Original Paper

MiR-410 Down-Regulates the Expression of Interleukin-10 by Targeting *STAT3* in the Pathogenesis of Systemic Lupus Erythematosus

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Key Words

Systemic lupus erythematosus (SLE) • MiR-410 • Interleukin-10 (IL-10) • *STAT3* • CD3+ T cells

Abstract

Background/Aims: Systemic lupus erythematosus (SLE) is a heterogeneous chronic inflammatory autoimmune disorder, in the pathogenesis of which miRNAs play a versatile function. The purpose of this study was to investigate the effect of miRNA-410 on the pathogenesis of SLE in T cells of SLE patients. **Methods:** Real-time PCR was used to test the mRNA levels of miRNA-410 in SLE patients and healthy controls. ELISA analysis was performed to examine the production levels of IL-10. Luciferase Assay was used to confirm the targeting effect of miRNA-410 on 3'UTR of *STAT3* mRNA. **Results:** We found that the expression level of miR-410 in T cells of SLE patients was decreased comparing to that in healthy controls, whereas overexpression of miR-410 significantly reduced the expression levels of IL-10. Furthermore, miR-410 suppresses the transcription activity of *STAT3* by binding directly to the 3'UTR of *STAT3* mRNA. Moreover, silence of *STAT3* down regulated IL-10 expression in CD3+ T cells. **Conclusion:** Our results demonstrate that miR-410 is the key regulatory factor in the pathogenesis of SLE by regulating the expression of IL-10 through targeting *STAT3*. These data suggest a novel function of miR-410 and bring new insight into understanding the complex mechanisms involved in SLE.

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Introduction

Systemic lupus erythematosus (SLE) is a heterogeneous chronic inflammatory autoimmune disorder, which is characterized by progressive involvement of multiple-organ systems with alternating clinical exacerbations and remissions [1, 2]. The pathogenesis of SLE is complex and confusing, involving interactions between environmental and various

aspects of the immune system. Serum interleukin-10 (IL-10) levels have been found higher in SLE patients, and both spontaneous and mitogen-induced IL-10 production is increased in peripheral blood mononuclear cells (PBMCs) of SLE patients [3]. Moreover, it has been demonstrated that IL-10 levels are strongly implicated in the pathogenesis of SLE [4, 5]. Except for IL-10, macrophages [6], hyper-activation of B and T cells, immune complex deposition with subsequent infiltration of neutrophils, reduced ability of immune complexes as well as apoptotic cell clearance, and defects in multiple immune regulatory networks, are found to contribute to organ inflammation and subsequent damage in SLE [7]. Also, Meroni et al. reported that new loci and genes were associated with the pathogenesis of SLE [8]. Current studies have clarified the aberrant molecular biological levels which were induced by environmental and epigenetic factors are thought to contribute to the pathogenesis of SLE [9]. Exploitation of these abnormalities has been proposed for development of therapeutic targets and biomarkers. Although several factors have been found to contribute to the pathogenesis of SLE, the mechanism of action and the target gene in SLE is still not fully understood. Thus, it makes sense to investigate the potential mechanism and screen the SLE-related gene in order to improve the diagnosis and the therapy of SLE.

MicroRNAs (miRNAs) are a class of 21- to 25-nucleotide single-stranded, endogenous, non-coding RNAs which regulate gene expression through binding to the mRNA 3' untranslated. It has been implicated in diverse cellular processes including inflammation factors [10], auto-antibodies [11], cell immunity [12], cell apoptosis, proliferation as well as differentiation [13]. Also, miRNAs have been associated with other different areas such as the immune response [14], neural development, DNA repair, and oxidative stress response [15]. Notably, it is confirmed that lots of diseases including SLE have been found to be regulated by the expression level of miRNAs [16]. Recently, exponentially growing evidence demonstrates that miRNAs play a versatile function in the pathogenesis of SLE [17-20]. Previously, our team has searched for miRNAs that may be involved in the pathogenesis of SLE in PBMCs from SLE patients by microarray analysis [21]. It was found that a total of 29 miRNAs were down-regulated in PBMCs of SLE patients as compared with healthy controls, in which miRNA-410 was likely involved in the signaling transduction pathways in regulating the pathogenesis of SLE.

