MicroRNA-126 deficiency enhanced the activation and function of CD4+T cells by elevating IRS-1 pathway

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Abbreviations:

MiR-126KD, microRNA-126 knock down
MACS, magnetic-activated cell sorting
FACS, fluorescence activated cell sorting
DSS, dextran sulfate sodium
IRS-1, insulin receptor substrate 1
Summary

Recent evidence showed that microRNA-126 (miR-126) has been involved in the development and function of immune cells, which contributed to the pathogenesis of related clinical diseases. However, the potential role of miR-126 in the development and function of CD4+ T cells remains largely unknown. Here we firstly found that the activation and proliferation, as well as the expression of IFN-γ, of CD4+ T cells from miR-126 knockdown (KD) mice using miRNA-Sponge technique were enhanced significantly in vitro, compared with those in CD4+ T cells from wild type (WT) mice. To further monitor the possible effect of miR-126 deficiency on the function of CD4+ T cells in vivo, we used dextran sulfate sodium (DSS)-induced murine model of acute autoimmune colitis and found that miR-126 deficiency could elevate the pathology of colitis. Importantly, the proportion of CD4+ T cells in splenocytes increased significantly in miR-126KD mice. Moreover, the expression levels of CD69 and CD44 on CD4+ T cells increased obviously and CD62L expression decreased significantly. Of note, adoptive cell transfer assay showed that the pathology of colitis was more serious in CFSE-labeled miR-126KD CD4+ T cells transferred group, compared with that in CFSE-labeled WT CD4+ T cells transferred group. Consistently, the expression levels of CD69 and CD44 on CFSE+ cells increased significantly. Furthermore, both the proliferation and IFN-γ secretion of CFSE+ cells also increased significantly in CFSE-labeled miR-126KD CD4+ T cells transferred group. Mechanistic evidence showed that the expression of insulin receptor substrate 1 (IRS-1), as a functional target of miR-126, was elevated in CD4+ T cells from miR-126KD mice, accompanied by altered transduction of ERK, AKT and NF-κB pathway. Our data revealed a novel role in which miR-126 was an intrinsic regulator in the function of CD4+ T cells, which provided preliminary basis for further exploring on the role of miR-126 in the development, function of CD4+ T cells and related clinical diseases.
Introduction

MicroRNA-126 (miRNA-126, miR-126) is an important member of microRNA families, localized within intron 7 of Epidermal growth factor-like domain-containing protein 7 (EGFL7), and highly expressed in endothelial cells of blood vessel, heart and lung, as well as regulates the development of angiogenesis, cardiovascular diseases and so on [1-5]. Some new evidence further showed that miR-126 also played an important role in the development of various cancers. For instance, Zhang et al. [6] have shown that miR-126 and miR-126* inhibited breast cancer metastasis by repressing the recruitment of mesenchymal stem cells and inflammatory monocytes. Other studies has also reported that the expression of miR-126 was downregulated in lung cancer, gastric cancer and ovarian cancer, indicating it was a new promising tumor suppressor gene [7-10].

Recently, significant progress also has been made in determining the role of miR-126 in the regulation of the immune-related diseases. For example, Feng et al. [11] reported that miR-126 expression was upregulated during the development of ulcerative colitis, accompanied by inhibition of its target gene IκBα. Zhang et al. [12] found that circulating miR-126 was a potential biomarker to predict the onset of type 2 diabetes mellitus in susceptible individuals. Importantly, more and more literatures have documented that miR-126 also played an important regulatory role in the function of various immune cells, such as plasmacytoid dendritic cells (pDCs), through changing the expression of vascular endothelial growth factor receptor 2 (VEGFR2) [13]. Moreover, Okuyama et al. [14] reported that miR-126 as a potential alternative to transcriptional factors regulated the function of B-cell myeloid progenitors. Interestingly, Zhao et al. [15] further found that the expression of miR-126 was upregulated in CD4+Th2 cells from systemic lupus erythematosus disease, in which miR-126 could regulate DNA hypomethylation through DNMT1. Similarly, our previous evidence also showed that miR-126 could regulate the function and induction of CD4+Foxp3+ regulatory T cells through PI3K/AKT pathway [16]. However, the exact roles and mechanisms of miR-126 involved in the development and function of CD4+T cells and their distinct subsets remain to be fully elucidated.

In present study, we firstly accessed the potential effect of miR-126 deficiency on the function of CD4+T cells from miR-126 knockdown (KD) mice using miRNA-Sponge technique. Our data showed that the activation and proliferation, as well as the expression of IFN-γ, of CD4+T cells from miR-126KD mice were enhanced significantly in vitro. Importantly, we found that miR-126 deficiency also could elevate the activation, proliferation and the expression of IFN-γ of CD4+T cells in vivo, which ultimately promoted the pathology of colitis in DSS-induced murine autoimmune colitis mode. Finally, we found that the expression of IRS-1, as a functional target of miR-126, was elevated in CD4+T cells from miR-126KD mice, accompanied by altered transduction of ERK, AKT and NF-κB pathway. Thus, our data revealed a novel role in which miR-126 was an intrinsic regulator in the function of CD4+T cells, which provided preliminary basis for further exploration on
the role of miR-126 in the development and function of CD4⁺T and their subsets, as well as related clinical diseases.
Materials and Methods

Mice

We established FVB/N miR-126 knockdown (KD) mice (8-10 week old, n=8) using pEGFP-C2-miR-126 sponge sequence with the help of Cyagen Biosciences Inc. All animals were housed under specific pathogen-free condition at Zunyi Medical College. And all animal experiments were performed according to the guidelines for the Care and Use of Laboratory Animals (Ministry of Health, PR China, 1998). And all the experimental procedures were approved by the ethical guidelines of Zunyi Medical College laboratory Animal Care and Use committee (permit number 2014026).

