Identification of novel microRNA signatures linked to acquired aplastic anemia

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ABSTRACT

Emerging evidence indicates that microRNA control and modulate immunity. MicroRNA have not been investigated in acquired aplastic anemia, a T-cell-mediated immune disease. Analysis of 84 microRNA expression levels in CD4+ and CD8+ T cells of patients with aplastic anemia revealed concurrent down-regulation of miR-126-3p, miR-145-5p, miR-223-3p, and miR-199a-5p (>3-fold change, $P<0.05$) in both T-cell populations, which were unique in aplastic anemia compared to other hematologic disorders. MiR-126-3p and miR-223-3p were down-regulated in CD4+ T effector memory cells, and miR-126-3p, miR-145-5p, and miR-223-3p were down-regulated in CD8+ T effector memory and terminal effector cells. Successful immunosuppressive therapy was associated with restoration to normal expression levels of miR-126-3p, miR-145-5p, and miR-223-3p (>2-fold change, $P<0.05$). In CD4+ and CD8+ T cells in aplastic anemia patients, *MYC* and *PIK3R2* were up-regulated and proved to be targets of miR-145-5p and miR-126-3p, respectively. MiR-126-3p and miR-145-5p knockdown promoted proliferation and increased interferon-γ and granzyme B production in both CD4+ and CD8+ T cells. Our work describes previously unknown regulatory roles of microRNA in T-cell activation in aplastic anemia, which may open a new perspective for development of effective therapy. *Clinicaltrials.gov identifier: NCT 01623167*

Introduction

Aplastic anemia (AA) is an acquired bone marrow disease characterized by trilineage marrow hypoplasia, a paucity of hematopoietic stem and progenitor cells, and pancytopenia of the peripheral blood due to immune cell attack on the bone marrow. The responsiveness of AA to immunosuppressive therapies remains the best evidence of an underlying immune pathophysiology: the majority of patients show hematologic improvement after only transient T-cell depletion by antithymocyte globulins.[1] Although immunity to hematopoietic progenitors by activated T cells has been considered to be responsible for the pathogenesis of AA, little is known about the molecular basis of T-cell activation.

Effector cells in AA have been identified by immunophenotyping as activated cytotoxic T cells expressing Th1 cytokines, especially γ-interferon (IFN-γ);[2] by oligoclonal expansion of CD8+ CD28- T cells, defined by flow cytometry for T-cell receptor (TCR) Vβ subfamilies; and using spectratyping to detect skewing of CDR3 length and sequencing of the *CDR3* region to define a molecular clonotype.[3] Polymorphisms in cytokine genes, associated with an increased immune response, are also more prevalent in AA.[4] Constitutive expression of T-bet, a transcriptional regulator that is critical to Th1 polarization, is present in the majority of AA patients.[5] Genome-wide transcriptional analysis of T cells from AA patients has implicated some components of innate immunity in AA, including Toll-like receptors and natural killer cells.[6] However, the precise mechanisms underlying activation of T cells in AA are still unclear.

MicroRNA (miRNA, miR) are a group of small, conserved, non-coding RNA molecules that primarily modulate gene expression at the post-transcriptional level by hybridization to complementary sequences in the 3′ untranslated region (3'UTR) of their corresponding mRNA.[7] miRNA bind to the ribonucleoprotein complex RNA-induced silencing complex, which in addition also binds to the 3′ UTR of complementary mRNA.[8] The double-stranded complex between miRNA and mRNA is then degraded, which leads to decreased protein translation.[9]

Approximately 30% of the human genome is estimated to be regulated by miRNA, and a single miRNA can potentially regulate hundreds of proteins.[10] More than 1,000 miRNA have been identified in mammals and implicated in a wide range of biological functions.[11] MiRNA contribute to the pathophysiology of a number of important human diseases such as cancer,[12] cardiovascular disease, and neurodegenerative disorders.[13,14] There is emerging evidence that miRNA play crucial roles in controlling and modulating immunity.[15] Dysregulation of miRNA can lead to autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease.[16-18] Normalization of dysregulated miRNA can be therapeutic in murine disease models. MiRNA thus represent novel molecular diagnostic markers and potential targets for therapeutics.[19]

We hypothesized that dysregulated miRNA expression might lead to aberrant T-cell activation in AA. In this work, we used quantitative reverse transcriptase polymerase chain reaction (RT-qPCR)–based approaches to assess miRNA expression in CD4+ and CD8+ T cells from AA patients. We demon-

strate that down-regulation of miR-126-3p and miR-145-5p promotes CD4^{+} and CD8^{+} T-cell activation by increasing *MYC* and *PIK3R2* expression levels and T-cell proliferation, of potential importance in the pathogenesis of AA. Our results provide a pharmacological rationale for the potential use of synthetic miRNA mimics to limit disease.

Methods

Patients and treatment

Blood samples were obtained after informed consent from 15 patients with severe AA and 11 age-matched healthy donors. The median age of AA patients was 41 years (range, 13-69 years). MiRNA expression levels of all 15 patients were analyzed at diagnosis. Standard criteria were used for the diagnosis of AA and the evaluation of disease severity.[20] From 15 AA patients, 12 samples were used to determine miRNA expression in lymphocyte subsets and three samples were used to determine miRNA expression in T-cell subsets. From 11 healthy donors, eight samples were used to determine miRNA expression in lymphocyte subsets, and three samples were used to determine miRNA expression in T-cell subsets. Serial samples were collected before and after immunosuppressive therapy in six cases. Blood samples from five patients with low-risk myelodysplastic syndrome (MDS) at diagnosis and five patients with red blood cell transfusion-dependent sickle cell

Table 1. Characteristics of the patients and healthy controls.