This study aimed at investigating the role of miRNA-410 in SLE T cells in the possible dysregulated biological processes and pathogenesis of SLE. We found that miRNA-410 by down-regulating the expression of IL-10 through targeting *STAT3*. This study brings new insight into clarifying the complex mechanisms involved in the pathogenesis of SLE and a novel biomarker for SLE diagnostics.

Materials and Methods

Human subjects: SLE patients and healthy controls

This research protocol was approved by Institutional Review Board of China Medical University, which all participants provided consent written informed. Twenty Chinese patients diagnosed with SLE were admitted to Shengjing Hospital of China Medical University and enrolled in our present study. They had never been treated with disease-modifying antirheumatic drugs or other immunosuppressive drugs. The diagnosis of the twenty patients was made according to the classification criteria of SLE developed by the American College of Rheumatology (Hochberg, 1997). Twenty healthy subjects who matched with the SLE patients by gender and age were recruited as controls.

PBLs (peripheral blood lymphocytes) and CD3+/CD4+ T cell isolation

PBLs were obtained from the SLE patients and healthy controls, using ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. Phosphate buffered saline (PBS) was added into the samples at the ratio of 1:1 before centrifugation. T cells were isolated by Ficoll-Hypaque Solution (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation from the venous blood. After cytometry, T cells (10^7 cells/80 μ l buffer) were resuspended in CD3+/CD4+ cells sorting magnetic beads buffer. CD3+ T cells were isolated by

magnetic-activated cell sorting (MACS) kit (CD3 MicroBeads, Miltenyi Biotec, Germany) according to the manufacturer's instructions. CD4+ T cells were also isolated by MACS kit (CD4 MicroBeads, Miltenyi Biotec, Germany). The CD3+ and CD4+ T cells collected were all cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco®, Grand Island, NY, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, HyClone Co., Logan, UT, USA).

Vector construction

Template cDNAs from our lab were used to construct vectors the oligonucleotide primers of which are listed as follows: mir-410 CDS: 5'-CCA GAA GCT TAT GTC CCA TCC GTC CTC A-3' (forward) and 5'-ACT TGG ATC CGC ACA ACC CTA GCA TCT TC-3' (reverse); The 3' untranslated region (3'UTR) of IL-10 was obtained by the amplification of genomic DNA using a forward primer (5'-CTC GCT AGC ACT TCT CTG GAG CAG ATA TT-3') and a reverse primer (5'-GGC GTC GAC TTA CGG TTC CTA TAT AAC G-3'). For the mutant construct, the seed sequence was mutated as follows: 5'-TGA CGA GGC TAA CAC AGA TGG-3' (forward) and 5'-GTG TTA GCC TCG TCA TTA ATA A-3' (reverse). The PCR product was cloned into the pUM-T simple vector (BioTeke Co., Beijing, China) and verified by sequencing. All PCR reactions were performed using Life Express RT-PCR machine (BIOER, Hangzhou, China). The entry clone plasmids, pcDNA3.1-mir-410 and pmirGLO-3'UTR were extracted using No endotoxin plasmid preparation kit (DP2802, BioTeke, Beijing, China) by inserting a T-mir-410/pcDNA3.1+ and a T-3'UTR, pmirGLO fragments, respectively.

RNA interference

To generate miR-410 overexpression and STAT3 silence CD3+ T cells, the clonal plasmids were harvested. For the anti-miR-410 group, a miR-410 inhibitor and a control siRNA were purchased from Gene Pharma (Shanghai, China) and then transfected into CD3+ T cells. The diluted miRNA-410(-) plasmid, miRNA-410, siRNA(-), siRNA(STAT3), anti-miR-410(-) and anti-miR-410 plasmids were added into Lipofectamine® 2000 Reagent (1:1 ratio, Invitrogen, Grand Island, NY, USA) and incubated for 20 min at room temperature. After that, the complex was added to CD3+ T cells and incubated for 4 h at 37°C. Cells were continue to be planted in 6-wells plates with Dulbecco's Modified Eagle's Medium (DMEM, Gibco®, Grand Island, NY, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS, HyClone Co., Logan, UT, USA) for 24 h at 37°C. Later, 50ng/ml PMA (P8139, Sigma-Aldrich, St. Louis, MO, USA) and 1μM ionomycin (1-0634, Sigma-Aldrich, St. Louis, MO, USA) were added into the cells for stimulation. Cells were collected after 24 h incubation.

