex, and enables the nuclear translocation of NF-κB for activation [Lang et al., 2003; Moynagh, 2009; Rakoff-Nahoum and Medzhitov, 2009]. The activation of NF-κB and MAPK then trigger pro-inflammatory or innate immune responses by induction of the expression of various inflammatory genes, including IL-1β, IL-6, and TNF-α [Lang et al., 2003; Rakoff-Nahoum and Medzhitov, 2009]. BCG has been demonstrated to induce pro-inflammatory gene transcription through the TLR2 and TLR4 mediated signaling pathway [Heldwein et al., 2003], among which the TLR-2 signaling pathway is one of the best characterized. TLR-2 was capable of sensing pathogens by recognizing the conserved structures of pathogen-associated molecular patterns (PAMPs) and activating the innate immune response, which in turn produce pro-inflammatory mediators, such as TNF-α, IL-8, IL-12, and IL-6, to induce an antigen-specific adaptive immune response [Takeda and Akira, 2004; Banerjee and Gerondakis, 2007; Huang et al., 2012]. Lipopolysaccharide (LPS) is a component of the cell wall of Gram-negative bacteria that binds to mammalian cell membrane molecules such as CD14 and toll-like receptors (TLRs). TLR-4 is thought to be the main receptor for LPS, binding of LPS to TLR-4 leads to the activations of several signaling cascades and drive the expression of pro-inflammatory cytokines that contribute to the pathogenesis of septic shock.

MicroRNAs (miRNAs) have recently emerged as important regulators of gene expression at the post-transcriptional level. miRNAs are highly conserved, and each mammalian miRNA may be capable of targeting multiple distinct mRNAs, vice verses, individual mRNA can be targeted by multiple miRNAs [Baek et al., 2008]. These suggest that the expressions of most mRNAs could be regulated by miRNAs to some extent. The miRNAs could therefore be important molecules similar to the transcription factors in controlling the protein content, fate and/or function of a cell [Baek et al., 2008]. With the dynamic nature of miRNAs, several lines of evidence have suggested that the expression of miRNAs was highly regulated in immune cells, in which miRNAs played roles of immune modulators, and were involved in regulating the signaling of TLR pathway [Taganov et al., 2006; Androulidaki et al., 2009; Schnitger et al., 2011; Chen et al., 2012]. In this regards, miR-92a has recently been demonstrated that it played a negative roles in regulation of TLR-triggered inflammatory responses in macrophages through a mechanism of targeting MKK4 [Lai et al., 2013]. Such a role of immunomodulation of miRNAs was also supported by the findings of rapidly increased expressions of miRNAs let-7e [Androulidaki et al., 2009], miR-146 [Taganov et al., 2006], miR-21 [Sheedy and O′Neill, 2008], miR-155 [O′Connell et al., 2007], and/or miR-181c [O′Connell et al., 2009], in monocytic cell lines or mouse macrophages in response to lipopolysaccharide (LPS) or other microbial components. These miRNAs were able to modulate inflammatory responses by targeting different components of TLR signaling pathways, such as TLR4, IL-1R-associated kinase 1/TNFR-associated factor 6, PDCD4, SCOS1/SHP1, and TNFSF11 (RANKL). In addition, down-regulated expressions of miRNAs were also demonstrated in some circumstances. For instance, the expression of miR-223 was significantly decreased in murine macrophages in response to LPS or poly(IratioC) stimulation, and the down-regulation of miR-223 led the activation of signal transducer and activator of transcription 3 (STAT3) [Chen et al., 2012]; similarly, a decreased expression of miR-125b was also found in the Jurkat cells following LPS stimulation [Tili et al., 2007]. In macrophages, Schnitger et al. [2011] found a MyD88 dependent up-regulation of miR-149 in murine primary macrophages in response to *Listeria. monocytogenes* (*L. monocytogenes*) infection, However, the underlying mechanisms of miRNAs in the regulation of immune responses against varied pathogens or stimuli remain poorly understood, which await further investigation. In this study, we analyzed the expression profile of miR-149 in mouse macrophages after TLR activation and demonstrated its possible functionality in the TLR-triggered immune response. We found that the expression level of miR-149 was down-regulated in response to the TLR ligands (LPS and BCG) in macrophages; the miR-149 was able to negatively regulate TLR-triggered inflammatory response of cytokine production through a mechanism of targeting the adaptor molecule, MyD88 of the TLR/MyD88 pathway. We thus identified that miR-149 was a novel immune modulator, which played a negative role in TLR-mediated innate responses.

MATERIALS AND METHODS

CELL CULTURE

The human embryonic kidney cell line 293T (Shanghai Institute of Biochemistry and Cell Biology, Shanghai, China) and murine macrophage RAW264.7 cells from American Type Culture Collection (Manassas, VA) were cultured and maintained in DMEM (for 293T) or RPMI 1640 (for RAW264.7) medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 1% pen/strep at 37°C in a humidified atmosphere of 5% CO_2 95% air.

IN VITRO MODEL OF BCG INFECTION AND LPS STIMULATION

To determine the miR-149 in the regulation of the TLR signaling pathway in vitro, lipopolysaccharide (LPS) and *Mycobacterium bovis* Bacillus Calmette-Guerlin (BCG) were used as ligands of TLRs (TLR4) in this study. *Mycobacterium bovis* BCG, Beijing strain (Center for Disease Control and Prevention of China [CCDC], Beijing, China) were grown at 37°C with shaking in Middle-brook 7H9 broth containing 10% albumin dextrose catalase supplement for 2 weeks, the bacterial cultures were then harvested by centrifugation at 500*g* for 10 min and the cell pellets were resuspended in the medium. The colony formation unit (CFU) of the culture was ascertained by plating

serially diluted cultures on Middlebrook 7H11 plates supplemented with OADC enrichment (BD Biosciences Shanghai, Shanghai, China), and the bacterial colonies were counted after 4 weeks of culture [Lewin et al., 2003].

The RAW264.7 cells were seeded in a 24-well plate at a density of at 10^6 cells/ml and cultured for overnight. Cells were then exposed to medium with 1 μg/ml LPS (Sigma, St. Louis, MO) or infected with BCG at a multiplicity of infection (MOI) of 10 for 3, 6, 12, or 24 h prior to be harvested for analysis.