#	Diagnosis	Age at Dx	Sex	Sampling status	Transfusion	IPSS	Treatment	3 months response	6 months response	Relapse	ANC	Hb	Platelets	ARC
1	SAA	69	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	CR	CR	Yes	0.12	7.3	17	26.6
2	SAA	30	F	Before Tx	Yes	NA	ATG+CsA+TPO-RA	PR	CR		0.09	6.7	9	43
3	SAA	56	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	NE/Death	NE		0	7.4	3	6.4
4	SAA	28	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	PR	PR		0.66	7.3	9	44.9
5	SAA	17	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	NR	NR		0.01	6.6	37	2.4
6	SAA	41	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	PR	PR		0.68	7.3	6	20.3
7	SAA	22	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	PR	CR		0.38	7.3	8	6.9
8	SAA	50	F	Before Tx	Yes	NA	ATG+CsA+TPO-RA	PR	PR	Yes	0.63	4.8	2	31.4
9	SAA	13	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	CR	CR		0.2	7.8	1	11.8
10	SAA	41	F	Before Tx	Yes	NA	ATG+CsA+TPO-RA	PR	PR	Yes	0.36	6.7	12	24.8
11	SAA	42	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	PR	PR	Yes	0.22	7.4	13	19.6
12	SAA	22	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	PR	PR		0.35	8.9	8	16.9
13	SAA	59	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	PR	PR	Yes	0.252	8.6	5	6.1
14	SAA	21	M	Before Tx	Yes	NA	ATG+CsA+TPO-RA	NR	PR		0.43	7.5	6	1.6
15	SAA	56	F	Before Tx	Yes	NA	ATG+CsA+TPO-RA	PR	PR	Yes	0.37	8	9	42.7
16	MDS	67	M	Before Tx	Yes	Int-1	Untreated	NA	NA	NA	79	10.6	40	89.7
17	MDS	42	M	Before Tx	Yes	Int-1	Campath	NR	NR	NA	3.76	8.1	47	59.3
18	MDS	27	M	Before Tx	Yes	Int-1	Campath	PR	NR	NA	0.39	10.6	66	44.5
19	MDS	46	M	Before Tx	Yes	Int-1	Campath	NR	NR	NA	0.27	6.9	14	2.3
20	MDS	49	F	Before Tx	Yes	Int-1	Campath	PR	PR	NA	0.9	10.6	36	72.4
21	SCD	56	F	After Tx	Yes	NA	Transfusion Program	NA	NA	NA	10.23	7.7	476	433.3
22	SCD	61	M	After Tx	Yes	NA	Transfusion Program	NA	NA	NA	6.93	7.6	320	176
23	SCD	50	M	After Tx	Yes	NA	Transfusion Program	NA	NA	NA	6.2	8.1	128	139.3
24	SCD	30	M	After Tx	Yes	NA	Transfusion + HU	NA	NA	NA	8.12	5.2	302	203
25	SCD	44	M	After Tx	Yes	NA	Transfusion Program	NA	NA	NA	3.17	7.3	269	304.6
26	Healthy	46	M	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
27	Healthy	40	M	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
28	Healthy	48	F	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
29	Healthy	54	F	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
30	Healthy	28	F	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
31	Healthy	32	M	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
32	Healthy	30	M	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
33	Healthy	57	M	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
34	Healthy	25	M	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
35	Healthy	33	M	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
36	Healthy	51	F	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA

SAA: severe aplastic anemia; MDS: myelodysplastic syndrome; SCD: sickle cell disease; HU: hydroxyurea; M: male; F: female; Tx: treatment; Dx: diagnosis; NA: not applicable; IPSS: International Prognostic Scoring System; Int-1: intermediate-1; ATG: antithymocyte globulins; CsA: cyclosporine; TPO-RA: thrombopoietin receptor agonist; CR: complete response; PR: partial response; NR: no response; NE: not evaluable; ANC: absolute neutrophil count; Hb: hemoglobin; ARC: absolute reticulocyte count.

disease (SCD) were used for comparison. The demographic and clinical characteristics of the 25 patients are summarized in Table 1. All AA patients received horse anti-thymocyte globulin + cyclosporine + eltrombopag on a clinical research protocol (clinicaltrials.gov, #NCT01623167). All human subjects were enrolled on clinical protocols approved by the NHLBI Institutional Review Board.

Flow cytometry and cell sorting

The gating strategies for sorting lymphocyte subsets in human or mouse samples, and T-cell subsets in human samples are summarized in *Online Supplementary Figure S1*.

RNA isolation

Total RNA, including small RNA, was isolated using the miRNeasy kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. RNA concentration was measured using a Nanodrop device (Peqlab, Erlangen, Germany).