Real-time PCR

The expression levels of miR-410 and IL-10 in different groups were validated using Real-time (RT) PCR in all cell samples (twenty SLE patients and twenty healthy controls). Total RNA was extracted using High purity total RNA extraction kit (RP1201, BioTeke Co, Beijing, China) according to the manufacturer's protocol. cDNAs were synthesized from total RNAs using Super M-MLV reverse transcriptase kit (PR6502, BioTeke, Beijing, China). The Reverse Transcription products were then amplified for PCR. Primers used in RT-PCR were as follows: miR-410: CCG CAC GAT ATA ACA CAG ATG (sense) and GTG CAG GGT CCG AGG TAT TC (antisense); U6: CTC GCT TCG GCA GCA CA (sense) and AAC GCT TCA CGA ATT TGC GT (antisense); IL-10: CGA TTT AGA AAG AAG CCC AA (sense) and TCA ACA GCT AGA AAG CGT GGT (antisense); STAT3: AAG GAC ATC AGC GGT AAG AC (sense) and GAG ATA GAC CAG TGG AGA CAC (antisense); β-actin: CTT AGT TGC GTT ACA CCC TTT CTT G (sense) and CTG TCA CCT TCA CCG TTC CAG TTT (antisense). SYBR Green (Solarbio, Beijing, China) was used for RT-PCR. Forward primer, reverse primer, and 10 ng template cDNA was prepared; the reaction condition for the RT was as follows: 25°C for 10 min, 42°C for 50 min, and 95°C 5 min. The PCR amplification conditions were: 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 20 s and 72°C for 30 s, then 4°C for 5 min. The PCR results were verified by varying the number of PCR cycles for each cDNA and set of primers. PCR reaction was performed using Exicycler™ 96 RT-PCR machine (Bioneer, Daejeon, Korea) with β-actin as a control. RT-PCR was performed at least in quadruplicate.

Western blot analysis

Anti-IL-10, anti-STAT3, anti-p-STAT3 and anti-β-actin were obtained from Santa cruz Biotechnology, Inc. (Dallas, Texas, USA). For western blot analyses, protein content was determined using BCA Protein Assay Kit (Beyotime Co., Shanghai, China) following manufacturer's instructions. 40 μg of proteins were separated

using 9% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Co., Billerica, MA, USA). The filters were hybridized with the appropriate primary antibodies overnight at 4°C. The relative amounts of the transferred proteins were quantified by scanning the autoradiographic films with a gel densitometer and normalized to the corresponding β -actin level. Quantitative analysis for Western blot was made by Gel-Pro-Analyzer software.

ELISA

The concentration of IL-10 in CD3+ T cells was determined with the Interleukin-10 (IL-10) ELISA Assay Kit (WHB-Bio., Shanghai, China) according to the manufacturer's protocol. All the samples and the standards diluted into different concentrations were added into the wells at the volume of 50 μ l. The absorbance of the wells was measured using microplate reader (BIOTEK, Winooski, VT, USA) at 450 nm, and the concentration levels of IL-10 was calculated from the standard curve.

Luciferase Assay

Luciferase assays were measured using Dual-Luciferase® Reporter (DLR™) Assay System (E1910, Promega, Madison, WI, USA) according to the manufacturer's instructions. In brief, the cells were cultured in 6-well plates and transfected with vectors, miR-410 plasmids or the miRNA mutant control using Lipofectamine 2000 reagent (Invitrogen, Grand Island, NY, USA). The firefly and *Renilla* luciferase activities were measured at 24 h after transfection using the dual-luciferase assay system (Promega, Madison, WI, USA).

Statistical analysis

Data were presented as the mean values $\pm$ standard deviation (SD) from at least three experiments. Statistical comparisons were analyzed by Bonferroni's multiple comparison tests or analysis of variance. $P < 0.05$ was considered as a statistically significant difference.

