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RESEARCH ARTICLE

MicroRNA *in vivo* precipitation identifies miR-151-3p as a computational unpredictable miRNA to target *Stat3* and inhibits innate IL-6 production

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MicroRNAs (miRNAs) function as important regulators in the immune response and inflammation. Several approaches have been reported to computationally predict miRNAs and their potential targets. However, there are still many miRNA–target interactions that are unpredictable by using the current computational algorithms. We established a miRNA *in vivo* precipitation method (miRIP) to identify unpredictable miRNAs with definite targets in these cells. Because Stat3 is a well-known transcription factor involved in innate immunity and inflammation, we utilized the miRIP method to identify miRNAs that bind *Stat3* mRNA in macrophages. Among the captured miRNAs, miR-151-3p was confirmed to interact with *Stat3* mRNA 3′-UTR and downregulate the Stat3 protein levels. LPS stimulation decreased miR-151-3p expression, thereby increasing IL-6 production. Therefore, we found that miR-151-3p inhibited LPS-induced IL-6 production by targeting *Stat3*. These data further confirmed miRIP as an efficient method to identify unpredictable miRNAs and explore miRNAs-mediated regulation in innate immunity and inflammation.
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INTRODUCTION

MicroRNAs (miRNAs) are small non-coding RNA molecules that play important roles in various physiological and pathophysiological processes, including immune response and inflammation.[1–3] As the first defense line against invading pathogens, the innate immune system is tightly regulated by a wide range of factors via post-transcriptional and epigenetic modifications.[4–6] Among these factors, miRNAs have been widely demonstrated to be involved in important signaling pathways in innate immune responses.[7–10] Several miRNAs play pro-inflammatory roles, typified by miR-155, and others are negative feedback regulators of inflammatory responses, such as miR-146a and miR-29.[11–13] Although the interaction between innate immune-related genes and miRNAs has been elucidated in many studies, the roles of miRNAs in regulating innate immune responses are far from fully understood. In addition, identifying miRNAs that target specific mRNA *in vivo* remains a significant challenge, as one miRNA can target multiple mRNAs and *vice versa*.

Many transcriptional factors have been identified as important initiators and regulators of innate immunity and inflammation. The superfamily of the signal transducer and activator of transcription (STAT) is well known in the field. Among the Stat superfamily, Stat3, predominantly activated by gp130-acting cytokines (for example, IL-6, IL-11, oncostatin-M, LIF), is critical in the signal transduction of cytokine function and the production of pro-inflammatory cytokines.[14] These cytokines bind to their receptors and induce the activation of Jak kinases, which activate Stat3. Then, Stat3 is phosphorylated, dimerized and translocated into the nucleus, where it can initiate and regulate the expression of target genes, such as *IL-6*. In addition to the increase of phosphorylated Stat3 (p-Stat3),

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total Stat3 protein is upregulated upon the stimulation of LPS and IL-6.[15,16] However, the epigenetic mechanism underlying the upregulation of total Stat3 protein in innate immune responses remain unclear.

Previous studies have focused on the regulation of the *Stat3* gene at the transcriptional level; for example, the *Stat3* gene itself is activated via IL-6 signaling through an IL-6 response element within the *Stat3* gene promoter that contains both a low-affinity Stat3-binding element and a cAMP response element, thus activating the Stat3 gene in cooperation with an unidentified CREB.[17] Post-transcriptional control has increasingly emerged as a prominent mechanism of Stat3 regulation, particularly through miRNA targeting. Multiple miRNAs, such as miR-17, miR-20a, miR-93, miR-106b, miR-125b and miR-199, were identified to target Stat3.[18–20] However, further investigation is needed to identify other *Stat3*-targeting miRNAs under physiological conditions, particularly in innate immunity.

The 3′-untranslated region (3′-UTR) sequence of mouse Stat3 mRNA contains ~1895 nucleotides, suggesting that Stat3 mRNA is targeted by many miRNAs. Identifying the true miRNAs that target Stat3 mRNA is a challenging issue. To characterize the *in vivo* mRNA:miRNA interactions that cannot be predicted by using the current computational algorithms, we established the miRIP method (miRNA *in vivo* precipitation) and used this method to identify miR-92a, which targets p21 mRNA.[21] In this study, by using biotin-tagged anti-sense oligonucleotides for Stat3 mRNA (Stat3 probe), we identified specific Stat3-interacting miRNAs. Among these miRNAs, miR-151-3p was confirmed to interact with *Stat3* mRNA and consequently downregulate the Stat3 protein levels in mouse macrophages in response to LPS stimulation. LPS stimulation decreased the level of miR-151-3p in mouse macrophages, leading to the upregulation of Stat3 protein level and promotion of the production of the pro-inflammatory cytokine IL-6. In this study, miRIP was further demonstrated to be an efficient method for identifying unpredictable interacting miRNAs in the immune system, and furthermore, miR-151-3p was found to be important in amplifying the initial innate immune responses upon pathogen invasion.

MATERIALS AND METHODS

Mice and cell cultures

Male C57BL/6 mice (6–8 weeks) obtained from Joint Ventures Sipper BK Experimental Animal were used to prepare primary mouse peritoneal macrophages. All animal experiments were conducted according to the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai. The RAW264.7 cell line was obtained from the American Type Culture Collection.

Primary mouse peritoneal macrophages were prepared and cultured, as previously described.[22] RAW264.7 cells were cultured and maintained in DMEM containing 10% FBS at 37 °C in 5% CO_2. All cell culture media and other reagents were entirely free of endotoxins.

miRNAs *in vivo* precipitation (miRIP)

miRIP was performed as previously described.[21] Briefly, RAW264.7 cells were transfected with a biotin-tagged specific probe or a control probe 24 h before the cells were coll (the probe sequences are listed in Supplementary Table S1). The cells were cross-linked using 1% formaldehyde for 10 min, equilibrated in glycine buffer for 5 min, washed three times with cold PBS, scraped with 1 ml of lysis buffer and incubated for 10 min. The lysis mixtures were sonicated (VCX130, SONICS, and MATERIALS) using the following parameters: 50% amplitude, 30 s of a constant pulse, and a 30-s pause for 10 min. Subsequently, the samples were centrifuged at 10 000*g* for 10 min. The supernatant lysate was incubated with 1 ml of M-280 beads (Invitrogen, Carlsbad, CA, USA) for 1 h under rotation. The beads sample mixture was washed twice with wash buffer, and finally incubated with 200 μl of lysis buffer for 2 h to reverse the formaldehyde cross-links. Subsequently, the RNA was extracted using TRIzol. After DNase I treatment, the RNA was repurified using TRIzol.

Exiqon multiplex miRNA arrays

Expression analysis of miRNAs was performed using the miRCURY LNA Universal RT microRNA PCR System (Exiqon, Vedbaek, Denmark) according to the manufacturer's instructions. Initial data analysis was performed using the software supplied with the real-time PCR instrument to obtain raw Ct values (Ct or Cq, depending on PCR instrument). Briefly, two miRCURY LNA Universal RT microRNA PCRs were performed in this study (GSE77873): one was for the RNA samples identified using the Stat3 specific probe P2, and the other was for the RNA samples identified using the control probe C5. First, the raw Ct value of all miRNAs in P2 and C5 samples were normalized to the Ct value of U6 in P2 and C5 samples, respectively, to yield the ΔCt value. Next, the ΔCt values of all miRNAs from the P2 sample were normalized to ΔCt values from the C5 sample to yield ΔΔCt. Finally, the $2^{-\Delta\Delta Ct}$ value was calculated for each miRNA as the relative level of a certain miR in the P2 probe sample compared with the C5 probe sample. If the P2/C5 ratio exceeded 10, then a specific interaction exists, and the miRNA is 'enriched'.

