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MicroRNA-21 and MicroRNA-148a Contribute to DNA Hypomethylation in Lupus CD4$^+$ T Cells by Directly and Indirectly Targeting DNA Methyltransferase 1

Wen Pan,*[,1] Shu Zhu,[†,1] Min Yuan,* Huijuan Cui,*[,‡] Lijia Wang,* Xiaobing Luo,*[,‡] Jia Li,* Haibo Zhou,*[,‡] Yuanjia Tang,*[,‡] and Nan Shen*[,‡]

Systemic lupus erythematosus is a complex autoimmune disease caused by genetic and epigenetic alterations. DNA methylation abnormalities play an important role in systemic lupus erythematosus disease processes. MicroRNAs (miRNAs) have been implicated as fine-tuning regulators controlling diverse biological processes at the level of posttranscriptional repression. Dysregulation of miRNAs has been described in various disease states, including human lupus. Whereas previous studies have shown miRNAs can regulate DNA methylation by targeting the DNA methylation machinery, the role of miRNAs in aberrant CD4$^+$ T cell DNA hypomethylation of lupus is unclear. In this study, by using high-throughput microRNA profiling, we identified that two miRNAs (miR-21 and miR-148a) overexpressed in CD4$^+$ T cells from both patients with lupus and lupus-prone MRL/lpr mice, which promote cell hypomethylation by repressing DNA methyltransferase 1 (DNMT1) expression. This in turn leads to the overexpression of autoimmune-associated methylation-sensitive genes, such as CD70 and LFA-1, via promoter demethylation. Further experiments revealed that miR-21 indirectly downregulated DNMT1 expression by targeting an important autoimmune gene, RASGRP1, which mediated the Ras–MAPK pathway upstream of DNMT1; miR-148a directly downregulated DNMT1 expression by targeting the protein coding region of its transcript. Additionally, inhibition of miR-21 and miR-148a expression in CD4$^+$ T cells from patients with lupus could increase DNMT1 expression and attenuate DNA hypomethylation. Together, our data demonstrated a critical functional link between miRNAs and the aberrant DNA hypomethylation in lupus CD4$^+$ T cells and could help to develop new therapeutic approaches. ***The Journal of Immunology*****, 2010, 184: 6773–6781.**

Epigenetic alternations in genomic DNA include cytosine methylation in CpG islands, which usually extend in the promoter and the first exon of genes. The DNA methylation, which is associated with gene silencing (1), is carried out by DNA methyltransferases (DNMTs). Three main types of DNMTs are involved in genomic DNA methylation: DNMT1, DNMT3A, and DNMT3B. Whereas DNMT1 preferentially replicates existing methylation patterns and maintains DNA methylation, DNMT3A and DNMT3B are responsible for establishing de novo DNA methylation (2). Epigenetic changes in DNA methylation have been implicated in the development of malignant and autoimmune diseases (3). Systemic lupus erythematosus (SLE) is a complex autoimmune disease characterized by chronic immune activation and multiple immunological phenotypes (4, 5). Epigenetic-DNA methylation abnormalities play an important role in the pathogenesis of SLE (6). DNA extracted from the T cells of patients with lupus is hypomethylated compared with the DNA from normal T cells. Various environmental factors, such as procainamide, hydralazine, UV light, aging, diet, and others, can prevent the replication of DNA methylation patterns during mitosis, resulting in the DNA demethylation in T cells and lupus-like autoimmunity (7–9). Such agents usually induce the overexpression of autoimmune-associated methylation-sensitive genes, such as TNFSF7 (CD70) and LFA-1, which confer an autoreactive status to T cells (10, 11). Adoptive transfer of T cells made autoreactive by treatment with DNA methylation inhibitors or by transfection with LFA-1 is sufficient to cause a lupus-like disease in unirradiated syngeneic mice (12, 13). All of these demonstrate that methylation inhibition is sufficient to mediate the pathogenesis of SLE.

MicroRNAs (miRNAs) are small (~22 nucleotides long), noncoding RNAs that mediate posttranscriptional silencing of target genes. In animals, miRNAs usually bind to complementary sites in the 3′ untranslated region (UTR) of target genes and regulate target gene expression by either translational inhibition, mRNA degradation, or both (14). miRNAs are involved in diverse biological processes, including development, cancer, autoimmunity, and so on (15–17). Dysregulation of miRNAs by several mechanisms has also been described in various disease states, including SLE (18–20). However, to the best of our knowledge, studies relevant to miRNAs and lupus CD4$^+$ T cell DNA hypomethylation are still lacking. Recent works have suggested some miRNAs can

*Joint Molecular Rheumatology Laboratory, Institute of Health Sciences and Shanghai Renji Hospital, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences and Shanghai Jiaotong University School of Medicine, [†]Laboratory of Immunity and Diseases, Institute of Health Sciences, and [‡]Key Laboratory of Stem Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China

[1]W.P. and S.Z. contributed equally to the work.

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Address correspondence and reprint requests to Dr. Nan Shen, Department of Rheumatology, Renji Hospital, Shanghai JiaoTong University School of Medicine, 145 Shan Dong Middle Road, Shanghai 200001, China. E-mail address: shennand@online.sh.cn

Abbreviations used in this paper: CDS, coding DNA sequence; DNMT, DNA methyltransferase; miRNA, microRNA; MUT, mutated; LN, lupus nephritis; P/N, positive/negative; siRNA, small interfering RNA; SLE, systemic lupus erythematosus; SLEDAI, SLE Disease Activity Index; UTR, untranslated region; WT, wild-type.

regulate DNA methylation by targeting the DNA methylation machinery. In lung cancer, the miR-29 family directly targets DNMT3A and DNMT3B, thereby leading to a reduction of global DNA methylation and the re-expression of DNA hypermethylated and silenced tumor suppressor genes (21–23). The discovery of the association between miRNAs and methylation regulation provides an insight into the roles of miRNAs in lupus $CD4^+$ T cell hypomethylation and the pathogenesis of SLE.

In this study, we used both miRNA microarray- and quantitative RT-PCR–based approaches to assess miRNA expression in $CD4^+$ T cells from the lupus-prone MRL/lpr mice and patients with lupus. Focusing on the highly elevated miRNAs (miR-21 and miR-148a) in disease, we subsequently demonstrated that these two miRNAs promoted $CD4^+$ T cell hypomethylation by indirectly and directly repressing DNMT1 expression, induced the overexpression of autoimmune-associated methylation-sensitive genes, and mediated the pathogenesis of SLE. These results therefore supported a previously unreported role of miRNAs in the aberrant DNA methylation of lupus T cells and provided a pharmacological rationale for the potential use of synthetic miRNAs inhibitors to attenuate DNA hypomethylation and limit the disease.

Materials and Methods

Mice

Female MRL/lpr lupus-prone mouse strain was purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). All mice were maintained under specific pathogen-free conditions in accordance with institutional guidelines. Splenic $CD4^+$ T cells and B cells were isolated from these mice at 5 and 16 wk of age (before and after autoimmunity is established) (24). Total RNA was isolated and used for miRNA microarray studies.