Results

Dysregulated miR-410 expression in CD3+ T cells of SLE patients comparing to Healthy controls

PBLs from 20 Chinese SLE patients and 20 Healthy controls were collected for this study. CD3+ T cells were isolated by magnetic-activated cell sorting (MACS) kit. RT-PCR analysis was used to explore miRNA-410 expression level in CD3+ T cells from SLE patients and Healthy controls. We found that the expression level of miRNA-410 in SLE patients was lower than that in Healthy controls ($**P < 0.01$, Fig. 1A). As we have known, in the subsets of T cells, CD4+ T cells which are important immune cells in the immune system, have been also used to study the microRNA regulation in SLE [22, 23]. Also, the miRNA-410 level was tested in CD4+ T cells (Fig. 1B). As we expected, the expression level of miRNA-410 in CD4+ T cells of SLE patients was lower than that in Healthy controls ($**P < 0.01$). It was also found that the miR-410 level was negatively correlated with the percentage of CD4+ T cells in SLE patients (Fig. 1C&D). Together with the results in Fig. 1A, our observations indicated that miR-410 expression is decreased in T cells especially in CD4+ T cells of SLE patients comparing with Healthy controls suggesting the close relations between miRNA-410 expression in T cells and the pathogenesis of SLE. For investigating the effects of miR-410 in overall T cells functions, CD3+ T cells were used to investigate the role of miR-410 in SLE for further study.

MiR-410 down-regulated the expression of IL-10 in CD3+ T cells of SLE patients

Cytokines are reported to play an important and diverse role in the pathogenesis of SLE, in which the level of IL-10 is a key factor for disease activity [24]. To evaluate whether the level of IL-10 in CD3+ T cells from SLE patients is associated with miR-410, first, miR-410 overexpression vectors as well as negative control vectors were transfected into CD3+ T cells. The mRNA levels of IL-10 in CD3+ T, miR-NC and miR-410 cells from SLE patients and healthy controls were examined by RT-PCR analysis. Data of healthy control groups in Fig. 2A showed that, the mRNA level of IL-10 was decreased slightly without statistical

Fig. 1. The expression levels of miR-410 in T cells of SLE patients and healthy control. (A) Relative miR-410 mRNA levels in CD3+ T cells of SLE patients and healthy control were tested by RT-PCR. (B) The expression levels of miR-410 in CD4+ T cells of SLE patients compared with healthy control. (C) The percentage change in CD4+ T cells in SLE patients compared with that in healthy control by FACS analysis. (D) Correlation between expression level of miR-410 and percentage of CD4+ T cells in SLE patients. The miR-410 level is negatively correlated with the percentage CD4+ T cells. $r = 0.5648$. SLE, systemic lupus erythematosus. **P < 0.01 versus Healthy control group.

differences in miR-410 CD3+ T cells comparing with that in control group; However, the mRNA of IL-10 was significantly increased in CD3+ T cells of SLE groups (from 1.00 ± 0.00 in Healthy control to 3.69 ± 0.39 in SLE group, $^{**}P < 0.01$), while overexpression of miR-410 down-regulated the mRNA level of IL-10 in half. As seen in T cells of SLE groups, there were no obvious differences between SLE and miR-NC cells, the relative mRNA level of IL-10 between miR-NC and miR-410 group was decreased from 3.76 ± 0.44 to 1.85 ± 0.29 ($^{**}P < 0.01$). The same trends with the result of RT-PCR, Western blot analysis showed that the protein level of IL-10 expression in SLE group was twice higher than that in Healthy control (from 1.00 ± 0.00 in control group to 2.16 ± 0.26 in SLE group, $^{**}P < 0.01$). Overexpression of miR-410 significantly down-regulated the protein level of IL-10 in miR-410 cells. The quantitative value of gray intensity analysis of IL-10 between miR-NC and miR-410 was 2.14 ± 0.26 vs. 0.72 ± 0.13 ($^{**}P < 0.01$, Fig. 2B). In addition, the IL-10 levels in supernatant of CD3+ T cells between Healthy control groups and SLE groups were tested using ELISA analysis, and data showed that the IL-10 level in supernatant of SLE group was increased more than three times than that in Healthy control (from 35.94 ± 4.14 to 140.61 ± 16.95 , $^{**}P < 0.01$). Likewise, overexpression of miR-410 decreased the IL-10 level in supernatant (from 144.94 ± 11.29 in miR-NC group to 55.72 ± 6.74 in miR-410 group, $^{**}P < 0.01$, Fig. 2C). These data suggest that miRNA-410 decreased the expression levels of IL-10 in CD3+ T cells.

