FEBS Letters

FEBS PRESS science publishing by scientists

MiR-21-5p regulates mycobacterial survival and inflammatory responses by targeting Bcl-2 and TLR4 in *Mycobacterium tuberculosis*-infected macrophages

Zhonghua Zhao, Jinzhu Hao, Xia Li, Yanfang Chen and Xiaoyan Qi

Department of Tuberculosis, Shandong Provincial Chest Hospital, Jinan, China

Correspondence
Z. Zhao, Department of Tuberculosis, Shandong Provincial Chest Hospital, Jinan 250101, Shandong, China
Tel: +8613188870890
E-mail: zhaozhonghuasd@163.com

(Received 6 April 2019, revised 7 May 2019, accepted 8 May 2019, available online 2 June 2019)

doi:10.1002/1873-3468.13438

Edited by Tamas Dalmay

To date, very little is known about the role of microRNA-21-5p (miR-21-5p) in *Mycobacterium tuberculosis* (M.tb)-infected macrophages. Here, we show that M.tb infection of RAW264.7 and THP-1 cells increases the expression of miR-21-5p. MiR-21-5p enhances M.tb survival and apoptosis, and attenuates the secretion of inflammatory cytokines, including interleukin (IL)-1β, IL-6, and tumor necrosis factor-α in M.tb-infected macrophages. Dual-luciferase reporter assay revealed that the 3′-UTR of B-cell lymphoma 2 (Bcl-2) or toll-like receptor 4 (TLR4) is a direct target of miR-21-5p. Enforced expressions of Bcl-2 or TLR4 partially attenuate the suppressive effects of miR-21-5p on cell viability and inflammatory cytokines, and effectively decrease bacterial burden. Therefore, the present study highlights a novel role for miR-21-5p in regulation of mycobacterial survival and inflammatory responses by targeting Bcl-2 and TLR4 in M.tb-infected macrophages.

Keywords: Bcl-2; macrophages; miR-21-5p; *Mycobacterium tuberculosis*; survival; TLR4

Tuberculosis (TB) caused by *Mycobacterium tuberculosis* (M.tb) is one of the main infectious diseases in the world [1,2]. The development of TB is closely related to the pathogenicity of M.tb in the host [3]. Generally, M.tb infects human macrophages and initiates diverse signal transduction pathways, including the nuclear factor-kappa B and mitogen-activated protein kinase signaling pathways, which induces the secretion and release of inflammatory cytokines [4,5]. However, the mechanisms underlying the M.tb survival and inflammatory responses in M.tb-infected macrophages are not fully elucidated. It is vital to elucidate the host-M.tb interactions to develop strategies and therapeutic methods for the control of M.tb.

miRNAs belong to evolutionarily conserved noncoding RNAs with a length of 19–25 nt, and can specifically bind the 3′-UTR of target mRNAs, which can cause inhibition of mRNA translation or degradation [6]. In recent decades, miRNA has also been verified as a potent regulator for the biology of macrophages [7]. More and more studies validated that microRNA-21-5p (miR-21-5p) is implicated with infectious or inflammatory diseases. For example, Ning *et al.* [8] reported that circulating miR-21 was up-regulated in the serum of patients with liver fibrosis and was positively associated with liver fibrosis and oxidation. Loboda *et al.* [9] demonstrated that miR-21 plays a dynamic role in inflammatory responses and accelerates organ failure and fibrosis. However, there have

Abbreviations
Bcl-2, B-cell lymphoma 2; CCK-8, cell counting kit-8; CFU, colony-forming unit; DMEM, dulbecco's modified eagle's medium; HEK293T, human embryonic kidney-293T; IL-1β, interleukin-1β; IL-6, interleukin-6; M.tb, *Mycobacterium tuberculosis*; miR-21-5p, microRNA-21-5p; miR-NC, negative control miRNA; PI, propidium iodide; SD, standard deviation; TB, tuberculosis; TLR4, toll-like receptor 4; TNF-α, tumor necrosis factor-α.

been few studies focusing on the expression and significance of miR-21-5p in M.tb-infected macrophages.

In the present study, we used RAW264.7 and THP-1 cells as *in vitro* macrophage model, and focused on the interactions between macrophages and M.tb, aiming to determine the function of miR-21-5p, and explore its downstream targets in M.tb-infected macrophages.

Materials and methods

Cell culture

The RAW264.7 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were retained in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY, USA), which contained l-glutamine (2 mM), penicillin G (100 $U \cdot mL^{-1}$), 10% FBS (Gibco, Carlsbad, CA, USA), and streptomycin (100 $mg \cdot mL^{-1}$). The cells were incubated in a cell incubator remained at 37 °C and a relative humidity of about 95% with 5% CO_2. The cells before the 30th generation were used for experimental research.

The human monocytic leukemia cell line THP-1 (ATCC) was cultured in RPMI 1640 medium containing 10% FBS and antibiotics. One hundred and sixty nanomolar per milliliter PMA (Sigma, StLouis, MO, Bloomington, MA, USA) was added to the culture to induce macrophage differentiation. After 48 h, the plates were washed thrice with 1 mL of PBS (Beyotime Institute of Biotechnology, Shanghai, China). The adherent macrophages were then washed three times with RPMI 1640 medium and maintained in a 37 °C humidified incubator containing 5% CO_2. Cells before the 30th generation were used for the experiments.

Human embryonic kidney-293T (HEK293T; Cell Bank of the Chinese Academy of Sciences, Shanghai, China) cells were cultured in DMEM. The cells were incubated at 37 °C in an atmosphere of 5% CO_2.

Cell infection

For all experiments, M.tb strains H37Ra and H37Rv (ATCC) were cultured in Middlebrook 7H9 broth with 10% BBL Middlebrook oleic acid-albumin-dextrose-catalase (OADC; BD Biosciences, Franklin Lakes, NJ, USA) at 37 °C under a 5% CO_2 atmosphere. Mid log phase cultures were aliquoted and stored at −80 °C. Mycobacteria were ground to generate a single bacterial suspension in RPMI 1640. Then RAW264.7 and THP-1 cells were placed on 12-well cell culture plates and were continuously infected with H37Ra and H37Rv at different MOIs at indicated time points.