Vector construction

The selected region (2723–3279) of *Stat3* mRNA 3′-UTR was amplified from normal mouse genomic DNA using the primers listed in Supplementary Table S1 and inserted into the *Hind III/Spe I* sites of the pMIR-Report construct (Ambion, Austin, TX, USA). The *Stat3* mRNA 3′-UTR mutant fragment was constructed and amplified by two PCR rounds. In the first round of PCR, Stat3 3′-UTR F primer and Stat3 3′-UTR Mut R primer were utilized for the clone of the first half of *Stat3* mRNA 3′-UTR mutant fragment, and Stat3 3′-UTR Mut F primer and Stat3 3′-UTR R primer were utilized for the second round. Then, the PCR products of the first round were mixed and diluted for use as a template in the second round of PCR. The Stat3 3′-UTR mutant fragment was finally amplified using Stat3 3′-UTR F/R primer and inserted into the *Hind III/Spe I*

sites of the pMIR-Report construct (Ambion). The coding fragment of *Stat3* was amplified using the primers listed in Supplementary Table S1 from normal mouse complementary DNA (cDNA) and inserted into the *Xho I/Hind III* sites of the pBflag construct (Ambion). All constructs were verified by direct sequencing.

Oligonucleotide transfection

RNA oligonucleotides were chemically synthesized and purified at Invitrogen. The sense sequence of mouse miR-151-3p mimics was 5′-CUA GAC UGA GGC UCC UUG AGG-3′ and the antisense sequence was 5′-UCA AGG AGC CUC AGU CUA GUU-3′. Negative control mimics were 5′-UUC UCC GAACGU GUC ACG UdTdT-3′ and 5′-ACG UGA CAC GUU CGG AGA AdTdT-3′. The sequence of the mouse miR-151-3P inhibitor was 5′-CCU CAA GGA GCC UCA GUC UAG-3′ and the sequence of the inhibitor control was 5′-CAG UAC UUU UGU GUA GUA CAA-3′. The Stat3 siRNAs mix was obtained from Genepharma (Shanghai, China). The transfections were performed using INTERFERin reagent (Polyplus-transfection, Illkirch, France) and Lipofectamine RNAiMAX transfection reagent (Invitrogen) according to the manufacturer's instructions. The final concentrations of Stat3 siRNAs, miR-151-3p mimics and inhibitor used were 20, 50 and 100 nM.

RNA isolation, reverse-transcription, and quantitative real-time polymerase chain reaction (RT-qPCR)

Total RNA was extracted from the cell lines and mouse primary macrophages using TRIzol reagent (Invitrogen). cDNA synthesis was performed using ReverTra Ace (TOYOBO, Osaka, Japan) and the All-in-One miRNA qRT-PCR Detection Kit (GeneCopoeia, Rockville, MD, USA), respectively, according to the manufacturer's instructions.

Amplification and detection were performed using the Light Cycler 480II/96 and the LightCycler2.0 (Roche, Rotkreuz, Switzerland). The miR-151-3p and *Stat3* mRNA levels were normalized to *U6* and *Actin*, respectively, to yield a $2^{-\Delta\Delta Ct}$ value for the relative expression of each transcript. The primers used are shown in Supplementary Table S1.

Luciferase reporter assay

Briefly, the cells plated in a 24-well plate were co-transfected with 50 nM miRNA mimics or 100 nM miRNA inhibitor, 25 ng of firefly luciferase reporter comprising Stat3 3′-UTR, and 6 ng of pRL-TK (Promega, Madison, WI, USA) using jetPRIME and jetPEI Macrophages (Polyplus-transfection, Illkirch, France). The cells were collected at 36 h after the last transfection and analyzed using a Dual-Luciferase Reporter Assay System (Promega).

Western blotting

Total protein was extracted after lysing the cells in RIPA buffer containing protease inhibitors. The protein samples were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 5% non-fat milk in TBS-T, the membranes were incubated with primary antibody. The following antibodies were used: anti-Erk (1:3000), anti-Phospho-Erk (1:3000), anti-p65 (1:3000), anti-Phospho-p65 (1:3000), anti-IKKβ (1:3000), anti-Phospho-IKKβ (1:3000), anti-IkBα (1:3000), anti-Phospho-IkBα (1:3000), anti-Stat3 (79D7) (1:3000), anti-Phospho-Stat3 (Tyr-705) (1:3000), anti-β-Actin (1:10 000), anti-Gapdh (1:10 000). Goat-anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:2000) and goat-anti-mouse IgG conjugated to horseradish peroxidase (HRP) (1:2000) (Cell Signaling Technology, Danvers, MA, USA) were used as the secondary antibodies. Protein was detected with image acquisition using Chemiluminescent Western Blot Scanner (Gene Company, Hong Kong, China).

Statistical analysis

All data are presented as the means ± s.d. Student's *t* test was used to analyze the difference between two experimental groups, and a P-value < 0.05 indicates statistical significance.

RESULTS

Identification of miR-151-3p as an unpredictable Stat3 mRNA-targeting miRNA

The 3′-UTR of *Stat3* mRNA is relatively long with ~1895 nucleotides, indicating that Stat3 mRNA is a potential target for miRNAs (Figure 1a). To explore whether miRNAs are involved in the regulation of Stat3 *in vivo*, miRIP was performed to purify miRNAs binding to *Stat3* mRNA. The *Stat3* mRNA probe was transfected into RAW264.7 cells to hybridize *Stat3* mRNA for 24 h. The interacting region of *Stat3* mRNA was purified using Streptavidin Dynabeads (M-280) and isolated using TRIzol reagent. The enriched region of Stat3 mRNA was confirmed by RT-PCR (Figure 1b), confirming the specificity of miRIP. The scope analysis of purified mRNA was also conducted by RT-PCR using multiple primers in different positions. As shown in Figure 1c, the majority segments purified by miRIP in RAW264.7 cells were ~500 bp around the probe binding site.