To figure out the role of miRNA-410 in the decrease of IL-10, anti-miRNA-410 (CD3+ T cells transfected with miRNA-410 inhibitor vector), miR-NC (CD3+ T cells transfected with an empty vector) and CD3+ T cells were used to compare the expression levels of IL-10 in

Fig. 2. miR-410 regulated the expression of IL-10 in CD3+ T cells of SLE patients. Overexpression of miR-410 down-regulated the expression levels of IL-10 in CD3+ T cells. CD3+ T Cells from both Healthy controls and SLE patients were transfected with miR-410/empty vectors and then stimulated with 50 ng/ml PMA as well as 1 μ M ionomycin for 24 h. Relative mRNA levels of IL-10 were tested by RT-PCR analysis (A). The protein levels of IL-10 were tested by Western blot analysis (B, above) and the quantitative analysis of gray intensity was calculated and showed in (B, below). The concentration of IL-10 in CD3+ T supernatant was tested by ELISA analysis (C). Inhibition of miR-410 increased the expression levels of IL-10 in CD3+ T cells. CD3+ T Cells from both Healthy controls and SLE patients were transfected with miR-410 inhibitor or empty vectors. Relative mRNA levels of IL-10 were tested by RT-PCR analysis (D). The protein levels of IL-10 were tested by Western blot analysis (E, above) and the quantitative analysis of gray intensity was calculated and showed in (E, below). The concentration of IL-10 in CD3+ T supernatant was tested by ELISA analysis (F). &P < 0.05 or &&P < 0.01 versus Healthy control group; *P < 0.05 or **P < 0.01 versus SLE miR-NC group. miR-NC, CD3+ T cells from Healthy control/SLE patients transfected with an empty (negative control) vector; miR-410, CD3+ T cells from Healthy control/SLE patients transfected with a miR-410 vector; anti-miR-410, CD3+ T cells from Healthy control/SLE patients transfected with a miR-410 inhibitor vector.

Fig. 3. miR-410 is a key mediator in the regulation of STAT3 expression in CD3+ T cells of SLE patients. (A) Transfection with miR-410 dramatically reduced the luciferase activity in CD3+ T cells transfected with the luciferase reporter vector containing the 3'-UTR of *STAT3* compared with those transfected with miRNA control groups. *STAT3* 3'-UTR, cells transfected with the vector containing the 3'-UTR of *STAT3*; vector, cells transfected with an empty vector; miR-410, cells transfected with a miR-410 vector; mu miR-410, cells transfected with a mutant miR-410 vector. (B) Overexpression of miR-410 down-regulated the phosphorylation and the expression level of STAT3 by Western blot analysis and the quantitative analysis of gray intensity was calculated and showed in (B).

both Healthy control groups and SLE groups. As seen in the results of Healthy control groups, there were no obvious differences between control and anti-miR-410 group, while in the SLE groups, the mRNA level of IL-10 was obviously increased in anti-miR-410 CD3+ T cells of SLE patients (from 3.17 ± 0.36 in miR-NC cells to 6.53 ± 0.67 in anti-miR-410 cells, ** $P < 0.01$, && $P < 0.01$, Fig. 2D). Western blot analysis showed that the expression levels of IL-10 in anti-miRNA-410 cells of SLE patients was also increased from 2.24 ± 0.30 to 4.41 ± 0.58 (** $P < 0.01$, && $P < 0.01$, Fig. 2E). Meanwhile, the IL-10 level in supernatant of anti-miRNA-410 cells of SLE patients was twice higher than that in miR-NC cells (140.14 ± 9.18 in miR-NC cells vs. 250.02 ± 20.56 in anti-miRNA-410 cells, ** $P < 0.01$, && $P < 0.01$, Fig. 2F). These data suggest that miRNA-410 regulated the expression levels of IL-10 in CD3+ T cells.

