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MicroRNA-21 can Regulate Apoptosis of CD4$^+$ T Cells in Systemic Lupus Erythematosus

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Abstract

Objectives: Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by increased apoptosis of T cells. MicroRNA (miR-)21 has been linked to apoptosis of T cells in SLE and was recently shown to be regulated by IL-21. Our objective was to investigate the effect of miR-21 on apoptosis in CD4$^+$ T cells and its possible regulation by IL-21 in SLE.

Methods: We investigated the capacity of IL-21 to induce miR-21 by stimulating CD4$^+$ T cells from SLE patients and healthy controls (HCs) with IL-21. The dependency of this induction upon STAT3 was investigated by inhibition of STAT3. MicroRNA-21's effects on apoptosis were assessed by overexpressing miR-21 in CD4$^+$ T cells and quantification of apoptotic and dead cells.

Results: Both primary and mature forms of miR-21 were induced by IL-21 in a STAT3-dependent manner in CD4$^+$ T cells. Induction of miR-21 by IL-21 and baseline expression levels of miR-21 were lower in CD4$^+$ T cells from SLE patients compared to HCs. PDCD4 expression levels were increased in CD4$^+$ T cells from SLE patients compared to HCs and correlated negatively with expression levels of miR-21. Finally, overexpression of miR-21 in CD4$^+$ T cells from SLE patients and HCs resulted in decreased apoptosis.

Conclusion: IL-21 can regulate miR-21 expression in CD4$^+$ T cells, and CD4$^+$ T cells from SLE patients have low levels of miR-21 and high levels of PDCD4 compared to HCs. These findings could in part explain the increased apoptosis seen in T cells from SLE patients.

Keywords: miR-21; Apoptosis; PDCD4; SLE; IL-21

Introduction

Systemic lupus erythematosus is clinically and pathologically heterogeneous disease. The pathogenesis of SLE is still unknown but the hallmarks of SLE are autoantibodies directed towards DNA- and RNA-associated molecules and defects in the regulation of apoptosis. These abnormalities result in inflammatory reactions in a range of organs such as the skin, kidneys, eyes and the central nervous system (CNS). SLE patients experience a relapsing disease pattern with flaring of symptoms such as rash, fever, hair loss, tiredness and in severe cases kidney failure and CNS affection [1-3].

Systemic lupus erythematosus is characterized by both increased apoptosis of leukocytes and defective clearance of apoptotic debris [4-7]. This increased apoptosis of leukocytes from SLE patients is reflected by their tendency to develop lymphopenias during disease flare. Several groups have studied and replicated this process but no clear cause for the increased apoptosis of lymphocytes has been found [7-9].

MicroRNA-21 (miR-21) is a key regulator of apoptosis during both a healthy and diseased state and has recently been implicated in SLE [10-12]. MicroRNAs are a class of small non-coding RNAs that are ubiquitously expressed in higher eukaryote cells and have emerged as crucial players in regulating immune responses in SLE [12,13]. They regulate gene expression post-transcriptionally by binding to target sequences on mRNAs, which results in mRNA degradation and/or suppression of mRNA translation. MicroRNA-21 targets several genes linked to apoptosis and functions as an oncomir in certain cancers where high levels of miR-21 suppress the tumor suppressor programmed cell death 4 (PDCD4) thus inhibiting apoptosis [14-16]. It has been speculated that miR-21 could play a role in the dysregulated apoptotic responses seen in SLE with increased apoptosis of T cells [12].

Several groups have studied the role of miR-21, but not its direct effects on apoptosis in SLE. Zhu et al. found expression of miR-21 to be decreased in renal biopsies from SLE patients compared to controls while Stagakis et al. reported higher levels of miR-21 in PBMCs and CD4$^+$ T cells from SLE patients compared to HCs [11,17]. Finally, Carlsen et al. found that plasma levels of circulating miR-21 were significantly increased in a Swedish cohort of SLE patients and marginally decreased in a Danish cohort of SLE patients [18]. Although apparently conflicting, these findings probably reflect the heterogeneic functions of miR-21 and that regulation of miR-21 is highly organ specific.

