European Journal of Inflammation

Original Research Article

European Journal of Inflammation
Volume 16: 1–10

DOI: 10.1177/2058739218798463
journals.sagepub.com/home/eji
SAGE

Baicalin attenuates lipopolysaccharide-induced pro-inflammatory cytokine expression in murine macrophage cell line RAW264.7 through miR-124-STAT3 axis

Bin Tang,[1] Xixi Wang,[2,3] Yuqing Zhu,[1] Xuhui Li[3] and Shukun Yao[4]

Abstract

Baicalin, a flavonoid isolated from *Scutellaria baicalensis* Georgi, has shown a wide range of anti-inflammatory, antioxidative, antiviral, and antitumor properties. However, the molecular mechanism of how baicalin exerts its effects, especially on inflammation regulation, has not been fully investigated. In this article, we report the effects of baicalin on the mouse macrophage cell line RAW264.7. Our results demonstrate that baicalin inhibits the production of inflammatory factors interleukin-6 and tumor necrosis factor-alpha upon lipopolysaccharide stimulation of macrophages. We observed that baicalin inhibits STAT3 activation through retarding its expression and phosphorylation. Interestingly, baicalin treatment promotes the elevation of miR-124 in lipopolysaccharide-treated macrophages. Overexpression of the miR-124 mimic in RAW264.7 reduced STAT3 expression and phosphorylation. Furthermore, inhibition of miR-124 attenuated the dysregulation of STAT3 and reduction of inflammatory factors upon baicalin treatment. Our results revealed the molecular mechanism that baicalin attenuates pro-inflammatory cytokine production through miR-124-STAT3 signaling pathway, suggesting that miR-124 is an important modulator in regulating anti-inflammation by baicalin in macrophages.

Keywords

baicalin, cytokine, inflammation, macrophage, miR-124, STAT3

Date received: 1 March 2018; accepted: 25 July 2018

Background

Baicalin (BA; 7-glucuronic acid, 5, 6-dihydroxyflavone, Figure 1(a)) has been demonstrated to exhibit anti-inflammatory and antioxidative effects on macrophages, lymphocytes, and leukocytes.[1,2] For example, BA inhibits T cell proliferation and suppresses the production of several inflammatory mediators.[3] In addition, BA inhibits the activation of leukocytes by preventing leukocyte adhesion.[4] Moreover, BA decreases production of pro-inflammatory factors and attenuates inflammation in an in vivo model of edema.[5]

Macrophages play important roles in regulating innate and chronic inflammation and have been

[1]International Medical Department, China-Japan Friendship Hospital, Beijing, China
[2]School of Basic Medicine, Beijing University of Chinese Medicine, Beijing, China
[3]Zhejiang Provincial Key Laboratory of Applied Enzymology, Yangtze Delta Region Institute of Tsinghua University, Zhejiang, Jiaxing, China
[4]Department of Gastroenterology, China-Japan Friendship Hospital, Beijing, China

Corresponding authors:
Xuhui Li, Zhejiang Provincial Key Laboratory of Applied Enzymology, Yangtze Delta Region Institute of Tsinghua University, Zhejiang, 705 Yatai Road, Jiaxing 314006, Zhejiang, China.
Email: lixuhui07@mails.tsinghua.edu.cn

Shukun Yao, Department of Gastroenterology, China-Japan Friendship Hospital, No. 2 Yinghua East Road, Beijing 100029, China.
Email: yao_sk@163.com

reported as important biological targets of BA.[6] Macrophages produce a wide range of inflammatory factors that participate in both beneficial and detrimental aspects of inflammation. Macrophages mainly respond to Gram-negative bacteria and their associated endotoxins (e.g. lipopolysaccharides (LPS); Lipid A) through Toll-like receptor (TLR) expressed on their surface.[7] LPS can trigger macrophages to produce a variety of inflammation mediators, including NO, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), prostaglandin E2, and thromboxane A2.[8] BA has been shown to exhibit inhibitory effects on inducible nitric oxide synthase (iNOS) expression and NO release in mouse macrophage cell line RAW264.7.[9] In addition, BA can inhibit the production of TNF-α and IL-6 by macrophages in macrophage-mediated diseases.[10]

STAT3, a major member of STAT family, is constitutively activated in inflamed cells and plays a pivotal role in cellular differentiation, proliferation, transcription, and survival.[11,12] Dysregulation of STAT3 is crucial in the initiation and maintenance of several inflammatory diseases. BA has been reported to function through nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways in macrophages;[9] however, the effects and detailed molecular mechanisms of BA on STAT3 regulation in macrophages have not been fully investigated until now.

MicroRNAs (miRNAs and miRs) are a class of small, noncoding, and single-stranded RNAs, which usually regulate gene expression through binding to the 3′-untranslated region (3′-UTR) of target messenger RNAs (mRNAs).[13] Recent reports indicate that most of the physiological and pathological processes, including inflammatory responses, are modulated by miRNAs at multiple levels.[14] Among them, functions of miRNAs in immunity by regulating immune cell differentiation, commitment, and maturation have been reported.[15,16] For example, miR-146a was able to regulate the immune responses of macrophages through the TLR signaling pathway.[17] In addition, miR-149 was able to negatively regulate TLR-triggered inflammatory responses in RAW264.7 cells in response to mycobacterium bovis Bacillus Calmette–Guérin (BCG) infection or LPS stimulation.[18]

miR-124 has also been reported to be involved in regulation of macrophages by inhibiting LPS-induced production of pro-inflammatory cytokines.[19,20] Previous reports have described how miR-124 targets STAT3 to inhibit the inflammatory response as well as tumor progression.[21,22] In this article, we have investigated the effects and molecular mechanism of BA on modulating the pro-inflammatory cytokine expression in RAW264.7 cells through the miR-124-STAT3 axis. Our results indicated that miR-124 is an important modulator in regulating the effects of BA on macrophages.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), penicillin, and streptomycin were purchased from Gibco (Carlsbad, CA, USA). BA and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). The compounds were diluted in dimethyl sulfoxide (DMSO; final concentration was $<0.1\%$) unless otherwise mentioned. Antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA) if not mentioned.