MiR-410 is a key mediator in the regulation of STAT3 expression in CD3+ T cells of SLE patients

It is reported that STAT3 is involved in SLE and the target of miR-124. Over-expression of miR-155 significantly increased IL-21 mediated STAT signaling in SLE [22, 25]. In order to find whether STAT3 is the target of miR-410, luciferase reporter assay was performed. The intact 3' UTR of *STAT3* was cloned into luciferase reporter plasmids, which were used for co-transfection with miR-410 or mutant-miR-410 as control into CD3+ T cells. Figure 3A showed that miR-410 significantly suppressed the luciferase activity of the 3' UTR reporter for *STAT3*, whereas mutations of the miR-410 binding sites abrogated the suppression of luciferase expression (Fig. 3A), which suggests that miR-410 suppresses the transcription activity of *STAT3* by directly bind to the 3' UTR of *STAT3* mRNA.

Furthermore, the phosphorylation and expression levels of STAT3 in the CD3+ T, miR-NC (CD3+ T cells transfected with an empty vector) and miR-410 (CD3+ T cells transfected with a miR-410 vector) cells were determined by Western blot analysis (Fig. 3B). As we expected,

Fig. 4. Silence of STAT3 down-regulated the expression of IL-10 in CD3+ T cells. Cells were first transfected with shRNA(STAT3)/shRNA-NC vectors and then stimulated with 50 ng/ml PMA as well as 1 μ M ionomycin for 24 h. (A) The relative expression levels of STAT3 mRNA in CD3+ T, shRNA-NC, shRNA(STAT3) cells were tested by RT-PCR. (B) The expression levels of STAT3 protein. The expression levels of STAT3 protein in CD3+ T, shRNA-NC, shRNA(STAT3) cells were compared by Western blot analysis and the quantitative analysis of gray intensity was determined. (C) Silence of STAT3 down-regulated the expression of IL-10. The protein levels of IL-10 were tested by Western blot analysis (C, above) and the quantitative analysis of gray intensity was calculated and showed in (C, below). The concentrations of IL-10 in supernatant of CD3+ T, shRNA-NC, shRNA(STAT3) cells were tested by ELISA analysis (D) ** $P < 0.01$ versus shRNA-NC group. CD3+ T, CD3+ T cells of SLE patients without RNA interference; shRNA-NC, CD3+ T cells transfected with an empty vector; shRNA(STAT3), CD3+ T cells transfected with STAT3-silence vector.

both the phosphorylation and the expression levels of STAT3 in miR-410 cells was lower than that in miR-NC or CD3+ T cells. There were no obvious differences between CD3+ T and miR-NC cells, the quantitative value of gray intensity analysis of p-STAT3 and STAT3 between miR-NC and CD3+ T was 0.99 ± 0.12 vs. 0.60 ± 0.06 and 1.06 ± 0.12 vs. 0.72 ± 0.11 (** $P < 0.01$, Fig. 3B). Taken together, these results indicate that miR-410 regulated the expression levels of STAT3 by directly bind to the 3' UTR of STAT3 mRNA in CD3+ T cells of SLE.

Silence of STAT3 down-regulated the expression of IL-10 in CD3+ T cells

It has been confirmed that T cell-derived IL-10 mediates myeloid inflammation in part via macrophage STAT3 in the inflammatory bowel diseases (IBDs) [26]. To investigate the relationship between STAT3 and IL-10 in CD3+ T cells of SLE, STAT3 was silenced in

CD3+ T cells by RNA interference. The mRNA level of STAT3 in CD3+ T, shRNA-NC (CD3+ T cells transfected with an empty vector) and shRNA(STAT3) (CD3+ T cells transfected with STAT3 silence vectors) cells were tested using RT-PCR analysis. As shown in Fig. 4A, the mRNA expression levels of STAT3 were 4-fold lower in the shRNA(STAT3) cells (0.27 ± 0.03), as compared with the CD3+ T (1.0 ± 0.00) and shRNA-NC (1.04 ± 0.11) cells. In addition, the expression levels of STAT3 in CD3+ T, shRNA-NC and shRNA(STAT3) cells were also examined by Western blot analysis. Fig. 4B showed that there was little STAT3 expression in the shRNA(STAT3) cells. The quantitative analysis of gray intensity revealed that STAT3 expression levels were also 4-fold lower in shRNA(STAT3) (0.23 ± 0.03) cells, as compared with the CD3+ T (1.00 ± 0.00) and shRNA-NC (0.97 ± 0.10) cells (Fig. 4B, $P < 0.01$).