MicroRNA-21 is regulated by multiple factors including growth factors and cytokines [19]. STAT3 has recently been shown to activate the transcription of miR-21 and expanding on this finding Van der Fits et al. demonstrated that IL-21 activates transcription of miR-21 in a STAT3-dependent manner in Sézary Syndrome (a cutaneous T cell lymphoma) [20,21]. IL-21 is produced by activated $CD4^+$ T cells and follicular T helper cells and is a key regulator of T, B, and NK cell activation [22-24]. IL-21 signals by phosphorylation of signal transducer and activator of transcription (STAT) molecules-mainly STAT3 as well as STAT1 and 5 [25-28]. IL-21 has been proposed as a pathological factor in SLE, and several groups have demonstrated increased plasma levels of IL-21 in SLE and higher IL-21 production in $CD4^+$ T cells from SLE patients compared to HCs [29-32]. Further, IL-21 has been shown to promote a lupus-like disease in mice through both $CD4^+$ T cell and B cell-intrinsic mechanisms [33].

We hypothesize that miR-21 regulates apoptosis in $CD4^+$ T cells from SLE patients and that its expression is in part controlled by IL-21. To investigate this we examine expression levels of miR-21 and PDCD4 and the capacity of IL-21 to induce miR-21 in $CD4^+$ T cells. We further overexpress miR-21 in $CD4^+$ T cells and quantify its impact on apoptosis in SLE patients and HCs.

Materials and Methods

Patients

All SLE patients (n=22) met the 1997 revised American College of Rheumatology SLE criteria [34,35]. Samples were collected at the outpatient clinic at the Department of Rheumatology, Aarhus University Hospital. Age and gender matched HCs (n=14) were collected from the Blood Center at Aarhus University Hospital. The SLE Disease Activity Index (SLEDAI) [36] and the Systemic Lupus International Collaborative Clinics/American College of Rheumatology (SLICC/ACR) Damage Index [37] were recorded at sampling. Patients receiving high dose prednisone (>7.5 mg/day) or biologics such as belimumab (Benlysta) were excluded from the study.

The study was approved by the Regionel Ethics Committee (VEK2004-800-2) and the Danish Data Protection Agency (2006-41-6098). All samples were obtained after informed consent and in accordance with the Declaration of Helsinki and the study was carried out in accordance with the principles of the International Conference on Harmonization guidelines for good clinical practice (GCP, 1996 revision).

Quantitative reverse transcriptase PCR

RNA for mRNA, pri-miRNA and miRNA analyses were isolated using the High Pure RNA Isolation Kit (Roche) and miRNeasy mini kit (Qiagen), respectively. The primary (pri-)miRNA expression levels of hsa-mir-15a, hsa-mir-21, hsa-mir-23b, hsa-mir-124, hsa-mir-125a, hsa-mir-126, hsa-mir-146a, hsa-mir-223 and mRNA levels of PDCD4 and PD-1 were determined using predesigned primer and probe mixes (Applied Biosystems) labeled with the FAM-BHQ system as fluorescence/quencher. Quantitative reverse transcriptase polymerase chain reaction (qPCR) was performed using the TaqMan RNA-to-CT 1-Step Kit (Applied Biosystems) on an MX3005P RQ-PCR machine (Stratagene), using the following cycle parameters: 15 minutes at 48°C, 10 minutes at 95°C and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. For normalization, RPS11 and ACTB were used.

The expression levels of the mature miRNA hsa-mir-21-5p were determined using predesigned primer and probe mixes (Applied Biosystems). Complementary (c) DNA was made using specific primers for each miRNA (as supplied in the kit) and the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems), using the following cycle parameters: 30 minutes at 16°C, 30 minutes at 42°C and 5 minutes at 85°C followed by a qPCR reaction using the following cycle parameters: 2 minutes at 50°C, 10 minutes at 95°C and 40 cycles 15 seconds at 95°C and 1 minute at 60°C. For normalization of miRNA levels, RNU6B and RNU43 were used.

For all experiments, individual samples were run in duplicate with a maximum acceptable inter-replicate difference of 1 cycle. Data was calculated using the ΔCt method and expressed relative to the geometric mean of the housekeeping genes.

Cell culture and cell purification

$CD4^+$ T cells from SLE patients and HC were cultured at 37°C and 5% CO_2 in cell culture media (RPMI-1640 supplemented with 10% fetal calf serum (FCS), streptomycin, penicillin, and glutamine) and stimulated with 50 ng/mL of IL-21 for 0, 1, 3, and 6 hours prior to RNA purification. $CD4^+$ T cells were purified using EasySep Untouched $CD4^+$ T cells (Stemcell). In respective experiments, cells were treated with 30 μM cucurbitacin i (CucI) (Sigma Aldrich) to selectively inhibit phosphorylation of STAT3.