GENERATION OF LENTIVIRAL PROVIRAL VECTOR EXPRESSING miR-149

Based on the sequence of mouse miR-149 (5′-UCUGGCUCCGUGUCUUCACUCCC-3′, MIMAT0000159) from miRBase database, a sense strand (5′-TCTGGCTCCGTGTCTTCACTCCCTTCAAGAGAGGGAGTGAAGACACGGAGCCAGATTTTTTC-3′) and an antisense strand (5′-AAGACCGAGGCACAGAAGTGAGGGAAGTTCTCTCCCTCACTTCTGTGCCTCGGTCTAAAAAAGAGCT-3′) of miR-149 were synthesized., and the restriction sites of *Hpa*I and *Xho*I were introduced at 5′-ends of these oligonucleotides, respectively. The mixture of the sense and anti-sense oligonucleotides was then used for production of the precursor of small hairpin RNA (shRNA) of miR-149 by temperature annealing approach as previously described [Wei et al., 2013]. Following a modification with appropriate restriction enzymes, the precursor was cloned into a miRNA expressing plasmid, pSicoR (Department of Biological Chemistry, School of Medicine, Fudan University, Shanghai, China) for generation of the lentiviral proviral vector expressing miR-149. pSicoR/miR-149 [Wei et al., 2013]. A negative control vector, pSicoR/NC was also generated using the same approach. The following respective sequences were unrelated to any gene in mouse and were used for generation of the control vector: sense strand: 5′-TTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTTC-3′, antisense strand: 5′-TCGAGAAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAAA-3′.

PRODUCTION OF LENTIVIRAL VECTOR AND INFECTION OF RAW264.7 MACROPHAGE

For production of a vesicular stomatitis virus-G (VSV-G) pseudotyped lentiviral vector, HEK 293T cells were seeded at a density of 1×10^6/well in six-well plates and cultured in DMEM/10% FBS without antibiotics for overnight. The medium was replaced with 1 ml serum/antibiotics free before the cells were co-transfected with proviral plasmid pSicoR/miR-149 (1.5 μg) or pSicoR/NC (1.5 μg), packaging plasmids of pCMV-VSV-G (0.5 μg) and pCMV-dR8.91 (1 μg) (Department of Biological Chemistry, School of Medicine, Fudan University, Shanghai, China) using TransLipid Transfection Reagent per the manufacturer instruction. The transfected cells were refreshed 2 ml of DMEM/10% FBS at 6 h post-transfection, and continually cultured for additional 48 h before the culture medium was harvested and filtered through a 0.45-μm poresize filter. The collected medium was then concentrated to 1/100 volume by ultracentrifugation with 50,000*g* at 4°C for 2.5 h using a SW28 rotor (Beckman Coulter, Fullerton, CA) and Sorvall Ultra 80® (Kendro Laboratory Products, Newtown, CT). The pellet of viral particle was resuspended in PBS, aliquoted and frozen at −80°C till use. The viral particles were titrated in 293T cells by counting EGFP-positive cells. The viruses generated from pSicoR/miR-149 and pSicoR/NC were designated as LV-miR-149 or LV-NC, respectively. For infection, RAW264.7 cells were seeded in a six-well plate 1 day prior to the infection, the cells were infected with LV-miR-149 or LV-NC at MOI of 10 at 24 h after the seeding.

RNA ISOLATION AND REAL-TIME QUANTITATIVE PCR

Small RNA and total RNA from different treated RAW264.7 cells were purified using the RNA purification kit per manufacturer's instruction (RNAiso for Small RNA, TaKaRa, Dalian, China) or TRIzol reagent (Invitrogen). The quality of RNA was assayed by calculation of the RNA integrity number (RIN) [Schroeder et al., 2006]. High quality RNA (RIN value was greater that 9.0) was used for reverse transcription of the first-strand cDNA synthesis by reverse transcription using M-MLV reverse transcriptase (TaKaRa). The sequence of the primer used for reverse transcription of mature miR-149 with stem-loop structure were miR-149 RT (5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGGGAGTGA-3′), which was designed based on the corresponding sequence from miRBase database (http://www.mirbase.org/). The quantitative real-time RT-PCR (qRT-PCR) was performed in the Roche lightcycler 2.0 using TaKaRa SYBR Green I kit (Takara); the thermal cycling condition for PCR was 95°C for 30 s, 40 cycles of 95°C for 5 s, 60°C for 20 s and 72°C for 20 s, followed by 40°C for 20 min. The primer sets used for RT-PCR of U6 control, miR-149, β-actin control, MyD88, NF-κB, IL-6, and TNF-α were listed in Table I. The controls were always included to normalize each reaction with respect to RNA integrity, sample loading and inter-PCR variations. The relative expression ratio was calculated from the real-time PCR efficiencies and the crossing point deviation of experimental samples versus controls [Pfaffl, 2001]. The specificity of PCR was determined by sequencing of the PCR products.

DUAL-LUCIFERASE REPORTER ASSAY

The wild-type mouse MyD88 3′-UTR luciferase reporter vectors were constructed by amplifying the mouse MyD88 mRNA 3′-UTR containing the potential binding sites and cloning it into the pMIR-report vector (Promega, Madison, WI) and miR-149 potential binding sites were mutated in the MyD88 UTR Mut vector. The following primers were designed based on GenBank database (NM_010851.2), and were used for amplification of wild-type and mutated 3′UTR of MyD88 mRNA: the sequence of common forward primer was 5′-CGGGACTAGTCCCCTGAAGATGACCCTGGGAG-3′, reverse primer for wild-type MyD88 mRNA 3′UTR was 5′-GGTCGACGCGTCGCCCTTCTTTTCTTTATTGAC-3′, reverse primer for mutated MyD88 mRNA 3′UTR was 5′-GGTCGACGCGTCTGCAGATGAGCATCAGTCTCAT-3′; restriction sites (underlined) of *Spe*I and *Mlu*I were introduced in the forward and reverse primers, respectively. HEK-293 cells were co-transfected with luciferase reporter plasmid (0.5 μg), pRL-TK (Promega, 0.1 μg), and the pSicoR/miR-149 or pSicoR/nc plasmid (0.5 μg). After 24 h, the cells were collected and used for luciferase assay using a Dual-Luciferase Reporter System (Promega) per the manufacturer's instruction. All samples were performed in triplicate within each experiment, and three independent experiments were conducted.