Exiqon multiplex miRNA array was performed to simultaneously analyze several hundred miRNAs (GSE77873). The top 18 enriched miRNAs are shown in Figure 1d, and miR-151-3p, the most robustly interacting miRNA, was confirmed by RT-PCR (Figure 1e). To examine whether the purified miRNAs could be predicted by computational algorithms, TargetScan, PicTar, PITA, miRanda and mirSVR were utilized to predict all potential miRNAs targeting a given mRNA region isolated using the Stat3-specific probe. Surprisingly, several robustly interacting miRNAs (>10-fold) identified using this technology could not be predicted by computational prediction algorithms, and several of the predicted miRNAs showed a weak interaction with Stat3 mRNA in RAW264.7 cells (Figure 1f and Supplementary Table S2). These data reveal that previous computational algorithms are defective to predict the truly enriched Stat3 mRNA

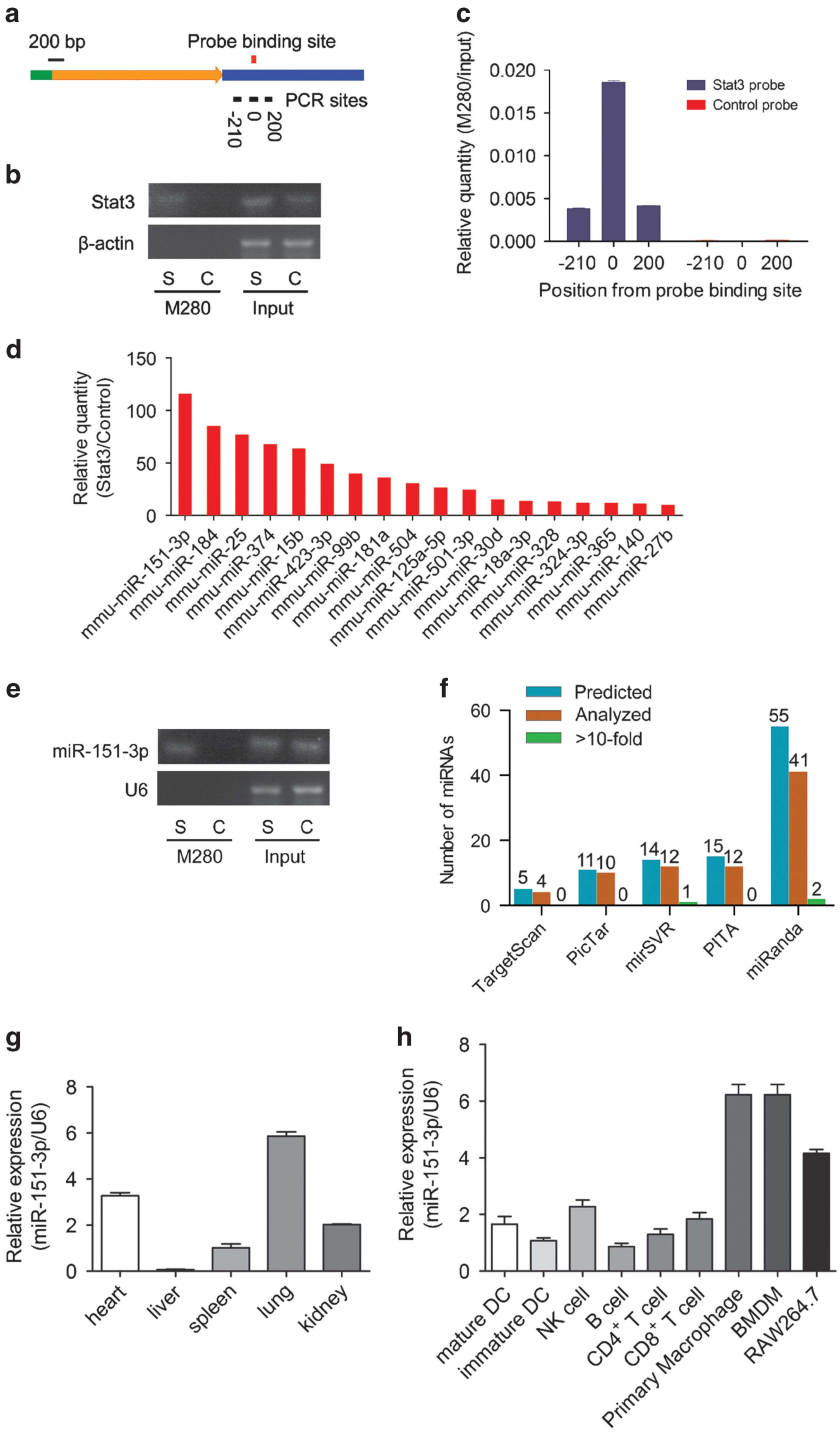

Figure 1 Identification of miR-151-3p as an unexpected *Stat3* mRNA-targeting miRNA. (**a**) *Stat3* mRNA and probe-binding sites. The green bar, 5′-UTR; the yellow arrow, coding sequence; the blue bar, 3′-UTR; the black bars and the numbers at the bottom denote regions analyzed using RT-qPCR; red bar, the probe binding sites. (**b**) RT-qPCR analysis of region '0' of Stat3 mRNAs from Stat3 probe and control probe affinity purified complex in RAW264.7 cells. S, Stat3 probe; C, Control probe. (**c**) RT-qPCR analysis of regions of *Stat3* mRNAs from Stat3 probe and control probe affinity purified complex in RAW264.7 cells. (**d**) Multiple miRNA array data of Stat3 probe and control probe affinity purified complex in RAW264.7 cells. The data are shown as normalized values for the enriched miRNAs (>10-fold). (**e**) RT-qPCR analysis of the most enriched miR-151-3p. S, Stat3 probe; C, control probe. (**f**) The comparisons of miRNAs identified by miRIP and predicted using TargetScan, PicTar, PITA, miRanda and mirSVR in the 500-bp region around probe-binding sites for *Stat3* mRNAs. (**g**) The expression of miR-151-3p in different organs was measured using RT-qPCR and normalized to the expression of U6. (**h**) The expression of miR-151-3p in different immune cells was measured using RT-qPCR and normalized to the expression of U6. Data are shown as the means ± s.d. ($n=3$) of one representative experiment. Similar results were obtained in at least three independent experiments.

binding miRNAs, and our *in vivo* miRIP assay can identify miR-151-3p as an unpredictable microRNA that binds Stat3 mRNA.

The distribution of miR-151-3p was further examined among different organs and different immune cells. The results showed that miR-151-3p was highly expressed in the lungs and heart and enriched in macrophages compared with other hematopoietic cells (Figures 1g and h). In addition, $CD4^+$ T cells, $CD8^+$ T cells and B cells were isolated from spleens of wild-type mice. The expression of miR-151-3p remained unchanged during the activation of $CD4^+$ T cells (Supplementary Figure 1a), $CD8^+$ T cells (Supplementary Figure 1b) and B cells (Supplementary Figures 1c and d). These results strongly indicated that miR-151-3p is more likely to participate in the function of macrophages instead of T/B cells.

miR-151-3p targets Stat3 mRNA and regulates Stat3 expression in macrophages

Several experiments were performed to confirm whether miR-151-3p regulates the expression of the target gene *Stat3*. Luciferase assay was conducted to confirm the interaction between miR-151-3p and the 3′-UTR of Stat3 mRNA. Luciferase reporter plasmids with a selected region (2723–3279) of *Stat3* 3′-UTR or *Stat3* 3′-UTR mutant (fragment 474–537 of the selected region were deleted) were constructed, and a dual-luciferase reporter assay was employed in RAW264.7 cells. The results indicated that the expression of luciferase reporter plasmid containing 3′-UTR of Stat3 was inhibited by co-transfection with miR-151-3p mimics, and the opposite result was obtained when transfected with miR-151-3p inhibitor (Figure 2a). In addition, no difference was observed between scrambled negative control mimics and miR-151-3p mimics in cells co-transfected with Stat3 3′-UTR mutant luciferase reporter plasmid, and a similar result was obtained after transfection with a miR-151-3p inhibitor (Figure 2b).