Western blot analysis was used to test the expression level of IL-10 after silence of STAT3 in CD3+ T cells. As we expected, silence of STAT3 suppressed the production of IL-10 in shRNA(STAT3) cells. There were no obvious differences between CD3+ T and shRNA-NC cells, the quantitative value of gray intensity analysis of IL-10 between shRNA-NC and shRNA(STAT3) was 1.08 ± 0.11 vs. 0.44 ± 0.05 ($**P < 0.01$, Fig. 4C). Identical to the result of Western blot analysis, silence of STAT3 also decreased the IL-10 level in supernatant of shRNA(STAT3) cells comparing to that in CD3+ T cells by ELISA analysis, and data showed that the IL-10 level in supernatant between shRNA-NC and shRNA(STAT3) was 144.64 ± 19.15 vs. 45.12 ± 6.43 ($**P < 0.01$, Fig. 4D). These data suggest that the production level of IL-10 was mediated by STAT3 in CD3+ T cells.

Discussion

SLE is considered as a potentially lethal systemic autoimmune disease with multi-organ damage. Since autoantibodies are elevated in the pathogenesis of SLE, auto-reactive B-cells as well as T-cells have been recognized to be important of it, in which T cells are critical players in SLE pathophysiology as they regulate B cell responses and also infiltrate target tissues, leading to tissue damage [27]. Therefore, they have been studied most intensively and extensively [28, 29]. On the other hand, recent discoveries have indicated that the aberrant expression of miRNAs is associated with the pathogenesis of SLE [30], which gives us new insights into the pathogenesis of SLE and might lead to the finding of new therapeutic targets.

In this study we investigated the expression of miR-410 in CD3+ T cells of twenty Chinese SLE patients and healthy controls to elucidate the role of miR-410 in the pathogenesis of SLE. We found that levels of miR-410 in CD3+ T cells of SLE patients were decreased comparing to that in healthy controls (Fig. 1). Moreover, the production level of IL-10 was increased in CD3+ T cells of SLE patients whereas overexpression of miR-410 decreased the IL-10 level in CD3+ T cells (Fig. 2). Also, we found that miR-410 regulated the levels of STAT3 directly in CD3+ T cells of SLE patients (Fig. 3). Luciferase activity analysis showed that miR-410 was found to directly bind to the 3' UTR of *STAT3* (Fig. 3) while silence of STAT3 decreased the level of IL-10 by Western blot analysis in CD3+ T cells of SLE patients (Fig. 4). Our results indicated that miR-410 plays an important role in the pathogenesis and development of SLE.

Exponentially growing evidence demonstrates that miRNAs play a versatile function in multiple biological and metabolic progresses although the functions are still unclear for a majority of identified miRNAs [31-34]. Recent investigations demonstrate that dysregulated expression of microRNA has been reported to be associated with the pathogenesis of SLE [35]. We found that the mRNA expression levels of miRNA-410 in SLE patients were lower than that in healthy controls, which showed that miRNA-410 may be a key factor in SLE development. Although, in previous studies, it was known that 29 miRNAs were identified differentially expressed in SLE patients, and the expression of miR-1246 was decreased in the activated B cells from SLE patients through the AKT-P53 signaling pathway [36], miR-410 was the first time to show its involvement in the deregulated signaling pathways related to SLE patients [21]. The miRNA also has been reported to function as a tumor suppressor

in human gliomas [37]. Thus, we can speculate that the expression of miR-410 is essential to the pathogenesis of SLE patients. However, it is needed to figure out the precise function of miR-410 and to unravel its regulatory mechanisms in SLE.