TABLE I. Primers Used for Quantitative RT-PCR

Primers	Sequence (5′–3′)	Annealing, T_m (°C)	Amplicon size (bp)
β-Actin	F: 5′-TTGTTACCAACTGGGACG-3′ R: 5′-CCAGAGGCATACAGGGAC-3′	66	214
MyD88	F: 5′-CACTCGCAGTTTGTTGGATG -3′ R: 5′-CCACCTGTAAAGGCTTCTCG-3′	58	185
IL-6	F: 5′-TGCCTTCTTGGGACTGAT-3′ R: 5′-TTGCCATTGCACAACTCTTT-3′	60	183
TNF-α	F: 5′-CCAGACCCTCACACTCAGAT-3′ R: 5′-GACAAGGTACAACCCATCG-3′	56	187
NF-κb1	F: 5′-TGCCAAAGAAGGACACGACAG-3′ R: 5′-GCCACCAGCAGCAGCAGACAT-3′	62	150
miR-149	F: 5′-ACACTCCAGCTGGCTCTGGCTCCGTGTCTTC-3′ R: 5′-CTCAACTGGTGTCGTGGA-3′	56	63
U6	F: 5′-CTCGCTTCGGCAGCACA-3′ R: 5′-AACGCTTCACGAATTTGCGT-3′	51	90

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

To determine the IL-6 and TNF-α level in the medium of RAW264.7 cell cultures, the culture medium was collected after treatment and centrifuged at 1,000*g* for 5 min to pellet the cell debris. The supernatant was harvested and stored at −80°C prior to analysis. Mouse IL-6 and TNF-α levels in the supernatant were determined with appropriate ELISA kits per the manufacturer′s instructions (Neobioscience Tech, Shenzheng, China). The protein concentration was determined by comparing the standard protein provided in the kits and expressed as ng/L.

WESTERN BLOTTING ANALYSIS

Protein extracts were prepared with RIPA lysis buffer in the presence of proteinase inhibitors. Western blotting analysis was performed using antibodies against MyD88 (1:500, Cell Signaling Technology, Danvers, MA) and β-actin (1:400, Beijing Biosynthesis Biotechnology Co, Beijing, China). The blots were then developed using the enhanced chemiluminescence (ECL) reagent (Amersham Biosciences, USA).

STATISTICAL ANALYSIS

All data collected in this study was obtained from at least three independent experiments for each condition. SPSS18.0 analysis software (PC version, SPSS, Inc., Chicago, IL) was used for the statistic analysis. Statistical evaluation of the data was performed by one-way ANOVA and *t*-test for comparison of differences between the two groups. $P < 0.05$ was set to represent a statistical difference, and a value $P < 0.01$ was set to represent a statistically significant difference. Data were presented as the mean ± standard deviations (SD).

RESULTS

DOWN-REGULATION OF miR-149 IN RESPONSE TO LPS AND BCG IN MACROPHAGES

Using a qRT-PCR assay, abundant miR-149 was found in macrophage RAW264.7 cells (mock in Fig. 1A,B). However, a dynamically decreased expression of miR-149 was detected with the time, when the cells were exposed to TLR ligands BCG (Fig. 1A) or LPS (Fig. 1B). Up to 20- and 5-fold reduced expression of miRNA-149 were detected in the RAW264.7 cells in response to BCG (Fig. 1A) and LPS (Fig. 1B) at 24 h post-stimulation, respectively. In contrast, a dynamic elevation of MyD88 expression was found in these cells stimulated with BCG (Fig. 1C) and LPS (Fig. 1D) with the time; the expression of MyD88 in this type of cell was elevated as high as about 22-fold in response to both of BCG and LPS at 24 h after the treatment in this study (Fig. 1C,D). These data strongly evidenced that miR-149 might be an important immunomodulator, which played a role in the negative role in TLR/MyD88 signaling pathway in macrophages. In response to BCG or LPS stimulation, the activated TLR signaling might trigger inflammatory responses by elevating expression of MyD88 a mechanism of reducing miR-149 expression in macrophages.

miR-149 NEGATIVELY REGULATES TLR-TRIGGERED INFLAMMATORY RESPONSE IN MACROPHAGES

To assess whether miR-149 was involved in the regulation of TLR/MyD88 triggered inflammatory response against microbial infection, lentiviral vector expressing miR-149 and control vector, LV-miR-149 and LV-NC were generated and used for infection of RAW264.7 cells, and the production of pro-inflammatory factors NF-κB1, TNF-α, and IL-6 were evaluated in the infected cells. The results showed in Figure 2 and Table II demonstrated that the infection of LV-miR-149 increased miR-149 expression by 48-fold in RAW264.7 cells, in comparison with the LV-NC infected cells (Fig. 2A); and the BCG or LPS induced productions of transcription factor NF-κB1 (Fig. 2B) and pro-inflammatory cytokines TNF-α (Fig. 2C) and IL-6 (Fig. 2D) were dramatically inhibited by the overexpression of miR-149 at the mRNA level, as well as at the protein level in macrophage RAW264.7 cells. These data suggested that miR-149 might play a negative role in the TLRs/NF-κB pathway, which sequentially down-regulated the expressions of its downstream pro-inflammatory mediators.

miR-149 DOWN-REGULATES MYD88 IN MACROPHAGES AT THE POST-TRANSCRIPTIONAL LEVEL

We next investigated the potential targets by which miR-149 modulates the TLR/MyD88 pathway in regulating the inflammatory response in macrophages. RAW264.7 cells were infected with LV-NC, LV-miR-149, and the total RNA and proteins were harvested at 24 h postinfection. Interestingly, miR-149 showed an inability to alter