Cell transfection

To up-regulate or down-regulate the expression of miR-21-5p in RAW264.7 and THP-1 cells, the mimics or inhibitors of miR-21-5p obtained from Guangzhou RuiBo Company (Guangzhou, Guangdong, China) were transfected into RAW264.7 and THP-1 cells at 50–60% confluence with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). pcDNA3.1-B-cell lymphoma 2 (Bcl-2), pcDNA3.1-toll-like receptor 4 (TLR4) and their empty vector were obtained from GeneCreate (Wuhan, Hubei, China). Cell transfections were performed using LipofectamineTM 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Quantitative real-time PCR (qRT-PCR) analysis was performed to evaluate the transfection effects.

qRT-PCR assay

The miRNA or mRNA expression was assessed using SYBR® Premix Ex Taq™ II (Tli RNaseH Plus; Takara Bio, Otsu, Shiga, Japan) on Mastercycler ep realplex4 (Eppendorf, Hamburg, Germany). The PCR conditions included an initial step at 95 °C for 120 s, followed by 45 cycles of amplification and quantification (95 °C for 15 s, 60 °C for 15 s), followed by a final extension at 68 °C for 20 s. U6 or GAPDH was used as an internal control. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Full primers are shown in Table 1.

Cell counting kit-8 assay

About 5×10^3 cells were seeded into the 96-well plate and transfected with mimics, inhibitor, or their negative control oligonucleotides, respectively. The culture medium was removed and 100 μL of cell counting kit-8 (CCK-8) medium (Dojindo, Tokyo, Japan) was added into the 96-well plate at indicated time points, and incubated at 37 °C for another 4 h. The absorbance at 450 nm was measured using a microplate reader (Bio-Rad, Richmond, CA, USA).

Table 1. Primers for qRT-PCR assay.

Gene symbol	Primers (5′–3′)
miR-21-5p	F: TGCGCTAGCTTATCAGACTGAT
	R: CCAGTGCAGGGTCCGAGGTATT
U6 SnRNA	F: GCGCGTCGTGAAGCGTTC
	R: GTGCAGGGTCCGAGGT
IL-1β	F: CCACAGACCTTCCAGGAGAATG
	R: GTGCAGTTCAGTGATCGTACAGG
IL-6	F: AGACAGCCACTCACCTCTTCAG
	R: TTCTGCCAGTGCCTCTTTGCTG
TNF-α	F: CTCTTCTGCCTGCTGCACTTTG
	R: ATGGGCTACAGGCTTGTCACTC
GAPDH	F: GTCTCCTCTGACTTCAACAGCG
	R: ACCACCCTGTTGCTGTAGCCAA

Flow cytometry assay

Cell apoptosis was detected using the Annexin V-FITC apoptosis detection kit on the basis of the manufacturer's instructions. In brief, cells of each sample were harvested after 24 h post-treatment and suspended in 500 μL of Annexin V binding buffer (1×). Annexin V-FITC (5 μL) and 5 μL of propidium iodide (PI) were added and incubated for 15 min in dark. The stained cells were evaluated by flow cytometry using a FACS Calibur (BD Biosciences, San Jose, CA, USA).

Colony-forming unit assay

To assay bacterial viability, cells were infected with M.tb at an MOI of 10 for 24 h at 37 °C, then washed six times with RPMI1640 to remove any extracellular bacteria, and incubated in fresh medium for indicated time points, the infected cells were lysed with sterile distilled water containing 0.06% SDS. Homogenates underwent 10-fold-serial dilution and each dilution was inoculated on 7H11 agar plates supplemented with 10% OADC and incubated at 37 °C for 3–4 weeks. Colony-forming units (CFUs) were calculated in triplicate using standard procedures.

ELISA

The concentration of the cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor-α (TNF-α) were determined using commercially available ELISA kits as per the manufacturer's instructions (ProZyme, Hayward, CA, USA). All spectrophotometric readings were performed with a microplate reader, and the concentration was presented as $pg{\cdot}mL^{-1}$ by comparing with respective standard curves. All experiments were repeated at least three times.

Western blot analysis

The Nuclear/cytoplasmic fractionation was separated using Cell Fractionation Kit (Cell Signaling Technology, Danvers, MA, USA) and the whole cell lysates were extracted using RIPA Buffer (Cell Signaling Technology) according to the manufacturer's instructions. Cell lysates with equivalent protein amounts (20 μg) were loaded, thereafter separated using SDS/PAGE, and then transferred to a poly vinylidene difluoride membrane. The membranes were blocked in PBS-Tween20 (pH 7.4, 0.5% Tween20) with 5% BSA (Dingguo Biological Products, Beijing, China), and then incubated overnight with the primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C. Then, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) at room temperature for 1 h, and at last visualized with an ECL kit (KeyGEN, Nanjing, China) according to the manufacturer' instructions.

Dual-luciferase reporter assay

B-cell lymphoma 2 or TLR4 3′UTR was amplified from cDNA of HEK293T cells and inserted into pGL-3 (Promega, Madison WI, USA) vector. The HEK293T cells were cotransfected with the wild-type (WT) 3′UTR of Bcl-2 or TLR4 containing the putative miR-21-5p binding sites and mutant Bcl-2 or TLR4 3′UTR with either negative control miRNA (miR-NC) or miR-21-5p mimics *via* Lipofectamine®2000 (Thermo Fisher Scientific, Inc.). After transfection, the cells were cultivated at 37°C, 5% CO_2 for 4 h. Then, the luciferase activities were confirmed using a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

Statistical analyses

All experiments were conducted no less than three times with each sample tested in triplicate. Data are presented as the mean ± standard deviation (SD). A two-tailed Student's *t*-test or a Mann–Whitney *U*-test was employed to evaluate the difference between the two groups. ANOVA followed by a *post hoc* Dunnett's test was used to determine statistical differences for multiple comparisons. Statistical analysis was performed using SPSS software 20.0 (IBM Corp, Armonk, NY, USA) and GRAPHPAD PRISM 5 software (GraphPad Software, Inc., La Jolla, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