Furthermore, RAW264.7 cells were transfected with miR-151-3p inhibitor or mimics. RT-PCR and western blot results showed that the miR-151-3p inhibitor or mimics did not affect the level of *Stat3* mRNA and that the Stat3 protein level was increased in the presence of the miR-151-3p inhibitor and decreased by miR-151-3p mimics in LPS stimulated macrophages (Figures 2c and d,Supplementary Figure 2a). Similar results were observed in primary peritoneal macrophages (Figures 2c and d,Supplementary Figure 2a). Therefore, miR-151-3p may target the *Stat3* mRNA 3′-UTR and then downregulate the expression of *Stat3* at the post-transcriptional level in macrophages.

LPS significantly decreases miR-151-3p and upregulates Stat3 protein levels in macrophages

The expression of miRNAs, such as miR-146a and miR-155, is downregulated after LPS stimulation. To confirm whether miR-151-3p is regulated by LPS stimulation in macrophages, RAW264.7 cells were stimulated with LPS, and the expression of miR-151-3p was analyzed using RT-PCR. miR-151-3p was rapidly downregulated in RAW264.7 cells within 30 min after LPS stimulation (Figure 3a). A similar downregulation pattern of miR-151-3p was observed in primary peritoneal macrophages and bone marrow-derived macrophages in response to LPS stimulation (Figures 3a and b). However, the expression of miR-151-3p in bone marrow-derived dendritic cells (BMDC), mouse embryo fibroblasts (MEF) and colon cancer cell line CT-26 did not fit this pattern (Figure 3b).

To examine the role of miR-151-3p, a Stat3 mRNA binding miRNA, in innate response and inflammation, we first analyzed pStat3 activation in macrophages (RAW264.7 cells and primary peritoneal macrophages) in response to LPS stimulation (Figure 3c and Supplementary Figure 2b). The results showed that both p-Stat3 and total Stat3 levels were increased upon LPS stimulation in macrophages, consistent with the results of previous studies.[13,14] The levels of p-Stat3 rapidly increased, peaking at 30 min and returned to relatively low levels by 60 min. An analysis of *Stat3* mRNA in cells stimulated with LPS was also performed. The results showed *Stat3* mRNA levels remained unchanged during the same period (Figure 3d), suggesting the posttranscriptional regulation of Stat3. Collectively, these results suggest that LPS stimulation rapidly decreases miR-151-3p, consequently leading to an increase in the Stat3 levels in macrophages posttranscriptionally, which eventually enhances Stat3 phosphorylation.

LPS-induced downregulation of miR-151-3p promotes Stat3-dependent gene expression in macrophages

To explore the physiological significance of the LPS-induced downregulation of miR-151-3p, we examined the expression of many Stat3-dependent genes and used Tnf as a Stat3 independent control gene. The results showed that many Stat3-dependent genes, such as *IL-6* (Figure 4a), *IL-1b* (Figure 4b), *Ccl12* (Figure 4c), *Ccl5* (Figure 4d) and *Socs3* (Figure 4e), were upregulated after the transfection of miR-151-3p mimics in LPS-stimulated macrophages compared with scrambled negative control mimics. Similar results were not observed for the expression of *Tnf-α* (Figure 4f). Consistently, miR-151-3p inhibitor-transfected macrophages decreased Stat3-dependent gene expression compared with scrambled negative control inhibitor (Figures 4a–e). On the basis of these results, the LPS-induced downregulation of miR-151-3p could play important roles in innate immunity by regulating Stat3.

IL-6 is not involved in instant Stat3 activation after LPS stimulation

As IL-6 is a strong stimulator of Stat3 activation, and LPS triggers IL-6 via the NF-κB pathway, the activation of Stat3 after LPS stimulation may be a subsequent event of IL-6 secretion. Thus, we examined whether the activation of Stat3 upon LPS stimulation resulted from autocrine or paracrine IL-6. Primary macrophages from $IL\text{-}6^{-/-}$ mice were stimulated with LPS and analyzed using western blotting. The results showed that the lack of IL-6 did not affect the phosphorylation of Stat3 within one hour of LPS stimulation (Figure 5a and

Supplementary Figure 2c). In addition, we neutralized IL-6 in LPS-stimulated macrophages and found that Stat3 could still be phosphorylated by LPS stimulation within one hour (Figure 5b and Supplementary Figure 2d). Moreover, we inhibited the transcription, translation of *IL-6* mRNA and secretion of IL-6 with cyclohexane (CHX), actinomycin D (ACTD) and

