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miR-181a and inflammation: miRNA homeostasis response to inflammatory stimuli in vivo

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ABSTRACT

Inflammatory stimuli are usually associated with homeostatic responses, which have an important function in protecting the body from excessive inflammatory damage. Previous studies reported the anti-inflammatory effect of miR-181a. The current study utilized two animal models of inflammation, induced by either lipopolysaccharides (LPS) or streptozotocin. We demonstrated that inflammatory stimuli significantly increase miR-181a expression, concurrently with inflammatory factors. In addition, the knock down of toll-like receptor 4 (TLR-4) by small interfering RNA in LPS-induced Raw264.7 cells significantly reduces the expression of both miR-181a and inflammatory factors. Furthermore, patients with inflammatory response show increased expression of miR-181a, which is strongly correlated with the expression of interleukin (IL)-1β, IL-6, and tumor necrosis factor alpha. These data indicate that the up-regulation of miR-181a may be associated with homeostatic response to inflammatory stimuli by TLR-4 pathway activation. Therefore, miR-181a may serve as a novel marker for inflammatory response.

1. Introduction

Inflammatory stimuli cause tissue injures and are always associated with homeostatic responses. Inflammations are usually resolved in a timely manner, resulting in the elimination of inflammatory cells and release of inflammatory factors from the injured tissue. Inflammatory responses have an important function in protecting the body from deleterious consequences. Several endogenous factors against inflammatory responses have been produced at various stages and distinct locations [1]. However, the detailed mechanism of these endogenous factors remains unclear.

MicroRNAs (miRNAs) are key players in regulating inflammatory response. Some miRNAs have anti-inflammatory functions and serve as negative-feedback regulator of inflammation. Inflammatory stimuli, such as toll-like receptor, interleukin (IL)-1β, or IL-13, can stimulate the up-regulation of miR-147, miR-146a, or miR-21, respectively. These miRNAs either reduce inflammatory factor expression or suppress inflammatory response [2–4]. miR-203 is up-regulated in lesional psoriatic skin, which can directly regulate the expression of pro-inflammatory cytokines in keratinocytes [5]. Pro-inflammatory stimuli reduce miR-181b expression, and miR-181b suppresses nuclear factor-kappaB (NF-κB)-mediated vascular inflammation by targeting importin-α3 [6]. miR-181a suppresses oxidized low-density lipoprotein-stimulated immune inflammatory responses in dendritic cells by targeting c-Fos [7]. miR-181a/b directly targets the down-regulation of p300/CBP-associated factor, a coactivator of tumor necrosis factor alpha (TNF-α), which provides negative-feedback regulation to inflammatory reactions in liver epithelial cells [8]. However, some miRNAs serve as positive regulator of inflammation. miR-19b exacerbates inflammatory activation by targeting NF-κB signaling [9]. miR-125a/b constitutively activates the NF-κB pathway by targeting TNF-α-induced protein 3, which can strength and prolong NF-κB activity [10]. Lipopolysaccharide (LPS)-stimulated miR-16 gene transcription promotes NF-κB-mediated transactivation of IL-8 by suppressing the silencing mediator for retinoid and thyroid hormone receptor [11]. In pancreatic cancer cells, miR-301a represses NF-κB-repressing factor to activate NF-κB, which in turn promotes miR-301a transcription [12].

A previous study has demonstrated that miR-181a has an anti-inflammatory effect in vitro. However, no direct evidence exists on whether miR-181a is involved in inflammation in vivo. Therefore, miR-181a changes in inflammatory diseases will be useful in understanding the exact role of miR-181a in vivo and will protect or deteriorate tissue injuries from inflammatory stimuli.

Abbreviations: LPS, lipopolysaccharides; STZ, streptozotocin; TNF-α, tumor necrosis factor alpha; IL-1α, interleukin 1 alpha; IL-1β, interleukin 1 beta; IL-6, interleukin 6.