Cell culture and cell viability assay

The mouse macrophage cell line, RAW264.7, was obtained from the American Type Culture Collection. The cell viability was assayed in 96-well plates. Briefly, 1×10^5 RAW264.7 cells were seeded in 96-well plates in DMEM medium supplemented with 5% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. After incubating at 37°C for 2 h, cells were replaced with fresh medium and treated with indicated concentrations of BA for 24 h. Then, the cell viabilities were determined using a Cell Counting Kit-8 assay (5 mg/mL for 3 h incubation; Dojindo Laboratories, Kumamoto, Japan).

miRs transfection in RAW264.7 cells

RAW264.7 was cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified incubator with 5% CO_2. When the confluence of RAW264.7 cells reached 85%, the cells were plated in six-well plates and supplemented with 2 μg plasmid per well. Cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according

to the manufacturer's instructions; 24 h after transfection, cells were viewed with a Leica DMIL fluorescence microscope and the following western blotting assays. Six random fields in each well (magnification 40×) were chosen for fluorescent analysis.

The miR-124 mimic (UAAGGCAAGAGGUG AAUGCCCAUU CACCGCGUGCCUUAUU), the control mimic (UUCUCCGAA GUGUCAC GUTTACGUGACACGUUCGGAGAATT), and the miR-124 inhibitor (GGCAUUCACCGCG UGCCUUA) were commercial products from Invitrogen.

RNA extraction and real-time quantitative reverse transcription polymerase chain reaction

Total RNA was extracted with TRIzol reagent and then synthesized to complementary DNA (cDNA) following the manufacturer's instructions (Invitrogen). Detection of the mature form of miRNAs was performed using the Hairpin-it miRNAs quantitative polymerase chain reaction (qPCR) Quantitation Assay as previously reported,[23] according to the manufacturer's instructions (GenePharma, Shanghai, China). The U6 small nuclear RNA was used as an internal control. The sequence of the primer for mmu-miR-124 (GenePharma) is given in the following:

5′-CUCUGCGUGUUCACAGCGGACCUUG AUUUAAU GUCUAUACAAUUAAGGCAC GCGGUGAAUGCCAAGAG-3′

Enzyme-linked immunosorbent assay measurements

The amount of IL-6 and TNF-α in the supernatants of macrophages was measured using enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, San Diego, CA, USA) as mentioned previously.[24] Briefly, 1×10^5 RAW264.7 cells were seeded in 96-well plates in DMEM medium supplemented with 5% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. After incubating at 37°C for 2 h, cells were replaced with fresh medium and challenged with 100 ng/mL LPS for 24 h. Then, cells were treated with 1, 5 and 20 μM BA in the presence of LPS. After 24 h, 100 μL culture medium was collected and used for the ELISA assay.

3′-UTR luciferase reporter assays

The 3′-UTR luciferase reporter plasmid of STAT3 was constructed by cloning the 3′-UTR region of the corresponding mRNA into the SpeI and HindIII sites of the pGL3-promoter plasmid (Promega, Madison, WI, USA) as previously reported.[19] The primers for amplifying the 3′-UTR of STAT3 were 5′-GAC TAGTCTCCTTTTTCAGCAGCTCGGG GTTGG-3′ (forward) and 5′-CCCAAGCTTCAGT TCGTCTTGGAGACAATGTCCTCACTGC-3′ (reverse). The 3′-UTR luciferase reporter plasmid of STAT3 and the miR-124 mimic were co-transfected into HEK-293T cells with Lipofectamine 2000 reagent according to the manufacturer's instructions (Invitrogen). After 24 h, cells were collected and luciferase activities were measured using the Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

Western blotting analysis to detect STAT3

RAW264.7 cells were challenged with 100 ng/mL LPS for 24 h. Then, cells were treated with 1 μM BA for 24 h in the presence of LPS. Next, cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and lysed on ice for 30 min in lysis buffer containing 20 mM Tris (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 135 mM NaCl, 2 mM dithiothreitol (DTT), 2 mM sodium pyrophosphate, 25 mM β-glycerophosphate, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Lysates were centrifuged (12,000×*g*) at 4°C for 15 min. Equal amounts of soluble proteins were electrophoresed on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene difluoride (PVDF) membranes. Protein levels and phosphorylation changes of STAT3 were detected using monoclonal anti-STAT3 and anti-phospho-Tyr705 STAT3 antibodies (1:1000; Cell Signaling Technology), respectively. β-actin (1:1000; Cell Signaling Technology) was used as a loading control. Membranes were rinsed with tris-buffered saline with Tween® 20 (TBST) for four times (10 min/time) and then incubated with the horseradish peroxidase–bound secondary antibody (1:5000) in a shaker for 1 h at room temperature. Finally, membranes were washed for four times

