

Article

## Rheumatoid Arthritis-Associated MicroRNA-155 Targets SOCS1 and Upregulates TNF- $\alpha$ and IL-1 $\beta$ in PBMCs

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**Abstract:** miR-155 plays a crucial role in proinflammatory activation. This study was carried out to assess the association of abnormal expression of miR-155 in peripheral blood of patients with Rheumatoid arthritis with the expression of TNF- $\alpha$  and IL-1 $\beta$ . Release of TNF- $\alpha$  and IL-1 $\beta$ , and expression of miR-155 were determined in RA peripheral blood or peripheral blood macrophages, followed by correlation analysis of the cytokines release and miR-155 expression. Furthermore, *in vitro* studies indicate that miR-155 inhibited the expression of SOCS1. Our results suggest that there is a correlation between the high-level expression of miR-155 and the enhanced expression of TNF- $\alpha$  and IL-1 $\beta$ . miR-155 targets and suppresses the expression of SOCS1, and the decrease of SOCS1 may lead to the upregulation of TNF- $\alpha$  and IL-1 $\beta$ .

**Keywords:** miR-155; rheumatoid arthritis; TNF- $\alpha$  and IL-1 $\beta$ ; SOCS1

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### 1. Introduction

Rheumatoid arthritis (RA) is an autoimmune disease with chronic synovial inflammation and about 1% of the world population suffers from this progressive joint destruction disease. The pathogenesis of RA is not fully understood, but it is thought that the interaction of genetic, immunological and environmental factors contributes to the disease [1]. In addition to the deposition of immune complexes (IC) of autoantigens and their antibodies, known as rheumatoid factor (RF), it was reported that T cells,

B cells and the orchestrated interactions of pro-inflammatory cytokines (CKs) play key roles in the pathophysiology of RA. The release of CKs, especially TNF- $\alpha$ , IL-6 and IL-1 $\beta$ , cause synovial inflammation. Furthermore, CKs promote the development of systemic effects, including production of acute-phase proteins (such as CRP), anemia of chronic disease, cardiovascular disease and so on [2,3]. However, little is known about the mechanism of the dysregulation of CKs expression in RA patients.

MicroRNAs (miRNAs) are small noncoding RNA molecules (18–22 nucleotides long) that regulate gene expression at the post-transcriptional level. miRNAs can bind the 3'-untranslated region of target messenger RNA (mRNA) to either block the target mRNA translation or to induce target mRNA degradation [4,5]. The expression of about 30% of human genes has been reported to be regulated by miRNAs [6]. Rapidly accumulating data suggest that miRNAs have important roles in immune responses and the development of autoimmunity [7–10].

Elevated levels of miR-146a, miR-155, miR-132, and miR-16 are observed in RA peripheral blood mononuclear cells (PBMCs); particularly miR-146a and miR-16 are associated with disease activity [11]. In addition, miR-146a overexpression contributes to the high levels of TNF- $\alpha$  in both peripheral blood (PB) and synovial fluid (SF) in RA [12]. On the contrary, miR-124a and miR-15a are down-regulated in synovial membrane in clinical and experimental arthritis [12–15]. Kurowska-Stolarska *et al.* evaluated the functional contribution of miR-155 in clinical and experimental arthritis models. It was shown that miR-155 is crucial for the proinflammatory activation of human myeloid cells and antigen-driven inflammatory arthritis [16]. Therefore, the miR-155-based therapeutic approaches may modulate the aberrant innate and adaptive autoimmunity associated with RA, and the details involved in the regulation of inflammatory activation in RA need urgently to be uncovered.

We have carried out experiments to confirm the abnormal expression of miR-155 in peripheral blood of patients with RA and to investigate further details of the dysregulation of miR-155 in RA on the expression of CKs, such as TNF- $\alpha$  and IL-1 $\beta$ .