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2. Materials and methods

2.1. LPS-induced animal model

Four-week old male Balb/c mice [specific pathogen-free (SPF) grade, Certified No. SCXK (Yue) 2008-0002] were obtained from Guangdong Medical Animal Center (Guang Zhou, China). Animals were kept in an environmentally controlled breeding room (temperature: 20 °C ± 2 °C; humidity: 60% ± 5%; 12 h dark/light cycle). The animals were fed standard laboratory chow diets with water ad libitum and fasted from 9:00 am to 3:00 pm before the experiments. The study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Institutional Animal Care and Use Committee of Tsinghua University. The protocol was approved by the Animal Welfare and Ethics Committee of Tsinghua University, China (No. 2011-XWD-CD). All surgeries were performed under sodium pentobarbital anesthesia, and efforts were made to minimize suffering. After the animals were housed for one week, they were intraperitoneally (IP) injected with 2 mg/kg of LPS (Sigma–Aldrich, USA). Blood was collected once from the orbital plexus at 0, 2, 4, 6, 8 and 12 h. Approximately 0.1 mL of whole blood was immediately used to extract miRNA. The remaining blood was used to separate the serum, which was isolated by centrifugation at 1500*g* and 4 °C for 10 min and stored at −80 °C until use for blood biochemical assays.

In a separate trial, animals received IP injection of 2 mg/kg of LPS. Abdominal macrophages were collected at 0, 3 and 6 h. The animals were euthanized by cervical dislocation. Afterward, 5 mL of phosphate buffer solution was given via IP injection in the abdominal cavity of the animals. Approximately 5 mL of the solution was collected from the abdominal cavity of the animals. The collected solution was centrifuged at 1000*g* and 4 °C for 5 min to obtain cell deposits. Supernatants were used to assay inflammatory factor levels, whereas deposits containing macrophage cells were used to extract miRNA.

2.2. Streptozotocin (STZ)-induced animal model

Four-week old male NIH mice [SPF grade, Certified No. SCXK (Yue) 2008-0002] were obtained from Guangdong Medical Animal Center (Guang Zhou, China). The raised conditions and experimental protocols of the animal were identical to that of the Balb/c mice described previously. Diabetic model was induced by IP injection of 100 mg/kg of STZ in NIH mice according to a previous method [13]. Rosglitazone (Sigma–Aldrich, USA) was orally administrated at 4 mg/kg. Normal and diabetic controls were treated with identical volume of distilled water to suspend the rosglitazone. After 4 weeks of treatment, fasting blood was collected from the animals for miRNA or mRNA extraction.

2.3. Cell culture and small interfering RNA (siRNA) transfection

Raw264.7 cells were obtained from the Cell Resource Center of Shanghai Institute for Biological Sciences (Chinese Academy of Sciences, China). The cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and incubated in a humidified atmosphere of 5% CO_2 at 37 °C. siRNAs were transiently transfected into cells with Lipofectamine 2000 reagent (Invitrogen, USA). siTLR-4-1: CAAUUCUGUUGCUUGUAUA; siTLR-4-2: ACAGCCUGAGACACUUAGA. siRNA duplexes with random sequence were used as negative control (UUG UAC UAC ACA AAA GUA CUG). After 6 h to 12 h of transfection, the medium was replaced with fresh medium containing 2 μg/mL of LPS (Lot. No. L4391, Sigma–Aldrich, USA). Cell and medium samples were collected for further biochemical assays (assaying levels of miR-181a and inflammatory factors) at 4 h after LPS induction. All samples were immediately stored at −80 °C for future biochemical assays.

2.4. Patients with inflammatory responses

Fifteen inflammatory patients and fifteen healthy controls were recruited in Shenzhen Traditional Chinese Medicine (TCM) Hospital. Age and sex were well matched between the groups. This study was approved by the Clinical Ethics Committee of Shenzhen TCM Hospital (20120515-16). Before the trial, the volunteers were informed and agreed on participating to this study. The inflammatory responses of the patients were mainly caused by rheumatoid arthritis. Approximately 2 mL of blood was obtained from patients with inflammatory responses, and used to measure miR-181a; serum was used to assay inflammatory factors. Correlationship analysis was conducted between miR-181a and inflammatory factors (IL-1β, IL6, and TNF-α).

2.5. Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) for mRNA and miRNA quantification

Total RNA was isolated using a Trizol reagent (Invitrogen). Real-time qRT-PCR was performed using a reverse transcription kit (Takara) and SYBR® Green PCR Master Mix (Toyobo). mRNA levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Reverse transcription of miRNA was conducted using the TaqMan miRNA reverse transcription kit (Applied Biosystems) according to the protocol of the manufacturer. The primers used are listed in Table 1.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Mouse IL-1α, IL-1β, IL6, and TNF-α, as well as human IL-1β, IL6, and TNF-α, were assayed using the QuickEIA™ ELISA method (DAKEWE Biotech Co., Ltd., Beijing, China). Protein was measured using the Bio-Rad protein assay kit. All samples were normalized with protein concentrations. The other steps were carried out according to the protocols of the kits.