Subjects

A total of 36 patients (33 female, 3 male; age 35 ± 13 y, disease duration 70.21 ± 66.14 mo [mean ± SD]) were included in the study. Thirty subjects (25 female, 5 male; age 32 ± 14 y [mean ± SD]) were used as healthy controls. The normal controls were matched with the patients for age, sex, and race. All patients fulfilled the American College of Rheumatology classification criteria for SLE. SLE activity was assessed with the SLE Disease Activity Index (SLEDAI). All participants are from Chinese Han population. The participants with concurrent infections were excluded from the study. For additional clinical information, refer to Table I. The study was approved by the Research Ethics Board of Renji Hospital. Peripheral blood samples were collected from each subject in tubes containing acid citrate dextrose formula A.

miRNA microarray analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA quality was assessed with an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA), and only samples with RNA integrity number >8 were used. For the miRNA expression analysis, TaqMan Low Density Assay v2.0 (Applied Biosystems, Foster City, CA) was used to detect and quantitate up to 585 mouse miRNAs using an Applied Biosystems real-time instrument, according to the manufacturer's protocol. In subsequent studies, the TaqMan kits (Applied Biosystems) specified for the quantification of miR-21 and miR-148a were used. Normalization was performed with snoRNA202 and RNU48 endogenous controls for mouse and human, respectively. Comparative real-time PCR was performed in triplicate, including no-template controls. Relative expression was calculated with the comparative cycle threshold method.

T cell culture and small interfering RNA transfection

Jurkat, a T cell leukemia line, was cultured in RPMI 1640 medium (Life Technologies, Rockville, MD) containing 10% FBS and 1% penicillin/streptomycin. For transfection, Jurkat was transfected with 3 μg FAM-labeled small interfering RNA (siRNA) using Amaxa Cell Line Nucleofector Kit V, with the Program X-05 (Lonza, Basel, Switzerland). Cells were analyzed by flow cytometry as early as 6 h postelectroporation. The transfection efficiency was above 85%. Cells were incubated at 37°C with 5% CO_2 for 72 h, and then mRNA or protein knockdown was assessed. PBMCs from healthy donors were isolated by density-gradient centrifugation, and human $CD4^+$ T cells were purified with a human $CD4^+$ T cell isolation kit II (Miltenyi Biotec, Auburn, CA, catalog number 130-091-155). The T cells were typically >94% $CD4^+$ cells. The cells were then cultured in RPMI 1640 medium (Life Technologies) containing 10% FBS and 1% penicillin/streptomycin. For transfection, $CD4^+$ T cells were prepared and transfected with 3 μg miRNA or siRNA using Amaxa nucleofection technology (Nucleofector I, Lonza; catalog number AAD-1001) with the V-24 program for high efficiency, according to the manufacturer's instructions (Human T cell Nucleofector Kit for unstimulated human T cells, Lonza; catalog number VPA-1002). Postelectroporation, cells were immediately transferred to the culture plates containing prewarmed LGM-3 medium (Lonza; catalog number CC-3211) supplemented with FCS and glutamine. The medium was changed after 6 h. T cells were activated with 5 μg/ml plate bound anti-CD3 (eBioscience, San Diego, CA; catalog number 16-0037) and 2 μg/ml anti-CD28 (eBioscience; catalog number 16-0289). Twenty-four hours later, DNMTs transcripts were measured relative to the housekeeping gene Rpl13a using real-time RT-PCR. Forty-eight hours posttransfection, DNMT protein expression were analyzed by Western blotting. For culture periods longer than 48 h, 5 ng/ml IL-2 (R&D Systems, Minneapolis, MN) was added to the LGM-3 culture medium. Quantitative PCR analysis of methylation sensitive genes was performed 72 h postnucleofection. RASGRP1 siRNA sequences are as follows: RASGRP1–siRNA-1: 5′-GGGAUGAGAUCACAGCCUA-3′; and RASGRP1–siRNA-2: 5′-AGAUUGCUGCGAGUUUUCCAU-3′. Negative control is a nonspecific siRNA provided by the manufacturer. The sequences are: sense, 5′-UUCUCCGAACGUGUCACGUTT-3′, antisense, 5′-ACGUGACACGUUCGGAGAATT-3′.

miRNA mimics and inhibitors

All miRNAs mimics and hairpin inhibitors were purchased from Dharmacon (Thermo Fisher Scientific). miRNA mimics are dsRNA oligonucleotides, and miRNA hairpin inhibitors are single-stranded oligonucleotides. Universal negative controls for both inhibitors and mimics are based on the sequences of cel-miR-67 in *Caenorhabditis elegans*. Cel-miR-67 has been confirmed to have minimal sequence identity with miRNAs in human, mouse, and rat. Mature sequence is: 5′-UCACAACCUCCUAGAAAGAGUAGA-3′. The combination of gain-of-function (mimic-induced downregulation) and loss-of-function (inhibitor-induced upregulation) experiments has demonstrated the miRNA-target relationships and allows miRNAs functional analysis.

Luciferase reporter assay

The psiCHECK-2 vector (Promega, Madison, WI) was used to clone the 3′ UTR of RASGRP1 or DNMT1 mRNA. The vector contains a multiple cloning region downstream of the stop codon of an SV40 promoter-driven Renilla luciferase gene. This allows expression of a Renilla transcript with the 3′ UTR sequence of interest. Renilla luciferase activity is then used to assess the effect of the 3′ UTR on transcript stability and translation efficiency. The psiCHECK-2 vector also contains a constitutively expressed firefly luciferase gene. Firefly luciferase is used to normalize transfections and eliminates the need to transfect a second vector control. For site-specific mutagenesis of the RASGRP1 reporter vector, the following primers were used: forward, 5′-ACTATTAATACTATTTAGAAATATCGATATTGGATCAGTGGCTT-3′; and reverse, 5′-AAGCCACTGATCCAATATCGATATTTCTAAATAGTATTAATAGT-3′. HeLa cells were seeded in the wells of a 96-well plate 1 d pretransfection and then transfected to each well with a mixture of 10 ng 3′ UTR luciferase reporter vector and 0.5 μl miRNA mimics. Twenty-four hours posttransfection, cells were lysed, and luciferase activity was measured on a luminometer (TR717, Applied Biosystems) by using the Dual-Luciferase Reporter Assay System (Promega). The ratio of Renilla luciferase to firefly luciferase was calculated for each well.

Real-time PCR

RNA samples were reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen) and oligo(dT) primers. To determine the quantity of mRNA, the cDNA was amplified by real-time PCR with SYBR Premix Ex Taq RT-PCR kit (Takara Bio, Shiga, Japan), and the housekeeping gene *Rpl13a* was used as the internal control. The SYBR Green assays were performed in duplicate or triplicate on a 7900HT real-time instrument (Applied Biosystems). The relative expression levels were calculated using the 2-ΔΔCt method. The primers used were: DNMT1 (forward 5′-ACGAGGATGAGAGGGAGGAG-3′, reverse 5′-GGCACTTTGGTGAGTTGAT-3′), DNMT3A (forward 5′-GCTGCACCTGGCCTTATG-3′, reverse 5′-CGTCTTTCAGGCTACGATCC-3′), DNMT3B (forward 5′-AGAGGGACATCTCACGGTTC-3′, reverse 5′-GGTTGCCCCAGAAGTATCG-3′), CD70

(forward 5′-TGGCTGTGGGCATCTGCT-3′, reverse 5′-ACATCTCCGTGGACCAGGTAT-3′), LFA-1 (forward 5′-CAGATTGAAGATGGGGTTGTC-3′, reverse 5′-CGGGACGATTTTGTAACATAGGTC-3′), RASGRP1 (forward 5′-CAGCTCCGAGCAGAAGTC-3′, reverse 5′-ACACAGCCCACCTATCACA-3′), and RPL13A (forward 5′-CCTGGAGGAGAAGAGGAAAGAGA-3′, reverse 5′-TTGAGGACCTCTGTGTATTTGTCAA-3′).