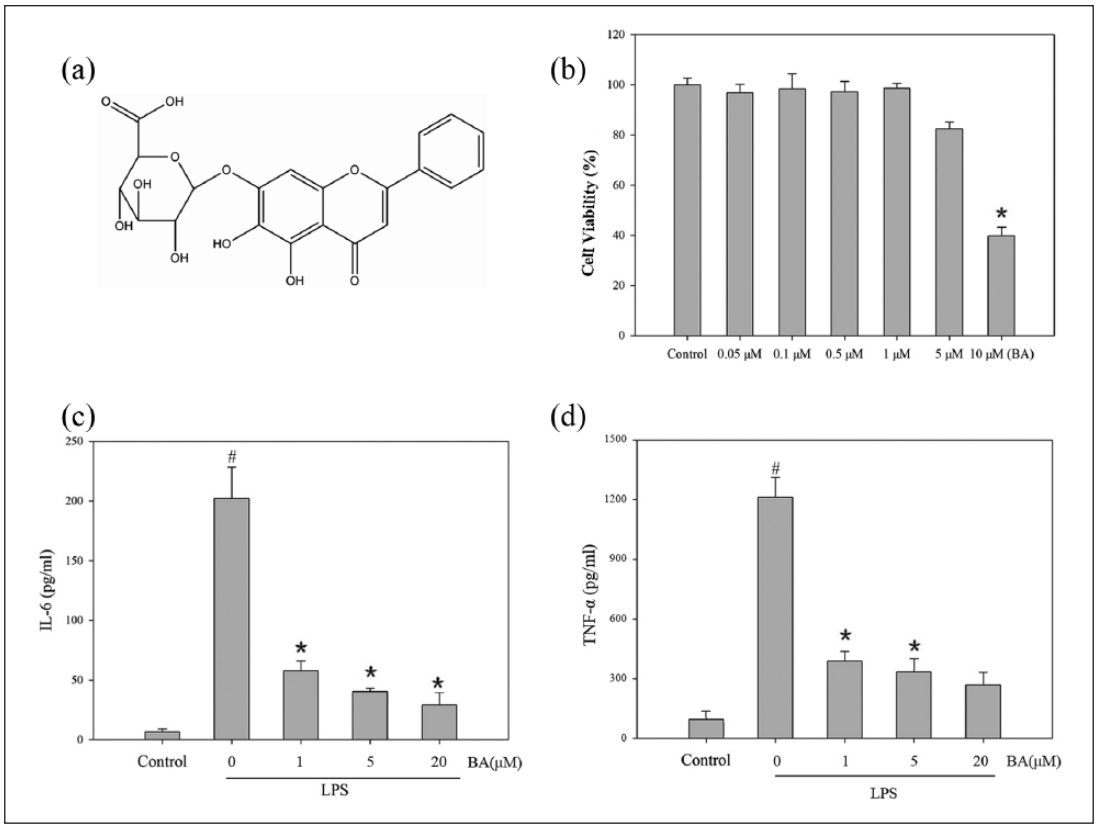

Figure 1. BA inhibits LPS-induced IL-6 and TNF-α production. (a) Chemical structure of BA and (b) toxicity assay of macrophages treated with BA. Murine macrophage RAW264.7 cells were cultured in the presence of BA (0.05, 0.1, 0.5, 1, 5, or 10 μM). The cellular toxicity is expressed as the percentage of OD450 value of each concentration of BA with control (DMSO). Data are presented as mean ± SD (*$P < 0.05$ vs DMSO). (c and d) ELISA analysis of LPS-induced IL-6 and TNF-α production in the presence of BA. RAW264.7 cells were challenged with 100 ng/mL LPS for 24 h. Then, cells were treated with BA (0, 1, 5, or 20 μM) for 24 h in the presence of LPS. Control indicated cells in the absence of any treatment (#$P < 0.05$ vs control; *$P < 0.05$ vs LPS).

(10 min/time), and then, chemiluminescence reagents were added for the visualization of the protein bands. Intensities of the digitally detected bands were evaluated by densitometry using Image-Pro Plus software (National Institutes of Health (NIH), USA).

Statistical analysis

All data are expressed as mean ± SD, and the Student's t-test was used to compare the statistical difference between two groups. P values <0.05 were considered to be statistically significant. At least three independent experiments have been performed.

Results

Macrophages produce a wide variety of inflammatory factors to regulate inflammation. IL-6 and TNF-α are two major pro-inflammatory cytokines which play important roles in the inflammatory process of macrophages. We first investigated the effects of BA on macrophage viability and production of inflammatory factors. The murine macrophage RAW264.7 cells were collected and treated with various concentrations of BA. Compared to the control group, BA showed slight toxicity on RAW264.7 cells viability under the concentration of 10 μM (Figure 1(b)). Next, we measured induction of IL-6 and TNF-α. RT-qPCR showed that LPS significantly increased expression of IL-6 and TNF-α in RAW264.7 cells compared to the control group; however, RT-qPCR analysis further revealed that treatment with BA reduced mRNA activation of IL-6 and TNF-α (data not shown). In addition, ELISA assays demonstrated that BA reduced the protein levels of IL-6 and TNF-α in a concentration-dependent manner ($P < 0.05$; Figure 1(c) and (d)). Although the effect at the higher concentrations may be at least partly

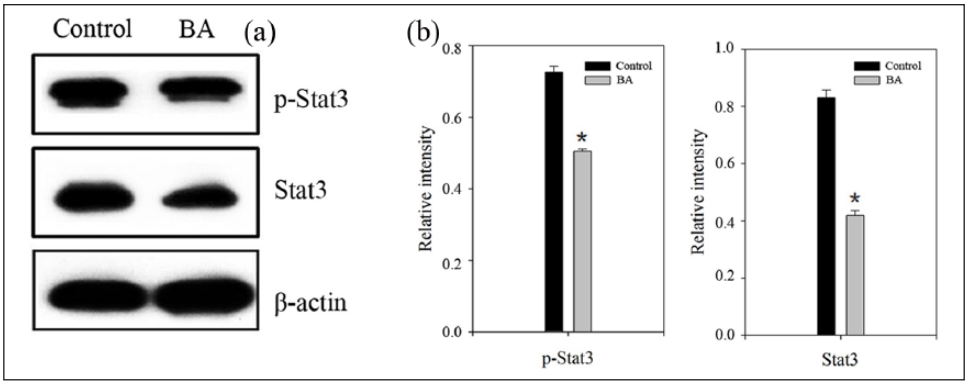

Figure 2. BA inhibited LPS-induced activation of STAT3 in RAW264.7 cells. (a) RAW264.7 cells were challenged with 100 ng/mL LPS for 24 h. Then, cells were treated with 1 μM BA for 24 h in the presence of LPS. Expression levels and phosphorylation changes of STAT3 were detected using monoclonal antibodies, respectively. β-actin was used as a loading control. (b) The calculated relative intensity of protein/β-actin by densitometry (*$P < 0.05$, compared with control).