3. Results

3.1. miR-181a expressed concurrently with inflammation factors in blood of LPS-induced mice

We used in vivo models of inflammation to examine the expression of miR-181a. Balb/c mice were injected with LPS to induce inflammatory response. The expression level of miR-181a in the blood was evaluated by qRT-PCR at different time points after LPS induction. The level of miR-181a in blood increased almost twofold at 2 and 4 h after LPS induction, as shown in Fig. 1A. The expression of miR-181a started to decrease at 6 h, but nonetheless 50% higher than the basal level. After 8 h of LPS induction, blood miR-181a dropped to the basal level. We also examined the expression of some inflammatory factors after LPS induction (Fig. 1B–E). Alongside miR-181a, the levels of IL-1α, IL-1β, IL-6, and TNF-α increased at an early time after LPS induction. However, the serum concentrations of these factors started to decline from 6 h of LPS induction. These data suggest that miR-181a experienced a transient increase in blood because of transient inflammatory stimuli.

Table 1
Primers used in this study.

Gene names	NCBI accession no.	Primers (5′ to 3′)	Sizes (bp)
Mouse IL-1α	NM_010554.4	Forward: TCTGCCATTGACCATCTC Reverse: ATCTTCCCGTTGCTTGAC	182
Mouse IL-1β	NM_008361.3	Forward: GTTCCCATTAGACAACTGC Reverse: GATTCTTTCCTTTGAGGC	199
Mouse IL-6	NM_031168	Forward: CTGCAAGAGACTTCCATCCAG Reverse: AGTGGTATAGACAGGTCTGTTGG	131
Mouse TNF-α	NM_013693.2	Forward: GGGCTTCCAGAACTCCA Reverse: GCTACAGGCTTGTCACTCG	213
Mouse GAPDH	NM_008084	Forward: TCTCCTGCGACTTCAACA Reverse: TGGTCCAGGGTTTCTTACT	178