Western blotting

Cells were directly lysed in 0.5% Triton buffer and separated by 10% SDS-PAGE. Immunoblot analysis was performed by the initial transfer of the proteins onto polyvinylidene difluoride filters using a Mini Trans-Blot apparatus (Bio-Rad, Hercules, CA) and followed by a blocking step with TBS containing 0.1% Tween 20 and 5% nonfat dried milk for 1 h at room temperature. The filters were incubated overnight with primary Abs and then washed. After further incubation with a secondary Ab conjugated with HRP for 1 h at room temperature, the filters were washed extensively, and the bands were visualized with ECL technology (Millipore, Bedford, MA). The same membrane was stripped and reprobed repeatedly with the other Abs. Abs directed against DNMT1 (ab13537), DNMT3A (ab13888), and DNMT3B (ab13604) were from Abcam (Cambridge, MA); Abs directed against RASGRP1 (H-120) (sc-28581), actin (sc-1615), and phosphorylated ERK1/2 (sc-7383) were from Santa Cruz Biotechnology (Santa Cruz, CA); Abs directed against Flag (M2, F7425) were from Sigma-Aldrich (St. Louis, MO); ERK1/2 (9102) was from Cell Signaling Technology (Beverly, MA). The volume tools of the software Quantity One (Bio-Rad) were used to quantitate the protein bands, according to the manufacturer's manual.

Bisulfite sequencing

Genomic DNA was isolated from the cells after miRNA transfection using the QIAamp DNA Blood Midi Kit (Qiagen, Valencia, CA) and was then treated with bisulfite using the Imprint DNA Modification Kit (Sigma-Aldrich). The bisulfite conversion-based PCR Primers were designed with the MethPrimer program: CD70, forward 5′-GAGGTTATGAATTTTGGGAGGATAT-3′ and reverse 5′-TCCCATCTCAACCTTTTACATAATTA-3′. PCR was performed using HotStar Taq DNA polymerase (Qiagen) under the following conditions: initial incubation at 95°C for 15 min, then 40 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s for the CD70 promoter. The PCR products were purified using the Wizard DNA Clean-up System (Promega), and then cloned into the pGEM-T Easy Vector I (Promega). Eight independent clones for each sample were picked, and the T7 and Sp6 primers were used to sequence inserted fragments.

Statistical analysis

The nonparametric Mann-Whitney *U* test was used to compare the groups. The unpaired *t* test was used to compare the reporter gene activity. The Spearman test was used for correlation analyses. Two-tailed *p* values <0.05 were considered statistically significant.

Results

Expression of miR-21 and miR-148a is upregulated in CD4$^+$ T cells from both lupus patients and MRL/lpr mice

To identify any differentially expressed miRNAs in the CD4$^+$ T cells of lupus, we used the MRL/lpr mouse strain first, which is a well-characterized animal model of lupus. These mice develop age-dependent glomerulonephritis, lymphadenopathy, and a number of autoantibodies, all of which are hallmarks of lupus in human (25). Most importantly, CD4$^+$ T cells of these mice show remarkable DNA hypomethylation, closely resembling those of human lupus (24). Because miRNAs are evolutionarily conserved and play critical roles in essential physiological events, we reasoned that a common set of miRNAs might be involved in the regulation of methylation in the two species. Using TaqMan Low Density Assay v2.0 (Applied Biosystems), we assayed the miRNA

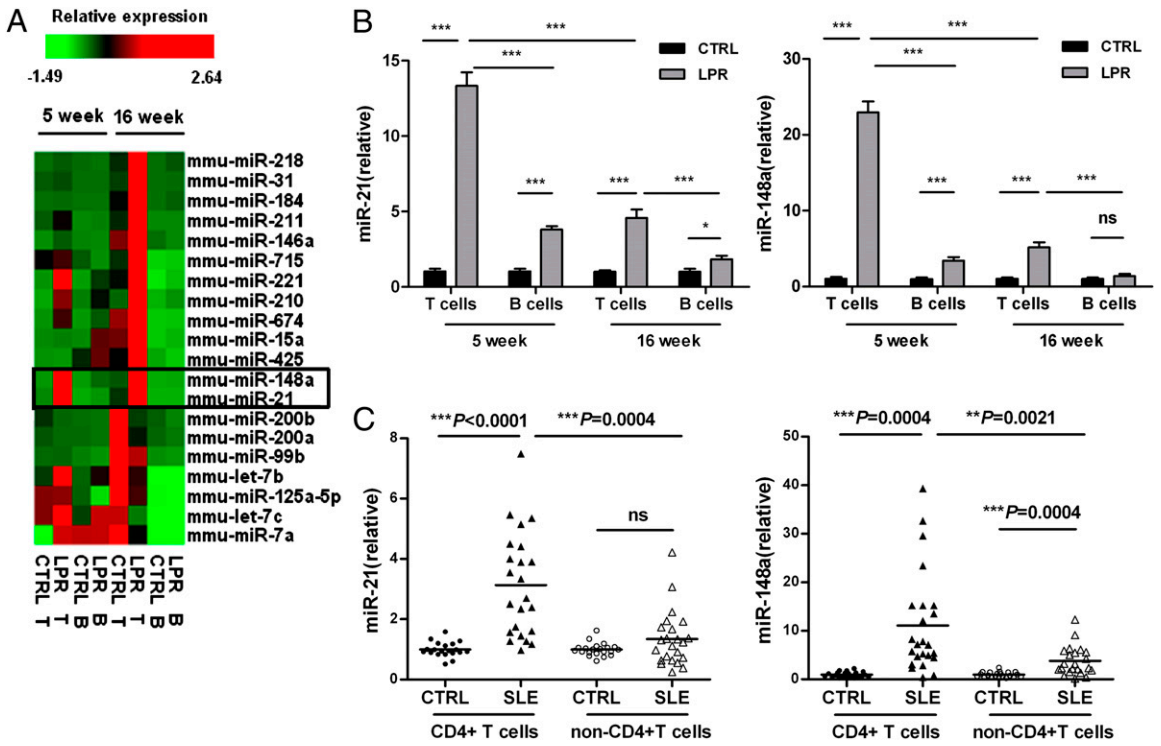

FIGURE 1. Expression of miR-21 and miR-148a is upregulated in CD4$^+$ T cells from both patients with lupus and MRL/lpr mice. *A*, Heat map of 20 differentially expressed miRNAs in splenic CD4$^+$ T cells and B cells isolated from MRL/lpr mice at 5 and 16 wk and normal controls. *B*, Quantitative PCR validation of miR-21 and miR-148a expression in splenic CD4$^+$ T cells and B cells isolated from MRL/lpr mice at 5 and 16 wk and normal controls. Data are representative of two independent experiments (means ± SEM); $n = 6$ mice per group. *C*, Quantitative PCR analysis of miR-21 and miR-148a expression in the CD4$^+$ T cells and the non-CD4$^+$ T cell fraction of PBMCs from patients with SLE ($n = 24$; 23 female, 1 male; age 33 ± 12 y [mean ± SD]) and normal controls ($n = 21$; 20 female, 1 male; age 31 ± 13 y [mean ± SD]). The relative expression levels in *B* and *C* were normalized to the expression of snoRNA202 and RNU48, respectively. Microarray data are available under accession number GSE21220 at the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE21220). $*p < 0.05$; $**p < 0.01$; $***p < 0.005$.

expression in the splenic $CD4^+$ T cells and B cells isolated from MRL/lpr mice at 5 and 16 wk of age (before and after autoimmunity is established), respectively. Of the 585 mouse miRNAs assayed, we selected miR-21 and miR-148a for further investigation for the following reasons: first, miR-21 and miR-148a were the best representative miRNAs among the group of elevated miRNAs in MRL/lpr mice. Notably, these two miRNAs showed greater increase in the $CD4^+$ T-cell population than in the B cell population, implying that their roles were closely related to the aberrance of $CD4^+$ T cells. Second, the extent of induction of miR-21 and miR-148a was greater in 5-wk-old MRL/lpr mice than in 16-wk-old MRL/lpr mice, in which autoimmunity is established. Thus, the upregulated expression of these miRNAs preceded the development of lupus, implying their critical roles in the establishment of autoimmunity (Fig. 1*A*, 1*B*). Third, previous reports have revealed that miR-148a inhibits DNMT3B expression by targeting the protein-coding region of its transcript (26), suggesting that miR-148a is probably involved in the regulation of DNA methylation in lupus.