related to the reduced cell viability, our results confirmed that BA displays anti-inflammatory effects in murine macrophage RAW264.7 cells.

STAT3 is an essential inflammatory signaling factor in the pathogenesis of inflammatory diseases. LPS can promote the activation and phosphorylation of STAT3 at Y705, which increased transcription of inflammatory factors. We then sought to examine the effects of BA on LPS-induced STAT3 activation. Our results showed that 1 μM BA treatment reduced the expression and phosphorylation of STAT3 at Y705 (Figure 2(a) and (b)). Moreover, BA treatment attenuated the phosphorylation of extracellular signal-regulated kinase (ERK), while the overall expression levels of ERK did not change significantly (data not shown). Therefore, these results indicate that BA specifically targets STAT3 to regulate LPS-induced macrophage activation.

As reported previously, miR-124 can directly regulate gene expression through binding to the 3′-UTR of STAT3 mRNAs, and the stability of STAT3 mRNA is regulated by miR-124.[19] We therefore wondered whether BA could exhibit effects on miR-124 expression in regulating STAT3 levels. Our results showed that 1 μM BA treatment significantly promoted the elevation of miR-124 levels compared with the LPS-treated group (Figure 3(a)). To directly assess the effect of miR-124 on the expression of STAT3, a miR-124 mimic was transfected into RAW264.7 cells. RT-qPCR and immunofluorescent analysis results confirmed the elevation of miR-124 levels in cells (Figure 3(b) and (c)). We also observed that the expression and phosphorylation of STAT3 decreased when cells were transfected with the miR-124 mimic (Figure 3(d) and (e)).

Since miR-124 can directly regulate gene expression through binding to the 3′-UTR of STAT3 mRNAs, we then assessed and investigated the 3′-UTR reporter activities of STAT3 by luciferase assays. We constructed a reporter plasmid by inserting the cDNA corresponding to the 3′-UTR of STAT3 into the firefly luciferase reporter plasmid. The 3′-UTR luciferase reporter plasmid of STAT3 and the miR-124 mimic were co-transfected into HEK-293T cells. As expected, transfected miR-124 mimic significantly decreased luciferase activity of the reporter plasmid containing the 3′-UTR of STAT3 (Figure 3(f)). These results support a notion that miR-124 exerts a predominant role as a regulator of STAT3 in the presence of LPS stimulation.

To further evaluate the role of miR-124 in modulating the effects of BA, miR-124 was knocked down using an miR-124 inhibitor in RAW264.7 cells. We then assessed the effects of miR-124 on the production of pro-inflammatory cytokines, including IL-6 and TNF-α. As shown in Figure 4(a) and (b), the reduction of IL-6 and TNF-α through 1 μM BA treatment was abolished when cells were pretreated with the miR-124 inhibitor. Further western blotting results showed that BA-stimulated reduction of STAT3 has been attenuated in the presence of miR-124 inhibitor (Figure 4(c) and (d)). Therefore, our results confirm that miR-124 plays a vital role in modulating the function of STAT3 in LPS-induced IL-6 and TNF-α production.