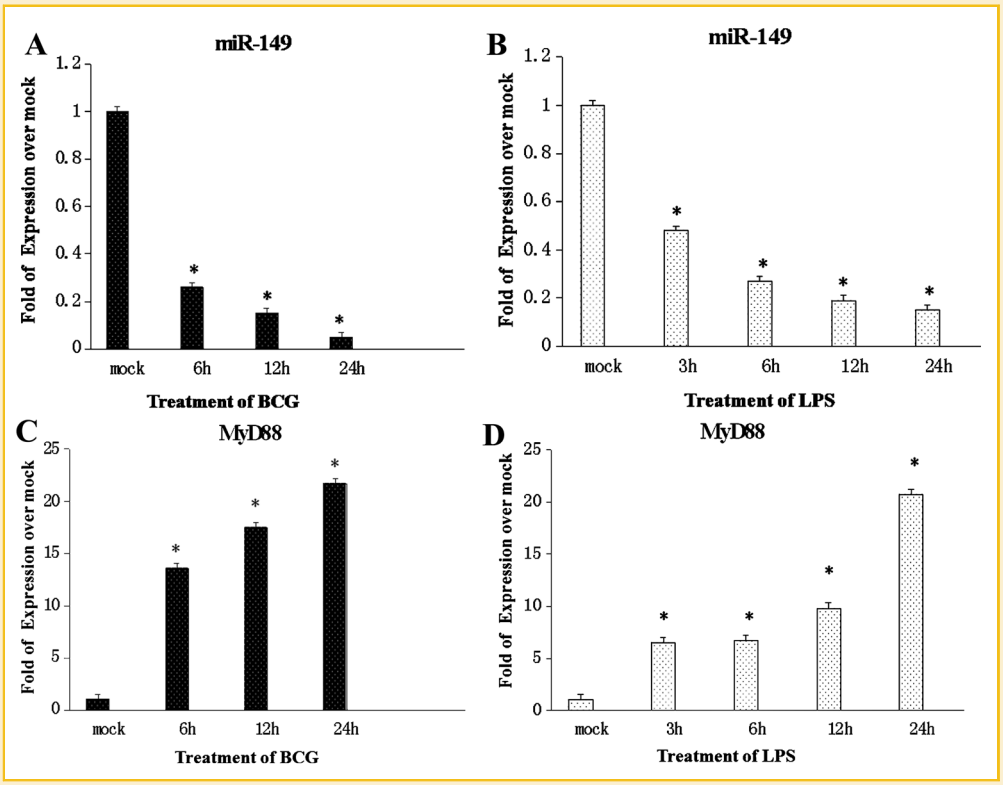

Fig. 1. Dynamic changes of miR-149 and MyD88 in macrophage RAW264.7 cells against BCG and LPS. A–D: Murine macrophage RAW264.7 cells were infected with BCG at MOI of 10 (A,C) or stimulated with LPS at concentration of 1 μg/ml (B,D) for indicated times. The expression of miR-149 (A,B) and MyD88 (C,D) were determined by a qRT-PCR assay. The expression was normalized to the expression of U6 for miR-149 (A,B) or β-actin for MyD88 (C,D) in each samples. Data are expressed as the mean ± SD ($n = 9$). All of the data shown above are representative of three independent experiments. $^{*}P < 0.05$.

MyD88 expression at mRNA level in these cells (Fig. 3A,C), however, it demonstrated a capacity to down-regulate MyD88 protein expression by 0.2-fold as compared with the LV-NC control (Fig. 3B). These data indicated that miR-149 had a potential to regulate immune response in macrophages through a mechanism in part by post-transcriptionally down-regulating MyD88 expression.

MYD88 IS A TARGET OF miR-149

Bioinformatic analysis using of miRanda (http://www.microrna.org/), PicTar (http://pictar.bio.nyu.edu/) and TargetScan (http://www.targetscan.org/) data bases revealed that a putative miR-149 binding site harbored in the 3′-UTR of MyD88 (Fig. 4A). To verify whether miR-149 is capable of targeting this potential site of MyD88, luciferase pMIR-report vectors containing either 3′-UTR for MyD88 or mutated 3′-UTR for MyD88, were generated (Fig. 4A). In order to achieve high transfection efficiency, 293T cells were co-transfected with pSicoR/miR-149 and the pMIR-report vector containing 3′-UTR or mutated 3′-UTR of MyD88, and a Luciferase reporter assay was performed at 24 h post-transfection. The luciferase assay demonstrated that the introduction of miR-149 was able to significantly repress the luciferase activity of 293T cells transfected with pMIR-report vector containing wild type 3′-UTR of MyD88, but not those transfected with the mutated 3′-UTR containing vector (Fig. 4B). This result suggested that miR-149 might inhibit MyD88 protein expression by directly targeting the 3′-UTR of MyD88 mRNA at post-transcriptional level.

DISCUSSION

In the present study, we reported a potential mechanism of miR-149 in regulation of TLR triggered inflammatory response against BCG and LPS in murine macrophage RAW264.7 cells. Our results demonstrated that abundant miR-149 was in the macrophage RAW264.7 cells; the addition of BCG or LPS dynamically reduced the miR-149 expression, along with a substantial increase of MyD88 expression in the cells; forced overexpression of miR-149 in RAW264.7 cells was associated with a significant decrease of MyD88 protein expression, as well as a reduction of the production of NF-κB 1, TNF-α, and IL-6 in response to BCG infection or LPS stimulation; moreover, miR-149 was able to directly target the 3′-UTR of MyD88 mRNA and post-transcriptionally regulated MyD88 protein expression. These data suggested that miR-149 might be an important modulator that played a negative role in TLR/MyD88 pathway in macrophages against mycobacteria infection, at least in part through a mechanism of targeting MyD88.

miRNAs have been demonstrated to play important roles in regulating TLR signaling at different levels by targeting multiple molecules involved in this pathway, including the expression of TLRs, adaptors and mediators of TLR signal pathway, and TLR-activated transcription factors [O′Neill et al., 2011]. For example, TLR4 was regulated by members of the let-7 miRNA family. The induction of let-7e expression could decrease the TLR4 expression on the cell surface of in liver-derived cholangiocytes [Chen et al., 2007]. miR-155 was found to be able to negatively regulate TLR signaling