MicroRNA overexpression

Lentiviral vectors were produced as previously described [38]. Verification of efficient miRNA expression was performed by transfection of $CD4^+$ T cells with the lentiviral transfer plasmid containing the miR-21 expression cassette, followed by quantification of miR-21 by qPCR. Activity of the over-expressed miR-21 was confirmed using a dual luciferase assay as described Bak et al. [38]. Briefly, HEK293T cells were seeded in a white 96-well plate (3000 cells/well) and the following day co-transfected with 10 ng/well of the psiCHECK-2 plasmid (Promega) encoding the firefly luciferase and renilla luciferase fused to a perfect target site for miR-21 and with 90 ng/well of a lentiviral transfer plasmid either encoding miR-21 and eGFP or eGFP alone. Two days post-transfection, luciferase activities were measures using the dual-glo luciferase assay system (Promega), and renilla levels were normalized to firefly levels.

The biological titer of the produced lentiviral vector preparations was determined using flow cytometric analysis of eGFP-expression in HEK293 cells transduced with serially diluted vectors. Titers were determined using the percentage of $eGFP^+$ cells in wells with 1-20% $eGFP^+$ cells. For transduction of PBMCs from SLE patients and HC, vector amounts were normalized according to the obtained titers in HEK293 cells and transductions were performed with equal inoculating volumes. Cells were treated with lentiviral vectors encoding miR-21 and eGFP, eGFP only, and phosphate buffered saline (PBS) as a control. Prior to transduction, PBMCs were stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours in 37°C and 5% CO_2 in cell culture media. Lentiviral vectors were then added directly to the cell culture and incubated for 24 hours. Subsequently the cells were washed with PBS and cultured in cell culture media supplemented with 2 ng/mL IL-2 (Proleukin®, Chiron) for 72 hours.

Apoptosis assay

Transduced cells were stained with Annexin V PE-Cy7 (eBioscience), Live/Dead Aquamine (Invitrogen), CD3 PerCP-Cy5.5 and CD4 APC-Cy7 according to manufacturer's recommendations. The samples were analyzed within 4 hours on an LSR Fortessa (BD Biosciences). Intact cells were selected using forward/side scatter and doublets were excluded using forward scatter height vs. area.

Statistics

Statistical analyses were performed using GraphPad Prism 6.0b for Mac (GraphPad Software). Non-paired, non-parametric data was analyzed by Mann-Whitney U test. Correlation of parametric data was tested using Pearson's correlation. Differences between data sets with multiple observations within each group were analyzed using repeated measures (RM) two-way ANOVA. In all tests the level of significance was a two-sided p value of less than 0.05.

Results

Induction of primary miR-21 by IL-21 is decreased in CD4+ T cells from SLE patients

To determine if IL-21 is capable of regulating miR-21 expression in CD4+ T cells we stimulated CD4+ T cells from SLE patients and HCs with IL-21 and measured the expression levels of the primary (pri-) transcript of miR-21.

Pri-miR-21 was induced above unstimulated levels (0 hours) after 1 hour of IL-21 stimulation in both SLE patients and HCs (Figure 1A). After 1 hour of IL-21 stimulation pri-miR-21 was upregulated 4.8 fold in HCs vs. 2.8 fold in SLE patients. The degree of pri-miR-21 induction was consistently higher in HCs compared to SLE patients throughout the time course. Furthermore, expression levels of pri-miR-21 in unstimulated cells were lower in CD4+ T cells from SLE patients compared to HCs.

MicroRNA-21 expression levels are decreased in CD4+ T cells from SLE patients

MicroRNAs are regulated at multiple steps - transcription being only one of them. Therefore, the levels of pri-miRNA and mature and functional miRNAs do not necessarily correlate [19,39]. We therefore examined the expression levels of the mature and functional form of miR-21 in purified unstimulated CD4+ T cells from SLE patients and HCs and upon IL-21 stimulation.

In agreement with our findings for pri-miR-21 the relative expression levels of the mature miR-21 were significantly decreased in untreated CD4+ T cells from SLE patients as compared to HCs (Figure 1B). Healthy controls had on average two-fold higher expression levels of miR-21 compared to SLE patients ($p<0.05$).