Preparation of Single-Cell Suspensions

We collected spleen from female wild type FVB/N mice (8-10 week old) and miR-126KD mice respectively. Then spleen was putted into gentle MACS C Tubes loading with 7 mL PBE (PBS+0.5% fetal bovine serum). The program of m_spleen_01 was selected and been taken for 56 seconds. After completion of the program, the cells were filtered by 200 mesh sterile cells strainer, spin down the whole cell suspension at 1200 rpm and 4°C for 10 minutes. Abandon supernatant and added 3 ml RBC pyrolysis liquid to resuspend cell pellet, on the ice for 15 minutes. Then, added 10 mL PBS suspension of splenocytes, being filtered by 200 mesh sterile cells strainer, repeated twice, into a new 15 mL centrifuge tube for next experimental protocol.

CD4^+CD62L^+ T cells purified by MACS

After the number of preparation of single-cell suspensions from mice spleen was counted, spin down the whole cell suspension at 1200 rpm and 4°C for 10 minutes. Abandon supernatant and resuspend cell pellet in 40 μL PBS for buffer per 10^7 total cells. Firstly, we added 10 μL of CD4^+T cells Biotin-Antibody Cocktail per 10^7 total cells, mix well and incubated in 4°C for 20 minutes, then added 30 μL 0.5% PBE, as the same time, added 20 μL Anti-Biotin MicroBeads per 10^7 total cells, mixed well and incubated in 4°C for 20 minutes, spun down the whole cells suspension at 1200 rpm and 4°C for 10 minutes. Abandon supernatant and added 2 mL 0.5% PBE to resuspend cell pellet for spare parts. Secondly, place LS Column in the magnetic field of suitable MACS Separator. Prepare column by rinsing with 2 mL of 0.5% PBE and applied cell suspension onto the column, then washed column with 2 mL of 0.5% PBE. Collect flow-through containing labeled cells, representing the enriched CD4^+T cells. Thirdly, we added 200 μL CD62 (L-selectin) MicroBeads and 800 μL 0.5% PBE, mixed well and incubated in 4°C for 20 minutes, spun down the whole cell suspension at 1200 rpm and 4°C for 10 minutes. Abandon supernatant and added 2 mL 0.5% PBE to resuspend cell pellet for spare parts. Lastly, place LD Column in the magnetic field of suitable MACS Separator. Prepare column by rinsing with 2 mL of 0.5% PBE and applied cell suspension onto the column, then washed column with 2 mL of 0.5% PBE. Collect flow-through containing unlabeled cells, then added 1 mL 0.5% PBE into LD Column twice, launched liquid with piston. Collect flow-through containing labeled cells, representing the enriched CD4^+CD62L^+T cells.
**T cells activation, proliferation and electroporation transfection**

All cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C. Coating with 20 μg/ml anti-CD3e (eBioscience, 16-0031-86) for 4°C overnight in plates and cells were co-stimulated with 4 ng/ml anti-CD28 (eBioscience, 16-0281-85) antibody and 10 ng/ml plus IL-2 (ProSpec, cyt-370-b). For transfection, cells were transfected with IRS-1 RNAi vector or IRS-1 RNAi negative vector with Mouse T cell Nucleofector Kit (Lonza). Proliferation of CD4⁺T cells after electroporation transfection was evaluated by Cell Counting Kit-8 (CCK8) kits (Boster Biological Technology Co., Ltd). All cells were harvested at indicated time points for following experiments.