IL-10 is found to be an immune-regulatory cytokine with pro- and anti-inflammatory functions, which plays a vital role in the pathogenesis of autoimmune disorders such as SLE, including regulation of the differentiation and the growth of B cells and auto-antibody production [38]. Although there were no changes in serum IL-10 and IL-17 levels in B10 cell transferred MRL/lpr SLE mice [39], in various cells, including T lymphocytes and monocytes/macrophages, IL-10 reduces the expression of pro-inflammatory cytokines, thus suppressing effector phenotypes [40]. However, the effects of IL-10 on B cells appear contradictory. IL-10 is reported to promote B-cell survival, differentiation, proliferation and antibody production in inflammation and disease [41]. It was found that increased level of IL-10 expression in SLE patients correlates with disease activity and antibody production, while blockade of IL-10 corrected the dysregulated cytokine responses [41] and facilitated clinical improvement in SLE patients [42]. Recently, this contradiction has been at least partially resolved. Increasing studies reported that serum IL-10 levels have been found higher in SLE patients and reduced expression of the IL-10 in SLE T cells could alter the effects of IL-10 on activated or memory T cells which are major sources of pro-inflammatory cytokines [23]. In the present study, we found that both the mRNA and the expression level of IL-10 were reduced by overexpression of miR-410 in CD3+ T cells of SLE patients. Also, the serum IL-10 level in CD3+ T cells was decreased in miR-410 overexpressing group. Furthermore, inhibition of miR-410 by transfection with miRNA-410 inhibitor reversed both the serum and the cellular levels of IL-10 in CD3+ T cells. These results confirmed that miRNA-410 regulated the expression levels of IL-10 in CD3+ T cells of SLE patients.

We then studied the mechanism of how miRNA-410 regulated the expression of IL-10 in SLE. STAT3 is a critical member of the STAT transcription factor family. It is reported that overexpression of miR-155 in CD4+ T cells increased STAT3 phosphorylation in response to IL-21 treatment in SLE [22]. Also, Hedrich et al. reported that in lupus T cells, the increasing level of IL-10 was promoted by STAT3 [43]. However, how these events are mechanistically regulated in SLE and involvement, if any, of miRNAs in these events is poorly understood. In our study, we found that miR-410 regulated the expression levels of STAT3 by directly bind to the 3'UTR of *STAT3* mRNA in CD3+ T cells of SLE. Moreover, overexpression of miRNA-410 decreased the phosphorylation as well as the expression level of STAT3 in CD3+ T cells of SLE patients. In SLE, the phosphorylation of STAT3 forms a homodimer, which translocates to the nucleus where it initiates transcription of target genes including the IL-10, IL-17A and IL-21. This forms an autocrine loop for their production via STAT3 phosphorylation [22, 43]. Thus, we can conclude that miRNA-410 regulated the inactivation of STAT3 by suppressing the phosphorylation as well as the expression level of STAT3 via directly targeting *STAT3* gene in CD3+ T cells of SLE patients.

In T cells of SLE patients, activation of STAT3 is increased, resulting in enhanced recruitment to regulatory regions and competitive replacement of STAT5, subsequently promoting IL-10 expression [43]. STAT3 was reported to play a central role in the regulation of pro-inflammatory effector cytokines, including IL-17A and IL-10, promoting effector T-cell phenotypes [44]. Furthermore, STAT3 is involved in IL-10 induction, which resulted in the proposal of STAT3-blocking strategies in autoimmune disorders and cancer. None of those studies, however, targeted the effect of STAT3 on the expression of IL-10 in SLE. In our study, we found that silence of STAT3 promoted the decrease of IL-10 both in CD3+ T cells and in cellular supernatant in of SLE patients. These results showed that STAT3 was the key factor in regulating the expression of IL-10 in CD3+ T cells. It was reported that the STAT signaling pathways are regulated through multiple levels, the intronic STAT-responsive element was reported to regulated the production of IL-10 in murine natural killer cells (through STAT4) and human T cells (through STAT5) [45, 46]. And the STAT-induced STAT inhibitors are confirmed to form a key negative feedback loop for regulation and attenuation of STAT signals. Cheng et al. also reported that the expression of STAT3 in esophageal cancer

cell lines was regulated by miRNA-21 through, at least partially, targeting STAT3 signaling pathway [25]. The particular mechanism of how the STAT3 signaling pathway is involved in the SLE is expected to be clarified by further research. Thus, our study confirmed that miRNA-410 regulated the expression of IL-10 via directly targeting *STAT3* in CD3+ T cells of SLE patients.

Conclusion

In conclusion, we demonstrate for the first time that miR-410 is the key regulatory factor in the pathogenesis of SLE, which regulated the expression of IL-10 via directly targeting *STAT3* in CD3+ T cells of SLE patients. These data suggest a novel function and a therapeutic application of miR-410 in SLE.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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