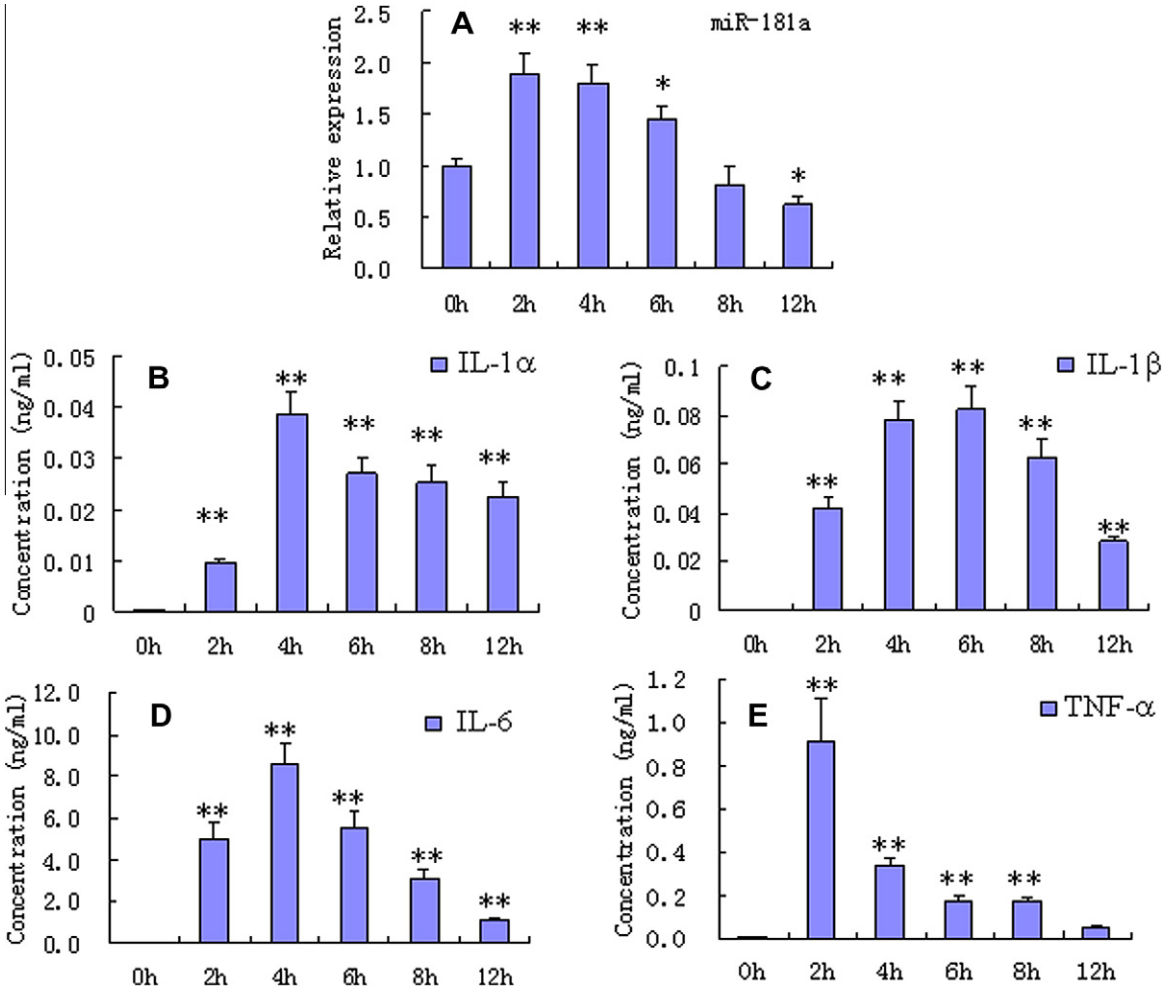

Fig. 1. Levels of miR-181a and inflammatory factors in blood of LPS-induced mice. Data are represented as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. values at 0 h, $n = 6$. miR-181a (A) and inflammatory factors: IL-1α (B), IL-1β (C), IL-6 (D), and TNF-α (E) show a transient increase in the blood of NIH mice with 12 h of LPS induction.

3.2. Levels of miR-181a and inflammation factors in abdominal macrophages of LPS-induced mice

In a separate trial, macrophages were collected from the abdominal cavities of Balb/c mice after LPS injection. miR-181a expression significantly increased at 3 and 6 h of LPS induction (Fig. 2A). Concurrently, a similar expression pattern of IL-1α, IL-1β, IL-6, and TNF-α was detected with increased inflammatory response in mice (Fig. 2B–E). These results further prove that a positive correlation exists between miR-181a and inflammatory factors in acute inflammation state. However, whether miR-181a changes in chronic inflammation state remains unknown.

3.3. Levels of miR-181a and inflammation factors in blood of STZ-induced diabetic mice

We established a mouse model with chronic inflammation state by STZ induction. This animal model showed classic syndromes of type 1 diabetes and low-grade chronic inflammatory responses. The blood miR-181a concentration in the