**Real-time PCR assay**

The conventional primers were obtained from Shanghai Sangon Biological Engineering CO. and the TaqMan probes of mir-126 (000386) and U6 (001793) were purchased from Life Technologies, the other reagents were from TAKARA Bio Inc.. Reverse transcriptase reactions and Real-time PCR assays were performed according to the manufacturer’s protocols. All Reverse transcriptase reactions, including no-template controls and Reverse transcriptase minus controls, were run in triplicate in BIO-RAD CFX96 (Bio-Rad Laboratories). The following primer sequence were used: IL-4 forward: 5’-AACGAGGTCAAGAGAAGA-3’, reverse: 5’-CCTTGAAGCCCTACAGA-3’; IL-6 forward: 5’-GGAGATCTGGGAATGAG-3’, reverse: 5’-AGGACTCTGGCTTTGCT-3’; IL-10 forward: 5’-IACGCCGGGGAACATTTATA-3’, reverse: 5’-AGGAGTCGGTTAGCAGTAG-3’; IL-12 forward: 5’-CCCCATTCTACTTCTCC-3’, reverse: 5’-ACGCACCTTTCTGGTGTTA-3’; IFN-γ forward: 5’-TCTGAGACATAAGCGCTAC-3’, reverse: 5’-TCTCAGCACATCTAGCCACT-3’; TGF-β forward: 5’-GGCGGTGCTGCTTTGTA-3’, reverse: 5’-TCCGAATGTCTGACGTATT-3’; TNF-α forward: 5’-CAGGGCCACCCACCGCTCTTC-3’, reverse: 5’-TTTGTGAGTGGAGGGTCGG-3'; IRS-1 forward: 5’-GGGTCTGCTACTCCT-3’, reverse: 5’-GGTATTGGTCACGGTT-3’. Gene expression levels were quantified using the BIO-RAD CFX96 detection system (Bio-Rad Laboratories). Relative expression was calculated with the comparative threshold cycle (Ct) method.

**Flow cytometry**

Surface markers of CD4⁺T cells were evaluated by flow cytometry (FCM) with Beckman Gallios (Beckman Coulter, Inc.). Flow cytometry was performed on Beckman Gallios (Beckman Coulter, Inc.) with Cell Quest Pro software using directly conjugated mAbs against the following markers: CD4-Perp-Cy5.5 (12-0041-82), CD62L-PE (17-5941-81), CD44-APC (17-5711-81), CD69-APC (17-0691-82), with corresponding isotype-matched controls (eBioscience). Ki-67-APC (50-5698-82), IL-4-APC (17-7041-81), IL17A-PC5.5 (45-7177-82), IFN-γ-PC5.5 (45-7311-82), p-AKT-APC (17-9715-42), p-ERK1/2-PE (12-9109-42), p-NF-κB (Cell Signaling Technology; no.3039), Anti-rabbit IgG Fab2-PE (Cell Signaling Technology; no.8885), before staining we used IC fixation buffer and permeabilization buffer (eBioscience) to destruct cells membrane. All cells were stained with these corresponding Abs (1:100) respectively at 4°C for 30 minutes and washed twice before
analysis, according to the manufacturer’s protocol.

**Induction of autoimmune colitis**

Make 3% dextran sodium sulfate (DSS) purchased from MP Biomedicals (Strasbourg, France) in drinking water. Mice were orally administered with distilled water. After drinking 10 days instead of normal drinking water to continue feeding, the mice were sacrificed on the 14th day (The mice were undrinking 3% DSS water for contrast). Then, the entire colon was removed and measured for length.

**Assessment of the severity of colitis and Histology**

Pathology and Histological scoring were using a previously validated scoring system [17,18]. Pathology was scored as follows: (a) weight loss (no change = 0; <5% = 1; 6–10% = 2; 11–20% = 3; >20% = 4), (b) feces (normal = 0; pasty, semiformed = 2; liquid, sticky, or unable to defecate after 5 min = 4), (c) blood (no blood = 0; visible blood in rectum = 1; visible blood on fur = 2), and (d) general appearance (normal = 0; piloerection = 1; lethargy and piloerection = 2; motionless, sickly = 4). For histological examination, the colons were removed, rolled around a cotton swab and fixed in 10% formaldehyde to be embedded in paraffin. The paraffin blocks were longitudinally sectioned serially with the thickness of 5 mm and were stained with hematoxylin and eosin (H&E) to allow histological examination of whole colons at 100× and 400× magnification by light microscopy. Histological was scored as follows: (a) inflammation severity: none = 0; slight = 1; moderate = 2; severe = 3, (b) depth of injury: none = 0; mucosal = 1; mucosal and submucosal = 2; transmural = 3, (c) crypt damage: none = 0; basal one-third damaged = 1; basal two-thirds damaged = 2; only surface epithelium intact = 3; entire crypt and epithelium lost = 4, (d) percentage of area involved: none = 0; 1% – 25% = 1; 26% – 50% = 2; 51% – 75% = 3; 76% – 100% = 4. The final scores are the averages of all individual scores of 6 pieces per colon.

**Adoptive transfer experiment**

In the adoptive transfer model, $10^6$ cells/mL CD4⁺CD62L⁺ T cells purified from WT or miR-126 KD mice by MACS were resuspended in 1640 medium. Then 2 μL CFSE was added into cells fluid per milliliter of liquid, followed by 2 mL PBS buffer solution. After 30 minutes at 37°C, 5× volume liquid PBE was added gently to stop staining. Cells were terminated respectively according to each mouse $10^7$ cells by tail intravenous injection to WT mice and 3% DSS water solution feeding. the mice were sacrificed on the 14th day.