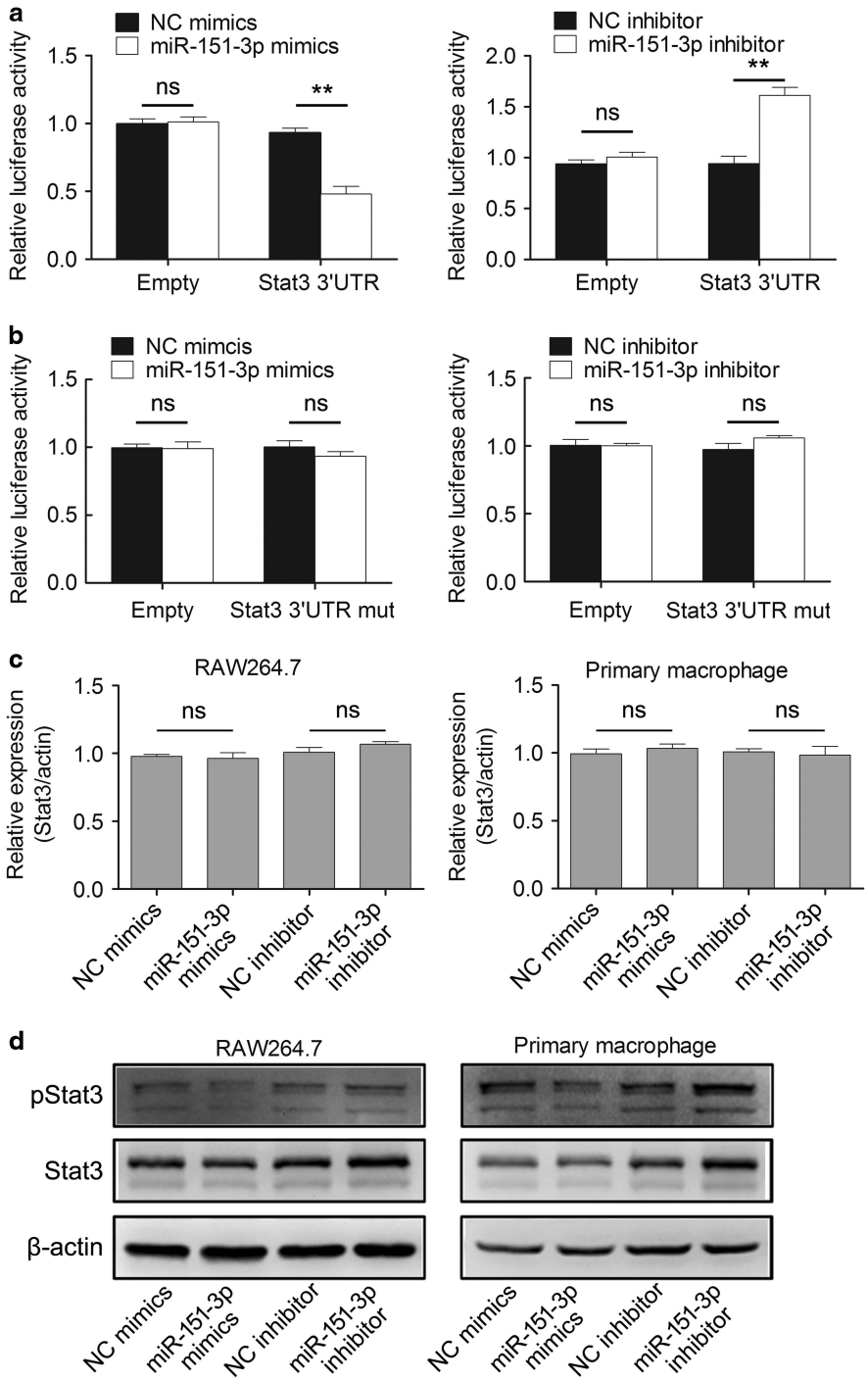

Figure 2 miR-151-3p targets *Stat3* mRNA and regulates Stat3 expression in macrophages. (**a** and **b**) Analysis of luciferase activity. RAW264.7 cells were co-transfected with pMIRfirefly luciferase reporter plasmids, pTK-Renilla luciferase plasmids, together with miR-151-3p inhibitor or mimics. After 36 h, firefly luciferase activity was measured and normalized to Renilla luciferase activity. (**c**) Effects of miR-151-3p on the endogenous *Stat3* mRNA level were analyzed using RT-qPCR in RAW264.7 cells and primary peritoneal macrophages. (**d**) Effects of miR-151-3p on the endogenous Stat3 protein and p-Stat3 levels after LPS stimulation for 30 min were analyzed by western blotting in RAW264.7 cells and primary peritoneal macrophages. The data are shown as the means ± s.d. ($n=3$) of one representative experiment. Similar results were obtained in at least three independent experiments. $^{**}P<0.01$, unpaired Student's *t*-test. NC, negative control oligonucleotides; ns, not significant.

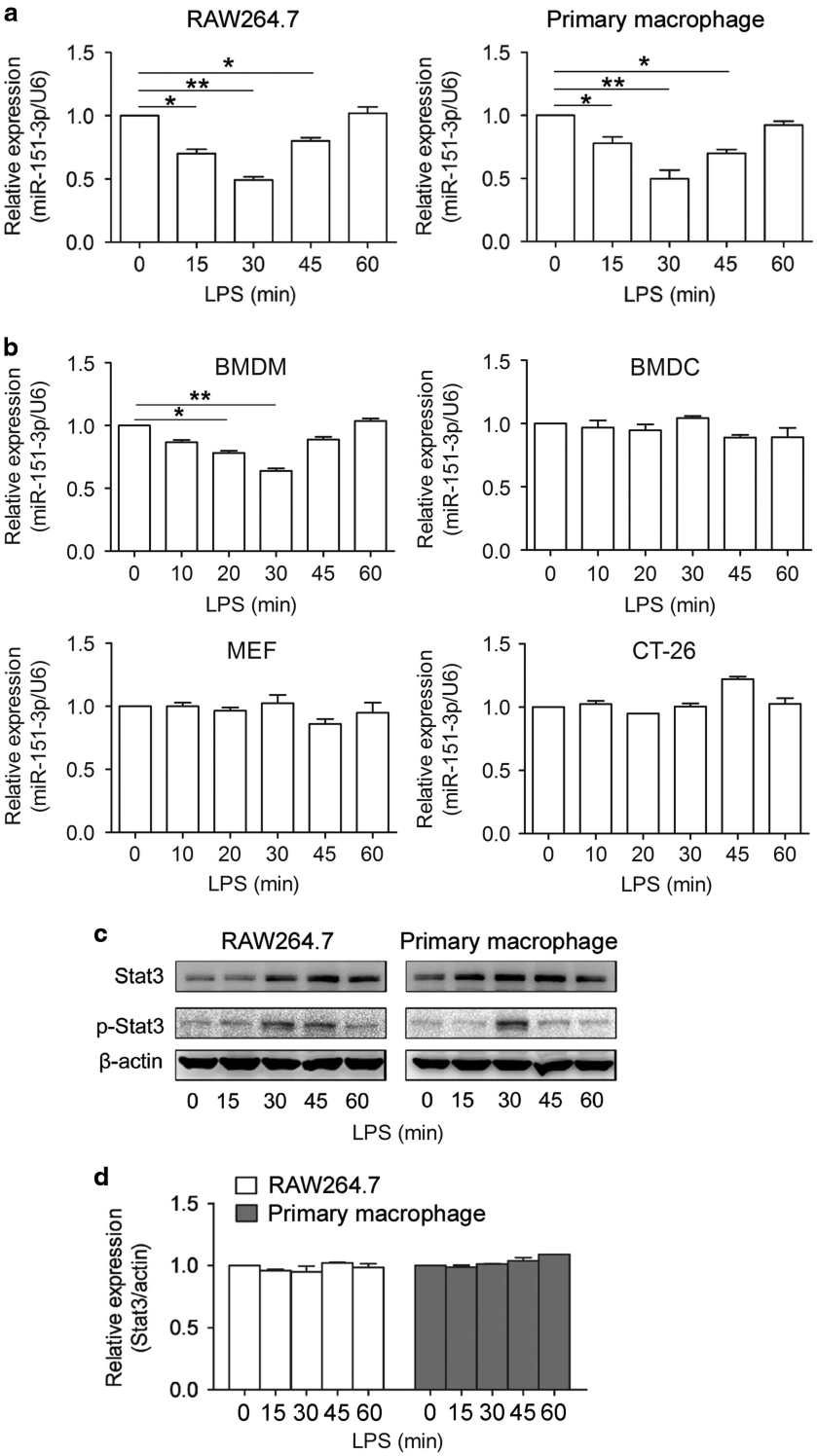

Figure 3 LPS stimulation downregulates miR-151-3p expression and upregulates Stat3 protein levels in macrophages. (**a**) RAW264.7 cells and primary peritoneal macrophages were stimulated with or without 100 ng/ml LPS for the indicated time points. The expression of miR-151-3p was measured using RT-qPCR and normalized to the expression of U6. (**b**) Bone marrow-derived macrophages (BMDM), bone marrow-derived dendritic cells (BMDC), mouse embryo fibroblasts (MEF) and the colon cancer cell line CT-26 were stimulated with or without 100 ng/ml LPS for the indicated time points. The expression of miR-151-3p was measured using RT-qPCR and normalized to the expression of U6. (**c** and **d**) The levels of p-Stat3 and Stat3 protein (**c**) and Stat3 mRNA (**d**) were analyzed by western blotting and RT-qPCR in macrophages stimulated with or without LPS (100 ng/ml) as indicated. Data are shown as the means ± s.d. ($n=3$) of one representative experiment. Similar results were obtained in at least three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$, unpaired Student's *t*-test.