## 2. Results

### 2.1. miR-155 Is Upregulated in PBMCs of Active RA Patients

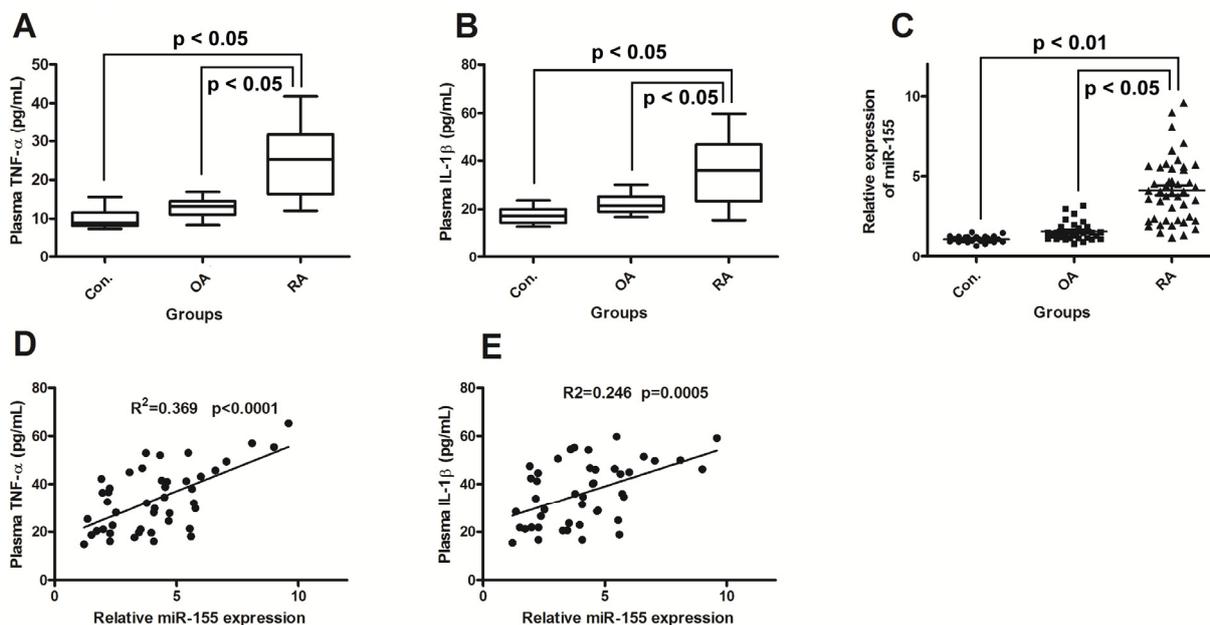
The demographic, clinical, and laboratory data of all subjects are summarized in Table 1. The patients with active RA ( $n = 45$ ) were seropositive for RF, ESR, anti-CCP (Table 1). Both TNF- $\alpha$  and IL-1 $\beta$ , which play important roles in RA, were significantly higher in the patients with RA than in the patients with OA or controls ( $p < 0.01$ ) (Figure 1A,B). In addition, IL-1 $\beta$  was significantly higher in the patients with OA than in control subjects ( $p < 0.05$ ) (Figure 1B), but the other parameters did not show any difference between these two groups ( $p > 0.05$ ). In the group of patients with RA, the median of the miR-155 expression in PBMCs was 4.13 (1.20–9.60). This was significantly higher than the expression level in the patients with OA (median 1.59 (0.84–3.20)) and in the control group (median 1.11 (0.7–1.56)) ( $p < 0.001$ ). However, the difference was not statistically significant between the patients with OA and control subjects ( $p = 0.06$ ) (Figure 1C).

**Table 1.** Demographic, clinical, and laboratory data of patients with rheumatoid arthritis, osteoarthritis, and controls.