T-cell and B-cell activation pathway-focused microRNA

polymerase chain reaction-array analysis

Using miScript miRNA PCR Array Human or Mouse T-Cell and B-Cell Activation (QIAGEN, Sabiosciences) with 84 miRNA (*Online Supplementary Table S1*), miRNA PCR array focused on T-cell and B-cell activation pathways was performed, according to the manufacturer's instructions. For each array, a minimum of 250 ng total RNA was retrotranscribed using the miScript II RT kit (QIAGEN) with HiSpec buffer, according to the manufacturer's instruction. Data were median-centered and submitted to hierarchical clustering using the Cluster 3 program with Pearson correlation as similarity metrics and centroid linkage clustering. Results were displayed as heatmaps generated using the TreeView program.[21]

Custom microRNA targets polymerase chain reaction-array analysis

To examine target genes of four selected miRNA, we designed the custom RT² Profiler™ microRNA Targets PCR Arrays with 84 target genes (*Online Supplementary Table S2*), based on target gene
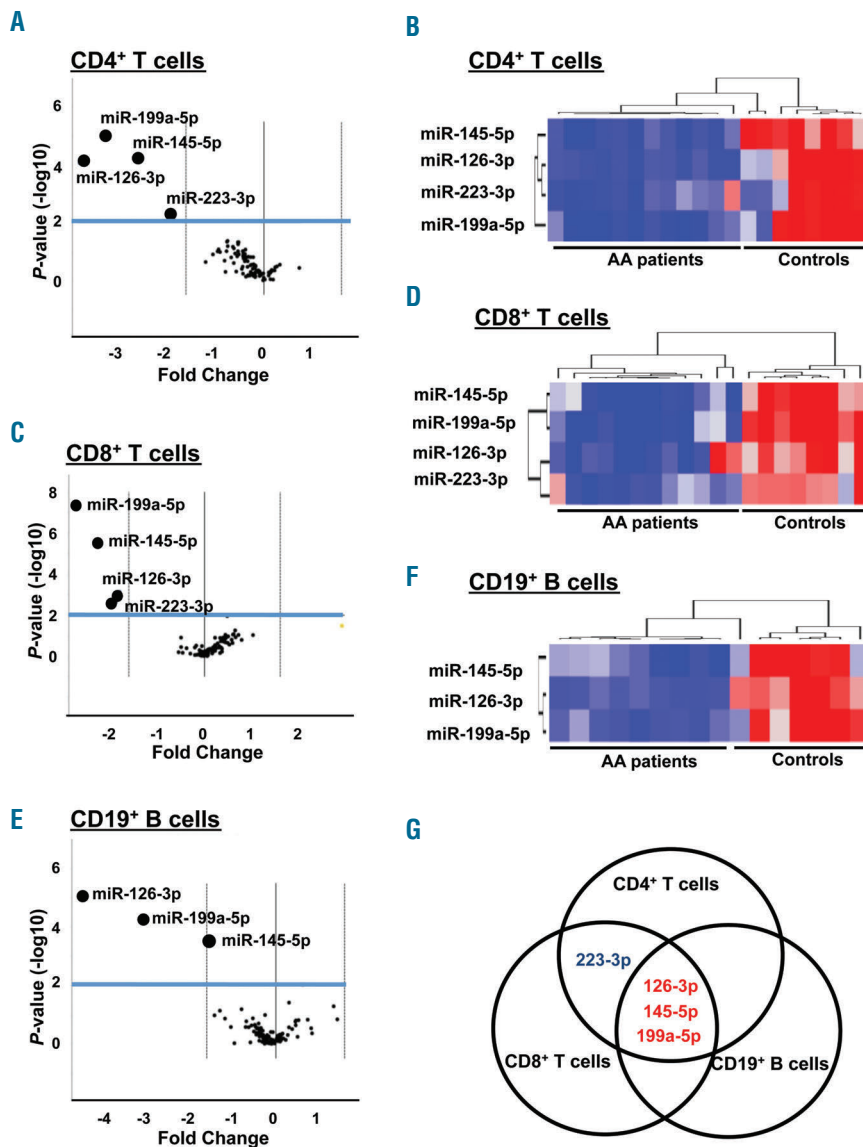

Figure 1. Distinct miRNA expression patterns in CD4+ and CD8+ T cells of AA patients. Volcano plots of results from 84 miRNA known to be involved in lymphocyte activation in (A) CD4+ and (C) CD8+ T cells (AA patients, n=12; healthy donors, n=8) and (E) CD19+ B cells (AA patients, n=9; healthy donors, n=7) using miRNA PCR-array. The *x*-axis is the estimated difference in expression measured in $\log_2$; vertical lines refer to a 3-fold difference in expression between the two groups. MiRNA highly expressed in AA or healthy donors are on the right or the left, respectively. The *y*-axis is the significance of the difference measured in $-\log_{10}$ of the *P*-value; the horizontal line represents our cutoff for significance at *P*<0.01. Hierarchical clustering of miRNA in (B) CD4+ T cells, (D) CD8+ T cells, or (F) CD19+ B cells was visualized by heatmap analysis. A red-blue color scale depicts normalized miRNA expression levels in Ct values (red: high, blue: low). (G) Venn diagram presentation of PCR array data.

prediction. For each array, 300 ng total RNA was retrotranscribed using the RT² First Strand kit (QIAGEN), according to the manufacturer's instruction.

Further experimental procedures are described in the *Online Supplementary Experimental Methods*.