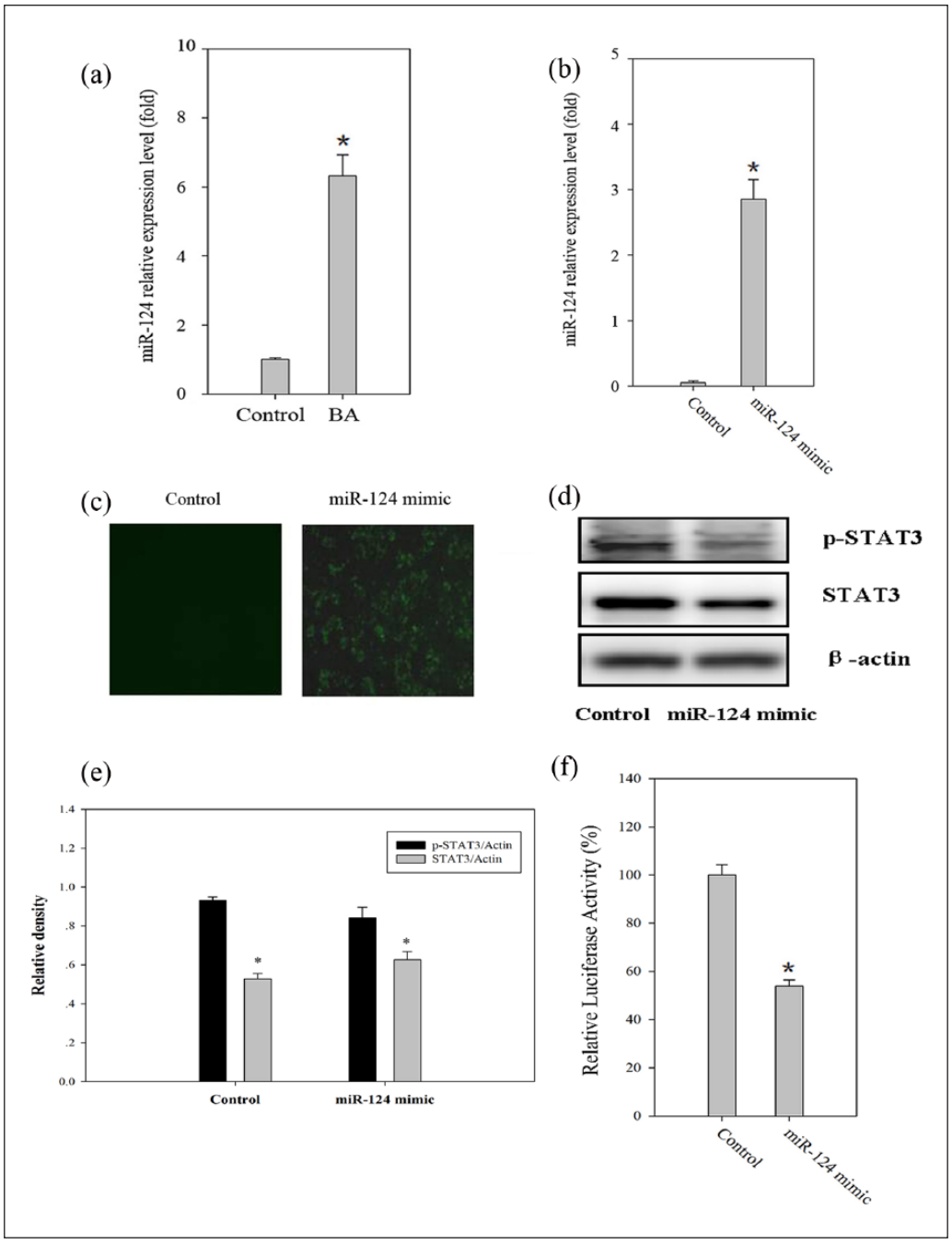

Figure 3. BA-stimulated upregulation of miR-124 to retard STAT3 activation. (a) RT-qPCR analysis of miR-124 in RAW264.7 cells stimulated with LPS in the presence of 5 μM BA. Control indicated the LPS-treated group. (b) RT-qPCR analysis of miR-124 in RAW264.7 cells after transfection with miR-124 mimic (*$P < 0.05$, compared with control mimic group). (c) Immunofluorescent analysis of the GFP-labeled miR-124 and control mimic (green) were used to indicate the transfection efficiency. (d) The miR-124 and control mimic were transfected in RAW264.7 cells with Lipofectamine 2000 in the presence of LPS. After 48 h, cells were collected and the expression levels of STAT3 were detected. (e) The calculated relative intensity of protein/β-actin by densitometry (*$P < 0.05$, compared with control mimic group). (f) The 3′-UTR luciferase reporter plasmid of STAT3 and the miR-124 mimic were co-transfected into HEK-293T cells. After 24 h, cells were collected and luciferase activities were measured (*$P < 0.05$, compared with the group transfected with control mimic).

Discussion

BA has been identified as having a wide range of anti-inflammatory, antioxidative, antiviral, and antitumor properties.[25,26] However, the molecular mechanism of how BA exhibits its effects, especially on inflammation regulation, has not been fully investigated. Several miRNAs have been found to regulate inflammation and immunity, which