M.tb infection increases miR-21-5p expression in human macrophages

Firstly, RAW264.7 and THP-1 cells were infected with H37Ra or H37Rv at MOI of 0 (PBS), 1, 5, and 10 for 12 h. We found that M.tb infection of RAW264.7 and THP-1 cells increased the expression level of miR-21-5p compared with PBS control (0 MOI) group in a concentration-dependent manner ($P < 0.01$, Fig. 1A). Notably, H37Ra or H37Rv had the most profound effect at an MOI of 10. Then, the miR-21-5p expression level was measured at 12, 24, 36, and 48 h, respectively, and M.tb infection time-dependently increased the miR-21-5p expression level in RAW264.7 and THP-1 cells ($P < 0.01$, Fig. 1B). Cells exhibited the maximum value of expression of miR-21-5p at 24 h; therefore, the macrophages were infected with H37Ra or H37Rv at an MOI of 10 for 24 h in the subsequent experiments. We further examined the effect of M.tb on cell viability using the CCK-8 assay. As shown in

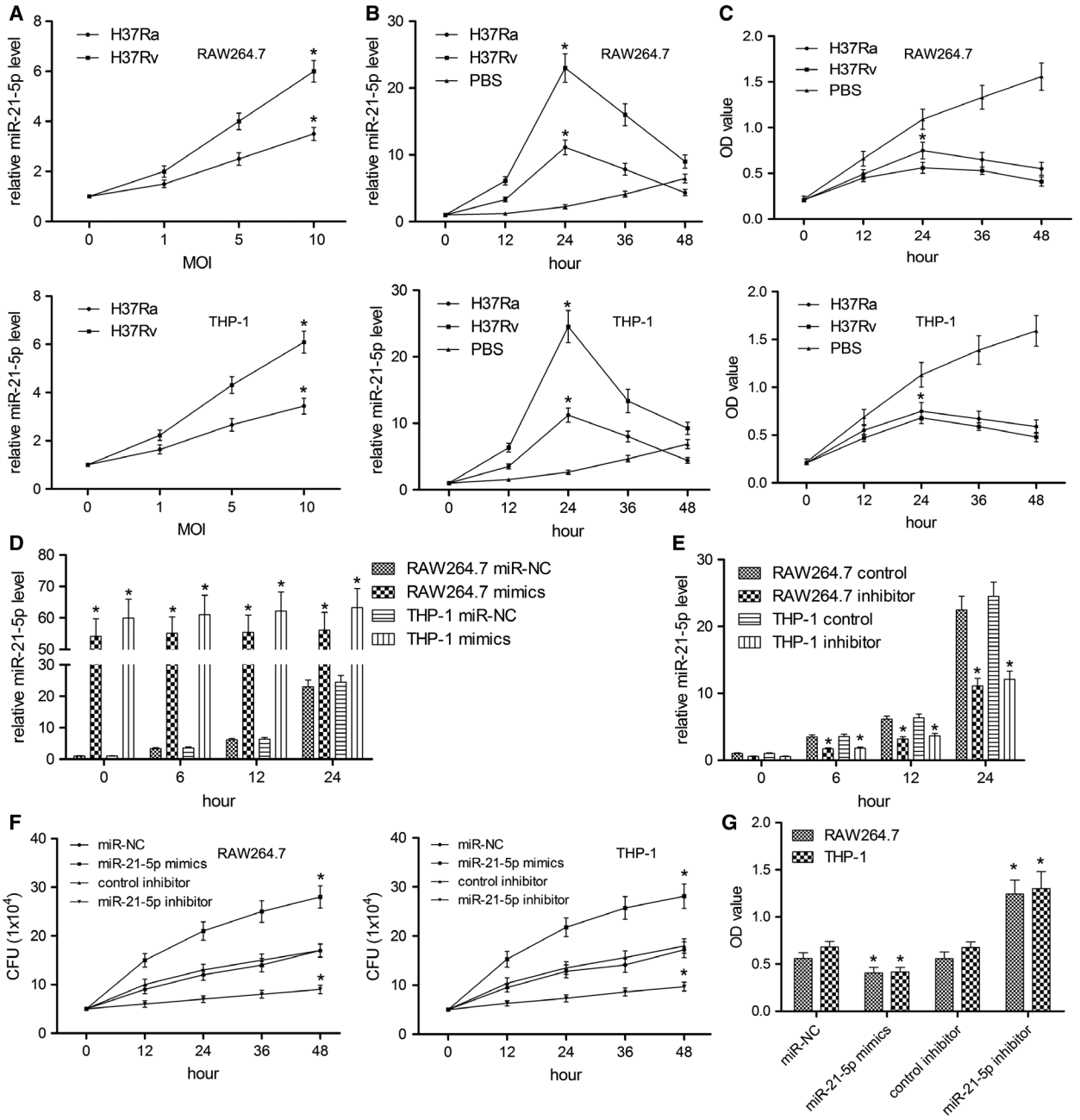

Fig. 1. Expression and effect of miR-21-5p induced by M.tb infection in macrophages. (A) RAW264.7 and THP-1 cells were infected with M.tb H37Ra or H37Rv at MOI of 0 (PBS), 1, 5, and 10 for 12 h; miR-21-5p expression level was measured by qRT-PCR. (B) RAW264.7 and THP-1 cells were infected with M.tb H37Ra or H37Rv at an MOI of 10 for different time points; miR-21-5p expression level was measured using qRT-PCR. (C) Cell viability was detected using the CCK-8 assay. (D) qRT-PCR validated transfection of miR-21-5p mimics in RAW264.7 and THP-1 cells. (E) qRT-PCR validated transfection of miR-21-5p inhibitor in RAW264.7 and THP-1 cells. (F) CFU assays were performed for evaluating the survival of H37Rv in RAW264.7 and THP-1 cells. (G) RAW264.7 and THP-1 cells were transfected with miR-21-5p mimics, or miR-21-5p inhibitor prior to H37Rv treatment at an MOI of 10 for 24 h. Cell viability was measured using the CCK-8 assay. All data represent means ± SD of three independent experiments. $*P < 0.01$ vs miR-NC or control inhibitor group.