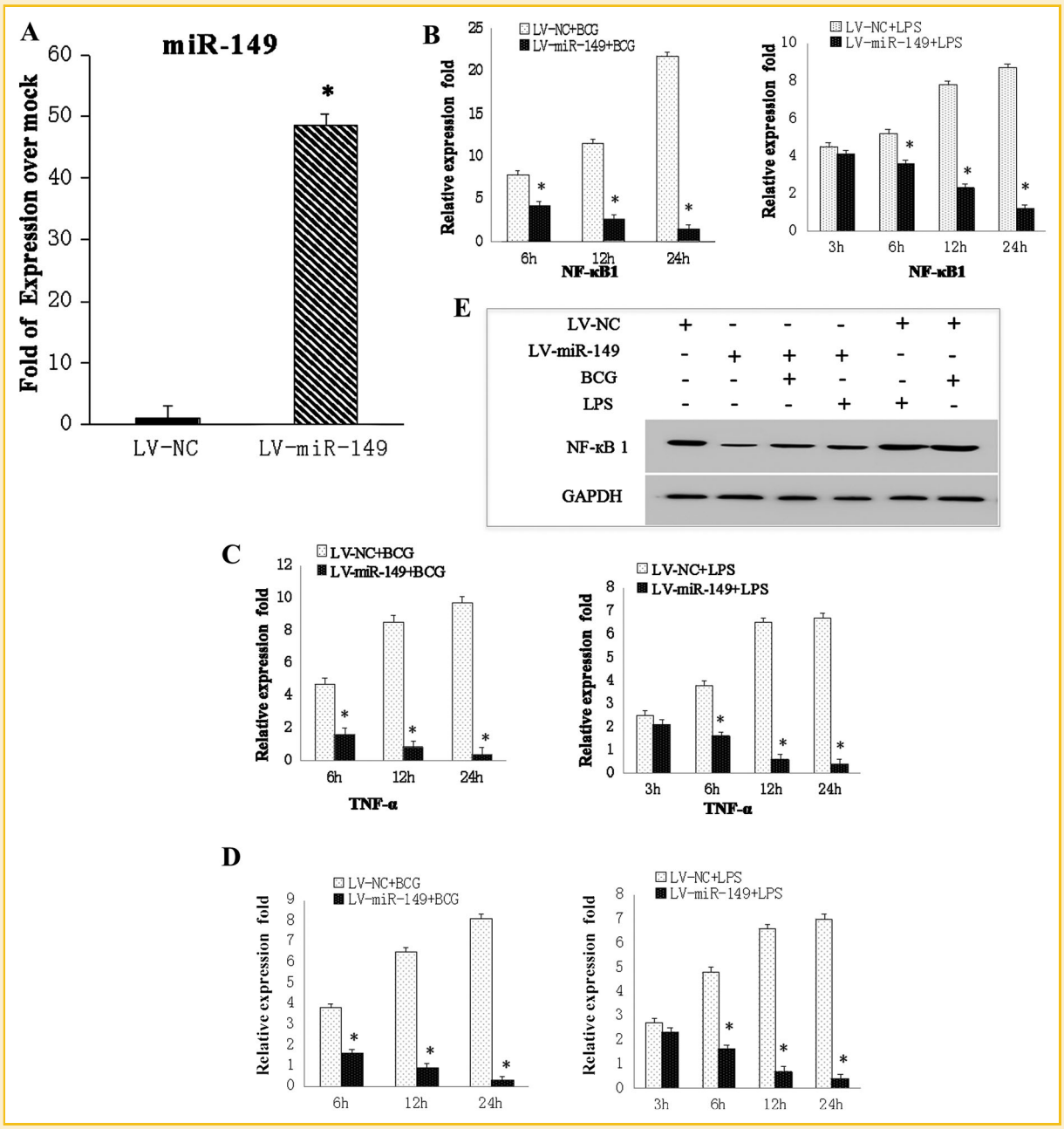

Fig. 2. The role of miR-149 in regulation of TLR-triggered inflammatory responses in macrophages against BCG and LPS. A: Overexpression of miR-149 in RAW264.7 cells infected with lentiviral vectors determined by a qRT-PCR assay. B–D: The expressions of NF-κB1 (B), TNF-α (C) and IL-6 (D) in lentiviral LV-miR-149 or LV-NC infected macrophage RAW264.7 cells in response to BCG infection (left panel in B, C, and D) or LPS stimulation (right panel in B, C, and D) at indicated time points were determined by a qRT-PCR assay. *Compared with the control group, $P < 0.05$. Results represented the mean ± SD from three independent triplicated experiments (N = 9).

TABLE II. Concentration of IL-6 and TNF-α in the Supernatant of Culture Determined by ELISA[a]

		IL-6 concentration (ng/L)			TNF-α concentration (ng/L)		
Stimulator	Infection	6 h	12 h	24 h	6 h	12 h	24 h
BCG	LV-NC	1,789.6 ± 93.6	3,246.5 ± 102.0	3,848.3 ± 129.1	1,468.8 ± 61.6	3,345.6 ± 138.3	3,916.4 ± 147.8
	LV-miR-149	853.7 ± 33.8*	1,246.8 ± 96.1*	1,562.8 ± 61.7*	843.7 ± 29.7*	1,456.8 ± 126.7*	1,693.8 ± 74.7*
LPS	LV-NC	1,564.5 ± 41.0	3,142.7 ± 87.1	4,613.8 ± 55.4	2,164.5 ± 105.3	3,847.8 ± 130.2	5,013.7 ± 84.8
	LV-miR-149	934.6 ± 39.1*	1,064.8 ± 66.9*	2,289.5 ± 55.5*	967.5 ± 76.6*	1,535.7 ± 48.8*	2,364.6 ± 143.5*

*$P < 0.05$ versus respective control values.
[a]The data represented the mean ± SD of nine samples from three independent experiments.