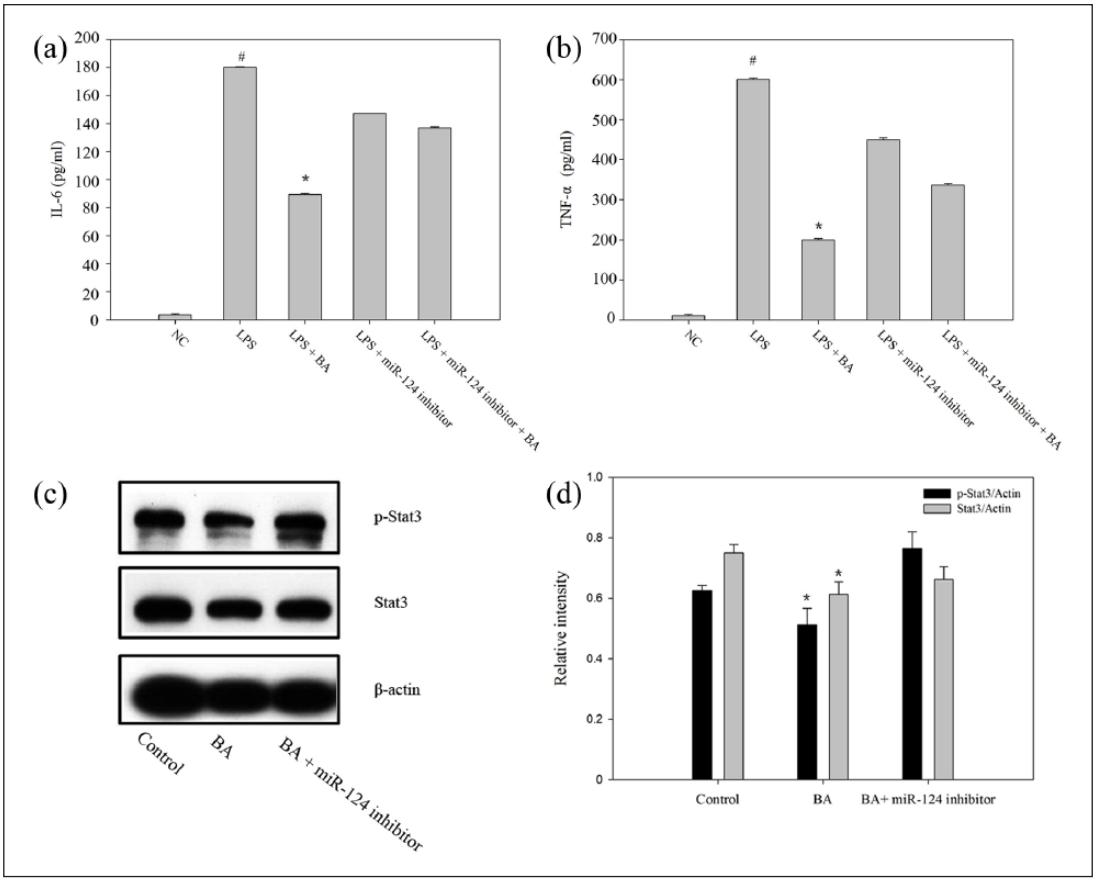

Figure 4. Inhibition of miR-124 expression attenuated the effects of BA. (a and b) ELISA analysis of LPS-induced IL-6 and TNF-α production in the presence of BA in RAW264.7 cells transfected with or without miR-124 inhibitors (#$P < 0.05$, compared with control; *$P < 0.05$, compared with LPS treatment group). (c) miR-124 inhibitors were transfected into RAW264.7 cells and then treated with BA in the presence of LPS for 24 h. Then, the expression levels and phosphorylation changes of STAT3 were detected using monoclonal antibodies, and β-actin was used as a loading control. Control group indicated cells treatment with LPS alone. (d) The calculated relative intensity of protein/β-actin by densitometry (*$P < 0.05$, compared with LPS treatment control).

can modulate the expression of target genes.[27] As reported previously, the 3′-UTR of mouse STAT3 mRNA contains two putative miR-124 target sites, and the stability of STAT3 mRNA is regulated by miR-124.[19] Since BA inhibits the expression of STAT3 in RAW264.7 cells, we therefore wondered whether BA could exhibit effects on miR-124 expression. Our results reported herein confirm that miR-124 is an important regulator of the anti-inflammatory effects of BA in macrophages. Specifically, BA exhibits its anti-inflammatory effects by modulating miR-124, which leads to attenuation of STAT3 and inflammatory factors IL-6 and TNF-α in RAW264.7 macrophage cells.

Macrophages mainly detect and respond to detection of Gram-negative bacteria and their associated endotoxins (e.g. LPS) through TLR receptor expressed on their surface. Therefore, uncontrolled TLR activation might result in immunological and inflammatory diseases, such as sepsis, autoimmune diseases, metabolic diseases, and cancer. BA exerts its anti-inflammatory effect mainly through suppressing macrophage activation. It has been reported to downregulate several inflammation-associated genes, such as inducible NO synthase, and consequently inhibit production of NO and prostaglandin E2 in macrophages.[10,28,29] BA inhibits the TLR4-linked NF-κB signaling pathway in LPS-induced RAW264.7 cells and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced rats experimental colitis.[30] BA has also been reported to attenuate cigarette smoke (CS)-induced inflammatory response in chronic obstructive pulmonary disease (COPD) rat models by inhibiting the production of IL-6, IL-8, and TNF-α.[31] In addition, BA can inhibit binding of a number of chemokines

to human leukocytes, which leads to reduction of the capacity of chemokines to induce cell migration.[32] In this article, we investigated the effects of BA on the murine macrophage cell line RAW264.7. Consistent with previous reports, we demonstrate that BA inhibits the production of IL-6 and TNF-α, two major inflammatory factors upon LPS stimulation of macrophages.

Besides, we observed that BA inhibits the expression and activation of STAT3, which is related to the production of IL-6 and TNF-α.[33] Specific chemical inhibitors targeting STAT3 have been reported to exhibit anti-inflammatory functions in primary peritoneal macrophages.[24] In this article, our results show that BA promotes the repression and phosphorylation of STAT3, similar to a previous report on the anti-inflammatory properties of BA.[34] Therefore, our results have identified that the effects of BA on anti-inflammatory pathways are directly related to STAT3 inhibition.

miR-124 is an important regulator of STAT3 expression in the context of inflammatory diseases, and several studies have identified the function of miR-124-controlled STAT3 axis in the regulation of innate immunity.[27] For example, Sun et al.[19] reported that miR-124 modulates LPS-induced cytokine production by targeting STAT3 in macrophages upon challenge with cholinergic agonists. miR-124 was also capable of targeting multiple components of the TLR signaling cascade, which might functionally impact the expression of its downstream target genes, including MyD88 and TRAF6, as well as inflammatory cytokines and chemokines.[20] In addition, miR-124 has been reported to modulate neuronal differentiation, downregulating canonical STAT3 signaling by targeting gp130, leukemia inhibitory factor receptor (LIFR), and ciliary neurotrophic factor receptor (CNTFR).[35] Our results demonstrate that BA promotes the expression of miR-124 in LPS-treated macrophages. Expression of the miR-124 mimic resulted in reduction of STAT3 levels. However, inhibition of miR-124 attenuated the induction of miR-124 expression upon BA treatment. Besides, the reduction of IL-6 and TNF-α through BA treatment was abolished when cells were pretreated with the miR-124 inhibitor. Though comparing LPS treatment group, miR-124 inhibitor also indicated the effects of inhibiting LPS-induced inflammatory cytokines production, which might be partly due to the toxicity of Lipofectamine 2000 transfection. Our results indeed confirmed that miR-124 is an important modulator in regulating the effects of BA in macrophages.