Fig. 1C, H37Ra or H37Rv Fig. 1C, H37Ra or H37Rv decreased cell viability in RAW264.7 and THP-1 cells, while PBS control showed normal cell viability ($P < 0.01$). Because H37Rv showed the markedly stronger effect than H37Ra, we selected H37Rv as the subject.

MiR-21-5p promotes the H37Rv survival in human macrophages

To figure out the biological effects of miR-21-5p on the M.tb-infected macrophages, the transient over-expression of miR-21-5p was established through transfection of miR-21-5p mimics (Fig. 1D) and the down-regulation of miR-21-5p over-expression was achieved *via* transfection of miR-21-5p inhibitor in the RAW264.7 and THP-1 cells prior to H37Rv treatment (Fig. 1E). CFU assays were performed for evaluating the survival of H37Rv. We found that miR-21-5p mimics significantly increased the number of CFUs of in H37Rv-infected RAW264.7 and THP-1 cells compared with the miR-NC group ($P < 0.01$, Fig. 1F). On the other hand, a significant decrease in the number of CFUs was also observed in H37Rv-infected RAW264.7 and THP-1 cells transfected with miR-21-5p inhibitor compared with control inhibitor group ($P < 0.01$, Fig. 1F). These results indicated that miR-21-5p significantly enhanced the survival of H37Rv in RAW264.7 and THP-1 cells.

MiR-21-5p affects H37Rv-infected macrophage viability and apoptosis

We further examined the effect of miR-21-5p on H37Rv-infected macrophage viability and apoptosis. RAW264.7 and THP-1 cells were transfected with miR-21-5p mimics, or miR-21-5p inhibitor prior to H37Rv treatment at an MOI of 10 for 24 h. Cell viability was measured using the CCK-8 assay. The apoptotic cell percentages were assessed using the Annexin V-FITC/PI staining assay. As shown in Fig. 1G, over-expression of miR-21-5p decreased cell viability in RAW264.7 and THP-1 cells, but inhibition of miR-21-5p increased cell viability ($P < 0.01$). As shown in Fig. 2A, compared with the control inhibitor group, H37Rv-infected RAW264.7 and THP-1 cells with miR-21-5p inhibitor showed lower apoptotic cell percentages ($P < 0.01$). Conversely, miR-21-5p mimics showed markedly higher apoptotic cell percentages compared with miR-NC ($P < 0.01$).

MiR-21-5p influences inflammatory cytokine secretion

In this work, the effects of miR-21-5p on inflammatory cytokines in H37Rv-infected RAW264.7 and THP-1 cells were examined. The qRT-PCR results showed that H37Rv infection alone significantly increased the mRNA expression levels of IL-1β, IL-6, and TNF-α in RAW264.7 and THP-1 cells, and miR-21-5p mimics suppressed the mRNA expression levels of IL-1β, IL-6, and TNF-α in the H37Rv-infected macrophages ($P < 0.01$, Fig. 2B–D). However, knockdown of miR-21-5p increased the mRNA expression levels of IL-1β, IL-6, and TNF-α in the H37Rv-infected macrophages ($P < 0.01$, Fig. 2B–D).

Using ELISA, we measured the secretion of inflammatory cytokines in the medium of RAW264.7 and THP-1 cells at 24 h after H37Rv infection. ELISA results showed that the levels of IL-1β, IL-6, and TNF-α were greatly up-regulated in the medium containing H37Rv-infected RAW264.7 and THP-1 cells with miR-21-5p inhibitor compared with the control inhibitor group ($P < 0.01$, Fig. 2E–G). In addition, the secretion levels of IL-1β, IL-6, and TNF-α were markedly repressed by miR-21-5p mimics in H37Rv–infected RAW264.7 and THP-1 cells compared with the miR-NC group ($P < 0.01$, Fig. 2E–G).

Bcl-2 or TLR4 is a direct target of miR-21-5p

In this work, we conducted bioinformatics analysis to predict the target genes of miR-21-5p using TargetScan and miRanda. Among these predicted target genes, Bcl-2 or TLR4 was notable due to their roles in cell viability and inflammatory response. Next, we carried out a dual-luciferase reporter assay to determine whether miR-21-5p targeted the 3′-UTR of Bcl-2 or TLR4 directly. We transfected HEK293T cells with a luciferase plasmid containing the WT or mutant 3′-UTRs of Bcl-2 or TLR4 in the pGL-3 vector. The results showed that the co-transfection of WT 3′-UTRs of Bcl-2 and miR-21-5p mimics into HEK293T cells led to a decrease of up to 65% in luciferase activity ($P < 0.01$, Fig. 3A), but the mutant reporter had nearly no effect on luciferase activity ($P > 0.05$, Fig. 3B).

To further confirm this interaction, we performed western blot to detect the expressions of Bcl-2 and TLR4 in the RAW264.7 and THP-1 cells transfected with miR-21-5p mimics, miR-21-5p inhibitor, and their respective controls. The results showed that miR-21-5p mimics strongly inhibited the expressions of Bcl-2 and TLR4 ($P < 0.01$, Fig. 3C), while in the miR-21-5p inhibitor group, the expressions of Bcl-2 and TLR4 were up-regulated than those in the control inhibitor group ($P < 0.01$, Fig. 3C).