Index	RA (n = 45)	OA (n = 32)	Controls (n = 25)
Gender			
M	13 (28.89%)	1 (3.13%)	2 (8.00%)
F	32 (71.11%)	31 (96.88%)	23 (92.00%)
Age (years)	42 (21–58)	55 (44–62)	39 (22–54)
Duration of disease (years)	7 (0.2–22)	7.5 (3.5–12.5)	—
DAS 28	5.7 (2.5–8.13)	—	—
HAQ score	44.6 (0.0–100.0)	—	—
Therapy			
Conventional	40 (86.96%)		
Anti-TNF- $\alpha$	6 (13.04%)		
ESR (mm/h) <sup>a</sup>	36 (5–107)	11 (7–17)	9 (6–12)
Anti-CCP (U/mL) <sup>a</sup>	105 (41–830)	9 (7.2–16.4)	7.1 (5.1–14.3)
TNF- $\alpha$ (pg/mL) <sup>ab</sup>	25.20 (12–41.8)	12.83 (8.4–16.9)	9.92 (7.4–15.6)
IL-1 (pg/mL) <sup>ab</sup>	36.27 (15.42–59.17)	22.45 (16.8–30)	17.56 (13.2–23.4)

All other data are presented as median (minimum–maximum) and statistically significant at  $p < 0.05$ . <sup>a</sup> Significantly higher in RA than in OA and controls. <sup>b</sup> Significantly higher in OA than in controls.

**Figure 1.** miR-155 upregulation correlates with increased production of TNF- $\alpha$  and IL-1 $\beta$  in Rheumatoid arthritis (RA) patients. (A) and (B): Increased expression of TNF- $\alpha$  and IL-1 $\beta$  in RA plasma. Results are expressed as pg/mL by an ELISA test; (C): MiR-155 is upregulated in PBMCs of active RA patients. Results in patients with RA (n = 45) and OA (n = 32) are shown as fold increase relative to the control group (n = 25); (D) and (E): Correlations between Relative miR-155 expression with the TNF- $\alpha$  or IL-1 $\beta$  expression. RA, rheumatoid arthritis; OA, osteoarthritis. Statistical significance was considered with a  $p$  value  $< 0.05$ .



### 2.2. miR-155 Upregulation Correlates with Increased Production of TNF- $\alpha$ and IL-1 $\beta$ in RA Patients

The correlations between miR-155 expression and the clinical and laboratory parameters of the RA patients were explored. In RA patients, miR-155 expression showed positive correlations with plasma TNF- $\alpha$  level ( $R^2 = 0.369$ ,  $p < 0.0001$ ) and IL-1 $\beta$  ( $R^2 = 0.246$ ,  $p = 0.0005$ ) (Figure 1D,E). In addition, positive correlations were also observed between miR-155 expression and ESR ( $R^2 = 0.371$ ,  $p = 0.022$ ) as well as DAS 28 ( $r = 0.372$ ,  $p = 0.009$ ) (Table 2). There was no significant difference in the expression of miR-155, TNF- $\alpha$ , and IL-1 $\beta$  between male and female patients (data not shown). No correlation was demonstrated between miR-155 expression and the other clinical or laboratory parameters of the patients with RA, including age of patients and disease duration (Table 2).

**Table 2.** Correlations between miR-155 expression and the various clinical and laboratory data of patients with rheumatoid arthritis ( $n = 45$ ).

Variables	Correlation	<i>p</i> -value
Age	−0.152	0.36
Disease duration	−0.478	0.098
Duration of morning stiffness	0.316	0.064
DAS 28	0.372	0.009 <sup>a</sup>
HAQ score	−0.015	0.76
RF	0.125	0.48
ESR	0.371	0.002 <sup>a</sup>
Anti-CCP	0.282	0.18
TNF- $\alpha$	0.369	<0.0001 <sup>a</sup>
IL-1 $\beta$	0.246	0.0005 <sup>a</sup>

<sup>a</sup> Data were analyzed by Spearman rank correlation test and the results were statistically significant at  $p < 0.01$ .