To confirm the differentially expression of miR-21 and miR-148a in $CD4^+$ T cells of human lupus, we next examined the expression of these two miRNAs in larger samples obtained from $CD4^+$ T cells of 24 patients with lupus and 21 healthy controls (Table I). As expected, the levels of the two miRNAs were remarkably increased in lupus $CD4^+$ T cells relative to those in healthy $CD4^+$ T cells. Notably, the extent of induction of miR-21 and miR-148a in the $CD4^+$ T cells was also significantly greater than that in the non-$CD4^+$ T cells, suggesting a specific association between the increased miRNAs expression and the aberrant $CD4^+$ T cell function in SLE (Fig. 1*C*).

We next performed analysis to observe for any correlation between the levels of miR-21 and miR-148a and clinical features. A direct positive correlation was observed between miR-21 levels and SLEDAI scores ($r = 0.4884$, $p = 0.0062$; data not shown), and also between miR-148a levels and SLEDAI scores ($r = 0.438$, $p = 0.0155$; data not shown). There is no significant difference of miRNA expression levels between the patients with lupus nephritis and those without. In addition, the expression of miR-21 correlated well with the expression of miR-148a in $CD4^+$ T cells of patients ($r = 0.5227$, $p = 0.003$; data not shown), suggesting the patients with the highest miR-21 levels also have a high miR-148a level. Moreover, we evaluated the influence of drugs on the expression of miR-21 and miR-148a. The doses of steroids were not associated with altered expression of these two miRNAs. Similarly, no obvious difference was observed after using secondary antirheumatic agents apart from steroids (data not shown). Taken together, the results confirmed that these two miRNAs are intrinsically upregulated in patients with lupus.

Table I. *Clinical features of patients with SLE*

Characteristic	Control ($n = 30$)	SLE ($n = 36$)
No. of male/female	5/25	3/33
Age (y)	32 ± 14	35 ± 13
Disease duration (mo)	NA	70.21 ± 66.14
SLEDAI score	NA	10 ± 5
Anti-dsDNA [P/N (n)[a]]	NA	18/15
LN [P/N (n)]	NA	20/16
Steroids (n)[b]		
≤10 mg/d	NA	2
10–40 mg/d	NA	19
>40 mg/d	NA	15
Secondary agents[c] [P/N (n)]	NA	24/12

Values are presented as mean ± SD, except where indicated otherwise.

[a]Because some patients were not examined, the number listed for the feature is less than the total number of patients.

[b]The dose of steroids presented in this article is that of prednisone. If other equivalent was taken, the dosage was converted (e.g., 40 mg methylprednisolone was equivalent to 50 mg prednisone).

[c]Some patients were receiving secondary antirheumatic agents, including chloroquine, cyclophosphamide, methotrexate, and azathioprine.

LN, lupus nephritis; P/N, positive/negative (for the feature listed).

miR-21 and miR-148a downregulate DNMT1 expression in Jurkat and $CD4^+$ T cells

DNA methylation is a postsynthetic event and, during mitosis, the methylation patterns are replicated by DNMT1 (27). Expression of DNMT1 is reduced in lupus $CD4^+$ T cells (28), and the inhibition of DNMT expression by 5-aza-dC results in passive DNA hypomethylation (29), suggesting a mechanism for the DNA hypomethylation in lupus. Therefore, to test whether upregulation of miR-21 and miR-148a was related to T cell hypomethylation in some way, we first examined whether miR-21 and miR-148a downregulated the expression of DNMTs. After transfecting Jurkat and primary $CD4^+$ T cells with miRNAs and controls, we measured the mRNA and protein levels of DNMTs by quantitative real-time PCR and Western blotting, respectively. Our quantitative PCR results showed that in $CD4^+$ T cells, transfection of miR-21 resulted in a 0.58-fold ($p < 0.01$) reduction of endogenous DNMT1 mRNA level. No statistically significant differences were found for DNMT3A and DNMT3B; transfection of miR-148a had

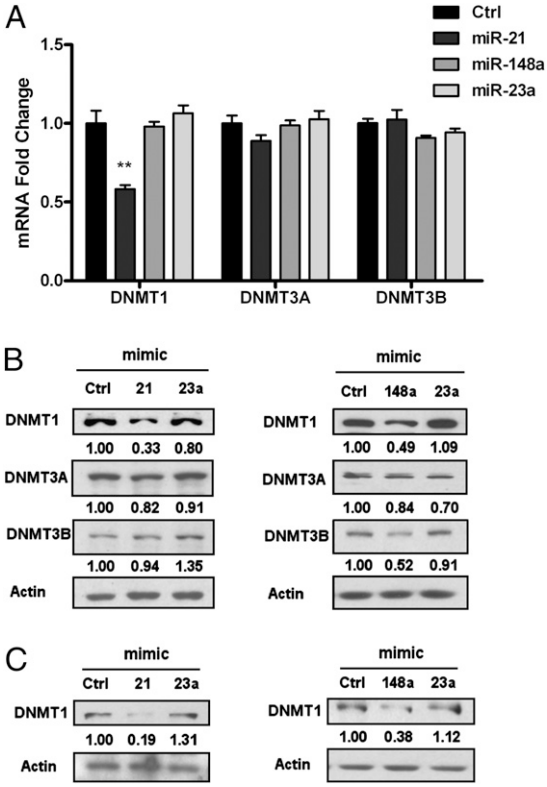

FIGURE 2. miR-21 and miR-148a downregulate DNMT1 expression in Jurkat and $CD4^+$ T cells. *A*, Quantitative PCR analysis of DNMT1, DNMT3A, and DNMT3B expression in primary $CD4^+$ T cells 24 h postnucleofection of miR-21, miR-148a, miR-23a, or control. Histograms show fold changes in mRNA expression with respect to the controls after normalization with the housekeeping gene RPL13A. *B*, Immunoblot analysis of DNMT1, DNMT3A, and DNMT3B expression in Jurkat cells 48 h postnucleofection of miR-21, miR-148a, miR-23a, or control. *C*, Immunoblot analysis of DNMT1 expression in primary $CD4^+$ T cells 48 h postnucleofection of miR-21, miR-148a, or control. Data are representative of four (*B*, *C*) and three (*A*) independent experiments (means ± SEM in *A*). $*p < 0.05$; $**p < 0.01$; $***p < 0.005$.

little effect on the DNMT mRNA expression (Fig. 2*A*). Western blotting analysis revealed that in Jurkat cells, transfection of miR-21 and miR-148a resulted in a remarkable reduction of DNMT1 protein expression. Reduction of DNMT3B protein levels was also observed in the presence of miR-148a, as reported (26). miRNA-23a was used as a nontargeting miRNA (Fig. 2*B*). In primary $CD4^+$ T cells, the protein expression of DNMT3A and DNMT3B was barely detectable. Overexpression of miR-21 and miR-148a also markedly reduced DNMT1 expression (Fig. 2*C*). Together, these data indicated that the upregulation of miR-21 and miR-148a expression can inhibit DNMT1 expression. Notably, miR-148a affected only DNMT1's protein level, suggesting that post-transcriptional control was involved.