Bcl-2 reverses the effects of miR-21-5p on the H37Rv survival and cell viability

Furthermore, we determined *in vitro* functional role of Bcl-2 in M.tb-infected RAW264.7 and THP-1 cells. Macrophages were transfected with Bcl-2-overexpressing

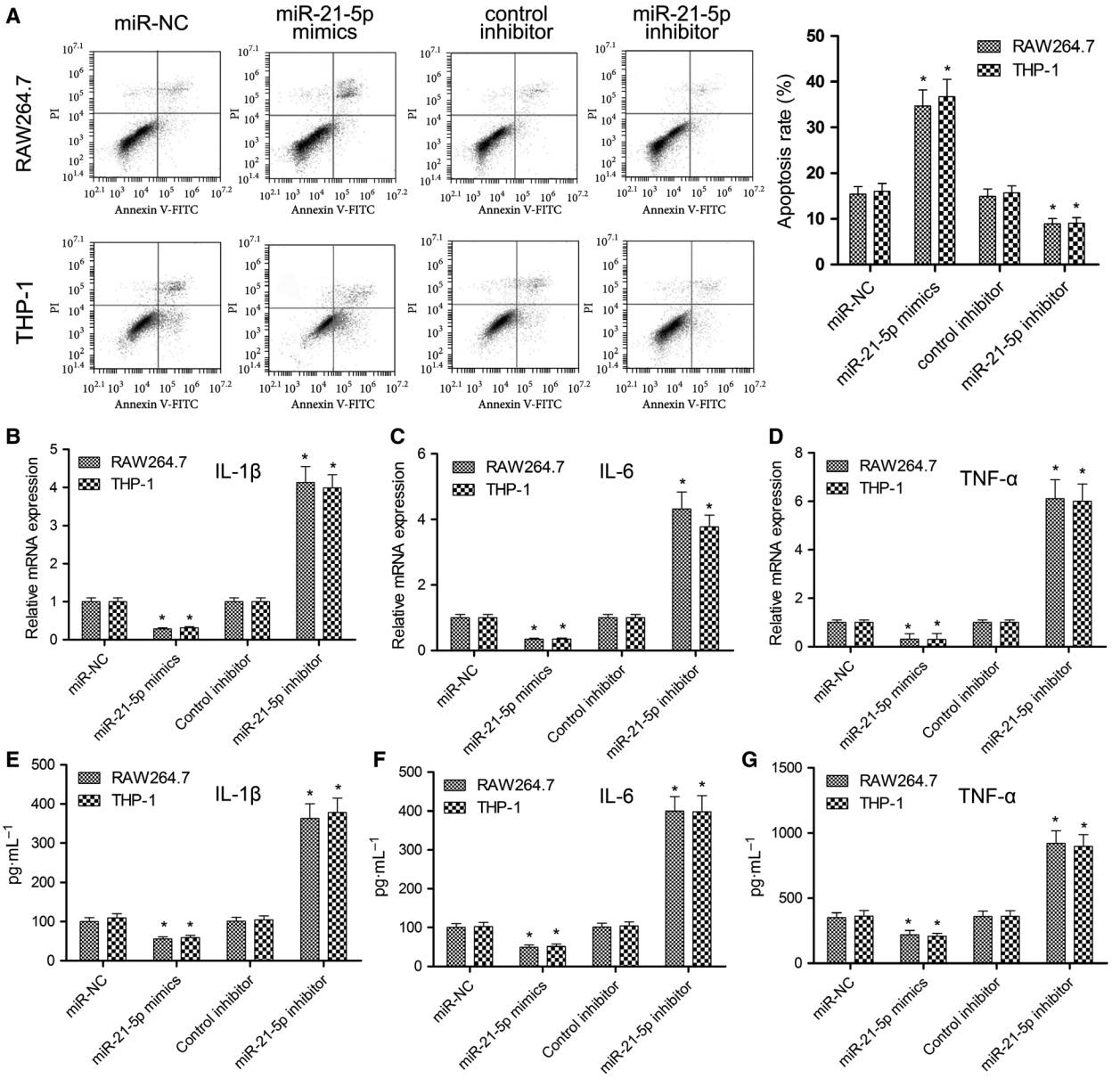

Fig. 2. MiR-21-5p influences cell apoptosis and inflammatory cytokine secretion in M.tb-infected macrophages. RAW264.7 and THP-1 cells were transfected with miR-21-5p mimics, or miR-21-5p inhibitor prior to H37Rv treatment at an MOI of 10 for 24 h. (A) The apoptotic cell percentages were assessed using the Annexin V-FITC/PI staining assay at 24 h postinfection. The mRNAs of inflammatory cytokines IL-1β (B), IL-6 (C), and TNF-α (D) were examined using qRT-PCR. The secretion of inflammatory cytokine IL-1β (E), IL-6 (F), and TNF-α (G) was examined using ELISA. All data represent means ± SD of three independent experiments. *$P < 0.01$ vs miR-NC or control inhibitor group.

vector pcDNA3.1-Bcl-2 (Fig. 4A,B). Cell viability was measured using the CCK-8 assay. CFU assays were performed for evaluating the survival of H37Rv. The CCK-8 assay showed that over-expression of Bcl-2 significantly improved miR-21-5p mimics-repressed cell viability compared with the vector control group ($P < 0.01$, Fig. 4C). In addition, over-expression of Bcl-2 also enhanced miR-21-5p inhibitor-increased cell viability compared with the vector control group ($P < 0.01$, Fig. 4D). CFU assays demonstrated that over-expression of Bcl-2 markedly repressed miR-21-5p mimics-increased H37Rv survival compared with the vector control group ($P < 0.01$, Fig. 4E). In addition, over-expression of Bcl-2 also aggravated miR-21-5p inhibitor-induced inhibition of H37Rv survival compared with the vector control group ($P < 0.01$, Fig. 4F).