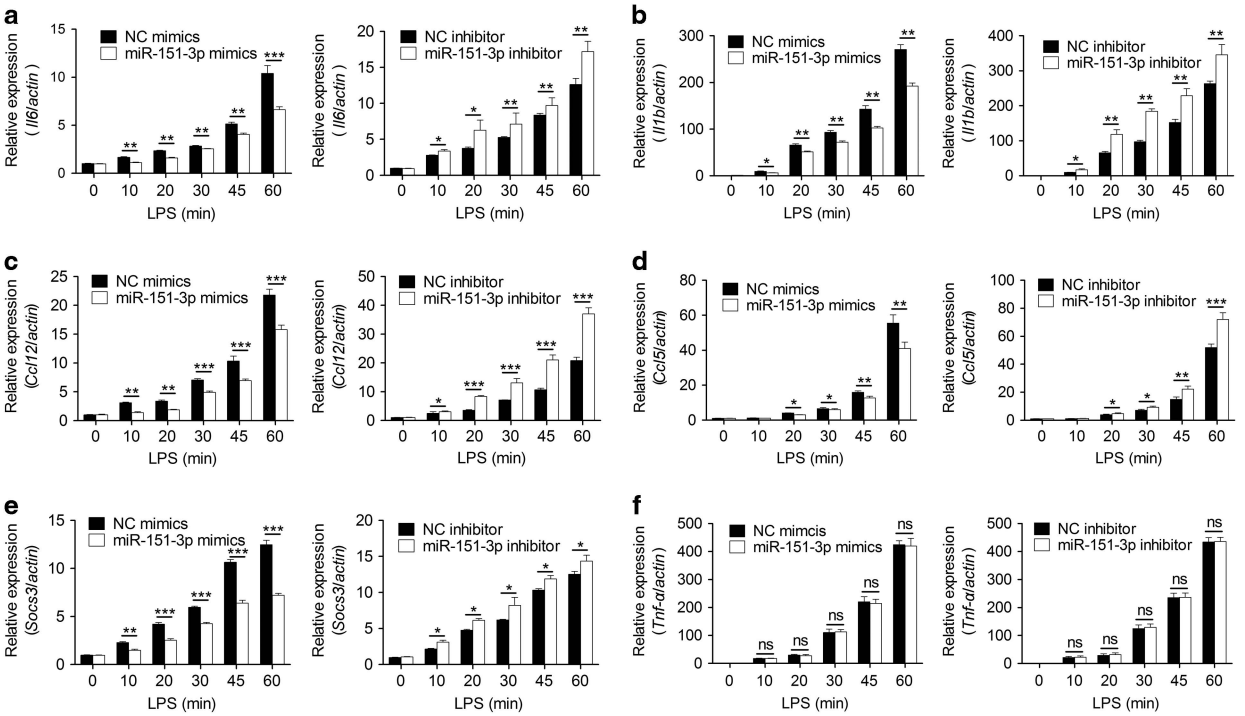

Figure 4 miR-151-3p downregulates Stat3 dependent genes in macrophages. Macrophages were stimulated with or without 100 ng/ml LPS for the indicated time points. The expression of *IL-6* (**a**), *IL-1b* (**b**), *Ccl5* (**c**), *Ccl12* (**d**), *Socs3* (**e**) and *Tnf-α* (**f**) was measured using RT-qPCR. Data are shown as the mean ± s.d. ($n=3$) of one representative experiment. Similar results were obtained in at least three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$, unpaired Student's *t*-test.

brefeldin A (BFA), respectively. Western blot results showed instant Stat3 phosphorylation in these cells upon LPS simulation (Figures 5c–e and Supplementary Figures 2e–g). In addition, the observed phenomenon exists within one hour after LPS stimulation, while the standard LPS/IL-6/Stat3 loop requires more than one hour,[23] suggesting that LPS may trigger instant Stat3 phosphorylation aside from the canonical LPS/NF-κB/IL-6/Stat3 loop. Taken together, these results convincingly demonstrated that the phosphorylation of Stat3 within one hour of LPS stimulation was not driven by IL-6. However, the detailed mechanism needs further elucidation.

miR-151-3p suppresses IL-6 production in LPS-stimulated macrophages by reducing Stat3 expression

As the upregulation of Stat3 post LPS stimulation was not induced by the secretion of IL-6, we further examined the role of Stat3 in LPS-induced cytokine production. We silenced Stat3 by using Stat3 siRNA, and the results indicated decreased IL-6 production upon LPS stimulation but had no effect on Tnf-α production (Figure 6a).

We further investigated whether the LPS-induced downregulation of miR-151-3p could influence IL-6 expression by targeting Stat3. The transfection of miR-151-3p mimics markedly decreased the levels of Stat3 protein and p-Stat3 in primary macrophages stimulated with LPS (Figure 6b and Supplementary Figure 2h). Conversely, increased levels of Stat3 and p-Stat3 expression were observed in macrophages treated with miR-151-3p inhibitor (Figure 6b and Supplementary Figure 2i). The upregulation of IL-6 upon LPS stimulation was markedly reduced in macrophages treated with miR-151-3p mimics, but the opposite phenomenon was observed in cells transfected with the miR-151-3p inhibitor (Figures 6c and d).

To further investigate whether the changes in IL-6 were due to the differences in the NF-κB signaling pathway between miR-151-3p and scrambled negative control mimic-transfected macrophages, we examined the activation of the NF-κB signaling pathway. No significant difference in the level of p-IKKβ, p-IkBα and p-p65 was observed between miR-151-3p-treated macrophages and scrambled negative control mimic-treated macrophages (Figure 6e). Similar results were obtained in inhibitor-transfected macrophages (Figure 6f). These results suggest that the changes in IL-6 are not induced by the classical NF-κB signaling pathway.

Next, both gain-of-function and rescue studies were performed to convincingly demonstrate the role of miR-151-3p in the regulation of IL-6 via Stat3 in macrophages. Indeed, miR-151-3p could downregulate Stat3 phosphorylation and activation. However, when Stat3 was overexpressed without 3′-UTR, miR-151-3p could no longer regulate Stat3 expression (Figure 6g and Supplementary Figure 2j) or *IL-6* transcription (Figure 6h). The overexpression of Stat3 by transfecting Stat3 expression plasmid in miR-151-3p mimic-transfected macrophages could rescue the suppression of IL-6 expression by miR-151-3p (Figure 6h). Therefore, miR-151-3p reduces IL-6 production upon LPS stimulation by suppressing Stat3 activation. The LPS-induced downregulation of miR-151-3p in