Results

Distinct patterns of microRNA expression in CD4+ and CD8+ T cells from patients with aplastic anemia

First, miRNA expression levels in CD4+ and CD8+ T-cell populations of AA patients were analyzed using a commercial miRNA platform. This platform allows measurement of 84 miRNA involved in lymphocyte activation. For this array, we tested 12 samples (#1 - #12) from 15 AA patients and eight samples (#26 - #33) from 11 healthy donors (Table 1). A threshold of 3-fold change (FC) in expression and a two-tailed *P* value cutoff of 0.01 were used to identify miRNA that were differentially expressed in the AA and control groups. Expression levels of four miRNA (miR-126-3p, miR-145-5p, miR-199a-5p, and miR-223-3p) among 84 miRNA were significantly down-regulated (>3 FC, *P*<0.01) in both CD4+ and CD8+ T cells of AA patients compared with those of healthy controls, as highlighted in volcano plots (Figure 1A,C and *Online Supplementary Table S3*). For comparison, CD19+ B cells were also examined in the same PCR array platform, showing significant down-regulation (>3 FC, *P*<0.01) of miR-126-3p, miR-145-5p, and miR-199a-5p (Figure 1E and *Online Supplementary Table S3*). We observed dysregulation of these four miRNA in the 12 AA patients relative to eight healthy donors (Figure 1B,D,F). Venn diagrams summarize expression profiles of the four miRNA in both T and B cells from AA patients compared with healthy controls: down-regulation of miR-126-3p, miR-145-5p, and miR-199a-5p in both T- and B-cell populations from AA patients and down-regulation of miR-223-3p only in T cells from AA patients (Figure 1G). Thus, three of the four miRNA expression patterns were similar across T- and B-cell lineages.

Comparison of microRNA expression patterns between patients with aplastic anemia or other hematologic disorders

To determine whether the miRNA dysregulation observed in AA was distinct from miRNA expression in other hematologic diseases, miRNA levels of CD4+ and CD8+ T cells from five low-risk MDS patients and five transfusion-dependent SCD patients (Table 1) were assessed using the miRNA PCR array platform. Disease-specific miRNA expression patterns were observed (*Online Supplementary Figures S2 and S3*). In both CD4+ and CD8+ T cells of MDS patients, miR-182-5p was up-regulated (>3 FC, *P*<0.01), whereas miR-199a-5p and miR-19a-3p were down-regulated (>3 FC, *P*<0.01), compared with healthy controls. However, decreased miR-126-3p and miR-223-3p (>3 FC, *P*<0.01) expression levels were seen only in CD8+ T cells. In both CD4+ and CD8+ T cells from SCD patients, miR-34a-5p expression was enhanced (>3 FC, *P*<0.01) and miR-199a-5p and miR-142-5p expression was decreased (>3 FC, *P*<0.01) compared with levels in healthy controls.

For validation, levels of expression of four miRNA (miR-

126-3p, miR-145-5p, miR-199a-5p, and miR-223-3p) in CD4+ and CD8+ T cells were measured by RT-qPCR. MiR-199a-5p expression was reduced in both CD4+ and CD8+ T cells from patients with AA, MDS, and SCD (>3 FC, *P*<0.05). Decreased expression of miR-223-3p (>3 FC, *P*<0.05) in CD4+ and CD8+ T cells was observed in patients with low-risk MDS. MiR-126-3p expression was normal in both CD4+ and CD8+ T cells from SCD patients, but it was down-regulated in CD8+ T cells from patients with MDS and CD4+ and CD8+ T cells from those with AA (>3 FC, *P*<0.05). In both CD4+ and CD8+ T cells from MDS patients, miR-145-5p expression was not significantly changed compared with that in healthy controls. Accordingly, concurrent down-regulation of miR-126-3p, miR-145-5p, miR-223-3p, and miR-199a-5p (>3 FC, *P*<0.05) in both CD4+ and CD8+ T cells was a distinguishing feature of AA (Figure 2A,B).

MicroRNA expression profiles in CD4+ and CD8+ T-cell subsets from patients with aplastic anemia

T cells from three AA patients and three healthy controls were sorted by flow cytometry: CD4+ T cells into three subsets [naïve (TN), central memory (TCM), and
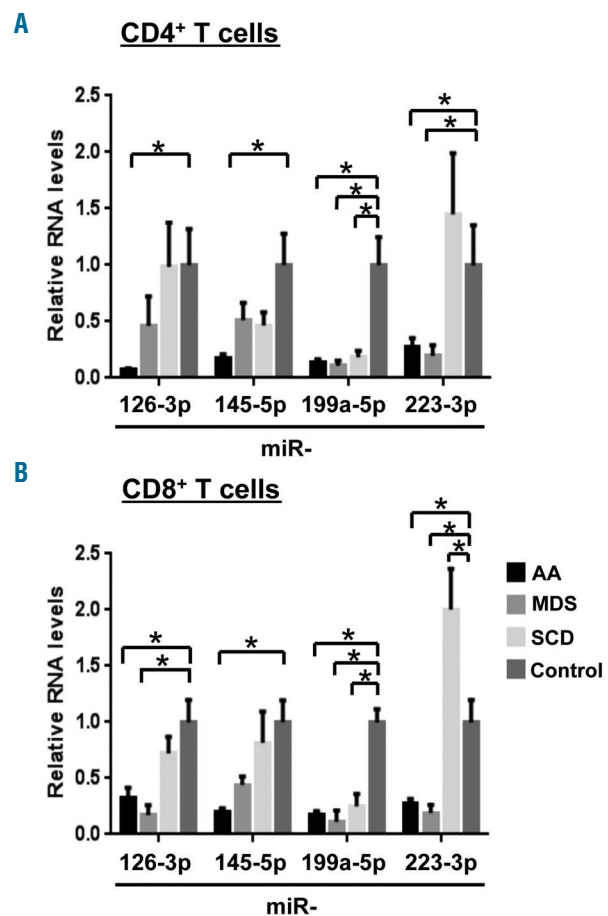