**Western blotting**

Western blotting was performed on cytosolic cellular extracts. Equal amounts of protein were resolved under reducing conditions on a 10% SDS–polyacrylamide gel. Protein migration was assessed using protein standards (Bio-Rad, CA). Transfer to a nitrocellulose membrane was performed for 1 hour at 250 mA using a wet transfer system. Equal protein loading was confirmed with Ponceau staining. The membrane was washed in 5% skim milk in PBS plus 0.05% Tween 20 (PBST) for 2 hours to block nonspecific protein-binding sites on the membrane. Immunoblotting
was performed using an mAb to IRS-1 (Cell Signaling Technology; no.2390), GAPDH (Cell Signaling Technology; no.4970) at a dilution of 1/800 in a nonfat milk-Tris buffer. The membrane was then washed in PBST and subsequently probed with a secondary anti-mouse or rabbit Ab conjugated to HRP (Cell Signaling Technology, no.7074) at a dilution of 1:2000. The signal was detected and analyzed using the chemiluminescence Imaging System (ChemiScope5600, CLINX, Shanghai, China). Each experiment was performed in triplicate.

**Construction of IRS-1 RNAi vector**

We designed synthesis the sequence of IRS-1 RNAi. Sense sequence: 5’-CAGACTCGAACTAT-TTCACAATTCA-3’, antisense sequence: 5’-TGAATTGTGAAATAGTTCGAGTCTG-3’ and then subcloned into *BamHI* and *EcoRI* sites of pLVX-shRNA1. plasmid DNA sequence was confirmed by sequencing.

**Statistical analyses**

Statistical analyses of the data were performed with GraphPad Prism TM(Graphpad Software Inc.) and the aid of analysis programs in SPSS16.0 software. All the data were presented as the mean X±S. Student’s *t*-test was used when two conditions were compared, and analysis of variance with Bonferroni or Newman-Keuls correction was used for multiple comparisons. Probability values of < 0.05 were considered significant.
Results

Mir-126 deficiency enhanced the activation and proliferation of CD4+T cells in vitro

To investigate the potential role of miR-126 in the function of CD4+T cells, we firstly detected the relative expression of miR-126 in various organs and tissues. As shown in Sfig. 1a, we found that the relative expression level of miR-126 was higher in spleen than that in other immune organs ($p<0.05$). However, we further found that the expression of miR-126 decreased significantly in CD4+T cells-deprived splenocytes (Sfig. 1b, $p<0.05$), indicating that miR-126 might be dominantly expressed in CD4+T cells. Then, we generated miR-126KD mice using miRNA-Sponge technique, according to our recent work [19]. As shown in Sfig. 2, the expression level of miR-126 in indicated organs and tissues decreased significantly in miR-126 KD mice, compared with those in WT mice ($p<0.05$). Importantly, our data further showed that the proportion of CD4+T cells increased significantly in splenocytes in miR-126KD mice compared with that in control mice (Sfig. 3a and b, $p<0.05$). These data suggested that miR-126 might be involved in the development and function of CD4+T cells.

Then, to further study the exact role of miR-126 in the function of CD4+T cells, we purified CD4+CD62L+ T cells from miR-126KD mice and observed the possible change on its activation and function. Expectedly, our result showed that the relative expression of miR-126 decreased significantly in CD4+T cells from miR-126KD mice, compared with WT mice group (Fig. 1a, $p<0.01$). Importantly, we found that the expression levels of surface active molecules, including CD44 and CD69, were elevated in CD4+T cells from miR-126KD mice stimulated by anti-CD3/anti-CD28 antibody (Fig. 1b and c, $p<0.05$). Conversely, these CD4+T cells expressed lower level of CD62L (Fig. 1b and c, $p<0.05$), indicating enhanced activation phenotype. We further detected the expression of intracellular proliferating nuclear antigen Ki-67 in CD4+T cells and found that the proportion of Ki-67 in CD4+T cells from miR-126KD mice increased significantly (Fig. 1d and e, $p<0.001$). To further confirm this finding, we also detected the proliferation of CD4+T cells stimulated by ConA and obtained similar results (Sfig. 4a and b, $p<0.01$). Finally, we also found the apoptosis proportion of CD4+T cells did not change significantly (Fig. 1f and g, $p>0.05$). All these data suggested that miR-126 deficiency could obviously enhance the activation and proliferation capacity of CD4+T cells in vitro.

Mir-126 deficiency affected the expression of cytokines in CD4+T cells

Next, we investigated the potential effect of miR-126 deficiency on cytokines expression of CD4+T cells. Realtime PCR assay showed that lower level expression of cytokines IL-4 and IL-10 in CD4+T cells from miR-126KD mice, compared with WT mice (Fig. 2a, $p<0.05$). Conversely, we found the expression levels of cytokines including IL-12, TGF-β, TNF-α and IFN-γ were higher in CD4+T cells from miR-126KD mice (Fig. 2a, $p<0.05$). Even though the level expression of cytokines IL-6 in CD4+T cells from miR-126KD mice did not change significantly, compared with
WT mice (Fig. 2a, p>0.05). To confirm this phenomenon, we further detected the proportion of relative cytokines of CD4⁺T cells by Flow cytometry. As shown in Fig. 2b and c, the proportion of IL-4⁺ cells in CD4⁺T cells from miR-126KD mice decreased significantly, compared with that in CD4⁺T cells from WT mice (p<0.01). Meanwhile, the proportion of IFN-γ⁺ cells increased noticeably (Fig. 2b and c, p<0.05). In addition, we found that the proportion of IL-17A⁺ cells in CD4⁺T cells from miR-126KD mice did not change significantly (Fig. 2b and c, p>0.05). Collectively, these data suggested that miR-126 deficiency also could alter secretion of related cytokines in CD4⁺T cells in vitro.