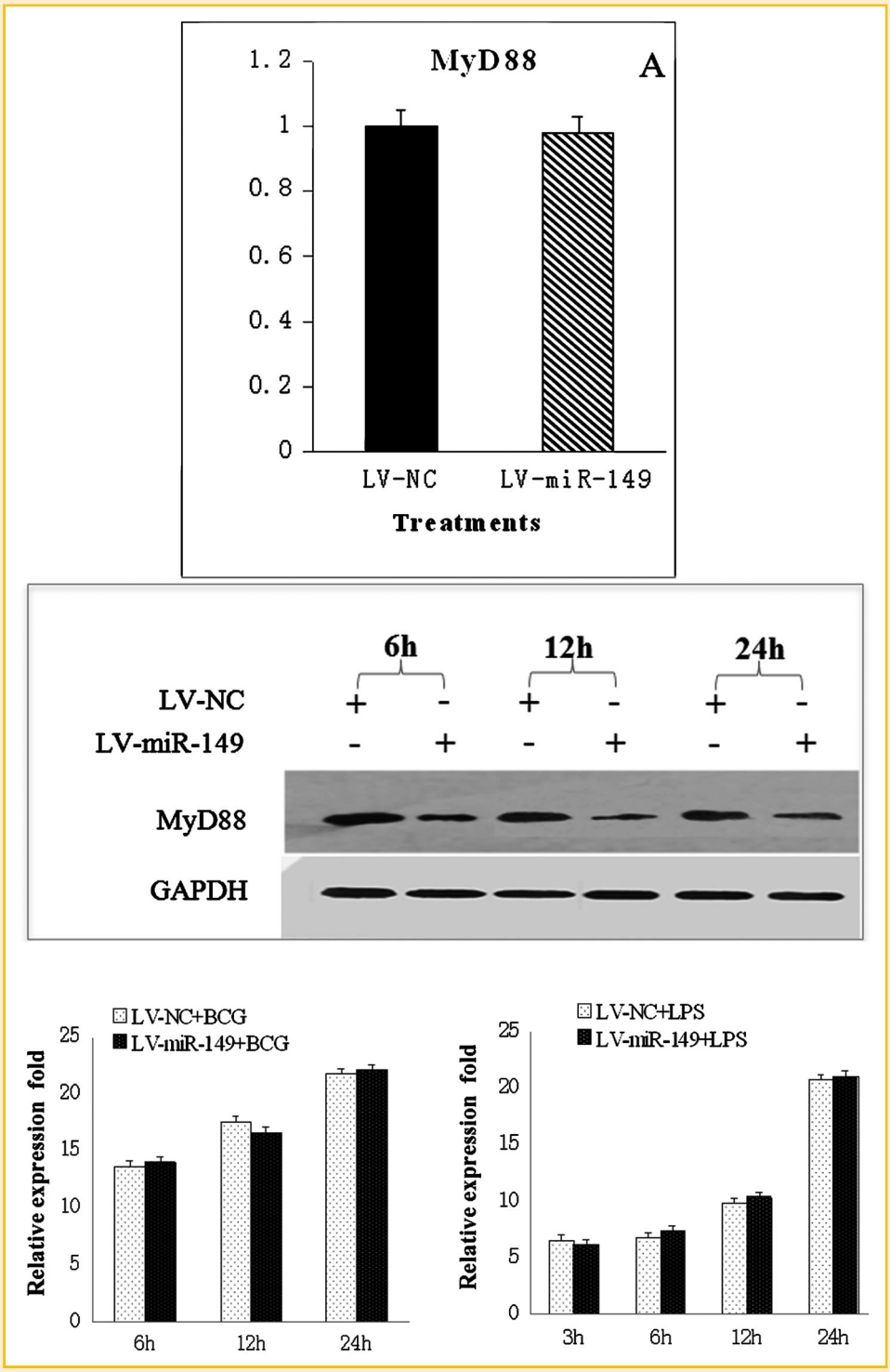

Fig. 3. miR-149 directly target MyD88 in RAW264.7 cells at the post-transcriptional level. A: The expression of MyD88 mRNA was detected by qRT-PCR in the RAW264.7 cells infected with LV-miR-149 or LV-NC. B: The expression of MyD88 was detected by immunoblotting using against anti-MyD88 antibody in the RAW264.7 cells, less abundant MyD88 protein was determined in cells overexpressing miR-149. C: The transcripts of MyD88 determined by a qRT-PCR in BCG or LPS treated RAW264.7 cells showed an inability of miR-149 to alter MyD88 expression at mRNA level.

pathway by targeting some key some signaling molecules including MyD88 [Tang et al., 2010]. Inhibition of miR-155 in dendritic cells (DCs) resulted in up-regulated expression of components in the p38 mitogen-activated protein kinase (mAPK) pathway [Ceppi et al., 2009]. NF-κB is the most important transcription factor in the TLR signaling pathway. miR-9, the TLR-responsive miRNA has been shown an ability to directly target NF-κB1 mRNA [Bazzoni et al., 2009]; and.miR-210 also has been demonstrated to be induced

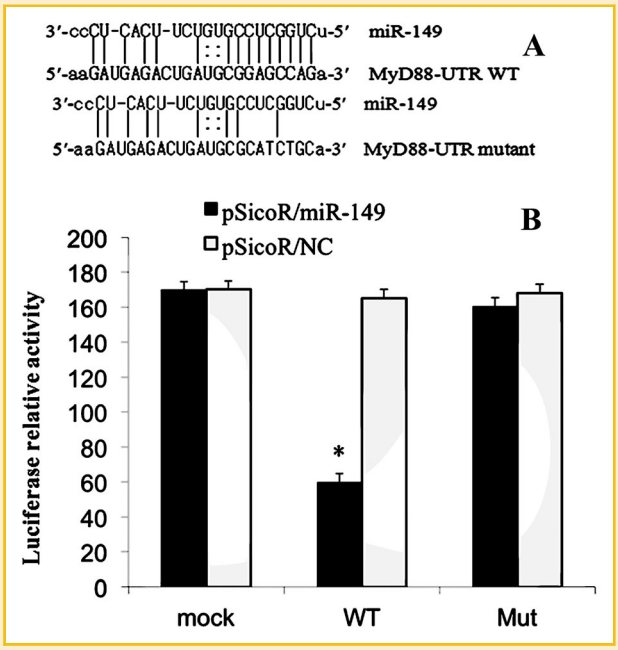

Fig. 4. Validation of MyD88 mRNA as a target of miR-149. A: Sketch and sequence of potential binding site of miR-149 in the 3′-UTR of MyD88 mRNA (top sequence) and mutated MyD88 3′-TUR (bottom sequence), the mutations were introduced into the binding site for generation of a mutated MyD88 3′-TUR. B: Validation of miR-149 target using MyD88 3′-UTR luciferase reporter. *Compared with pSicoR/nc group, $P < 0.05$. Results represented the mean ± SD from three independent triplicated experiments (N = 9).

by LPS and able to target NF-κB1 mRNA [Qi et al., 2012]. Similarly, some miRNAs have been revealed abilities to directly target pro-inflammatory mediators. For instance, miR-365 and miR-142-3p were demonstrated to target IL-6 mRNA, and were capable of reducing the endotoxin-induced mortality in mice [Sun et al., 2011; Xu et al., 2011], while miR-29 was able to suppress immune responses against intracellular pathogens by targeting IFN-γ [Ma et al., 2011]. In this study, we found that miR-149 was capable of negatively regulating inflammatory response in macrophage RAW264.7 cells against BCG infection or LPS stimulation, partially through a mechanism of directly targeting MyD88 in the TLR/NF-κB pathway.