miR-21 indirectly downregulates DNMT1 expression by targeting its upstream regulator, RASGRP1

Generally, miRNAs repress protein-coding gene expression through sequence-specific base pairing with the 3′ UTRs of target transcripts. Because no miR-21 binding site on Dnmt1 3′ UTR was predicted, and reporter gene assay also revealed no difference in luciferase activity posttransfection with miR-21 or control (data not shown), we reasoned that miR-21 probably indirectly downregulated DNMT1 expression. Importantly, previous works have demonstrated that DNMT1 level is regulated by the Ras–MAPK pathway (30–32), so reduced Ras-MAPK signaling in lupus may be responsible for the downregulated expression of DNMT1 and the cellular hypomethylation status (8, 28, 30). We therefore used miRNA target-prediction databases to search for miR-21 targets in the upstream signaling pathway of DNMT1. Of the predicted targets, RASGRP1 was chosen as a good candidate. It activates the Ras-MAPK cascade and regulates T cell development (33, 34). The dysregulation of RASGRP1 in mice results in an SLE-like disorder (35).

To test the hypothesis that miR-21 inhibited DNMT1 expression by targeting RASGRP1, we next confirmed that RASGRP1 was a direct target of miR-21. By cotransfecting HeLa cells with reporter vectors (a wild-type RASGRP1 3′ UTR or a mutant RASGRP1 3′ UTR) and miRNAs, we observed a remarkable reduction of the wild-type 3′ UTR reporter gene expression in the presence of miR-21. In contrast, no obvious change in the mutant 3′ UTR reporter plasmid expression was observed (Fig. 3*A*, 3*B*). Transfecting Jurkat cells with miR-21 resulted in a significant repression of endogenous RASGRP1 expression (at both mRNA and protein levels), as well as its downstream Ras-MAPK signaling (Fig. 3*C*). These experiments identified Rasgrp1 as a direct target of miR-21. Next, we investigated whether the inhibition of DNMT1 by miR-21 was mediated by RASGRP1. If so, the inhibition of RASGPR1 expression would have the same effect as the overexpression of miR-21. Knockdown of RASGRP1 expression

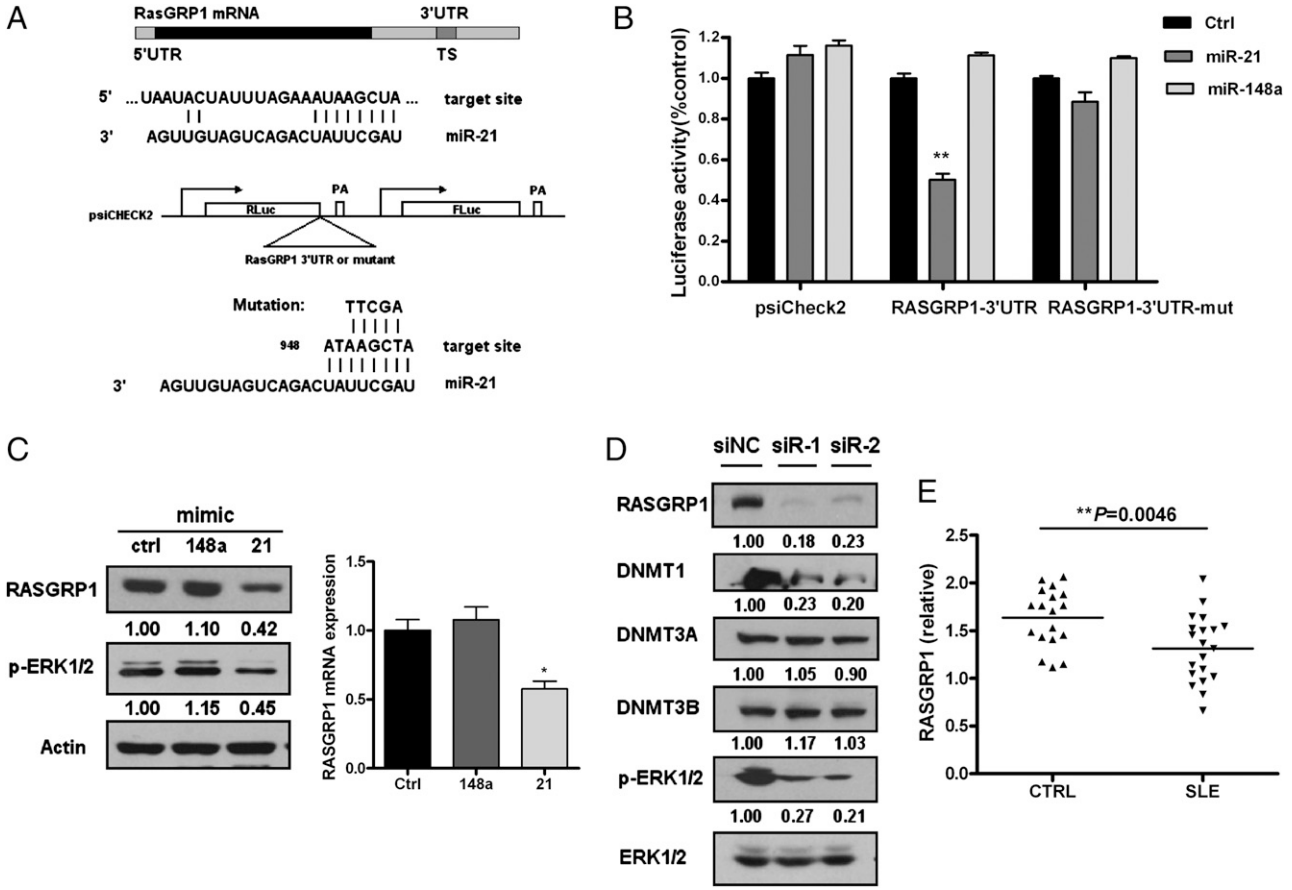

FIGURE 3. miR-21 indirectly downregulates DNMT1 expression by targeting DNMT1 upstream regulator RASGRP1. *A*, Schema of the WT and the MUT (23) RASGRP1 3′ UTR reporter vector, indicating the interaction sites between miR-21 and the 3′ UTR of RASGRP1. *B*, Dual luciferase assay of HeLa cells cotransfected with the reporter vectors containing the RASGRP1 WT or MUT 3′ UTR and miR-21 or controls. The Renilla luciferase activity was normalized to the firefly luciferase activity. The data were shown as relative luciferase activity of miR-21 transfected cells with respect to the controls from three independent experiments. *C*, Immunoblot analysis of RASGRP1, phosphorylated ERK1/2 (p-ERK1/2), and total ERK1/2 expression in Jurkat cells 48 h postnucleofection of miR-21 or controls (*left panel*). Quantitative PCR analysis of RASGRP1 expression in Jurkat 24 h postnucelofection of miR-21 or controls (*right panel*). *D*, Immunoblot analysis of RASGRP1, p-ERK1/2, ERK1/2, DNMT1, DNMT3A, and DNMT3B expression in Jurkat after the transfection of two siRNAs directed against RASGRP1 (siR-1 and siR-2) or control. *E*, Quantitative PCR analysis of the RASGRP1 expression in lupus $CD4^+$ T cells ($n = 24$) and normal controls ($n = 21$). Data are representative of three independent experiments (means ± SEM). $*p < 0.05$; $**p < 0.01$. MUT, mutated; WT, wild-type.