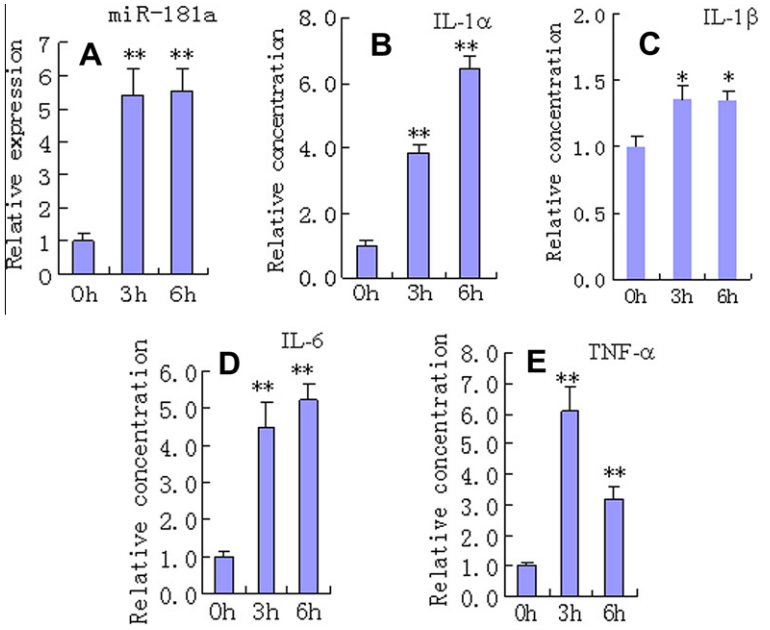

Fig. 2. Levels of miR-181a and inflammatory factor in the abdominal macrophages of LPS-induced mice. Data are expressed as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. values at 0 h, $n = 6$. miR-181a (A) and inflammatory factors: IL-1α (B), IL-1β (C), IL-6 (D), and TNF-α (E) show a significant increase in macrophages in the abdominal cavities of NIH mice with 6 h of LPS induction.

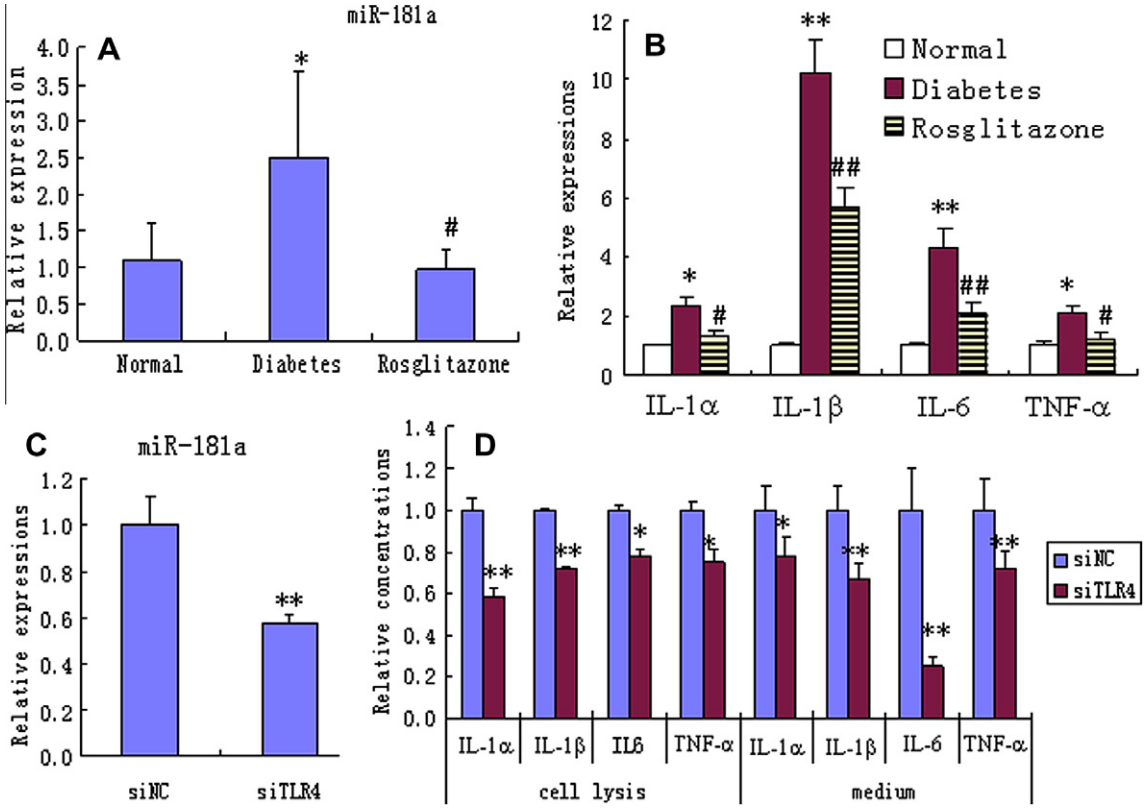

Fig. 3. (A) and (B) Levels of miR-181a and inflammatory factor in blood of STZ-induced mice. Data are expressed as mean ± SD, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. normal controls, $^{\#}P < 0.05$ $^{\#\#}P < 0.01$ vs. diabetic controls, $n = 6$. (C) and (D) Effects of siTLR-4 on miR-181a and inflammatory factor levels in Raw264.7 cells. Data are represented as mean ± SD, $^{*}P < 0.05$ $^{**}P < 0.01$ vs. negative control (siNC), $n = 3$.