Both BCG and LPS can activate TLR/MyD88 signaling pathway and promote the expression of pro-inflammatory cytokines. In this study, RAW264.7 cells exposed to BCG exhibited a dynamic of mRNA expressions of NF-κB1, TNF-α, and IL-6 (Fig. 2), along with the increased expression of MyD88 and decreased expression of miR-149 with the time (Fig. 1). Such finding may imply an ability of BCG to inhibit miR-149 expression in macrophage, and consequentially elevate the MyD88 protein expression, which in turn, increase the expression of pro-inflammatory cytokines in response to BCG infection (Fig. 2). Of note, in the presence of BCG or LPS, the pro-inflammatory factors of MyD88 downstream genes were stimulated at the early time points regardless of the transfection of plasmid expressing miR-149. However, the expressions were gradually declined with the time, and eventually switched to an inhibitory expression at late time points. The blunted response might imply that the accumulated overexpression of miR-149 efficiently targeted the MyD88, which in turn inhibited the expression of its downstream genes, such as NF-κB 1, TNF-α, and IL-6, even in the presence of TLR ligands BCG or LPS. This finding clearly suggested that miR-149 played a negative role in the TLR/NF-κB pathway in macrophages.

Several lines of evidence have suggested that miR-149 plays multiple roles in the cell proliferation, as well as pathogenesis of including tumors, and infectious diseases [Hariharan et al., 2005; Li et al., 2011; Luo et al., 2011; Chen et al., 2012; Pan et al., 2012; Dominguez, 2013; Lai et al., 2013; Wang et al., 2013]. However, whether miR-149 plays a regulatory role in immune responses has not been extensively demonstrated. Schnitger et al. demonstrated that miR-149 was one of most up-regulated miRNAs in bone marrow derived macrophages in response to *Listeria monocytogenes* infection [Schnitger et al., 2011]. Interestingly, the study reported by Schniter et al. demonstrated a MyD88 dependent up-regulation of miR-149 in macrophages in response to *L. monocytogenes* infection, which differed from the finding in the present study that BCG down-regulated miR-149 expression in the murine macrophage RAW264.7 cell line. Two possibilities might cause the discrepancy: firstly primary macrophages were used in the study performed by Schniter et al., which a macrophage transformed cell line (RAW264.7) was employed in this study; secondly, *L. monocytogenes* is a Gram-positive bacterium, the cell wall of *M. tuberculosis* has characteristics of both Gram-positive and negative, despite both of them are intracellular pathogens [Fu and Fu-Liu, 2002]. On the other hand, a significantly decreased miR-149 was detected in peripheral blood of patients with active Crohn′s disease [Wu et al., 2011]. In this study, we also found that miR-149 was able to negatively regulate TLR-triggered inflammatory cytokine production probably through a mechanism of directly targeting MyD88 involved in the TLR/NF-κB pathway in alveolar macrophages. We have thus for the first time identified a novel mechanism by which the TLR-mediated miR-149 reduction regulates the inflammatory responses in macrophage against mycobacteria infection, and the infection of BCG or LPS stimulation was able to down-regulate miR-149 expression in RAW264.7 cells. However, whether this down-regulation of miR-149 was MyD88 dependent need to be further determined by using gain- and/or loss-function approaches.

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REFERENCES

Androulidaki A, Iliopoulos D, Arranz A, Doxaki C, Schworer S, Zacharioudaki V, Margioris AN, Tsichlis PN, Tsatsanis C. 2009. The kinase Akt1 controls macrophage response to lipopolysaccharide by regulating microRNAs. Immunity 31:220–231.

Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. 2008. The impact of microRNAs on protein output. Nature 455:64–71.

Banerjee A, Gerondakis S. 2007. Coordinating TLR-activated signaling pathways in cells of the immune system. Immunol Cell Biol 85:420–424.

Bazzoni F, Rossato M, Fabbri M, Gaudiosi D, Mirolo M, Mori L, Tamassia N, Mantovani A, Cassatella MA, Locati M. 2009. Induction and regulatory function of miR-9 in human monocytes and neutrophils exposed to proinflammatory signals. Proc Natl Acad Sci USA 106:5282–5287.

Ceppi M, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, Santos MA, Pierre P. 2009. MicroRNA-155 modulates the interleukin-1 signaling pathway in activated human monocyte-derived dendritic cells. Proc Natl Acad Sci USA 106:2735–2740.

Chen XM, Splinter PL, O'Hara SP, LaRusso NF. 2007. A cellular micro-RNA, let-7i, regulates Toll-like receptor 4 expression and contributes to cholangiocyte immune responses against Cryptosporidium parvum infection. J Biol Chem 282:28929–28938.

Chen Q, Wang H, Liu Y, Song Y, Lai L, Han Q, Cao X, Wang Q. 2012. Inducible microRNA-223 down-regulation promotes TLR-triggered IL-6 and IL-1beta production in macrophages by targeting STAT3. PLoS ONE 7:e42971.

Dominguez G. 2013. Deciphering the epigenetic network in colorectal cancer. J Pathol 229:1–3.

Fu LM, Fu-Liu CS. 2002. Is *Mycobacterium* tuberculosis a closer relative to Gram-positive or Gram-negative bacterial pathogens? Tuberculosis (Edinb) 82:85–90.

Hariharan M, Scaria V, Pillai B, Brahmachari SK. 2005. Targets for human encoded microRNAs in HIV genes. Biochem Biophys Res Commun 337:1214–1218.

Heldwein KA, Liang MD, Andresen TK, Thomas KE, Marty AM, Cuesta N, Vogel SN, Fenton MJ. 2003. TLR2 and TLR4 serve distinct roles in the host immune response against *Mycobacterium bovis* BCG. J Leukoc Biol 74:277–286.

Huang S, Rutkowsky JM, Snodgrass RG, Ono-Moore KD, Schneider DA, Newman JW, Adams SH, Hwang DH. 2012. Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. J Lipid Res 53:2002–2013.