Figure 2. Comparison of expression levels of specific miRNA in other hematologic diseases. RT-qPCR analysis of miR-126-3p, miR-145-5p, miR-199a-5p, and miR-223-3p expression in (A) CD4+ and (B) CD8+ T cells from AA (n=12), low-risk MDS (n=5), and SCD (n=5) patients, and healthy controls (n=8). MiRNA relative expression was obtained by normalizing to RNU-2 expression. *P<0.05 [two-way analysis of variance (ANOVA)].

effector memory (TEM)] and CD8+ T cells into four sub-sets [TN, TCM, TEM, and T effector (TE)]. Expression of the four down-regulated miRNA in AA patients was measured in individual T-cell subsets by RT-qPCR in order to identify those cells responsible for differential expression of the four miRNA. Among CD4+ T cells, miR-126-3p and miR-223-3p were down-regulated in a TEM population in AA patients (Figure 3A, *P*<0.05), compared with controls. In CD8+ T cells, down-regulation of miR-126-3p, miR-145-5p, and miR-223-3p was present in both TEM and TE populations in AA (Figure 3B, *P*<0.05). However, no significantly different miR-199a-5p expression levels were detected in either CD4+ or CD8+ T-cell subsets. Thus there was a clear tendency of down-regulation of miR-126-3p, miR-145-5p, and miR-223-3p from TN toward TEM or TE populations in CD8+ T cells from AA patients.

Altered microRNA expression patterns after immuno-suppressive therapy

To address the status of miRNA expression levels after immunosuppressive therapy, we sorted CD4+ and CD8+ T cells from six AA patients after immunosuppressive thera-py (#1, #7, #9, #10, #11, and #12) and measured miRNA expression by RT-qPCR. All six patients achieved partial or complete responses (Table 1). Analysis of the four miRNA (miR-126-3p, miR-145-5p, miR-199a-5p, and miR-223-3p) showed restoration of expression levels of miR-126-3p, miR-145-5p, and miR-223-3p (>2 FC, *P*<0.05) after successful immunosuppressive therapy as compared to the same cases tested before immunosuppressive therapy (Figure 4A,B). The percentage restoration of three miRNA (miR-126-3p, miR-145-5p, and miR-223-3p) after immunosuppressive therapy, relative to levels in healthy controls, were 54%, 96%, and 57% in CD4+ T cells, and 93%, 109% and 94% in CD8+ T cells, respectively. However, recovery of miR-199a-5p expression was not seen, suggesting that miR-199a-5p is regulated by a cellular mechanism distinct from the other three miRNA.

MicroRNA target gene prediction and validation in CD4+ and CD8+ T cells

We generated a list of pathways likely to be specifically controlled by the four miRNA that were differentially expressed in AA and healthy controls. Using the web-
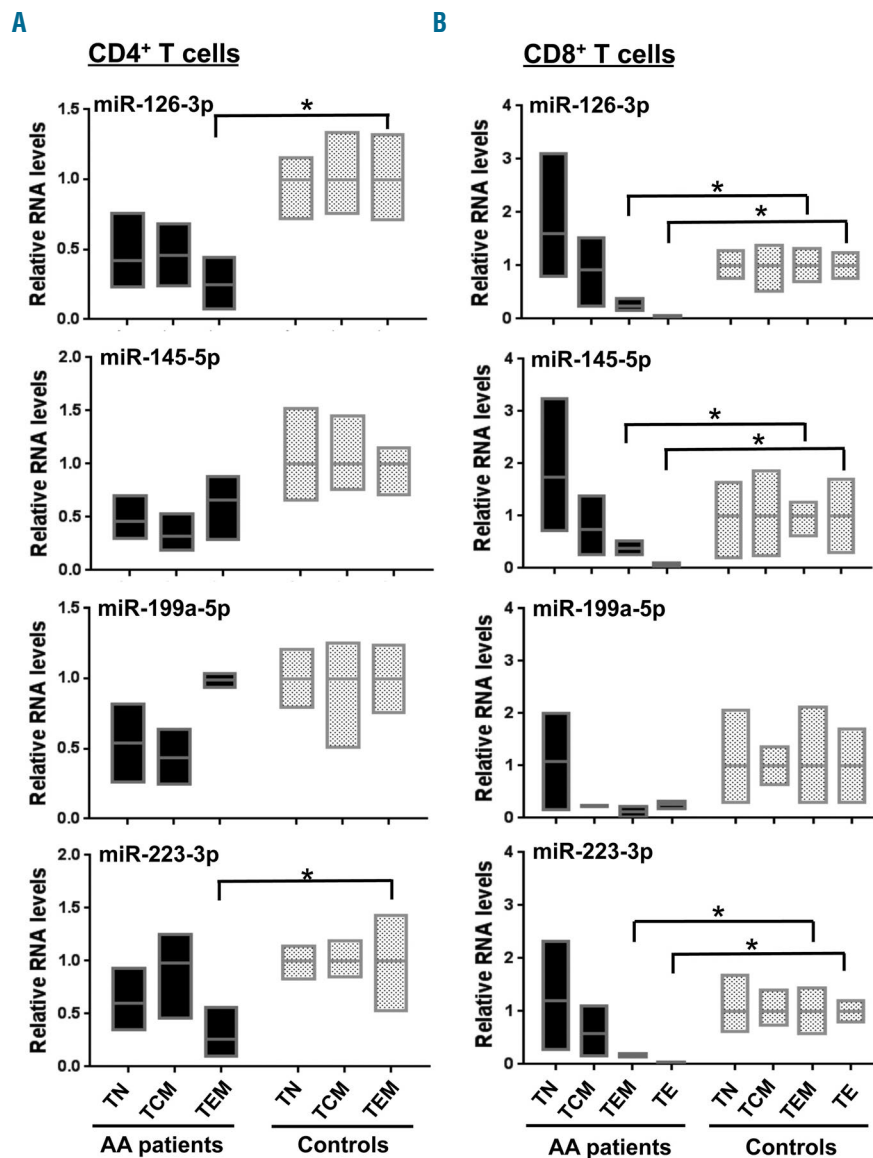