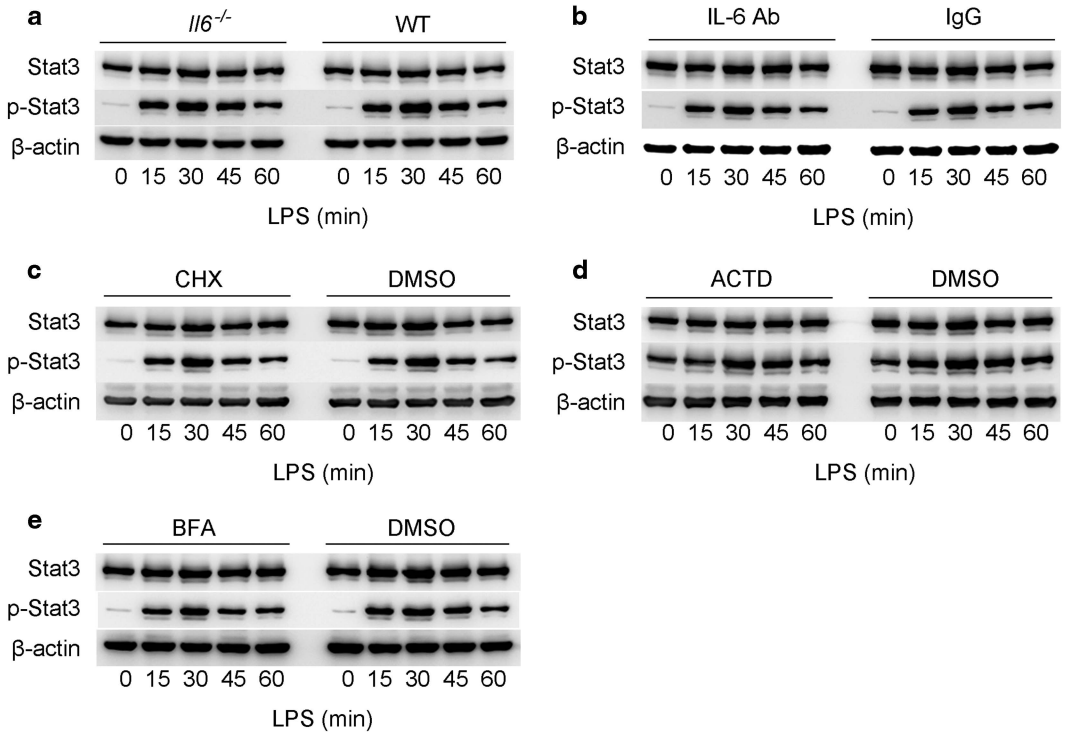

Figure 5 LPS activates Stat3 in an IL-6 independent manner. (**a**) Primary macrophages from IL-$6^{-/-}$ and WT mice were treated with LPS (100 ng/ml) for the indicated time points. p-Stat3 and Stat3 were analyzed using immunoblotting. (**b**) Primary macrophages were treated with IL-6 antibody or mouse normal IgG and subsequently treated with LPS for the indicated time points. p-Stat3 and Stat3 were analyzed through immunoblotting. (**c**–**e**) Primary macrophages were treated with CHX, ACTD or BFA for eight hours and then stimulated with LPS (100 ng/ml) for the indicated time points. p-Stat3 and Stat3 levels were analyzed using immunoblotting.

macrophages contributes to the production of IL-6 by increasing Stat3 protein levels and consequently amplifying the Stat3 pathway in inflammatory responses.

DISCUSSION

Exploring miRNA–mRNA interactions remains as a significant challenge because of the limited knowledge of the rules governing these processes and the high false-positive rate of computational algorithms.[24] Technologies have been developed to identify the targets of certain miRNA and extensively explored over the past decade.[25–30] However, there are few experimental methods for purifying miRNA regulomes. For example, microRNA capture affinity technology (miR-CATCH)[31] and the identification of miRNAs targeting a single gene by applying short biotinylated DNA anti-sense oligonucleotide mix[32] cannot reflect the physiological state *in vivo*. Here, in continuation of a previous work concerning identification of unexpected miRNAs in human cancer cells,[21] several unexpected miRNAs targeting mouse *Stat3* mRNA were identified using a miRIP approach. Among these miRNAs, miR-151-3p was confirmed to directly target Stat3 mRNA.

Until recently, miR-151-3p could not be predicted using bioinformatics tools, even with the loosest parameters. Such results indicated that miR-151-3p was unpredictable using most bioinformatics tools. However, with more evolving bioinformatics tools, the prediction of miRNA:mRNA interactions would become increasingly precise. Other than miR-151-3p, miRNAs such as miR-27b and miR-184, also impact the expression of mouse Stat3, although not as significantly as miR-151-3p, thus confirming the accuracy and efficiency of the method.

MicroRNA-151-3p was primarily expressed in the lungs and enriched in macrophages compared with its expression in other hematopoietic cells. The expression levels of miR-151-3p in T cells and B cells were relatively lower than that in macrophages and almost remained unchanged during the activation of CD4+ T cells, CD8+ T cells, and B cells; however, we cannot conclude that miR-151-3p has no function in T/B cells. The influence of miR-151-3p on T/B cells function needs further investigation. As an organ susceptible to various potential pathogens, it is reasonable that the lung possesses the highest miR-151 expression to maintain homeostasis.

MicroRNA − 151-3p was also markedly decreased in LPS-induced macrophages, and the down-regulation of miR-151-3p led to the up-regulation of Stat3, which further promoted the subsequent production of many Stat3-dependent genes, such as the pro-inflammatory cytokine IL-6. The level of miR-151-3p after LPS stimulation diminished at 30 min, began to recover at 45 min, and finally returned to the baseline levels at 60 min. We assume that this finding may have biological significance, as the overactivation of STAT3/IL6 signaling could be avoided in this manner. The recovery of miR-151-3p restrains IL-6 production, and thus the risk of the harm resulting from uncontrolled immune responses may be minimized. In this