Lai L, Song Y, Liu Y, Chen Q, Han Q, Chen W, Pan T, Zhang Y, Cao X, Wang Q. 2013. MicroRNA-92a negatively regulates TLR-triggered inflammatory response in macrophages by targeting MKK4. J Biol Chem 288:7956–7967.

Lang CH, Silvis C, Deshpande N, Nystrom G, Frost RA. 2003. Endotoxin stimulates in vivo expression of inflammatory cytokines tumor necrosis factor alpha, interleukin-1beta, -6, and high-mobility-group protein-1 in skeletal muscle. Shock 19:538–546.

Lewin A, Freytag B, Meister B, Sharbati-Tehrani S, Schafer H, Appel B. 2003. Use of a quantitative TaqMan-PCR for the fast quantification of mycobacteria in broth culture, eukaryotic cell culture and tissue. J Vet Med B Infect Dis Vet Public Health 50:505–509.

Li D, Chen P, Li XY, Zhang LY, Xiong W, Zhou M, Xiao L, Zeng F, Li XL, Wu MH, Li GY. 2011. Grade-specific expression profiles of miRNAs/mRNAs and docking study in human grade I-III astrocytomas. OMICS 15:673–682.

Luo Z, Zhang L, Li Z, Jiang C, Dai Y, Liu X, Zheng Y, Yu H, Xiang J, Li G. 2011. miR-149 promotes epithelial-mesenchymal transition and invasion in nasopharyngeal carcinoma cells. Zhong Nan Da Xue Xue Bao Yi Xue Ban 36:604–609.

Ma F, Xu S, Liu X, Zhang Q, Xu X, Liu M, Hua M, Li N, Yao H, Cao X. 2011. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma. Nat Immunol 12:861–869.

Medvedev AE. 2013. Toll-like receptor polymorphisms, inflammatory and infectious diseases, allergies, and cancer. J Interferon Cytokine Res 33:467–484.

Moynagh PN. 2009. The Pellino family: IRAK E3 ligases with emerging roles in innate immune signalling. Trends Immunol 30:33–42.

O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. 2007. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci USA 104:1604–1609.

O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. 2009. Inositol phosphatase SHIP1 is a primary target of miR-155. Proc Natl Acad Sci USA 106:7113–7118.

O'Neill LA, Bowie AG. 2007. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling. Nat Rev Immunol 7:353–364.

O'Neill LA, Sheedy FJ, McCoy CE. 2011. MicroRNAs: The fine-tuners of Toll-like receptor signalling. Nat Rev Immunol 11:163–175.

Pan SJ, Zhan SK, Pei BG, Sun QF, Bian LG, Sun BM. 2012. MicroRNA-149 inhibits proliferation and invasion of glioma cells via blockade of AKT1 signaling. Int J Immunopathol Pharmacol 25:871–881.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45.

Qi J, Qiao Y, Wang P, Li S, Zhao W, Gao C. 2012. microRNA-210 negatively regulates LPS-induced production of proinflammatory cytokines by targeting NF-kappaB1 in murine macrophages. FEBS Lett 586:1201–1207.

Rakoff-Nahoum S, Medzhitov R. 2009. Toll-like receptors and cancer. Nat Rev Cancer 9:57–63.

Schnitger AK, Machova A, Mueller RU, Androulidaki A, Schermer B, Pasparakis M, Kronke M, Papadopoulou N. 2011. Listeria monocytogenes infection in macrophages induces vacuolar-dependent host miRNA response. PLoS ONE 6:e27435.

Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, Lightfoot S, Menzel W, Granzow M, Ragg T. 2006. The RIN: An RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7:3.

Sheedy FJ, O'Neill LA. 2008. Adding fuel to fire: microRNAs as a new class of mediators of inflammation. Ann Rheum Dis 67(Suppl3):iii50–iii55.

Sun Y, Varambally S, Maher CA, Cao Q, Chockley P, Toubai T, Malter C, Nieves E, Tawara I, Wang Y, Ward PA, Chinnaiyan A, Reddy P. 2011. Targeting of microRNA-142-3p in dendritic cells regulates endotoxin-induced mortality. Blood 117:6172–6183.

Taganov KD, Boldin MP, Chang KJ, Baltimore D. 2006. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci USA 103:12481–12486.

Takeda K, Akira S. 2004. TLR signaling pathways. Semin Immunol 16:3–9.

Takeda K, Kaisho T, Akira S. 2003. Toll-like receptors. Annu Rev Immunol 21:335–376.

Tang B, Xiao B, Liu Z, Li N, Zhu ED, Li BS, Xie QH, Zhuang Y, Zou QM, Mao XH. 2010. Identification of MyD88 as a novel target of miR-155, involved in negative regulation of Helicobacter pylori-induced inflammation. FEBS Lett 584:1481–1486.

Tili E, Michaille JJ, Cimino A, Costinean S, Dumitru CD, Adair B, Fabbri M, Alder H, Liu CG, Calin GA, Croce CM. 2007. Modulation of miR-155 and miR-125b levels following lipopolysaccharide/TNF-alpha stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 179:5082–5089.

Wang F, Ma YL, Zhang P, Shen TY, Shi CZ, Yang YZ, Moyer MP, Zhang HZ, Chen HQ, Liang Y, Qin HL. 2013. SP1 mediates the link between methylation of the tumour suppressor miR-149 and outcome in colorectal cancer. J Pathol 229:12–24.

Wei J, Huang X, Zhang Z, Jia W, Zhao Z, Zhang Y, Liu X, Xu G. 2013. MyD88 as a target of microRNA-203 in regulation of lipopolysaccharide or Bacille Calmette-Guerin induced inflammatory response of macrophage RAW264.7 cells. Mol Immunol 55:303–312.

Wu F, Guo NJ, Tian H, Marohn M, Gearhart S, Bayless TM, Brant SR, Kwon JH. 2011. Peripheral blood microRNAs distinguish active ulcerative colitis and Crohn's disease. Inflamm Bowel Dis 17:241–250.

Xu Z, Xiao SB, Xu P, Xie Q, Cao L, Wang D, Luo R, Zhong Y, Chen HC, Fang LR. 2011. miR-365, a novel negative regulator of interleukin-6 gene expression, is cooperatively regulated by Sp1 and NF-kappaB. J Biol Chem 286:21401–21412.