Mir-126 deficiency promoted the pathology of DSS-induced autoimmune colitis.

It is well known that DSS-induced autoimmune colitis is an important animal model platform for the exploration on immune reaction in inflammatory bowel disease in vivo, in which effector CD4⁺ T cells play a critical role in the pathology of colitis [20]. Then, to further investigate whether miR-126 deficiency could affect the activation and function of CD4⁺T cells in vivo, we established murine DSS-induced autoimmune colitis (Fig. 3a) and observed the possible change on pathology of colitis. As shown in Fig. 3b and c (p<0.05), the clinical and histological scores indicated that the liquid and sticky defecate seriously correlated with weight loss significantly in miR-126KD mice group, compared with that in WT mice group. And mucosal epithelium appeared larger ulcer in miR-126KD mice group. Moreover, the infiltration of inflammatory cells also increased obviously. Importantly, we found that the percentage and total cell number of CD4⁺T cells in splenocytes increased obviously in miR-126KD mice group compared with those in WT mice group (Fig. 3d and e, p<0.05). Further analysis showed that the expression level of CD62L on CD4⁺T cells decreased significantly (Fig. 3f and g, p<0.05). And the level of CD44 in CD4⁺T cells in miR-126KD mice group increased obviously (Fig. 3f and g, p<0.05), indicating elevated activation phenotype. Combining these data suggested that miR-126 deficiency could promote the pathology of DSS-induced autoimmune colitis, which might be related to elevated activation and function of CD4⁺T cells.

MiR-126 deficiency endowed CD4⁺T cells to aggravate the pathology of DSS-induced autoimmune colitis

To verify the effect of miR-126 deficiency on the activation and function of CD4⁺T cells in vivo, we purified and labeled CD4⁺CD62L⁺T cells from miR-126KD mice with CFSE, then adoptively transferred these cells into normal WT mice through tail vein (Fig. 4a). Our data showed that the length of colon was significantly shortened in miR-126KD CD4⁺T cells transferred group, compared with that in WT CD4⁺T cells transferred group (Fig. 4b and c, p<0.05). Clinical and Histological examination showed that the liquid and sticky defecate seriously correlated with weight loss significantly in miR-126KD CD4⁺T cells transferred group, compared with that in WT CD4⁺T cells...
transferred group. Furthermore, intestine tissues damage was aggravated and inflammatory cells infiltration significantly increased in miR-126KD CD4\(^+\)T cells transferred group (Fig. 4d and e, \(p<0.05\)). Notably, we found that the expression level of CD62L on CFSE\(^+\) cells decreased significantly in miR-126KD CD4\(^+\)T cells transferred group (Fig. 4f and g, \(p<0.05\)). And, the level of CD44 on CFSE\(^+\) cells increased obviously (Fig 4h and i, \(p<0.05\)), which was consistent with our above data.

Next, we further analyzed the possible change on the proliferation and cytokine secretion of CD4\(^+\)T cells in vivo. As shown in Fig. 4j and Fig. 4k, the proportion of Ki-67 positive, a kind of proliferating cell nuclear antigen, in CFSE\(^+\) cells was significantly elevated in miR-126KD CD4\(^+\)T cells transferred group, compared with that in WT CD4\(^+\)T cells transferred group (\(p<0.05\)). Furthermore, we found that the proportion of IFN-\(\gamma\)\(^+\) in CFSE\(^+\) cells also increased significantly (Fig. 4l and m, \(p<0.05\)). Consistent with our above data, the proportion of IL-4\(^+\) in CFSE\(^+\) cells decreased obviously (Fig. 4l and m, \(p<0.01\)). Collectively, all of these data demonstrated that miR-126 deficiency endowed CD4\(^+\)T cells with enhanced activation and function capacity to aggravate the pathology of DSS-induced autoimmune colitis.

**MiR-126 deficiency altered the expression of IRS-1**

To elucidate the potential molecular mechanism which miR-126 deficiency enhanced the activation and function of CD4\(^+\)T cells, we used a miRNA target detection program (http://www.microrna.org) to predict the potential target genes of miR-126 and selected thirteen potential candidate genes including PMM1, Tom1, PIK3CD, RGS3, Tsc1, IRS1, ADAM9, EGFL7, Vcam1, Phf7, SLC39a6, HIP1 and PARP16. Then, the relative expression of these indicated genes on CD4\(^+\)T cells between WT mice and miR-126KD mice were analyzed. As shown in Fig. 5a, among these thirteen candidate genes, the expression of IRS-1 was significantly upregulated in CD4\(^+\)T cells from miR-126KD mice compared with that from WT mice.