by siRNAs directed against RASGRP1 (siR-1 and siR-2) led to a marked decrease in the Ras-MAPK signaling as well as DNMT1 expression. No obvious difference was observed for DNMT3A and DNMT3B (Fig. 3*D*). To determine the RASGRP1 expression in lupus clinical samples, we designed RT-PCR primers within common sequence of all splice variants of RASGRP1 and performed quantitative analysis (36). Results revealed that the mRNA level of RASGRP1 was significantly reduced in lupus $CD4^+$ T cells, which was consistent with the decreased Ras-MAPK signaling in lupus (Fig. 3*E*). Together, our data suggested that miR-21 indirectly downregulated DNMT1 expression by targeting its upstream regulator RASGRP1.

miR-148a directly downregulates DNMT1 expression by targeting the protein coding region of its transcript

In contrast to miR-21, a putative miR-148a binding element has been identified in the 3′ UTR of DNMT1 transcript. Therefore, we cloned the DNMT1 3′ UTR into the psiCHECK-2 reporter vector and performed dual luciferase assays in HeLa cells. Unexpectedly, only a marginal reduction of DNMT1 3′ UTR reporter gene expression was observed in the presence of miR-148a (Fig. 4*A*). A possible reason is that the miR-148a binding site in DNMT1 3′ UTR is close to the stop codon, whereas effective sites preferentially reside in the 3′ UTR, but not too close to the stop codon (37).

Because miR-148a did repress DNMT1 protein expression in both Jurkat and primary $CD4^+$ T cells, we reasoned that miR-148a had other target sites beyond the confines of 3′ UTR. Recent works have identified increasing numbers of functional miRNA binding sites in the coding regions of mammalian genes (26, 38, 39). Using RNA22, which is an ideal tool for determining miRNA targets in gene coding DNA sequence (CDS) (40), we predicted a potential miR-148a target site in the CDS of DNMT1 (Fig. 4*B*). To examine whether DNMT1 was a direct target of miR-148a, we cotransfected HeLa cells with miR-148a and an expression vector containing the CDS sequence for DNMT1 downstream of a Flag peptide (pcDNA3.1-Flag-DNMT1-CDS-WT) and performed immunoblotting analysis for Flag. Notably, DNMT1 was cloned in-frame with the Flag-coding sequence, which was verified by sequencing. As expected, the overexpression of miR-148a resulted in a reduction of Flag-tagged DNMT1 expression in a dose-dependent manner. Moreover, mutation of 10 nucleotides within the putative target site in the DNMT1 CDS (pcDNA3.1-Flag-DNMT1-CDS-MUT) almost completely abrogated this miRNA-148–mediated repression, confirming the specificity of the action. Importantly, mutating this site did not change the frame of the fusion protein, as was verified by sequencing and immunoblotting analysis (Fig. 4*C*). All of these data suggested that miR-148a directly inhibited DNMT1 protein expression through interaction with the protein coding region of DNMT1 transcript.

miR-21 and miR-148a induce the overexpression of autoimmune-associated methylation-sensitive genes in $CD4^+$ T cells

In lupus, a number of methylation-sensitive T cell genes that are linked to T cell autoreactivity are overexpressed. These genes, including CD70 and LFA-1, are also demethylated and overexpressed post-treatment with hypomethylating agents in $CD4^+$ T cells. CD70, a T cell costimulatory molecule encoded by the TNFSF7 gene, is overexpressed in $CD4^+$ T cells from patients with lupus and increases B cell costimulation and subsequent Ig overproduction (11). LFA-1 (CD11a) is overexpressed in subsets of autoreactive T cells in patients with lupus and also contributes to the development of lupus (12).

As described above, miR-21 and miR-148a inhibited DNMT1 expression. Because the inhibition of DNMT1 results in passive

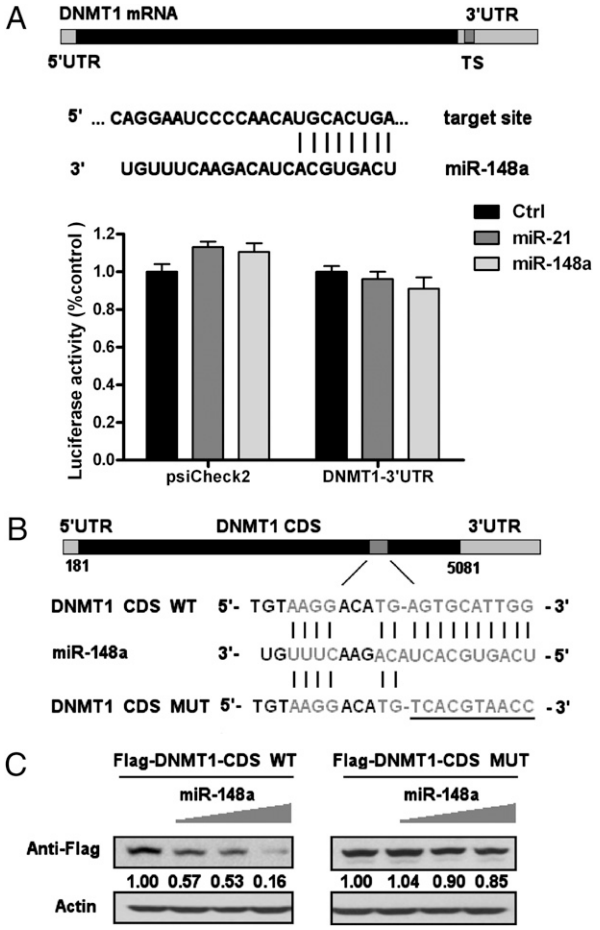

FIGURE 4. Mir-148a indirectly downregulates DNMT1 by targeting the protein coding region of its transcript. *A*, Schema of the binding site of miR-148a in DNMT1 3′ UTR. Dual luciferase assay of HeLa cells cotransfected with the reporter vector carrying WT DNMT1 3′ UTR and miR-148a or controls. The data were shown as relative luciferase activity of miR-148a–transfected cells with respect to the controls from three independent experiments. *B*, Schema of the miR-148a putative target site in human DNMT1 CDS and alignment of miR-148a with DNMT1 WT and MUT CDS showing complementary pairing. The mutated nucleotides are underlined. *C*, Immunoblot analysis of Flag expression in HeLa cells cotransfected with a Flag-tagged WT DNMT1 CDS construct (pcDNA3.1-N-Flag-WT) or DNMT1 CDS with a mutated recognition element (pcDNA3.1-N-Flag-MUT) along with increasing dosages of miR-148a. Data are representative of three independent experiments. MUT, mutated; WT, wild-type.

DNA hypomethylation (29) and the overexpression of methylation-sensitive genes like CD70 and LFA-1 in $CD4^+$ T cells, we next examined whether miR-21 and miR-148a could induce the overexpression of CD70 and LFA-1. To test the hypothesis, we transfected primary $CD4^+$ T cells with miR-21 and miR-148a, and then measured the mRNA levels of CD70 and LFA-1 4 d post-transfection. Results showed that in $CD4^+$ T cells, transfection of miR-21 induced 2.3-fold ($p < 0.05$) and 2.0-fold ($p < 0.05$) increases of CD70 and LFA-1 mRNA levels; the transfection of miR-148a gave rise to 1.8-fold ($p < 0.05$) and 1.6-fold ($p < 0.05$) induction, respectively (Fig. 5*A*). Furthermore, reports have shown that the promoters of CD70 and LFA-1 were demethylated in response to DNMTs inhibitors, ERK pathway inhibitors, or in T cells from patients with lupus (10, 11). In this study, we used bisulfite sequencing to determine the methylation status of the CD70 promoter posttransfection of $CD4^+$ T cells with these two miRNAs. Results showed that the methylation level of CD70 promoter was significantly reduced, consistent with its increased

gene expression posttransfection of miR-21 and miR-148a (Fig. 5*B*). A linear correlation analysis demonstrated that the expression of CD70 and LFA-1 correlated well with the expression of miR-21 and miR-148a in the $CD4^+$ T cells of patients with lupus (Fig. 5*C*).