Upon IL-21 stimulation SLE patients had significantly reduced expression levels of miR-21 (Figure 1C). At all time points after IL-21 stimulation miR-21 expression levels were lower in SLE patients compared to HCs. However, both SLE patients and HCs showed a similar increase in expression levels of miR-21 increasing by 1.5 and 1.6 fold, respectively, after one hour of stimulation.

IL21-induced miR-21 expression is STAT3-dependent

Interleukin 21 is a member of the IL-2 family of cytokines and signals through its own distinct receptor and the common gamma-chain. Receptor ligation induces phosphorylation of several STAT molecules of which STAT3 is the dominant [25,40]. To investigate whether the observed effects on miR-21 could be attributed to alternate signal transduction not dependent on STAT3 we employed a specific STAT3 phosphorylation inhibitor (Cucurbitacin I, CucI). PBMCs from HCs, preincubated with and without CucI, were stimulated with IL-21 for the time points indicated and miR-21 expression levels were measured.

Inhibition of STAT3 phosphorylation using CucI resulted in a significant decrease in the induction of miR-21 from 1.6-fold in the controls to 0.3-fold in the CucI-treated CD4+ T cells after one hour of IL-21 stimulation ($p<0.05$, RM two-way ANOVA) (Figure 1D). Throughout the time course, levels of miR-21 were consistently and significantly lower in the CucI-treated cells compared to controls ($p<0.01$). This confirmed that the effects observed on miR-21 are mainly mediated by a STAT3-dependent pathway.

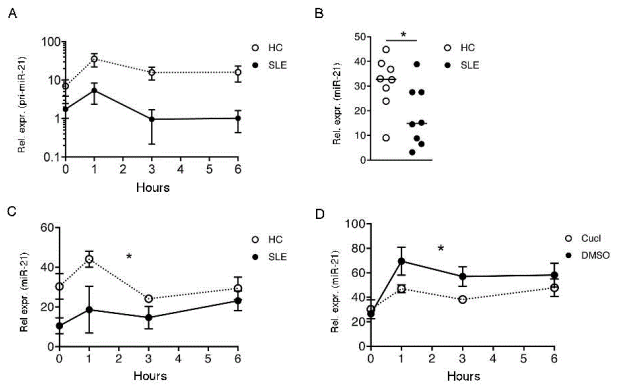

Figure 1: (A) IL-21-mediated induction of primary (pri-) miR-21. Relative expression levels of pri-miR-21 in CD4+ T cells from SLE patients (n=3) and HCs (n=5). Purified CD4+ T cells were stimulated with IL-21 for up to six hours, as indicated. Graphs show mean ± SEM. (B) Relative expression levels of miR-21 in CD4+ T cells from SLE patients and HCs (both n=8). Bar indicates median. *p<0.05 (Mann-Whitney's U test). (C) Relative expression levels of miR-21 in CD4+ T cells from SLE patients and HCs (both n=3) stimulated with IL-21 for the time points indicated. Graph shows mean ± SEM. *p<0.05 (RM two-way ANOVA). (D) Relative expression levels of miR-21 in CD4+ T cells from HCs (n=3) stimulated with IL-21 for the time points indicated incubated with or without the STAT3 phosphorylation inhibitor Cucurbitacin I (CucI). As a control the carrier for CucI (DMSO) was included to exclude that it by itself could affect miR-21 expression levels. Graph shows mean ± SEM. *p<0.05 (RM two-way ANOVA).

Expression of miR-21-targeted gene programmed cell death 4 (PDCD4) is increased in SLE patients compared to HCs

Systemic lupus erythematosus is a disease characterized by both defects in apoptotic clearance but also increased apoptosis of T cells [7-9]. MicroRNA-21 has multiple effects but has been shown to be a significant regulator of apoptosis. The miRNA exerts these anti-apoptotic effects by repressing the pro-apoptotic tumor suppressor molecule PDCD4 [14-16]. As we found decreased levels of miR-21 in CD4$^+$ T cells of SLE patients we wanted to investigate if the target gene PDCD4 was correspondingly increased. We did this by measuring the mRNA levels of PDCD4 in purified CD4$^+$ T cells from SLE patients and HCs.