Functions of miRNAs in immunity by regulating immune cell differentiation, commitment, and maturation have been reported. Although we have identified the function of miR-124 on modulating the effects of BA, our investigations have not excluded other potential candidate regulator of miRNAs. For example, miR-155 also plays key roles in both innate and adaptive immune responses.[27] miR-155 directly regulates the SH2 domain-containing inositol-5′-phosphatase (SHIP)-1, which might be related to the regulation of STAT3. Moreover, miR-20a is another negative regulator of STAT3, as it has been reported to bind the 3′-UTR of STAT3 mRNA and repress STAT3 expression.[36] The possibilities of miRNAs as therapeutic entities have been explored, and anti-miR compounds that inhibit specific miRNAs have the potential to become a whole new class of drugs. For example, sequestration of miR-122 with antisense constructs targeting the miRNA significantly reduced the replication of hepatitis C virus (HCV) RNA, suggesting that miR-122 may present a target for antiviral intervention. Clinical trials using anti-miR-122 against HCV are currently ongoing.[37] Moreover, some other studies demonstrated that BA inhibits LPS-induced inflammation through NF-kB signaling pathway.[33–36] It was found in a few studies that BA may alleviate inflammation via inhibiting other cytokines IL-17 and IL-33. All these studies did not reveal the molecular linkage of cytokines IL-6 and TNF-a with miR-124-STAT3 signaling pathway in relation to BA treatment in macrophages.

Therefore, BA attenuates pro-inflammatory cytokine production through miR-124-STAT3 signaling pathway, suggesting that miR-124 is an important modulator in regulating anti-inflammation by BA in macrophages. Aside from its anti-inflammatory properties, BA may also act as an antioxidant, reducing reactive oxygen species including hydroxyl radicals, superoxide anion radicals, and hydrogen peroxide.[2] Further investigations of the potential roles of miR-124 in regulating the antioxidant signaling pathway might be needed in the future. In summary, we proposed the possible molecular mechanisms that BA may exert pro-inflammatory effect on cytokines through miR-124/STAT3 signaling pathways (Figure 5).

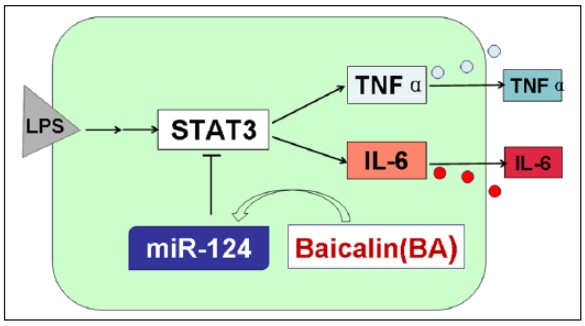

Figure 5. Baicalin (BA) may exert anti-inflammatory effect on cytokines through miR-124/STAT3 signaling pathways in murine macrophages. Previous studies have demonstrated that miR-124 mediates anti-inflammatory action through inhibiting the translation of the STAT3 mRNA and subsequent IL-6 production, as STAT3 activation leads to an increase in IL-6 transcription and production. LPS activates STAT3 pathways triggering the production of TNF-α and IL-6. miR-124 decreases STAT3 and its phosphorylation, resulting in the decrease in IL-6 at the transcription. Our results support a notion that BA upregulates miR-124 to retard STAT3 activation, while inhibition of miR-124 expression in turns attenuated the effects of BA.

Based on the above observations, our investigations identified miR-124 as a potential therapeutic target in macrophages and expanded our understanding of the effects of BA on the treatment of inflammatory diseases.

Authors' Contribution

The experimental design, data analysis, and manuscript preparation were done by Bin Tang, Xixi Wang, and Yuqing Zhu. B.T. and X.W. contributed equally to this work.

Acknowledgements

We greatly thank Prof. Yao's lab members for their discussions and suggestions on this manuscript. This study was proposed and supervised by Xuhui Li and Shukun Yao.

Availability of data and material

All data of this study are available.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval and Informed consent

This study was approved by Research Ethics Board of China-Japan Friendship Hospital. All participants had signed the consent forms of this study.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work is supported by the Research Fund of China-Japan Friendship Hospital (No. 2017-2-QN-18).

ORCID iD

Xuhui Li https://orcid.org/0000-0001-7933-174X

References

1. Wang JY, Chuang HN, Chiu JH et al. (2006) Effects of Scutellaria baicalensis Georgi on macrophage-hepatocyte interaction through cytokines related to growth control of murine hepatocytes. *Experimental Biology and Medicine* 231: 444–455.
2. Sahebkar A (2012) Baicalin as a potentially promising drug for the management of sulfur mustard induced cutaneous complications: A review of molecular mechanisms. *Cutaneous and Ocular Toxicology* 31: 226–234.
3. Krakauer T, Li BQ and Young HA (2001) The flavonoid baicalin inhibits superantigen-induced inflammatory cytokines and chemokines. *FEBS Letters* 500: 52–55.
4. Shen YC, Chiou WF, Chou YC et al. (2003) Mechanisms in mediating the anti-inflammatory effects of baicalin and baicalein in human leukocytes. *European Journal of Pharmacology* 465: 171–181.
5. Li L, Zeng H, Shan L et al. (2012) The different inhibitory effects of Huang-Lian-Jie-Du-Tang on cyclooxygenase 2 and 5-lipoxygenase. *Journal of Ethnopharmacology* 143: 732–739.
6. Medzhitov R (2008) Origin and physiological roles of inflammation. *Nature* 454: 428–435.
7. Biswas SK and Lopez-Collazo E (2009) Endotoxin tolerance: New mechanisms, molecules and clinical significance. *Trends in Immunology* 30: 475–487.
8. Dobrovolskaia MA and Vogel SN (2002) Toll receptors, CD14, and macrophage activation and deactivation by LPS. *Microbes and Infection* 4: 903–914.
9. Liu LL, Gong LK, Wang H et al. (2008) Baicalin inhibits macrophage activation by lipopolysaccharide and protects mice from endotoxin shock. *Biochemical Pharmacology* 75: 914–922.
10. Chen YC, Shen SC, Chen LG et al. (2001) Wogonin, baicalin, and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide. *Biochemical Pharmacology* 61: 1417–1427.
11. Gao H and Ward PA (2007) STAT3 and suppressor of cytokine signaling 3: Potential targets in lung inflammatory responses. *Expert Opinion on Therapeutic Targets* 11: 869–880.