Previous works showed that miR-126 could regulate the biological characters of various cells including cancer cells through IRS-1 [21-23]. And IRS-1 also was reported played an important role in the function of various immune cells [24]. Then, we performed sequence alignment analysis and expectedly found that miR-126-binding sits in the 3’UTR of IRS-1 (Fig. 5b). Next, we further detected the protein level of IRS-1 in CD4\(^+\)T cells from miR-126KD mice. As shown in Fig. 5c, the level of IRS-1 protein in CD4\(^+\)T cells from miR-126KD mice was increased significantly, compared with that in WT mice (\(p<0.05\)). Finally, to verify the role of IRS-1 in the effect of miR-126 deficiency on the activation and function of CD4\(^+\)T cells, we also transfected IRS-1 RNAi into CD4\(^+\)T cells from miR-126KD mice. As shown in Fig. 5d, the relative expression of IRS-1 in CD4\(^+\)T cells in IRS-1 RNAi transfected group decreased significantly, compared with that in control group (\(P<0.01\)). Furthermore, the proliferation of CD4\(^+\)T cells in IRS-1 RNAi transfected group also decreased obviously (Fig. 5e, \(P<0.05\)). Importantly, we also found that the expression level of CD69
and CD44 in CD4⁺T cells decreased significantly (Fig. 5f–i, p<0.05), indicating downregulation of IRS-1 could impair the activation of CD4⁺T cells in miR-126KD mice. To confirm this phenomenon, we also detected the expression of related cytokines in CD4⁺T cells. Consistently, we found that the expression of IFN-γ in CD4⁺T cells in IRS-1 RNAi transfected group decreased significantly compared with those in control group (Fig. 5j and k, p<0.05). Conversely, the expression level of cytokines IL-4 was higher in CD4⁺T cells in IRS-1 RNAi transfected group (Fig. 5j and k, p<0.01). Collectively, our data indicated that miR-126 deficiency enhanced the activation and function of CD4⁺T cells, which was closely due to the upregulation expression of its target IRS-1.

**MiR-126 deficiency altered the transduction of related signaling pathway in CD4⁺T cells.**

It is well known that multiple signaling pathways were related to the activation and function of CD4⁺T cells, including ERK, AKT, NF-κB and so on [25-29]. Moreover, IRS-1 was also reported closely related to the transduction of these signaling pathways [30-33]. Thus, to elucidate whether miR-126 deficiency affected the activation and function of CD4⁺T cells would be related to the change on these signaling pathway, the expression levels of phosphorylation of AKT, ERK and NF-κB were analyzed in CD4⁺T cells purified from miR-126KD mice and WT mice respectively. As shown in Fig. 6a and b, the expression level of phosphorylation of ERK in CD4⁺T cells from miR-126KD mice was elevated compared with that from WT mice (p<0.05). Moreover, we found that the level of phosphorylation of AKT in these CD4⁺T cells increased significantly (Fig. 6a and b, p<0.01). Finally, we analyzed the expression level of phosphorylation of NF-κB in CD4⁺T cells from miR-126KD mice and obtained similar result (Fig. 6a and b, p<0.01). Combing these results suggested that the effects of miR-126 deficiency on the activation and function of CD4⁺T cells were closely related to the altered transduction of ERK, AKT and NF-κB signaling pathway (Fig. 6c).
Discussion

In this study, it is the first time for us to investigate the potential effect of miR-126 deficiency on the function of CD4+ T cells. Our data showed that miR-126 deficiency could obviously enhance the activation and proliferation, as well as IFN-γ secretion, of CD4+ T cells in vitro and in vivo. Furthermore, miR-126 deficiency could endow CD4+ T cells to promote the pathology of autoimmune colitis inflammation. Finally, we found that IRS-1, a functional target of miR-126, was upregulated in CD4+ T cells with miR-126 deficiency, accompanied by altered transduction of ERK, AKT and NF-κB pathway.

CD4+ T cells were an important subpopulation of T cells, which played a central role for innate immune responses, maintaining balance of anti-inflammatory and proinflammatory responses, promoted activation of antigen-presenting cells (APCs) and B cells as well as promoted accumulation of immune cells [34-38]. More and more evidence demonstrated distinct miRNA molecule played critical regulatory roles in development and function of various immune cells including CD4+ T cells, which affected the pathogenesis and development of related clinical diseases [39-45]. For example, Zeng et al. [46] reported that down-regulation of miR-451a affected the activation and proliferation of CD4+ T cells by targeting the transcription factor myelocytomatosis oncogene (Myc) in dilated cardiomyopathy (DCM) patients, which contributed to the immunopathogenesis of DCM. Our newly research work also reported that miR-7 deficiency altered the proportion and the absolute number of CD4+ T cells in bronchoalveolar lavage (BAL) meanwhile related to ameliorated pathologies of acute lung injury [47]. In present study, we extended previous finding by demonstrating that miR-126 deficiency could obviously elevate the activation and proliferation, as well as IFN-γ secretion, in CD4+ T cells, indicating miR-126 might be a novel negative factor in CD4+ T cells function. Similarly, Okuyama et al. [14] reported that miR-126 was a critical regulator in the development and function of B cells. Combining these literatures might highlight the fact that miR-126 was an important intrinsic regulator in the generation and biological function of immune cells. It would be noticed that our previous work reported that miR-126 could be involved in the induction and function of CD4+ Th2 cells. Interestingly, Zhao et al. [15] also reported that miR-126 was highly expressed in CD4+ Th2 cells from systemic lupus erythematosus (SLE) patients. Similarly, in present study, we found that miR-126 deficiency could alter the expression of IFN-γ and IL-4, two critical representative cytokines for Th1 and Th2 subsets, suggesting that miR-126 also was critical for the biology of distinct CD4+ T cell subsets. Therefore, successive research work on the possible role of miR-126 in these CD4+ T cell subsets such as CD4+Th1 or Th2 cells was much important for verification on the exact biological role of miR-126 in immune system.