In correspondence with the low levels of miR-21, expression levels of PDCD4 were significantly elevated in CD4$^+$ T cells from SLE patients compared to HCs (Figure 2A). Expression levels were approximately 73% higher in SLE patients vs. HCs (0.26 vs. 0.15, respectively).

MicroRNA-21 and PDCD4 expression levels correlated inversely when examining SLE patients and HCs combined ($p<0.01$, $\sigma=-0.74$) (Figure 2B) supporting that miR-21 negatively regulates expression of PDCD4. Examining SLE patients and HCs individually, miR-21 and PDCD4 expression levels correlated significantly in SLE patients but not in HCs.

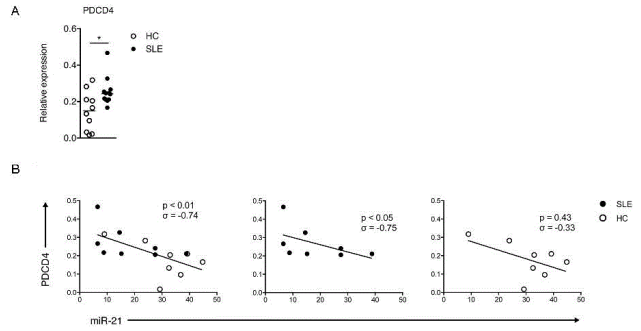

Figure 2: (A) Relative expression levels of PDCD4 in CD4$^+$ T cells from SLE patients (n=10) and HCs (n=12). Bar indicates median. $^*p<0.05$. (Mann-Whitney's U test). (B) Linear regression plots and correlation of relative expression levels of miR-21 vs. PDCD4 in SLE patients (black dots) and HCs (empty dots) individually and combined. Statistical analysis was done using a Spearman correlation.

Overexpression of miR-21 results in decreased apoptosis in CD4$^+$ T cells

As we have demonstrated that CD4$^+$ T cells from SLE patients have decreased levels of miR-21 and correspondingly increased levels of targeted PDCD4 mRNA compared to HCs, we wanted to evaluate the apoptotic effects of overexpressing miR-21 in these cells. We did this by overexpressing miR-21 in PBMCs from SLE patients and HC (both n=6) using a lentiviral vector encoding miR-21 and eGFP and subsequently staining the cells for CD3, CD4, Annexin V (AnnV) and a viability marker (Live-Dead) (Figure 3A).

Overexpression of miR-21 led to significantly reduced apoptosis in CD4$^+$ T cells from both SLE patients and HCs (Figure 3B). The percentage of apoptotic cells in CD4$^+$ T cells from SLE patients dropped from 3.7% to 1.9% vs. 2.3% to 1.2% in HCs (both $p<0.05$, paired T test). Further, SLE patients tended to have higher levels of apoptotic (AnnV$^+$/Live-Dead$^-$) CD4$^+$ T cells both in the GFP controls and in the miR-21 overexpressing samples compared to CD4$^+$ T cells from HCs. No significant differences in the levels of dead cells for GFP controls and miR-21-overexpressing cells between SLE patients and HCs were observed.

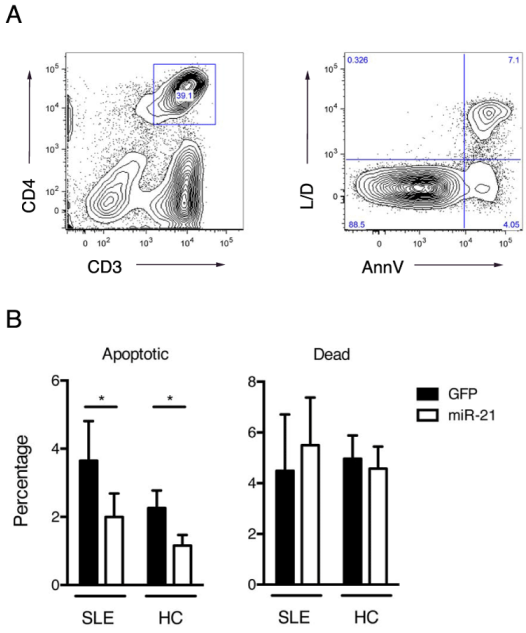

Figure 3: (A) Representative flow diagram of lentivirally transduced CD4$^+$ T cells overexpressing miR-21 and GFP. In brief, cells were prestimulated with CD3/CD28 before being transduced with lentiviral vectors encoding miR-21. The cells were allowed to rest 3 days post-transduction in culture media supplemented with IL-2. The cells were then stained using CD3, CD4, Annexin V and Live/Dead viability stain. (B) Percentage of apoptotic and dead CD4$^+$ T cells in SLE patients and HCs (both n=6). Apoptotic cells were defined as Annexin V$^+$ and Live/Dead$^-$, and dead cells were defined as Annexin V$^+$ and Live/Dead$^+$. Statistical analysis was done using a paired T test for paired data and Mann-Whitney's U test for non-paired data. Graphs show mean ± SEM. $^*p<0.05$.