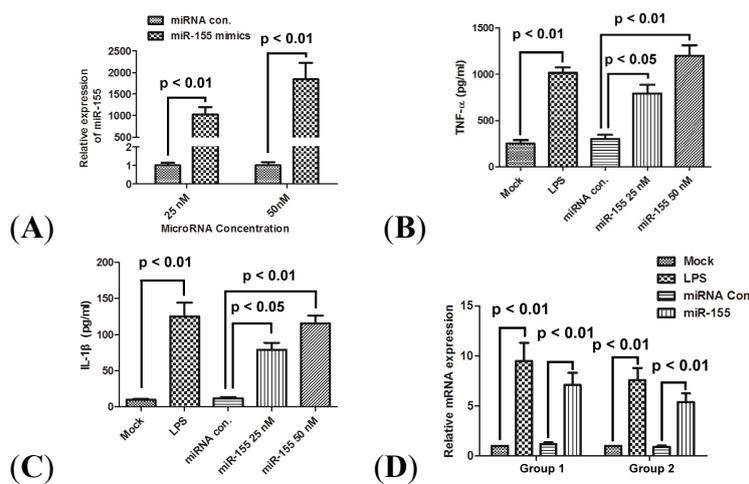
### 2.3. miR-155 Promotes Production of TNF- $\alpha$ and IL-1 $\beta$ in Human Macrophage Cells

To explore the correlation of miR-155 expression with the production of TNF- $\alpha$  and IL-1 $\beta$ , the effect of miR-155 overexpression on the TNF- $\alpha$  and IL-1 $\beta$  was determined in primary PBMCs. miR-155 was overexpressed in human macrophages by transfecting 25 or 50 nM miR-155 mimics (Figure 2A). The miR-155 mimics induced the macrophage cells to express significantly more TNF- $\alpha$  and IL-1 $\beta$  at both protein level (Figure 2B,C) ( $p < 0.01$ ) and mRNA level (Figure 2D) ( $p < 0.01$ ), while the control microRNA did not. The miR-155 mimics had a dose-dependent effect on the stimulation of TNF- $\alpha$  and IL-1 $\beta$  release.

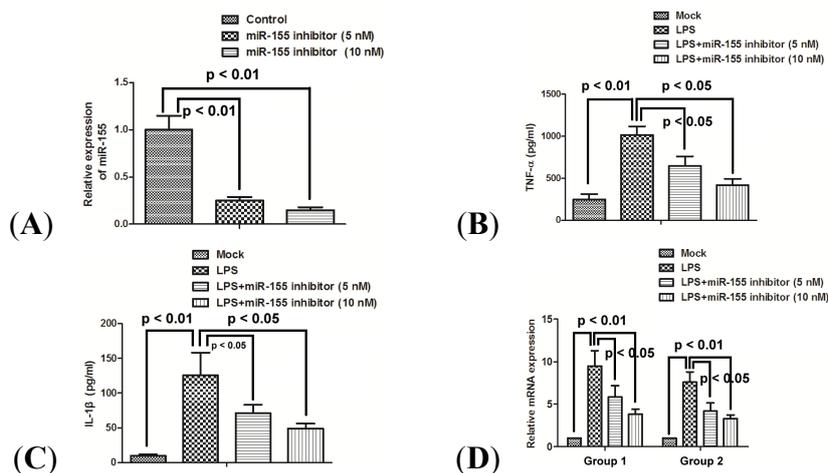
### 2.4. miR-155 Inhibitor Reduces the Production of Proinflammatory Cytokines Induced by LPS

In order to address the effect of miR-155 on the proinflammatory CKs induction in response to LPS, the expression levels of TNF- $\alpha$  and IL-1 $\beta$  were measured after miR-155 was knocked down in a macrophage inflammatory response model. The miR-155 knockdown was achieved by transfecting a synthetic miR-155 inhibitor. The miR-155 expression was significantly suppressed in a dose-dependent manner (Figure 3A) ( $p < 0.01$ ). Furthermore, the high-level expression of TNF- $\alpha$  and IL-1 $\beta$  induced by LPS was also significantly inhibited at both protein level (Figure 3B,C) ( $p < 0.05$  respectively) and mRNA level (Figure 3D) ( $p < 0.05$ ), along with the miR-155 suppression.