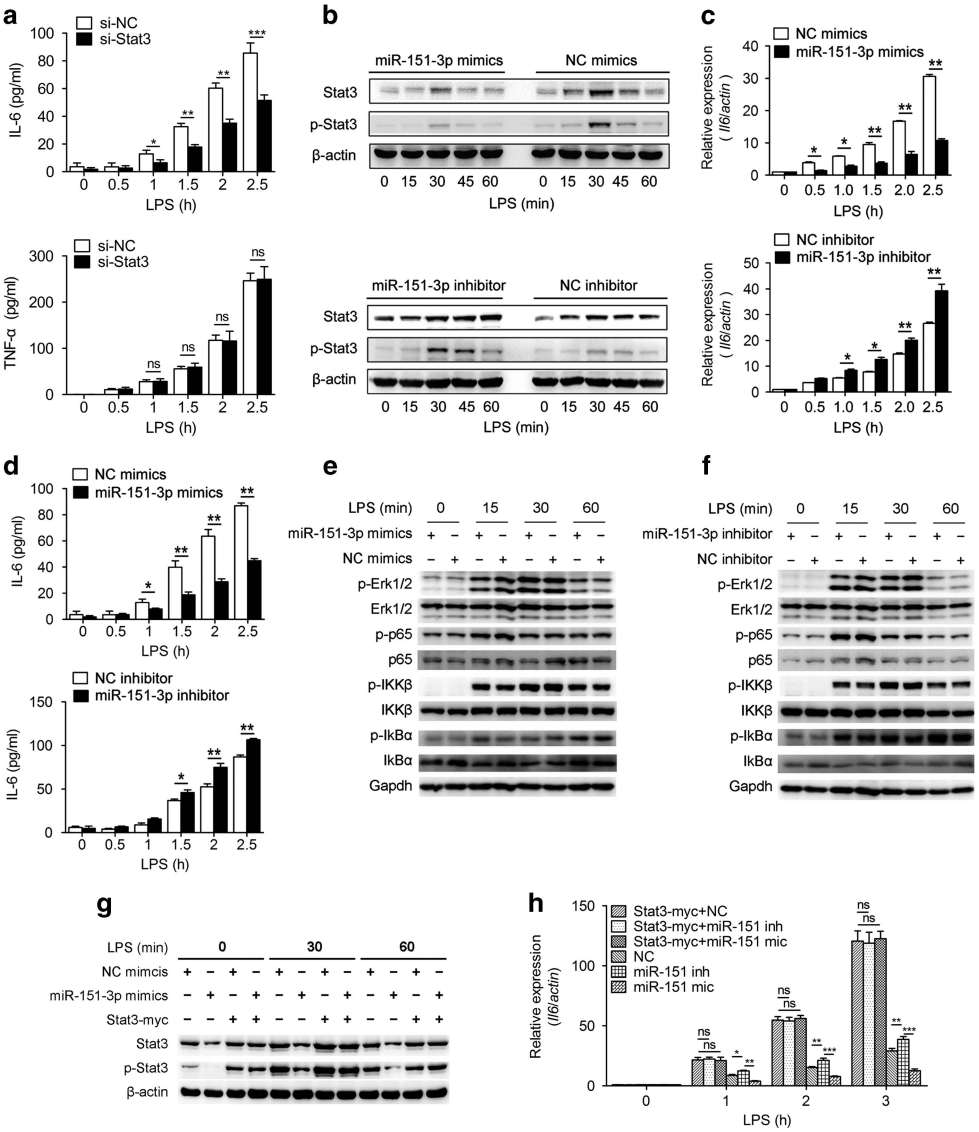

Figure 6 miR-151-3p negatively regulates LPS-induced IL-6 production in primary macrophages by targeting *Stat3*. (**a**) Primary macrophages were transfected with Stat3 siRNA or a scrambled negative control. Quantification of IL-6 and Tnf-α in cell culture supernatant were determined using ELISA after stimulation with LPS (100 ng/ml) for the indicated time. (**b**) Primary macrophages were transfected with mimics or inhibitors of miR-151-3p, then stimulated with LPS (100 ng/ml) for the indicated time points. p-Stat3 and Stat3 levels were analyzed by immunoblotting. (**c** and **d**) Primary macrophages were transfected with miR-151-3p mimics or inhibitors. After 36 h, the cells were stimulated with or without LPS (100 ng/ml). *IL-6* was measured at the indicated time points using RT-qPCR (**c**) and ELISA assay (**d**). (**e** and **f**) Representative immunoblot analysis of phosphorylated Erk, total Erk, phosphorylated p65, total p65, phosphorylated IKKβ, total IKKβ, phosphorylated IkBα, total IkBα and GAPDH from primary macrophages transfected with miR-151-3p mimics (**e**) or inhibitors (**f**) and then stimulated with 100 ng/ml LPS for the indicated time points. (**g** and **h**) Primary macrophages were transfected with negative control, miR-151-3p mimics, negative control plus the Stat3-myc overexpression plasmid as indicated. After 48 h, the cells were stimulated with LPS (100 ng/ml) for the indicated time points. The levels of Stat3 and p-Stat3 were analyzed by immunoblotting (**g**), and the levels of *IL-6* mRNA were measured using RT-qPCR (**h**). The data are shown as the means ± s.d. ($n=3$) of one representative experiment. Similar results were obtained in at least three independent experiments. NC, scrambled negative control oligonucleotides. *$P<0.05$; **$P<0.01$; ***$P<0.001$, unpaired Student's *t*-test.

case, we speculated that miR-151-3p maintains Stat3-dependent pro-inflammatory cytokines from activation, thus avoids casual inflammation in a healthy state, and that during infection, miR-151-3p enables the efficient clearance of pathogens, prevents the over-production of pro-inflammatory response and may participate in inflammation resolution. In general, miR-151-3p functions in maintaining macrophage homeostasis in a basal state and restricts harmful inflammatory responses after infection or injury.

Stat3 acts as a hinge in various signaling pathways in immune cells and inflammation responses and to some extent maintains the immune homeostasis. The upstream signal

sources of Stat3 (IL-6, IL-21, IL-23, IL-10 and so on) and the downstream targets of Stat3 (IL-6, IL-17, IL-10 and so on) confer Stat3 a dual role in anti-inflammatory and pro-inflammatory responses. Furthermore, Stat3 is also pivotal in different immune cell type maintenance functions.[33] Stat3 plays an important role in Th2 cell responses[34] and functions in Treg cell differentiation.[35] In addition, Stat3 was crucial for Th17 differentiation through IL-23 and IL-6 signals.[36]

In addition to T cells, Stat3 plays important roles in myeloid cells, particularly in DC and macrophage differentiation and function.[37,38] Most of the functions of Stat3 were realized by phosphorylation, methylation or acetylation modifications at different positions, but total Stat3 protein is rarely observed in innate immune responses.[15,16] Previous data have revealed relatively high levels of Stat3 phosphorylation in RAW264.7 cells at 30 min after LPS stimulation,[39] similar to our results. Our current study showed that total Stat3 continued to increase at the protein level, but the *Stat3* mRNA level did not change in macrophages upon LPS treatment, implying the post-transcriptional regulation of total Stat3 protein during this process. In addition, we investigated the roles of both IL-6 in LPS-induced instant Stat3 phosphorylation and Stat3 in LPS-induced cytokine production. We convincingly demonstrated that the phosphorylation of Stat3 within one hour of LPS stimulation was not driven by IL-6 secretion and that Stat3 does play an important role in LPS-induced IL-6 secretion aside from the canonical LPS/NF-κB/IL-6 signaling pathway. Although multiple miRNAs, including miR-17, miR-20a, miR-93, miR-106b, miR-125b, miR-199 and miR-223, target *Stat3* mRNA,[18–20] it is likely that *Stat3* mRNA is targeted by many other miRNAs based on the long 3′-UTR of Stat3. In this study, several unexpected miRNAs targeting Stat3 mRNA were identified, and a new Stat3 targeting miRNA (miR-151-3p) was verified to directly interact with Stat3 mRNA and function in inflammatory responses.

The fine regulation of pattern recognition receptors is indispensable for innate immunity.[40] In recent years, both post-transcriptional and epigenetic modifications participate in the sophisticated regulatory network of the immune system.[5,6] In addition to these modifications, non-coding RNAs and microRNAs have important functions in innate immune responses and inflammation.[3,41] Unlike regulators, such as cytokines or other noncoding RNAs, miRNAs function primarily as a buffer to maintain the balance of immune responses. For example, the downregulation of miR-497 by IL-17 results in high HIF-1α expression and the increased production of IL-1β and IL-6 by astrocytes in EAE mice.[42] In addition, microRNAs might be used by pathogens to attenuate human immunity in chronic viral infections.[43,44] For miR-151-3p, the miRIP fished miRNA examined in human cancers [45] and early mouse embryo development,[46] the function in innate immunity is far from clear, reflecting a lack of target gene information. Although the detailed mechanism of the novel fast Stat3 phosphorylation pathway in our study remains unclear, the enhanced production of pro-inflammatory cytokine IL-6 was NF-κB independent.

In summary, using the miRIP method, we identified computationally unpredictable miRNAs targeting mouse Stat3 mRNA and showed that miR-151-3p is a negative regulator of innate immune responses and inflammation by inhibiting IL-6 expression by downregulating the Stat3 and p-Stat3 levels. Thus, we speculated that at steady state, miR-151-3p restricts Stat3 and maintains macrophages in a tolerant state; under infection, miR-151-3p participates in preventing the innate immune system from over-activation. The findings of our current study in immune cells further confirmed that miRIP is an efficient method for identifying miRNAs that bind target mRNAs in the immune system and expands its application in the identification of previously unexpected miRNAs in cancer.

CONFLICT OF INTEREST

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