STZ-induced diabetic NIH mice was significantly increased upon STZ induction (Fig. 3A). However, this increase was attenuated by rosglitazone, an anti-diabetic drug in the thiazolidinedione class. Similarly, the mRNA expressions of blood IL-1α, IL-1β, IL-6, and TNF-α was induced by STZ, and declined upon rosglitazone treatment (Fig. 3B) These results suggest that miR-181a is up-regulated in chronic inflammation state even in low-grade inflammation.

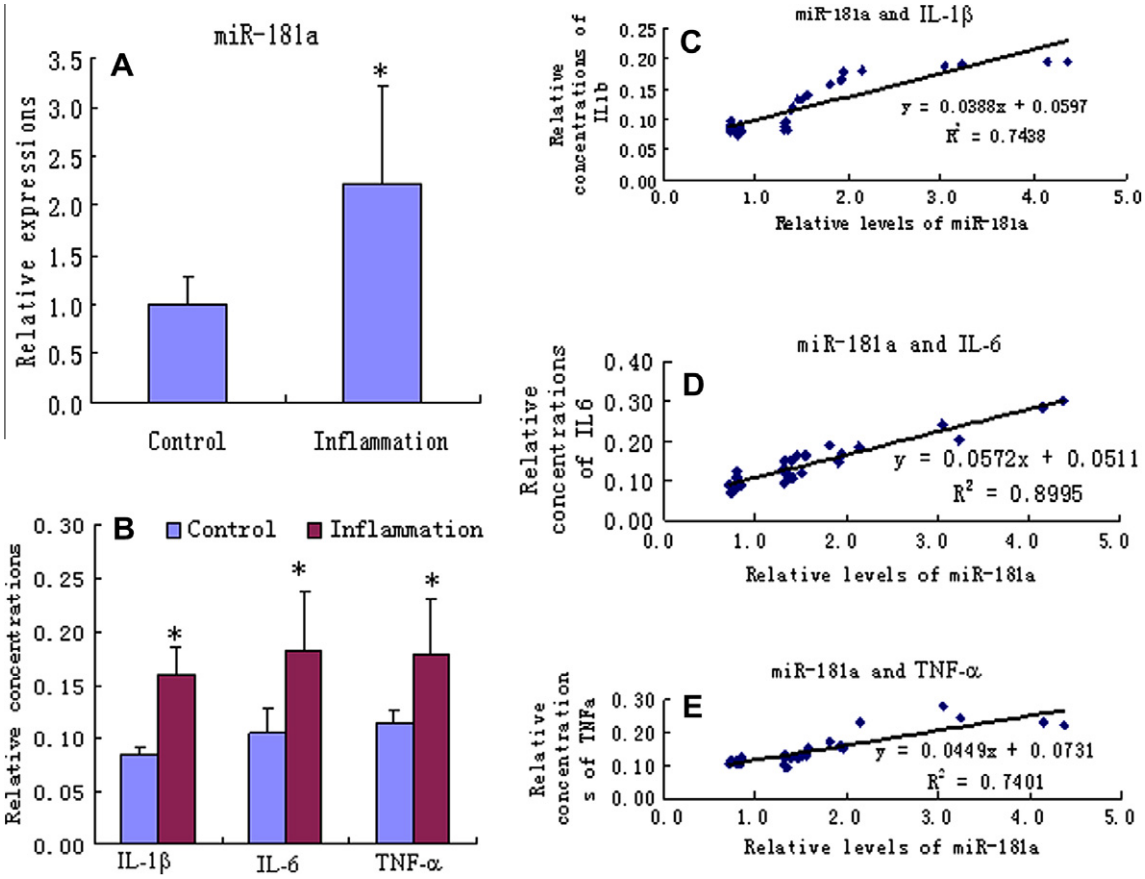

Fig. 4. Levels of miR-181a (A) and inflammatory factors (B) in blood of patients with non-inflammatory and inflammatory responses. Data are represented as mean ± SD, $^{*}P < 0.05$ vs. non-inflammatory controls, $n = 15$. Positive correlationship between miR-181a and inflammation factors: IL-1β (C), IL-6 (D), and TNF-α (E) levels in patients with inflammatory response.