**Figure 2.** miR-155 promotes production of TNF- $\alpha$  and IL-1 $\beta$  in human PBMCs. (A) Fold changes of miR-155 expression were determined by qRT-PCR after the transfection of miR-control, or miR-155 mimics at 25 or 50 nM for 24 h; (B) and (C) Expression of TNF- $\alpha$  and IL-1 $\beta$  in supernatant of PBMCs 24 h post LPS treatment, or 24 h post transfection with 25 nM miR-155 mimics, 50 nM miR-155 mimics or 50 nM miRNA control. Results were expressed as pg/mL by an ELISA test; and (D) mRNA expression of TNF- $\alpha$  (group 1) and IL-1 $\beta$  (group 2) in PBMCs 24 h after LPS treatment or 24 h post transfection with 25 nM miR-155 mimics, 50 nM miR-155 mimics or 50 nM miRNA control. All results are the average of three independent experiments. Statistical significance is shown.



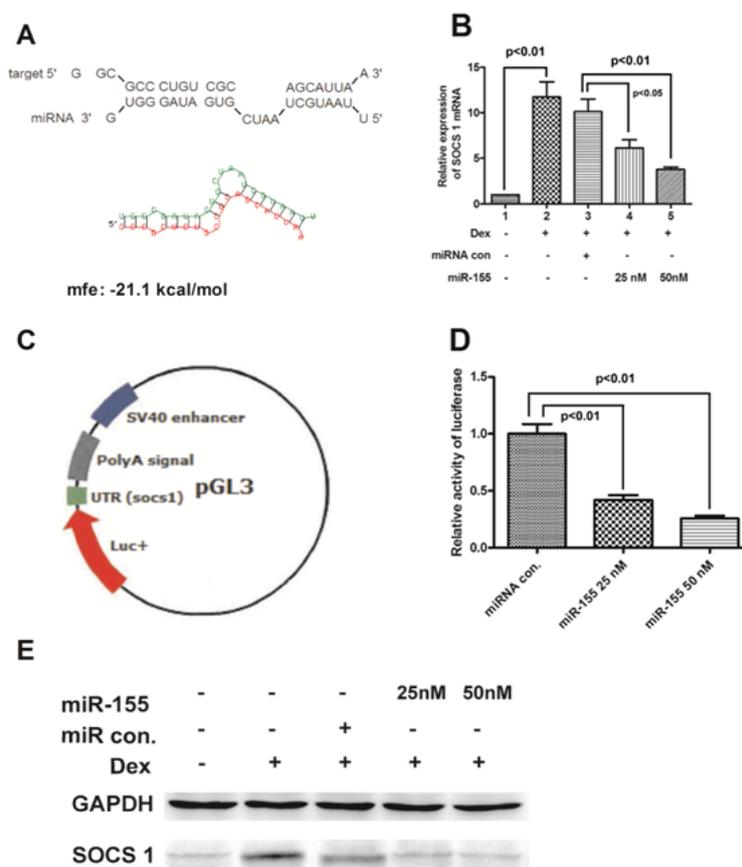
**Figure 3.** miR-155 inhibitor reduces the production of proinflammatory cytokines induced by LPS. (A) Fold changes in miR-155 expression were determined by RT-qPCR 24 h post transfection with 50 nM miRNA inhibitor control, 25 or 50 nM miR-155 inhibitor; (B) and (C) Expression of TNF- $\alpha$  and IL-1 $\beta$  in supernatant of PBMCs 24 h after LPS treatment, or (and) 24 h post transfection with 5 nM miR-155 inhibitor or 10 nM miR-155 inhibitor. Results were expressed as pg/mL by an ELISA test; and (D) mRNA expression of TNF- $\alpha$  (group 1) and IL-1 $\beta$  (group 2) in PBMCs 24 h after LPS treatment, or (and) 24 h post transfection with 10 nM miRNA inhibitor control, 5 nM miR-155 inhibitor or 10 nM miRNA inhibitor. All results are the average of three independent experiments. Statistical significance is shown.