Previous literatures documented that the change on biological function of CD4+ T cells were closely related to the development of inflammatory bowel disease (IBD) [48,49]. Moreover, accumulating evidence showed that the irreplaceable role of distinct miRNA molecules in the occurrence and development of IBD [50-52]. Such as, Runtsch et al. [53] reported that miR-146a...
was involved in constraining intestinal barrier function. Moreover, miR-146a deficiency was resistance to DSS-induced colitis. In our study, we found that miR-126 deficiency could significantly promote the pathological change of colitis in DSS-induced autoimmune colitis model mice. Simultaneously, the percentage and total number of CD4\(^+\) T cells, displayed elevated activation phenotype, increased obviously in DSS-induced autoimmune colitis model mice. Most importantly, adoptive cell transfer assay further showed that miR-126 deficiency could endow CD4\(^+\) T cells with elevated activation, proliferation and IFN-\(\gamma\) secretion capacity to aggravate the pathology of colitis in DSS-induced autoimmune colitis model. In line with our finding, Holmkvist et al. [20] reported that the state of activation and function of CD4\(^+\) T cells were closely correlation with the development of T-cell-mediated immune colitis. Combining these data strongly suggested that miR-126 might be a novel potential regulator in the development of autoimmune colitis, at least partially through regulating the function of CD4\(^+\) T cells. Hence, further studies on the correlation between miR-126 expression and clinical IBD patients, which did not investigated in current study, were much valuable for the exploration on the exact role of miR-126 in the development of clinical IBD.

The insulin receptor substrate-1 (IRS-1), a distinct member of IRS family, is an important factor for the biological character of some type cells including cancer cells, which was closely related to transduction of ERK, AKT and NF-\(\kappa\)B pathway [30]. To immune cells, Li et al. [54] reported that overexpression of IRS-1 could protect T cells from activation-induced cell death (AICD). Moreover, Stentz et al. [55] found that upregulation of IRS-1 was involved in activation of CD4\(^+\) T cells in response to hyperglycemia. In present study, our data showed that, in the condition of miR-126 deficiency, the expression of IRS-1 in CD4\(^+\) T cells was elevated, which was consistent with previous finding that miR-126 could regulate the biological character of various cells through IRS-1. Furthermore, we found that miR-126 deficiency also altered the transduction of ERK, AKT and NF-\(\kappa\)B pathway. Given the fact that the important roles of these signal pathway in the activation, proliferation and cytokine secretion of CD4\(^+\) T cells, we presumed that miR-126 deficiency affected the activation and function of CD4\(^+\) T cells, which was related to upregulation of IRS-1 and subsequently altered transduction of related signaling pathway. However, because of the complex of miRNA-targets networks, whether miR-126 regulated the activation and function of CD4\(^+\) T cells through controlling the expression of other potential molecules still remains to be fully elucidated in the future.

Altogether, our present data revealed the unknown biological role of miR-126, as a novel intrinsic negative regulator, in the function of CD4\(^+\) T cells, which providing a new fundamental basis for further exploring the role of miR-126 in the development and function of immune cells and their subsets, as well as the occurrence and development of related diseases.
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Conflict of Interest

All authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Reference


Figure legends

Figure 1. Mir-126 deficiency enhanced the activation and proliferation of CD4+T cells. CD4+CD62L+ T cells purified by MACS from splenocytes in miR-126KD mice and WT mice (8-10 weeks old, n=6) respectively. Next, cells were cultured in the presence of anti-CD3 (20 μg/ml) /anti-CD28 (4 ng/ml) antibody plus IL-2 (10 ng/ml) for 72 hrs. Then, the relative expression of miR-126 was detected by Realtime PCR assay (a). The expression levels of CD69, CD62L and CD44 on CD4+T cells were analyzed by FACS (b) and calculated (c). Proliferation of CD4+T cells from miR-126KD mice and WT mice were assessed by Ki-67 staining (d) and calculated (e). (f) The apoptosis of CD4+T cells also was analyzed by FACS and calculated. One representative data of three independent experiments was shown. *p<0.05, ***p<0.001.