12. Yang XO, Panopoulos AD, Nurieva R et al. (2007) STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *Journal of Biological Chemistry* 282: 9358–9363.
13. O'Connell RM, Rao DS and Baltimore D (2012) microRNA regulation of inflammatory responses. *Annual Review of Immunology* 30: 295–312.
14. Krol J, Loedige I and Filipowicz W (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nature Reviews Genetics* 11: 597–610.
15. Tsitsiou E and Lindsay MA (2009) microRNAs and the immune response. *Current Opinion in Pharmacology* 9: 514–520.
16. Essandoh K, Li Y, Huo J et al. (2016) MiRNA-mediated macrophage polarization and its potential role in the regulation of inflammatory response. *Shock* 46: 122–131.
17. Li S, Yue Y, Xu W et al. (2013) MicroRNA-146a represses mycobacteria-induced inflammatory response and facilitates bacterial replication via targeting IRAK-1 and TRAF-6. *PLoS ONE* 8: e81438.
18. Xu G, Zhang Z, Xing Y et al. (2014) MicroRNA-149 negatively regulates TLR-triggered inflammatory response in macrophages by targeting MyD88. *Journal of Cellular Biochemistry* 115: 919–927.
19. Sun Y, Li Q, Gui H et al. (2013) MicroRNA-124 mediates the cholinergic anti-inflammatory action through inhibiting the production of pro-inflammatory cytokines. *Cell Research* 23: 1270–1283.
20. Ma C, Li Y, Li M et al. (2014) microRNA-124 negatively regulates TLR signaling in alveolar macrophages in response to mycobacterial infection. *Molecular Immunology* 62: 150–158.
21. Xu S, Zhao N, Hui L et al. (2016) MicroRNA-124-3p inhibits the growth and metastasis of nasopharyngeal carcinoma cells by targeting STAT3. *Oncology Reports* 35: 1385–1394.
22. Lu Y, Yue X, Cui Y et al. (2013) MicroRNA-124 suppresses growth of human hepatocellular carcinoma by targeting STAT3. *Biochemical and Biophysical Research Communications* 441: 873–879.
23. Tan Y, Zhang B, Wu T et al. (2009) Transcriptional inhibition of Hoxd4 expression by miRNA-10a in human breast cancer cells. *BMC Molecular Biology* 10: 12.
24. Yuan S, Cao S, Jiang R et al. (2014) FLLL31, a derivative of curcumin, attenuates airway inflammation in a multi-allergen challenged mouse model. *International Immunopharmacology* 21: 128–136.
25. Altavilla D, Squadrito F, Bitto A et al. (2009) Flavocoxid, a dual inhibitor of cyclooxygenase and 5-lipoxygenase, blunts pro-inflammatory phenotype activation in endotoxin-stimulated macrophages. *British Journal of Pharmacology* 157: 1410–1418.
26. Bitto A, Squadrito F, Irrera N et al. (2014) Flavocoxid, a nutraceutical approach to blunt inflammatory conditions. *Mediators of Inflammation* 2014: 790851.
27. Haghikia A, Hoch M, Stapel B et al. (2012) STAT3 regulation of and by microRNAs in development and disease. *JAK-STAT* 1: 143–150.
28. Cui L, Feng L, Zhang ZH et al. (2014) The anti-inflammation effect of baicalin on experimental colitis through inhibiting TLR4/NF-κB pathway activation. *International Immunopharmacology* 23: 294–303.
29. Zhu W, Jin Z, Yu J et al. (2016) Baicalin ameliorates experimental inflammatory bowel disease through polarization of macrophages to an M2 phenotype. *International Immunopharmacology* 35: 119–126.
30. Hoensch HP and Weigmann B (2018) Regulation of the intestinal immune system by flavonoids and its utility in chronic inflammatory bowel disease. *World Journal of Gastroenterology* 24: 877–881.
31. Li L, Bao H, Wu J et al. (2012) Baicalin is anti-inflammatory in cigarette smoke-induced inflammatory models in vivo and in vitro: A possible role for HDAC2 activity. *International Immunopharmacology* 13: 15–22.
32. Dai SX, Zou Y, Feng YL et al. (2012) Baicalin down-regulates the expression of macrophage migration inhibitory factor (MIF) effectively for rats with ulcerative colitis. *Phytotherapy Research: PTR* 26: 498–504.
33. Zhao J, Yu H, Liu Y et al. (2016) Protective effect of suppressing STAT3 activity in LPS-induced acute lung injury. *American Journal of Physiology* 311: L868–L880.
34. Qi Z, Yin F, Lu L et al. (2013) Baicalein reduces lipopolysaccharide-induced inflammation via suppressing JAK/STATs activation and ROS production. *Inflammation Research* 62: 845–855.
35. Makeyev EV, Zhang J, Carrasco MA et al. (2007) The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing. *Molecular Cell* 27: 435–448.
36. Zhang M, Liu Q, Mi S et al. (2011) Both miR-17-5p and miR-20a alleviate suppressive potential of myeloid-derived suppressor cells by modulating STAT3 expression. *Journal of Immunology* 186: 4716–4724.
37. van Rooij E, Purcell AL and Levin AA (2012) Developing microRNA therapeutics. *Circulation Research* 110: 496–507.