Figure 3. MiRNA expression in CD4+ and CD8+ T cell subsets of AA patients. RT-qPCR analysis of miR-126-3p, miR-145-5p, miR-199a-5p, and miR-223-3p expression in (A) CD4+ and (B) CD8+ T cell subsets from AA patients (n=3) and healthy controls (n=3). The TE population in CD4+ T cells was not examined, due to the low number of cells in healthy controls. Relative expression of miRNA was calculated by normalizing to RNU-2 expression. *P*<0.05 [two-way analysis of variance (ANOVA)].

based computational tool DIANA-miRPath, we performed *in silico* analysis of putative interactions among the four miRNAs and common signaling pathways. This computational tool estimates the impact of co-expressed miRNA in biological pathways.[22] Pathway enrichment analysis in AA patients showed putative gene network interactions with the four miRNA, enriched significantly for signaling pathways of cancer, Toll-like receptor, mammalian target of rapamycin, and PI3K-Akt (*Online Supplementary Table S4*). Candidate target genes of the four miRNA were predicted and selected by using integrated analyses based on four computational algorithms and the Ingenuity Knowledge Base (IPAKB). From these predictions, we designed a custom PCR Array which included 84 experimentally validated or highly predicted target genes of these four miRNA (*Online Supplementary Table S2*).

In CD4+ T cells, the custom PCR Array analysis showed elevated expression levels of *MYC* and *ETS1* (>1.5 FC, $P<0.05$), as compared with levels in healthy controls (Figure 5A), whereas increased expression levels of *PIK3R2* and *PAK4* (>1.5 FC, $P<0.05$) were observed in CD8+ T cells (Figure 5G). Up-regulation of *MYC* (1.66 ± 0.25 FC, $P<0.05$) in CD4+ (Figure 5B) and *PIK3R2* (2.26 ± 0.21 FC, $P<0.05$) in CD8+ T cells (Figure 5H) was confirmed by RT-qPCR.

In order to experimentally validate that *MYC* and *PIK3R2* were targets of miRNA in CD4+ and CD8+ T cells, inhibition experiments using anti-miR-145-5p and anti-miR-126-3p were performed with freshly isolated CD4+ and CD8+ T cells from healthy donors. After exposure to each specific antagomir, gene and protein expression levels of *MYC* in CD4+ T cells and *PIK3R2* in CD8+ T cells were measured by RT-qPCR and immunoblot, respectively. Knockdown efficiencies were $62\% \pm 5\%$ for miR-145-

5p in CD4+ T cells and $54\% \pm 1\%$ for miR-126-3p in CD8+ T cells (Figure 5C,I). After knockdown of miR-145-5p, both RNA and protein expression levels of *MYC* were increased in CD4+ T cells (1.20 ± 0.02 FC, $P<0.05$) (Figure 5D,E). Similarly, miR-126-3p knockdown up-regulated both RNA and protein expression levels of *PIK3R2* in CD8+ T cells (1.62 ± 0.14 FC, $P<0.05$) (Figure 5J,K). To study direct interactions between miRNA and sites on the 3' UTR, we used two commercially available plasmids containing either *MYC* or *PIK3R2* 3' UTR inserted downstream of the firefly luciferase reporter gene, and renilla luciferase gene for normalization. In CD4+ or CD8+ T cells transiently transfected with the *MYC* 3' UTR or *PIK3R2* 3' UTR construct and selected mimic miRNA or miRNA control, significant inhibition of luciferase activity was observed. Compared with levels in controls, miR-145-5p caused an $19\% \pm 2.9\%$ decrease in *MYC* 3' UTR luciferase activity in CD4+ T cells, and miR-126-3p caused a $27\% \pm 2.4\%$ decrease in *PIK3R2* 3'UTR luciferase activity in CD8+ T cells ($P<0.05$, Figure 5F,L). As confirmation, we searched miR-145-5p and miR-126-3p target sequences in *MYC* 3'UTR and *PIK3R2* 3'UTR by TargetScan. Highly preserved target sequences of the two miRNA were identified in *MYC* 3'UTR and *PIK3R2* 3'UTR, respectively (*Online Supplementary Figure S4*).