2.5. miR-155 Targets SOCS1 Gene and Suppresses Its Expression

To investigate the details of the overexpression of proinflammatory CKs induced by miR-155, we screened the candidate target genes of miR-155 by PicTAR, Target Scan and miRanda softwares. A total of 83 overlapping genes was predicted by the three tools (data not shown), and SOCS 1 was on the top of the list (Figure 4A). The SOCS1 mRNA expression was strongly induced by Dex, and the induction was dramatically reduced by miR-155 in a dose-dependent fashion (Figure 4B) ( $p < 0.05$  for 25 nM and  $p < 0.01$  for 50 nM miR-155 mimics). In addition, the SOCS1 protein expression level was also decreased by miR-155 (Figure 4E). A luciferase reporter plasmid containing 3' UTR of SOCS1 was utilized to further evaluate the down-regulation of SOCS1 expression by miR-155 (Figure 4C). The luciferase activity induced by LPS decreased significantly when miR-155 mimics were transfected into cells at concentrations of 25 and 50 nM (Figure 4D) ( $p < 0.01$  respectively).

**Figure 4.** miR-155 targets 3' UTR of SOCS1 gene and suppresses its expression. (A) has-miR-155 /SOCS1 alignment by miRanda analysis; (B) and (E) miR-155 suppressed SOCS1 expression at mRNA level. After cells were transfected with miRNA control (50 nM) or miR-155 (25 or 50 nM) and were treated with 100 nM Dex for 24 h, the mRNA levels (B) and protein levels (E) of SOCS1 were quantitatively determined in PBMCs; (C) Schematic diagram of pGL30-con reporter plasmid with 3' UTR of SOCS1; and (D) Relative luciferase activity in PBMCs 24 h post transfection with 50 nM miRNA control, or 25 or 50 nM miR-155. All results are the average of three independent experiments. Statistical significance is shown.



### 3. Discussion

miRNAs have been indicated to play important roles in the regulation of immune responses and the development of autoimmune disorders [17,18]. Reports have confirmed the implication of miR-155 in the differentiation and activation of cells of both the innate and the adaptive immune systems [19]. miR-155 is involved in myeloid cell differentiation and the response to TLR ligands in mice [20–22]. It also mediates regulatory roles in T-cell homeostasis and in the development of B cells in germinal centers [10,23,24]. Recently, miR-155 has been implicated in several autoimmune diseases, including RA. Upregulated expression of miR-155 has been observed in PBMCs and synovial membrane cells of RA patients [11,25]. Further study by Kurowska-Stolarska *et al.* demonstrated that upregulated miR-155 promoted the production of proinflammatory cytokines by targeting Src homology 2-containing inositol phosphatase-1 in RA SF CD14+ Cells [16]. However, functional details of miR-155 in the immunopathogenesis of RA remain obscure. Furthermore, it is not clear whether the upregulated miR-155 is correlated with the dysregulated release of CKs, especially TNF- $\alpha$  and IL-1 $\beta$ , which cause synovial inflammation, systemic effects and cardiovascular disease [2,3].

We now present in this study that the expression of miR-155 in PBMCs of patients with RA is significantly higher than in patients with OA and in healthy subjects (Figure 1C), in agreement with previously reported results [11], as further confirmed by the association of miR-155 with RA. In another aspect of the problem, we have also found significantly high levels of TNF- $\alpha$  and IL-1 $\beta$  in peripheral blood of RA patients (Figure 1A,B); TNF- $\alpha$  and IL-1 $\beta$  is produced mainly by monocytes and macrophages in RA patients [26]. Furthermore, we determined the association of the upregulated miR-155 with the high levels of TNF- $\alpha$  and IL-1 $\beta$ . It was thus shown that there are significant correlations between the miR-155 upregulation in PBMCs and the production of TNF- $\alpha$  and IL-1 $\beta$  in peripheral blood of RA patients (Figure 1D,E), which have not been previously reported.