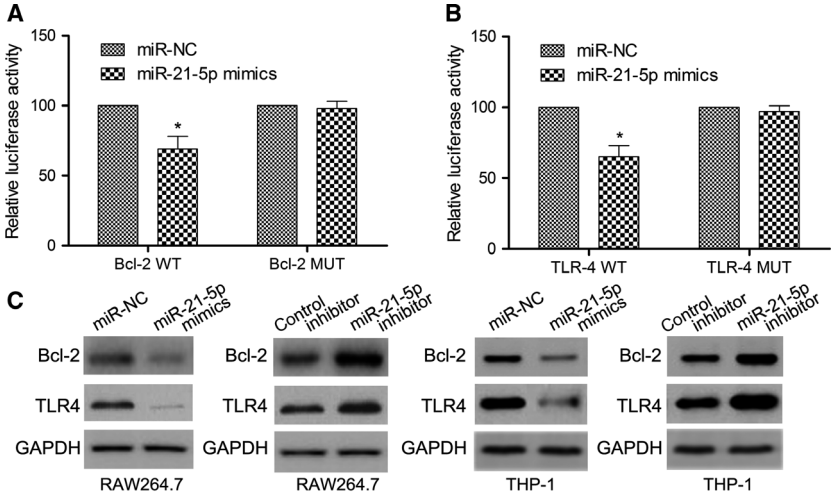

Fig. 3. Bcl-2 or TLR4 is a direct target of miR-21-5p. (A) Dual-luciferase reporter assays helped determine the effect of miR-21-5p on the WT or MUT 3′-UTR activity of Bcl-2 in HEK293T. (B) Dual-luciferase reporter assays helped determine the effect of miR-21-5p on the WT or MUT 3′-UTR activity of TLR4 in HEK293T. (C) RAW264.7 and THP-1 cells were transfected with miR-21-5p mimics, or miR-21-5p inhibitor prior to H37Rv treatment at an MOI of 10 for 24 h. Western blot was used to detect the expressions of Bcl-2 and TLR4 proteins. Data of A and B are presented as mean ± SD of three independent experiments. $*P < 0.01$ vs miR-NC or control inhibitor group.

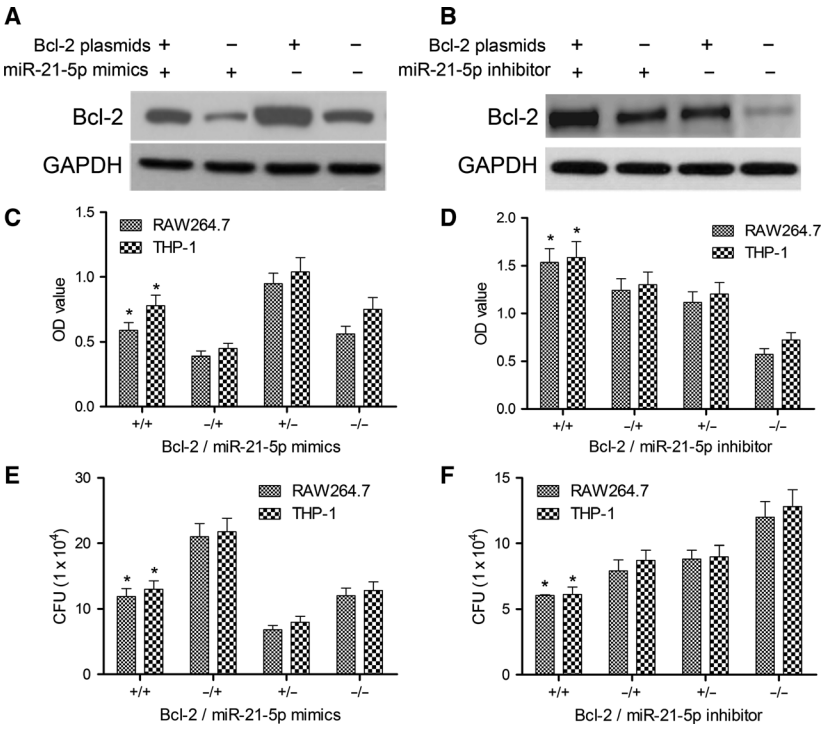

Fig. 4. Bcl-2 over-expression reverses the effects of miR-21-5p. (A, B) Macrophages were transfected with pcDNA3.1-Bcl-2, and then western blot was used to detect the expression of Bcl-2. (C, D) Cell viability was detected using the CCK-8 assay. (E, F) CFU assays were performed for evaluating the survival of H37Rv. Data of C–F are presented as mean ± SD of three independent experiments. $*P < 0.01$ vs miR-NC or control inhibitor group.

TLR4 attenuates the repressive effects of miR-21-5p on inflammatory cytokines

To identify whether miR-21-5p regulated the expressions of inflammatory cytokines by regulating TLR4, we further investigated the effect of TLR4 over-expression using pcDNA3.1-TLR4. H37Rv–infected RAW264.7 and THP-1 cells were transfected with miR-21-5p mimics, miR-21-5p inhibitor and their

respective controls, and then further treated with pcDNA3.1-TLR4 or its vector control (Fig. 5A,5). We found that up-regulation of TLR4 ameliorated the inhibitory effects of the miR-21-5p mimics on the secretion of IL-1β, IL-6, and TNF-α (Fig. 5C,5). Up-regulation of TLR4 further enhanced miR-21-5p inhibitor-induced the secretion of IL-1β, IL-6, and TNF-α (Fig. 5C,5). Overall, these data demonstrate that miR-21-5p regulates inflammatory cytokine secretion by targeting TLR4 in H37Rv -infected macrophages.

Discussion

Emerging evidence has shown that miR-21-5p is expressed in some cell types and its expression closely relates to inflammation, antiviral response and adaptive immunity. Several microbial infections can up-regulate miR-21-5p expression. Ning *et al.* [10] demonstrated that inhibition of miR-21 can inhibit inflammation and promote the recovery of spinal cord injury by down-regulating IL-6R/JAK-STAT signaling pathway.