Functional assessment of microRNA down-regulated in T cells from patients with aplastic anemia

Expression levels of *MYC* in CD4+ T cells and *PIK3R2* in CD8+ T cells were increased in AA patients, in which miR-145-5p and miR-126-3p appeared to be regulators. As miR-199a-5p and miR-223-3p were down-regulated in MDS or SCD patients, we focused on two miRNA associated with AA, miR-126-3p and miR-145-5p, in fur-
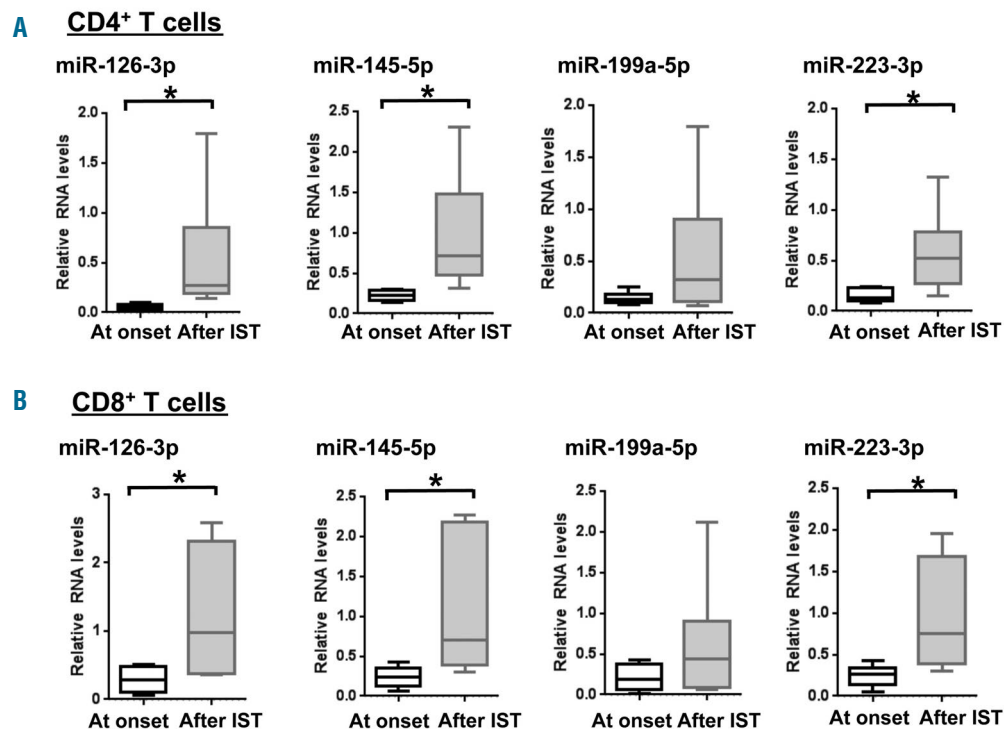

Figure 4. MiRNA expression changes after immunosuppressive therapy (IST). RT-qPCR analysis of miR-126-3p, miR-145-5p, miR-199a-5p, and miR-223-3p expression in (A) CD4+ and (B) CD8+ T cells from AA patients at onset (n=6) and after IST (n=6). Relative expression of miRNA was calculated with respect to RNU-2 expression. Relative expression levels were normalized to those of healthy controls. *P<0.05 (Student *t*-test).

ther functional experiments. Next, we tested whether miR-126-3p and miR-145-5p down-regulation affected proliferation of T cells. Purified CD4^{+} and CD8^{+} T cells from healthy controls were labeled with CFSE and transfected with anti-miR-126-3p and/or anti-miR-145-5p, followed by stimulation with anti-CD3/CD28 beads. When median fluorescence intensity of CFSE was assessed by flow cytometry on day 4, enhanced proliferation was observed in both CD4^{+} and CD8^{+} T cells co-transfected with anti-miR-126-3p and anti-miR-145-5p, as compared to those transfected with miR-negative control: 8887 ± 6 in CD4^{+} T cells and 2857 ± 54 in CD8^{+} T cells by co-transfection with anti-miR-126-3p and anti-miR-145-5p; 11842 ± 176 in CD4^{+} T cells and 4047 ± 157