To further investigate this correlation, we have reproduced the results *in vitro*, in PBMCs separated from normal volunteers without known infection. Over-expression of miR-155 mimics significantly upregulated the production of TNF- $\alpha$  and IL-1 $\beta$  at both protein and mRNA levels in PBMCs (Figure 2B–D). On the other hand, our data demonstrated that the miR-155 knockdown could suppress the TNF- $\alpha$  and IL-1 $\beta$  production induced by LPS (Figure 3B–D). Therefore, based on the *in vivo* and *in vitro* results presented in this study, upregulated miR-155 contributes to the high-level production of TNF- $\alpha$  and IL-1 $\beta$  in PBMCs.

Because a single type of miRNA can regulate the expression of multiple genes via partly complementary binding to mRNA of target genes, and form a regulatory network, the precise regulatory mechanism of miR-155 on the proinflammatory cytokines in RA needs to be explored in detail. A total of 83 overlapping candidate target genes of miR-155 were predicted through three different analysis programs, and SOCS1 was at the top of the list (Figure 4A). The knockdown of SOCS1 by miR-155 has been confirmed in Treg cells during thymic differentiation [23], in virus-infected macrophages [27], and in breast cancer cells [28]. Therefore, we focused on the possible involvement of SOCS1 on the miR-155-upregulated overexpression of TNF- $\alpha$  and IL-1 $\beta$  in human PBMCs.

The present study determined the regulation of miR-155 on the SOCS1 expression in the PBMC model. The SOCS1 expression at both protein and mRNA levels was inhibited by miR-155 in a dose-dependent manner (Figure 4B,E). A luciferase gene fused to the 3' UTR of SOCS1 has been

down-regulated after miR-155 transfection (Figure 4C,D). Therefore, miR-155 targets the 3' UTR region of SOCS1 gene and suppresses its expression in PBMCs. Moreover, the higher level of miR-155 leads to a decreased expression of SOCS1, which may contribute to the increased production of TNF- $\alpha$  and IL-1 $\beta$  in patients with RA.

## 4. Experimental Section

### 4.1. Subjects

A group of 45 patients with RA were recruited in this study from the outpatient clinics of the Department of Physical Medicine at the Shengjing Hospital of China Medical University. All patients with RA fulfilled the American College of Rheumatology (ACR) classification criteria [29]. In addition, a group of 32 patients fulfilling the ACR classification criteria of knee OA were also included [30]. The control group contained 23 healthy women and two healthy men. All subjects included in the study were chosen to be free of diabetes, renal, hepatic, or malignant diseases to exclude the potential influence on miRNA expression pattern. Informed consent was obtained from all subjects, and the study was approved by the ethics committee of China Medical University.

### 4.2. Assay for TNF- $\alpha$ and IL-1 $\beta$

Four milliliters of blood samples in EDTA tubes were used for miRNA extraction, and TNF- $\alpha$  and IL-1 $\beta$  assays. The levels of TNF- $\alpha$  and IL-1 $\beta$  in plasma and PBMCs supernatant were measured by an enzyme immunoassay using the human TNF- $\alpha$  and IL-1 $\beta$  ELISA Kit (Sino Biological Inc., Beijing, China).