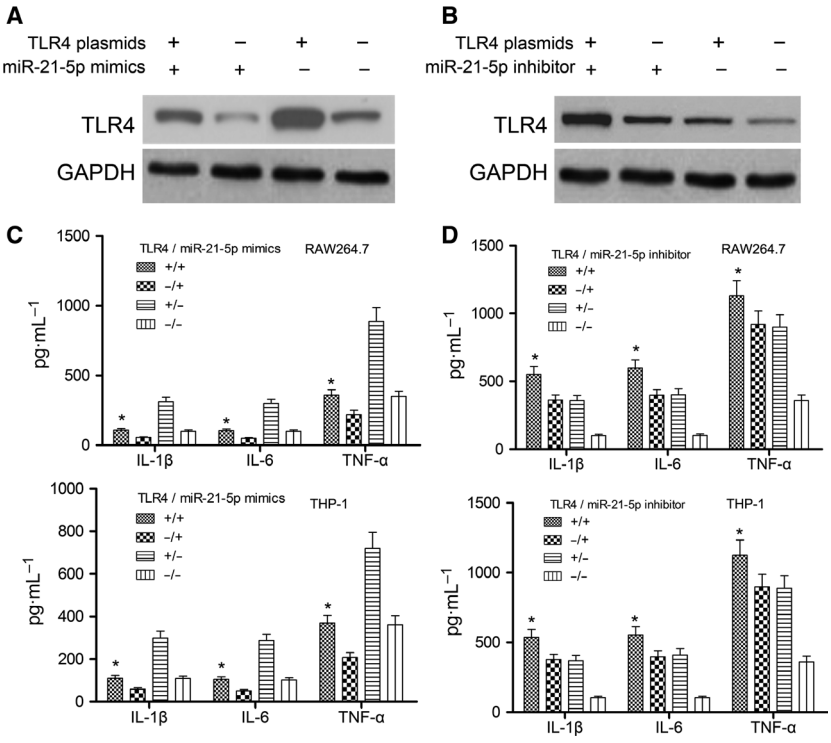

Fig. 5. TLR4 over-expression attenuates the repressive effects of miR-21-5p on inflammatory cytokines. H37Rv-infected RAW264.7 and THP-1 cells were transfected with miR-21-5p mimics, miR-21-5p inhibitor and their respective controls, and then further treated with pcDNA3.1-TLR4 or its vector control. (A, B) western blot was used to detect the expression of TLR4. (C, D) The secretion of inflammatory cytokines (IL-1β, IL-6, and TNF-α) was examined using ELISA. Data of C and D are presented as mean ± SD of three independent experiments. $^{*}P < 0.01$ vs miR-NC or control inhibitor group.

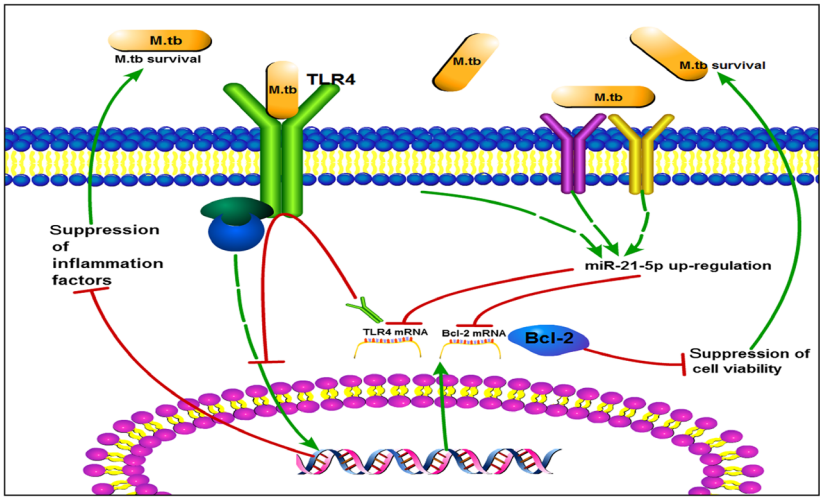

Fig. 6. Schematic representation of our study illustrating how miR-21-5p regulates M.tb survival and inflammatory responses by targeting Bcl-2 and TLR4 respectively in M.tb-infected macrophages.

Chen *et al.* [11] suggested that down-regulation of miR-21 reduced podocyte apoptosis in diabetic nephropathy by regulating TIMP3 expression and inhibiting inflammation. Yang *et al.* [12] found that miR-21 attenuated inflammation and maladaptive remodeling, and suggested that miR-21 may function as a novel potential therapeutic target for myocardial infarction. Nevertheless, whether miR-21-5p regulates M.tb-induced inflammatory responses in macrophages remains largely unknown. To this end, we decided to investigate the role of miR-21-5p in the regulation of the M.tb survival and inflammation of macrophage within the context of M.tb infection.

Our results showed that miR-21-5p was actively manipulated by M.tb to ensure its survival in macrophages, and miR-21-5p inhibited cell viability and promoted cell apoptosis in the M.tb-infected macrophages. Similarly, Ghorpade *et al.* [13] reported that over-expression of miR-155 in RAW264.7 cells promoted apoptosis and increased intracellular bacterial load. In addition, Zhang *et al.* [14] reported that enhanced miR-32-5p expression promoted the survival of M.tb in human macrophages infected with M.tb. Maybe, miR-21-5p was induced by M.tb *via* multiple pathways. In addition, over-expression of miR-21-5p attenuated the secretion of inflammatory cytokines in the M.tb-infected macrophages. Our results suggested that miR-21-5p plays a vital role in the regulation of the M.tb-induced inflammatory response.

It is well known that the Bcl-2 family can affect the balance between cellular apoptosis and autophagy. There are antiapoptotic Bcl-2 family members such as Bcl-2 that effectively block apoptosis [15]. Till now, the role of Bcl-2 in the inhibition of macrophages apoptosis has been widely reported [16–18]. In this study, dual-luciferase reporter assay showed that the 3′-UTR of Bcl-2 was a direct target of miR-21-5p, and miR-21-5p negatively regulated the expressions of Bcl-2. In addition, enforced expression of Bcl-2 significantly attenuated the suppressive effects of miR-21-5p on cell viability, inhibited cell apoptosis, and prevented M.tb survival, which has been shown in the schematic representation (Fig. 6).

TLR4 is increased during infection and inflammation in immune cells [19]. While TLR4 recognizes pathogens, stimulates phagocytosis, and coordinates innate and adaptive immunity, activation of TLR4 leads to exaggerated inflammation and tissue injury [20]. *Mycobacterium tuberculosis* triggers an inflammatory immune response *via* the activation of TLRs [21]. However, in the absence of TLR signaling, M.tb grows unchecked to cause systemic infection [22]. In the present study, we found that miR-21-5p negatively regulated the expression of TLR4 and enforced expression of TLR4 attenuated the repressive effects of miR-21-5p on the secretion of inflammatory cytokines in the M.tb-infected macrophages. These results may suggest that miR-21-5p regulates the M.tb-induced inflammatory response partially *by* targeting TLR4, which has been shown in the schematic representation (Fig. 4E).

In conclusion, the present study demonstrated that the miR-21-5p was induced by M.tb infection, and miR-21-5p accelerated macrophage apoptosis and increased M.tb survival partially by targeting Bcl-2 and TLR4. These findings offer exciting new therapeutic targets for alternative host-specific adjunct therapies in TB treatment.