Figure 2. Mir-126 deficiency altered the expression of cytokines in CD4+T cells. CD4+CD62L+ T cells purified by MACS from splenocytes in miR-126KD mice and WT mice (8-10 weeks old, n=6) were cultured in the presence of anti-CD3 (20 μg/ml) /anti-CD28 (4 ng/ml) antibody plus IL-2 (10 ng/ml). 72 hrs later, the relative expression of cytokines including IL-4, IL-6, IL-10, IL-12, TGF-β, TNF-α and IFN-γ were detected by Realtime PCR and calculated (a). (b) The expression levels of IFN-γ, IL-4 and IL-17A in CD4+T cells were analyzed by FACS and calculated (c). One representative data of three independent experiments was shown. * p<0.05, ** p<0.01, NS, no significance.

Figure 3. Mir-126 deficiency promoted the pathology of CD4+T cell-mediated autoimmune colitis. MiR-126KD mice and WT mice (8-10 weeks old, n=6) were treated with 3% DSS water for 10 days. (a) Schematic representation of the animal study. (b) Clinical and Histological scores. (c) The pathology of colitis was observed by H&E staining. Magnification: 100x and 400x. Arrows indicated intestinal tissues loss. (d) The proportion of CD4+T cells in spleen was analyzed by FACS and calculated. (e) The total cell number of CD4+T cells was calculated. (f) The expression levels of CD62L and CD44 in CD4+T cells also were analyzed by FACS and calculated (g). One representative data of three independent experiments was shown. * p<0.05.

Figure 4. MiR-126 deficiency endowed CD4+T cells to aggravate the pathology of DSS-induced autoimmune colitis. CD4+CD62L+ T cells were purified by MACS from miR-126KD mice and WT mice respectively. Then, 1×10^7 cells were labeled by CFSE and adoptively transferred into normal WT mice (8-10 weeks old, n=6) through tail vein. One day later, mice were treated with 3% DSS water for 10 days. (a) Schematic representation of the animal study. (b) The morphology of intestine and its length was calculated (c). (d) Clinical and Histological scores. (e) The histopathology of intestine was observed.
by H&E staining. Magnification: 100× and 400×. Arrows indicated intestinal tissues loss. (f-m) The expression levels of CD62L, CD44, Ki-67, IL-4 and IFN-γ in CD4⁺T cells were analyzed by FACS and calculated respectively. One representative data of three independent experiments was shown. *p<0.05.

Figure 5. The expression of IRS-1 in CD4⁺T cells.
CD4⁺CD62L⁺T cells were purified by MACS from miR-126KD mice and WT mice (8-10 weeks old, n=6) respectively. Then, cells were cultured in the presence of anti-CD3 (20 μg/ml) /anti-CD28 (4 ng/ml) antibody plus IL-2 (10 ng/ml) for 48 hrs. The relative expression of indicated genes, including PMM1, Tom1, PIK3CD, RGS3, Tsc1, IRS1, ADAM9, EGFL7, Vcam1, Phf7, SLC39a6, HIP1 and PARP16, were analyzed by Realtime PCR assay and calculated. (a) CD4⁺CD62L⁺T cells were purified by MACS from FVB/N6 miR-126KD mice and WT mice respectively. Then, cells were cultured in the presence of anti-CD3 (20 μg/ml) /anti-CD28 (4 ng/ml) antibody plus IL-2 (10 ng/ml) for 48 hrs. The expression of IRS-1 protein was determined by Western blot and calculated. CD4⁺CD62L⁺T cells were purified by MACS from miR-126KD mice, Then, cells were transfected with p-IRS-1 RNAi (5 ng) and cultured in the presence of anti-CD3 (20 μg/ml) /anti-CD28 (4 ng/ml) antibody plus IL-2 (10 ng/ml) for 48 hrs. (d) The expression of IRS-1 was analyzed by Realtime PCR. (e) Call Counting Kit-8 assay. The expression levels of CD69 and CD44 were analyzed by FACS and the percentage was calculated. (f-i) The expression levels of IFN-γ and IL-4 were analyzed by FACS and the percentage was calculated. One representative data of three independent experiments was shown. *p<0.05.

Figure 6. The effect of miR-126 deficiency on the transduction of related signaling pathway.
CD4⁺CD62L⁺T cells purified by MACS from miR-126KD mice and WT mice (8-10 weeks old, n=6) respectively. Then, cells were cultured in the presence of anti-CD3 (20 μg/ml) /anti-CD28 (4 ng/ml) antibody plus IL-2 (10 ng/ml) for 48 hrs. (a) The expression levels of p-ERK, p-AKT, and p-NF-κB were determined by FACS and calculated. (b) Schematic representation of the underlying mechanism of miR-126 deficiency on the activation and function of CD4⁺T cells. miR-126 deficiency leads to upregulation of IRS-1, which could successively alter the transduction of AKT, ERK and NF-κB signaling pathway.
Figure 3

(a) mR-126 KD / WT mice

(b) Analyzed

Day 10

Day 14

(c) Control

DSS-induced colitis

WT

100x

mR-126KD

100x

(d) WT

mR-126KD

(e) WT

mR-126KD

(f) WT

mR-126KD

DSS-induced colitis

338x572mm (300 x 300 DPI)