3.4. Effects of siTLR-4 on miR-181a and inflammatory factors in LPS-induced Raw264.7 cells

We demonstrated that both acute and chronic inflammatory responses induce the up-regulation of miR-181a. We will subsequently clarify the signaling pathway that controls the miR-181a expression in inflammation state. Toll-like receptor 4 (TRL-4) modulates immune response and inflammatory diseases. We examined whether TRL-4 is involved in regulating the expression of miR-181a and inflammatory factors. We used an LPS-induced Raw264.7 cell model, which can activate TRL-4 signaling pathway. Specific siRNA against TRL-4 was transfected into Raw264.7 cells. We performed qRT-PCR to assay the expression of miR-181a and inflammatory factors. TRL-4 siRNA decreased the miR-181a mRNA expressions compared with negative control (siNC), as shown in Fig. 3C. TRL-4 siRNA also significantly decreased the expression level of IL-1α, IL-1β, IL-6, and TNF-α (Fig. 3D). These results suggest that TRL-4 signaling may mediate the expression of miR-181a and inflammatory factors.

3.5. Levels of miR-181a and inflammation factors in blood of patients with inflammatory diseases

We examined the expression of miR-181a in patients with inflammatory responses. A significant increase of miR-181a expression was observed in patients with inflammatory responses compared with healthy controls with non-inflammatory responses (Fig. 4A). The up-regulation of IL-1β, IL-6, and TNF-α was also evident in patients with inflammatory responses compared with healthy controls (Fig. 4B). Afterward, we analyzed the expression correlation between miR-181a and inflammatory factors. miR-181a showed a highly positive relationship with IL-1β, IL-6, and TNF-α in patient blood. These results indicate that miR-181a may serve as a novel biomarker for inflammatory diseases.

4. Discussion

Recent studies have demonstrated that miRNA are key players in regulating inflammatory responses. Previous studies have indicated that miR-181a has an anti-inflammatory effect in vitro. However, whether miR-181a is involved in inflammation in vivo remains unclear. In the current study, we used LPS- and STZ-induced mice models to monitor acute and chronic inflammatory states in vivo. We demonstrated that miR-181a is up-regulated in the blood and abdominal macrophages, concurrently with inflammatory factors at the early stage of inflammatory response. Moreover, miR-181a up-regulation was detected in patients with inflammatory responses, which is strongly correlated with the expression of inflammatory factors IL-1β, IL-6, and TNF-α. These results suggest that miR-181a is involved in regulating inflammatory response in vivo, and may serve as a novel biomarker for inflammatory diseases.

The expression of miR-181a and inflammatory factors declined at the late stage of inflammatory response (Fig. 1A). This decrease may be associated with the decreased number of white cells because most white cells in the blood moved into the peripheral tissues in the late stage of inflammatory response. The degradation of LPS and the decrease of inflammatory factors also lowered the

requirement of miR-181a homeostasis responses to inflammatory stimuli in the late stage of inflammatory response. Thus, the transient increase of miR-181a at the early stage of inflammatory response indicates that miR-181a can be a homeostatic response to inflammatory stimuli, which may be useful in protecting tissues from excessive injury.

LPS binds to TLR-4 receptor and triggers the development of acute inflammation [14]. This study demonstrated that the knockdown of TRL-4 expression by siRNA significantly reduces the expression of miR-181a and inflammatory factors IL-1α, IL-1β, IL-6, and TNF-α in LPS-induced Raw264.7 cells. Diabetes is associated with low-grade chronic inflammation [15,16], and is mediated by the TLR-4 pathway activation [17]. Rosglitazone is a classic antidiabetic drug that activates peroxisome proliferator-activated receptor γ and enhances insulin sensitivity [18]. In particular, rosglitazone has anti-inflammatory effects by attenuating the TLR-4 signaling pathway [19]. Thus, both rosglitazone and TLR-4 siRNA have similar effects on attenuating the expression of miR-181a, indicating that TLR-4 signaling may be involved in regulating miR-181a expression during inflammatory response.

This study demonstrated that miR-181a may be associated with homeostatic responses to inflammatory stimuli, and miR-181a compensate inflammatory injures in vivo. Blood miR-181a may serve as a new marker in individuals with inflammatory responses, and miR-181a can be a new target for the development of anti-inflammatory drugs. Using bioinformatic approaches, we discovered that multiple inflammatory factors can be miR-181a putative targets. However, further studies needs to be conducted to clarify the detailed mechanisms of miR-181a in regulating inflammatory response.

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