### 4.3. Isolation, Culturing and Treatment of PBMCs

Venous blood samples for PBMCs isolation were donated from healthy adults without acute infection. The study protocol was approved by the ethics committee of China Medical University (Shenyang, China). PBMCs were isolated from whole blood using Ficoll-Paque (Biochrom, Berlin, Germany) density gradient centrifugation. Washed cells were resuspended in RPMI medium containing 10% FCS (Invitrogen, Carlsbad, CA, USA) without the addition of antibiotics. Cells were adjusted to  $2 \times 10^6$  cells/mL and cultured in flat-bottom, 12-well, cell-culture plates (Costar, Bodenheim, Germany) at 37 °C with 5% CO<sub>2</sub>. Dexamethasone (Dex) (Sigma-Aldrich, St. Louis, MO, USA) was resolved in RPMI medium with 0.5% ethanol; Lipopolysaccharides from *Escherichia coli* (LPS) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in PBS at a concentration of 1 mg/mL and utilized in RPMI medium at a final concentration of 100 ng/mL. miR-155 mimics [27], miR-155 inhibitor [27] (GMR-miR™, Shanghai, China), microRNA inhibitor, enhanced single-stranded oligonucleotide with chemical modification) and miRNA control were purchased from GenePharma (GenePharma, Shanghai, China). PBMCs were transfected with miR-155, miRNA control or miR-155 inhibitor, using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 24 h transfection, Dex or LPS were added to the PBMCs for 24 h.

#### 4.4. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR)

Total RNA was extracted from tissue samples by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) analysis of the TNF- $\alpha$ , IL-1 $\beta$  and SOCS1 mRNA in PBMCs was performed using SYBR Green with the LightCycle 2.0 (Roche, Mannheim, Germany). All RNA expression levels were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase). miRNA extraction from PBMCs was performed by using the mirVana miRNA Isolation Kit (Ambion, Austin, TX, USA). Quantification of miR-155 expression was conducted using the mirVana qRT-PCR miRNA Detection Kit (Ambion, Austin, TX, USA), and the U6 small nuclear RNA was used as internal control.  $\Delta\Delta C_t$  method was used for relative quantification [31].

#### 4.5. Protein Isolation and Western Blot Analysis

Whole cell extracts were prepared by a standard protocol, and proteins were detected by western blot analysis using polyclonal (Rabbit, Sigma-Aldrich, St. Louis, MO, USA) anti-SOCS1 antibody (Abcam, Cambridge, UK) or polyclonal (Rabbit, Cambridge, UK) anti-GAPDH antibody (Abcam, Cambridge, UK). Goat anti-rabbit IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL, USA) and ECL detection systems (Super Signal West Femto; Pierce, Rockford, IL, USA) were used for detection.

#### 4.6. Luciferase Activity Assay

pGL30-con reporter plasmid with the 3' UTR of SOCS1 was kindly provided by Professor Xiaohe Li at Inner Mongolia Medical University (Huhehaote, China). Briefly,  $3.5 \times 10^5$  PBMCs were transfected with 50 nM miRNA control, 25 or 50 nM miR-155, and 0.5  $\mu$ g pGL3-con reporter plasmid using Lipofectamine 2000 following the manufacturer's instructions. The medium was replaced with DMEM containing 10% FBS at 6 h post-transfection. At 24 h post-transfection, cells were harvested for analysis. Luciferase activity was determined by using a Promega luciferase assay system (Promega, Madison, WI, USA). Experiments were carried out in triplicate, and means were determined.

#### 4.7. Statistical Analysis

Statistical analyses were performed using SPSS16.0 software (IBM SPSS, Armonk, NY, USA). Correlations between miR-155 expression and the various clinical and laboratory data of patients with rheumatoid arthritis were analyzed using the Spearman rank correlation. The TNF- $\alpha$ , IL-1 $\beta$ , SOCS1 or miRNA expression between two groups were analyzed by Student's *t* test. A *p* value < 0.05 was considered statistically significant.

### 5. Conclusions

In summary, the present study has demonstrated the correlations between the upregulated miR-155 and high levels of TNF- $\alpha$  and IL-1 $\beta$  in the peripheral blood from RA patients and in PBMCs *in vitro*.

Further determination of targeting SOCS1 by miR-155 in PBMCs *in vitro* suggests a possible mechanism of high production of TNF- $\alpha$  and IL-1 $\beta$  in RA.

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### Conflicts of Interest

The authors declare no conflict of interest.

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