Acknowledgment

We are thankful to the other members in our lab for their suggestions.

Author contributions

ZZ and JH designed the study, analyzed the results and revised the manuscript. JH, XL and YC performed the experiments and wrote the manuscript. YC and XQ analyzed the data and prepared the Figures. All authors reviewed the manuscript.

References

1 Gagneux S (2018) Ecology and evolution of *Mycobacterium tuberculosis*. *Nat Rev Microbiol* **16**, 202–213.
2 Pai M, Behr MA, Dowdy D, Dheda K, Divangahi M, Boehme CC, Ginsberg A, Swaminathan S, Spigelman M, Getahun H, *et al.* (2016) Tuberculosis. *Nat Rev Dis Primers* **2**, 16076.
3 Cambier CJ, Falkow S and Ramakrishnan L (2014) Host evasion and exploitation schemes of *Mycobacterium tuberculosis*. *Cell* **159**, 1497–1509.
4 Yu Z, Zhang C, Zhou M, Li Q, Li H, Duan W, Li X, Feng Y and Xie J (2017) *Mycobacterium tuberculosis* PPE44 (Rv2770c) is involved in response to multiple stresses and promotes the macrophage expression of IL-12 p40 and IL-6 via the p38, ERK, and NF-κB signaling axis. *Int Immunopharmacol* **503**, 19–329.
5 Wang CH, Chou PC, Chung FT, Lin HC, Huang KH and Kuo HP (2017) Heat shock protein70 is implicated in modulating NF-κB activation in alveolar macrophages of patients with active pulmonary tuberculosis. *Sci Rep* **7**, 1214.
6 Eulalio A, Huntzinger E and Izaurralde E (2008) Getting to the root of miRNA-mediated gene silencing. *Cell* **132**, 9–14.

7 Squadrito ML, Etzrodt M, De Palma M and Pittet MJ (2013) MicroRNA-mediated control of macrophages and its implications for cancer. *Trends Immunol* **34**, 350–359.
8 Ning ZW, Luo XY, Wang GZ, Li Y, Pan MX, Yang RQ, Ling XG, Huang S, Ma XX, Jin SY, *et al*. (2017) MicroRNA-21 mediates angiotensin II-induced liver fibrosis by activating NLRP3 inflammasome/IL-1β axis via targeting Smad7 and Spry1. *Antioxid Redox Signal* **27**, 1–20.
9 Loboda A, Sobczak M, Jozkowicz A and Dulak J (2016) TGF-β1/Smads and miR-21 in renal fibrosis and inflammation. *Mediators Inflamm* **2016**, 8319283.
10 Ning SL, Zhu H, Shao J, Liu YC, Lan J and Miao J (2019) MiR-21 inhibitor improves locomotor function recovery by inhibiting IL-6R/JAK-STAT pathway-mediated inflammation after spinal cord injury in model of rat. *Eur Rev Med Pharmacol Sci* **23**, 433–440.
11 Chen X, Zhao L, Xing Y and Lin B (2018) Down-regulation of microRNA-21 reduces inflammation and podocyte apoptosis in diabetic nephropathy by relieving the repression of TIMP3 expression. *Biomed Pharmacother* **108**, 7–14.
12 Yang L, Wang B, Zhou Q, Wang Y, Liu X, Liu Z and Zhan Z (2018) MicroRNA-21 prevents excessive inflammation and cardiac dysfunction after myocardial infarction through targeting KBTBD7. *Cell Death Dis* **9**, 769.
13 Ghorpade DS, Leyland R, Kurowska-Stolarska M, Patil SA and Balaji KN (2012) MicroRNA-155 is required for *M. bovis* BCG mediated apoptosis of macrophages. *Mol Cell Biol* **32**, 2239–2253.
14 Zhang ZM, Zhang AR, Xu M, Lou J and Qiu WQ (2017) TLR-4/miRNA-32-5p/FSTL1 signaling regulates mycobacterial survival and inflammatory responses in *Mycobacterium tuberculosis*-infected macrophages. *Exp Cell Res* **3523**, 13–321.
15 Kale J, Osterlund EJ and Andrews DW (2018) BCL-2 family proteins: changing partners in the dance towards death. *Cell Death Differ* **25**, 65–80.
16 Sun XX, Zhang SS, Dai CY, Peng J, Pan Q, Xu LF and Ma XL (2017) LukS-PV-regulated MicroRNA-125a-3p promotes THP-1 macrophages differentiation and apoptosis by down-regulating NF1 and Bcl-2. *Cell Physiol Biochem* **44**, 1093–1105.
17 Halder P, Kumar R, Jana K, Chakraborty S, Ghosh Z, Kundu M and Basu J (2015) Gene expression profiling of *Mycobacterium tuberculosis* Lipoarabinomannan-treated macrophages: A role of the Bcl-2 family member A1 in inhibition of apoptosis in mycobacteria-infected macrophages. *IUBMB Life* **67**, 726–736.
18 Casalino-Matsuda SM, Nair A, Beitel GJ, Gates KL and Sporn PH (2015) Hypercapnia inhibits autophagy and bacterial killing in human macrophages by increasing expression of Bcl-2 and Bcl-xL. *J Immunol* **194**, 5388–5396.
19 Qureshi ST, Gros P and Malo D (1999) Host resistance to infection: genetic control of lipopolysaccharide responsiveness by TOLL-like receptor genes. *Trends Genet* **15**, 291–294.
20 Underhill DM and Goodridge HS (2012) Information processing during phagocytosis. *Nat Rev Immunol* **12**, 492–502.
21 Mortaz E, Adcock IM, Tabarsi P, Masjedi MR, Mansouri D, Velayati AA, Casanova JL and Barnes PJ (2015) Interaction of pattern recognition receptors with *Mycobacterium tuberculosis*. *J Clin Immunol* **35**, 1–10.
22 van der Vaart M, van Soest JJ, Spaink HP and Meijer AH (2013) Functional analysis of a zebrafish myd88 mutant identifies key transcriptional components of the innate immune system. *Dis Model Mech* **6**, 841–854.