Potential alleviation of hypomethylation in $CD4^+$ T cells from patients with lupus by transfection with miR-21 and miR-148a inhibitors

The results described above demonstrated that miR-21 and miR-148a inhibited DNMT1 expression and promoted $CD4^+$ T cell hypomethylation by targeting a critical protein in the upstream signaling cascade of DNMT1 or directly targeting DNMT1 CDS. Therefore, we investigated whether the manipulation of miR-21 and miR-148a levels could alleviate the hypomethylation of $CD4^+$ T cells in patients with lupus. To this end, we isolated $CD4^+$ T cells obtained from three patients and electroporated T cells with miR-21 and miR-148a hairpin inhibitors that specifically target and irreversibly bind to the endogenous miRNAs. As expected, miR-21 and miR-148a inhibitors increased DNMT1 protein expression (Fig. 6*A*) and reduced the mRNA levels of methylation-sensitive CD70 and LFA-1 (Fig. 6*B*). These results implied that the manipulation of miR-21 and miR-148a levels could potentially alleviate the DNA hypomethylation of $CD4^+$ T cells in patients with lupus.

Discussion

In recent years, there has been an increasing interest in the role of epigenetic modifications in the etiology of human disease. For instance, aberrant hypermethylation of CpG islands of tumor suppressor genes and the resulting transcriptional silencing are associated with malignant transformation in cancer (41). Mutations in the DNMT3B cause the facial anomalies syndrome. Rett syndrome involves mutations in the methylation-dependent transcriptional repressor MeCP2 (42). In SLE, epigenetic factors are important in the onset of the disease. Since Richardson et al. (43) reported the first evidence that T cells from patients with active lupus were shown to exhibit globally hypomethylated DNA, the role of aberrant DNA methylation patterns in the development of SLE has attracted more attention. DNA methylation is a fundamental determinant of chromatin structure, with potent suppressive effects on gene expression. Abnormalities of the DNA methylation system in lupus result in an aberrant increase of autoimmune-associated gene expression, which is implicated in the development of SLE. Although multiple signaling pathways and various environmental factors are known to regulate DNA hypomethylation in lupus, the role of noncoding RNAs is poorly understood. In this work, we used T cell samples from the MRL/lpr mouse strain and human SLE patients and identified two miRNAs (miR-21 and miR-148a) that were significantly upregulated in lupus, preferentially expressed in $CD4^+$ T cells, and increased more remarkably in the establishment of autoimmunity. Mechanistically, these two miRNAs were linked to DNA hypomethylation in lupus by directly or indirectly inhibiting the expression of DNMT1 and therefore implicated in the pathogenesis of SLE. Our work, for the first time, indicates that miRNAs are indeed involved in the much more

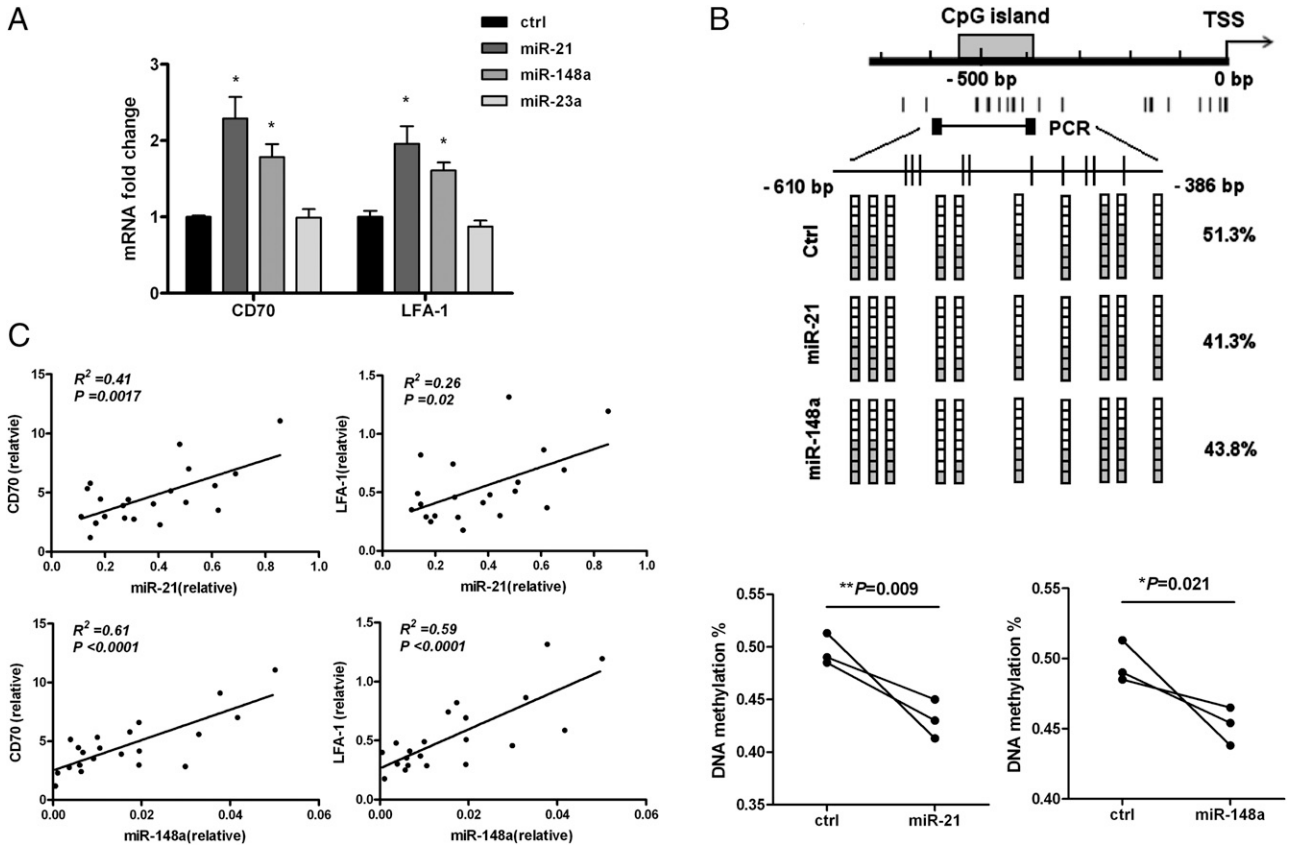

FIGURE 5. miR-21 and miR-148a induce the overexpression of autoimmune- associated methylation-sensitive genes in $CD4^+$ T cells. *A*, Quantitative PCR analysis of CD70 and LFA-1 expression in primary $CD4^+$ T cells 72 h postnucleofection of miR-21, miR-148a or controls. *B*, Schematic diagram of CD70 promoter region. The CpG island is depicted, and each vertical bar illustrates a single CpG. Eight single clones are represented for each sample. Gray and white squares represent the methylated and unmethylated CpG, respectively. The percentage of methylation is presented. Results from one independent experiment are shown (*top*). Data are from three independent experiments (means ± SEM in *A* and *B*). *C*, The linear correlation analysis between the expression of CD70 and LFA-1 and the expression of miR-21 and miR-148a in $CD4^+$ T cells of patients with lupus ($n = 24$). $*p < 0.05$; $**p < 0.01$.