Discussion

We here demonstrate a functional role for miR-21 in regulating apoptosis in CD4$^+$ T cells from SLE patients. We show that miR-21 is increased in response to IL-21 treatment through a STAT3-dependent pathway. CD4$^+$ T cells from SLE patients have lower levels of miR-21 and correspondingly higher levels of PDCD4 compared to HCs. Finally, overexpression of miR-21 led to decreased apoptosis in CD4$^+$ T cells from SLE patients and HCs.

We show that IL-21 is capable of regulating miR-21 in a STAT3-dependent manner in both SLE patients and HCs. This upregulation

was seen in both the primary transcript and the mature form of miR-21, reflecting that this upregulation was a direct result of increased transcription and further that this transcription is dependent on STAT3. This is in line with recent work by other groups who demonstrated that STAT3 regulates miR-21 [20,21,41].

Several groups have examined different aspects of miR-21 in SLE. We find that CD4$^+$ T cells from SLE patients have lower levels of both the primary and mature forms of miR-21. In line with our findings, Zhu et al. found that SLE patients had low expression levels of miR-21 in renal biopsies compared to controls [17]. Carlsen et al. found that serum levels of circulating miR-21 was marginally decreased in one SLE cohort and increased in the control cohort compared to HCs [18]. Using microarrays, two studies of separate cohorts of SLE patients showed that expression levels of miR-21 in CD4$^+$ T cells from SLE patients were significantly increased compared to HCs [11,42]. Both studies also found that miR-21 expression levels correlated positively with SLEDAI. While this is seemingly in conflict with our findings of decreased miR-21 levels, our cohort of SLE patients had a median SLEDAI of 3 (with interquartile range 0-5) thus mostly resembling the inactive SLEDAI subgroup (defined by SLEDAI<8) in this study but with an even lower SLEDAI. Thus, given that miR-21 correlates strongly with SLEDAI this discrepancy presumably reflects that our cohort generally has very low SLEDAI and furthermore that miR-21 expression levels vary depending on tissue-specific functions and the microenvironment in which it exists and is examined. In addition, one of the studies withheld treatment for 24 hours prior to blood sampling [11]. The effect of withdrawing treatment upon microRNA levels is uncertain and given that our patients continuously received appropriate treatment we are unable to control for or compare the effect of this intervention.

The negative regulation of PDCD4 by miR-21 has been demonstrated in two cancer studies and in several other diseases including SLE [11,14-16,43]. PDCD4 has been shown to act as a tumor suppressor by direct protein-protein interactions with eukaryote initiation factors (eIFs) thus regulating both mRNA translation and gene transcription [44]. While most of the current studies have focused on role of miR-21 in inhibiting apoptosis and promoting proliferation in cancer, its role in promoting apoptosis is less studied. We find that CD4$^+$ T cells from SLE patients have increased numbers of apoptotic cells and that overexpression of miR-21 by lentiviral transduction significantly decreases apoptosis of CD4$^+$ T cells in both SLE patients and HCs. In line with our findings, one study demonstrated that overexpression of miR-21 increased proliferation of T cells from healthy donors [11]. Another study examined the effect of inhibiting miR-21 in Sézary Syndrome and showed that inhibition of miR-21 leads to increased apoptosis of CD4$^+$ T cells, thus also supporting our findings.

In conclusion, we demonstrate a functional role for miR-21 in the regulation of apoptosis in CD4$^+$ T cells from SLE patients. We show that miR-21 can be regulated by IL-21 in a STAT3-dependent manner, and CD4$^+$ T cells from SLE patients have significantly lower levels of miR-21 and higher levels of PDCD4 compared to HCs. Finally, overexpression of miR-21 significantly reduces apoptosis in CD4$^+$ T cells. Combined, these findings highlight a role for miR-21 and PDCD4 in SLE pathology.

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