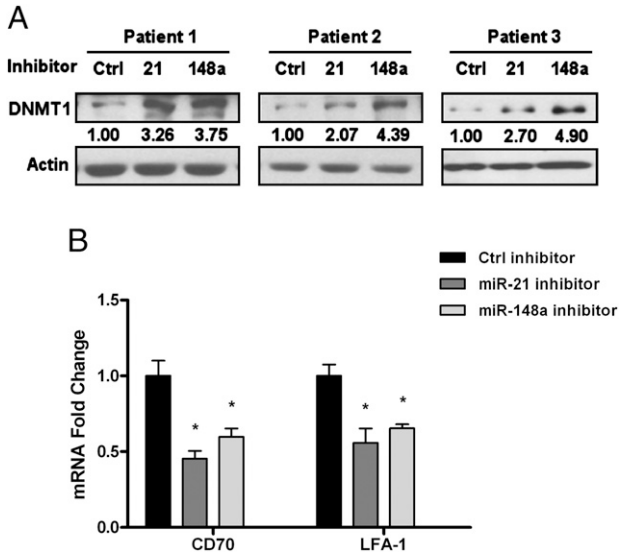

FIGURE 6. Potential alleviation of hypomethylation in $CD4^+$ T cells from patients with lupus by transfection with miR-21 and miR-148a inhibitors. *A*, Immunoblot analysis of DNMT1 protein expression in $CD4^+$ T cells of lupus patient ($n = 3$) 48 h postnucleofection of miR-21, miR-148a hairpin inhibitors, or control. *B*, Quantitative PCR analysis of CD70 and LFA-1 expression in $CD4^+$ T cells of a patient with lupus 72 h posttransfection with miR-21, miR-148a inhibitors or control. Data are from three independent experiments (means ± SEM in *B*). $*p < 0.05$; $**p < 0.01$.

complex regulatory network of DNA hypomethylation in lupus. This identification contributes to our understanding of a new pathway of the disease.

Ever since the discovery of miRNAs, tremendous effort has been devoted to determining the biological functions of miRNAs and their relevance to diseases. Dysregulation of miRNAs has been associated with certain human diseases, such as leukemia and heart disease (44). Although we are still at an early stage in understanding their impact on immunity, miRNAs are changing the way we think about the development of the immune system and regulation of immune functions (45). miRNAs are implicated in establishing and maintaining the cell fate of immune cells (e.g., miRNA-181a and miRNA-223), and they are involved in innate immunity by regulating TLR signaling and ensuing cytokine response (e.g., miRNA-146). Moreover, miRNAs regulate central elements of the adaptive immune response, such as Ag presentation (e.g., miRNA-155) and TCR signaling (e.g., miRNA-181a). Our work, by elucidating the presence of a miRNA signature and its contribution to alterations of methylation status in T cells of patients with SLE, now extends the role of miRNAs in the pathogenesis of autoimmune diseases.

Previous studies have reported that miR-21 is a principal regulator that controls major cell functions in various physiological and pathological process. In a large-scale profiling of miRNA expression in 540 human samples derived from 363 specimens representing six types of solid tumors and 177 respective normal control tissues (46), miR-21 was the only miRNA upregulated in all types of the analyzed tumors. Abundant miR-21 may be a general, albeit not universal, feature of tumor cells. Interestingly, high levels of miR-21 may not only characterize cancer cells but also represent a common feature of pathological cell growth or cell stress. For example, miR-21 is upregulated in several models of mouse hypertrophic heart, including thoracic aortic banding (47). However, a miR-21 knockout mouse has not yet been generated, and the current knowledge of miR-21 functions in human autoimmune diseases is still very limited. Our work extends the role of miR-21 in the pathogenesis of autoimmune diseases, with an emphasis on its regulation of lupus T cells hypomethylation.

Interestingly, miR-21 indirectly inhibited DNMT1 expression by targeting RASGRP1, a known critical regulator of the upstream Ras-MAPK signaling cascade of DNMT1, indicating a transcriptional control; miR-148a directly inhibited DNMT1 expression by targeting the protein coding region of its transcript, suggesting a posttranscriptional control mechanism. These results indicate that miRNAs exert multiple levels of regulation on DNMT1 expression and highlight the importance of stringently regulating DNMT1. These findings also fit the emerging concept that miRNAs fine-tune gene expression to precisely modulate essential biological processes and provide a mechanistic view of miRNA-based regulation on DNA hypomethylation in lupus.

Recently, Richardson et al. (48) has also demonstrated that regulatory sequences on the inactive X chromosome demethylate in T cells from women with lupus, contributing to CD40L overexpression uniquely in women. CD40L is a B cell costimulatory molecule encoded on the X chromosome. This is the first work to add X chromosome demethylation to the mechanism predisposing women to lupus. It would also be of interest to compare the effect of the miR-21 and miRNA-148 hairpin inhibitors on CD40L surface expression other than CD70 and LFA-1.

Even as miR-21 and miR-148a have emerged as regulators of the DNA hypomethylation in lupus $CD4^+$ T cells, a critical question about the putative mechanisms upregulating these two miRNAs in lupus remains unknown. The human miR-21 gene is relatively well characterized and mapped to chromosome 17q23.2; however, there is no clear correlation between the amplification of miR-21 genomic locus and its elevated expression in cancer, suggesting that aberrant expression of this miRNA occurs at either the transcriptional or the posttranscriptional level or both. Recent study demonstrated that miR-21 was among several miRNAs strongly induced in ovarian cell line OVCAR3 by treatment with a demethylating agent 5-AZA and therefore suggested that the hypomethylation could be the mechanism responsible for its overexpression in vivo (49). For miR-148a, reports have demonstrated that its promoter embeds in rich CpG islands, and treatment with DNA demethylating agent could induce its remarkable increase in lymph node metastatic cancer cells (50). Further research is clearly required to investigate the epigenetic mechanisms of miR-21 and miR-148a induction in disease.

Due to the strong association between the levels of miR-21 and miR-148a and clinical disease activity, they may serve as new disease biomarkers. Besides this, as miRNAs provide quantitative regulation of genes, rather than on-off decision, they can be thought of as fine-tuning a cell's responses to external influences. Consequently, manipulation of miRNA levels may lead to novel therapeutic strategies to combat SLE. Interestingly, when miR-21 and miR-148a inhibitors were introduced into patients' $CD4^+$ T cells, the expression of DNMT1 was increased, and several methylation-sensitive genes were decreased. These results imply that miR-21 and miR-148a levels may be manipulated to provide useful therapeutic interventions for SLE. Further work on knockout and transgenic animal models would help us to identify the in vivo function of miR-21 and miR-148a in autoimmune diseases.

In conclusion, we provided evidence for a previously unknown key regulatory mechanism by discovering that dysregulation of miR-21 and miR-148a has been involved in SLE, which inhibited DNMT1 expression and promoted DNA hypomethylation in lupus $CD4^+$ T cells. To the best of our knowledge, this is the first report on regulation of the cellular methylation status by miRNAs in lupus. These findings also have potentially therapeutic implications, as the inhibition of miR-21 and miR-148a expression partially reversed the hypomethylation status in lupus $CD4^+$ T cells. Therefore,

this work will deepen our knowledge of how miRNA-mediated mechanisms contribute to the pathogenesis of lupus and help us to identify new therapeutic approaches to SLE.

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Disclosures

The authors have no financial conflicts of interest.

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