in CD8^{+} T cells by transfection with negative control ($P<0.05$, Figure 6A,B). Knockdown of miR-126-3p and miR-145-5p decreased the numbers of TN cells and increased TEM cells among CD4^{+} and CD8^{+} T cells ($P<0.05$, Figure 6C, *Online Supplementary Table S5*). Consistently with more differentiated phenotypic features, knockdown of miR-126-3p and miR-145-5p increased production of effector molecules, granzyme B (GZMB) and IFN-γ production in CD4^{+} and CD8^{+} T cells, assessed by intracellular staining ($P<0.05$, Figure 6D,E, *Online Supplementary Table S6*). These results indicate promoted differentiation and functionality of effector cells as a result of reduced miRNA. Knockdown of miR-126-3p and miR-145-5p increased interleukin-2 (IL-2)
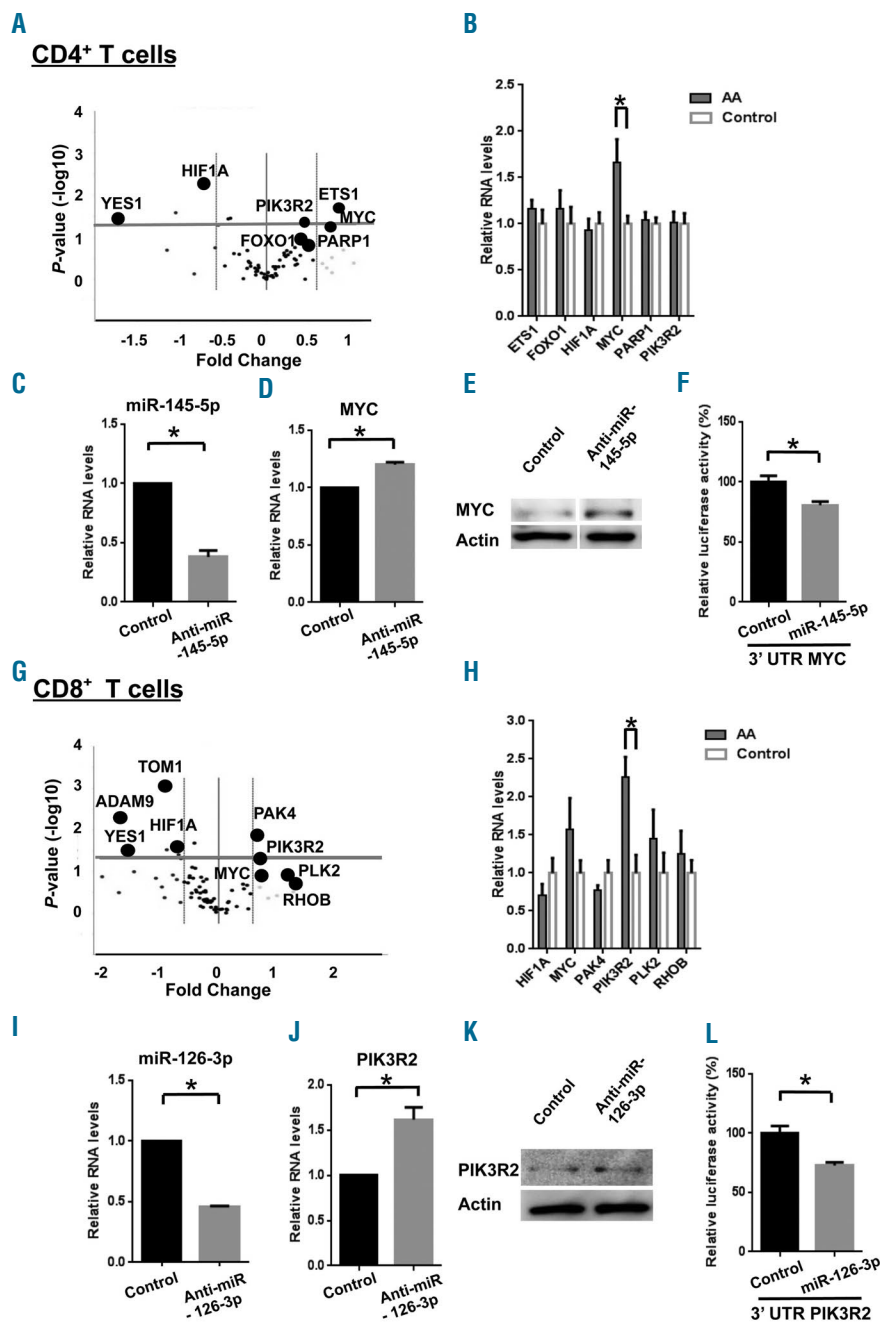

Figure 5. MiRNA target gene prediction and validation in CD4^{+} and CD8^{+} T cells. Volcano plots represent relative expression levels of 84 mRNA analyzed using miRNA Targets PCR Arrays. These 84 mRNA were predicted or experimentally validated to be targets of four miRNA (miR-126-3p, miR-145-5p, miR-199a-5p, and miR-223-3p) in (A) CD4^{+} and (G) CD8^{+} T cells from AA patients (n=6) and healthy donors (n=6). The x-axis is estimated difference in expression measured in log$_2$; vertical lines refer to a 1.5-fold difference in expression between the two groups. mRNA highly expressed in AA or healthy donors are on the right or the left, respectively. The y-axis is the significance of the difference measured in $-\log_{10}$ of a *P*-value; the horizontal line indicates our cutoff for significance at *P*<0.05. RT-qPCR analysis of several target gene expression in (B) CD4^{+} or (H) CD8^{+} T cells from AA patients (n=12) and healthy controls (n=8) *P*<0.05 [two-way analysis of variance (ANOVA)]. After treatment of CD4^{+} T cells with anti-miR-145-5p, relative expression of (C) miR-145-5p or (D) *MYC* was measured by RT-qPCR 24 h later while (E) MYC protein expression was assessed by immunoblot analysis 48 h later. After treatment of CD8^{+} T cells with anti-miR-126-3p, relative gene expression of (I) miR-126-3p or (J) *PIK3R2* and (K) protein expression of PIK3R2 were measured in similar manners. (F, L) Dual luciferase reporter assay. The relative luciferase activity of *MYC* 3' UTR or *PIK3R2* 3' UTR construct was measured by co-transfection with miR-145-5p in CD4^{+} T cells or miR-126-3p in CD8^{+} T cells, respectively. Data are shown as percentages of controls (cells co-transfected with *MYC* 3' UTR or *PIK3R2* 3' UTR construct and control miRNA, respectively). The relative activity of firefly luciferase expression was normalized to renilla luciferase activity. Relative RNA expression of miRNA or mRNA was calculated by normalizing to RNU-2 or β-actin expression, respectively. Data are from three independent experiments (means ± SEM). *P*<0.05 (Student *t*-test).
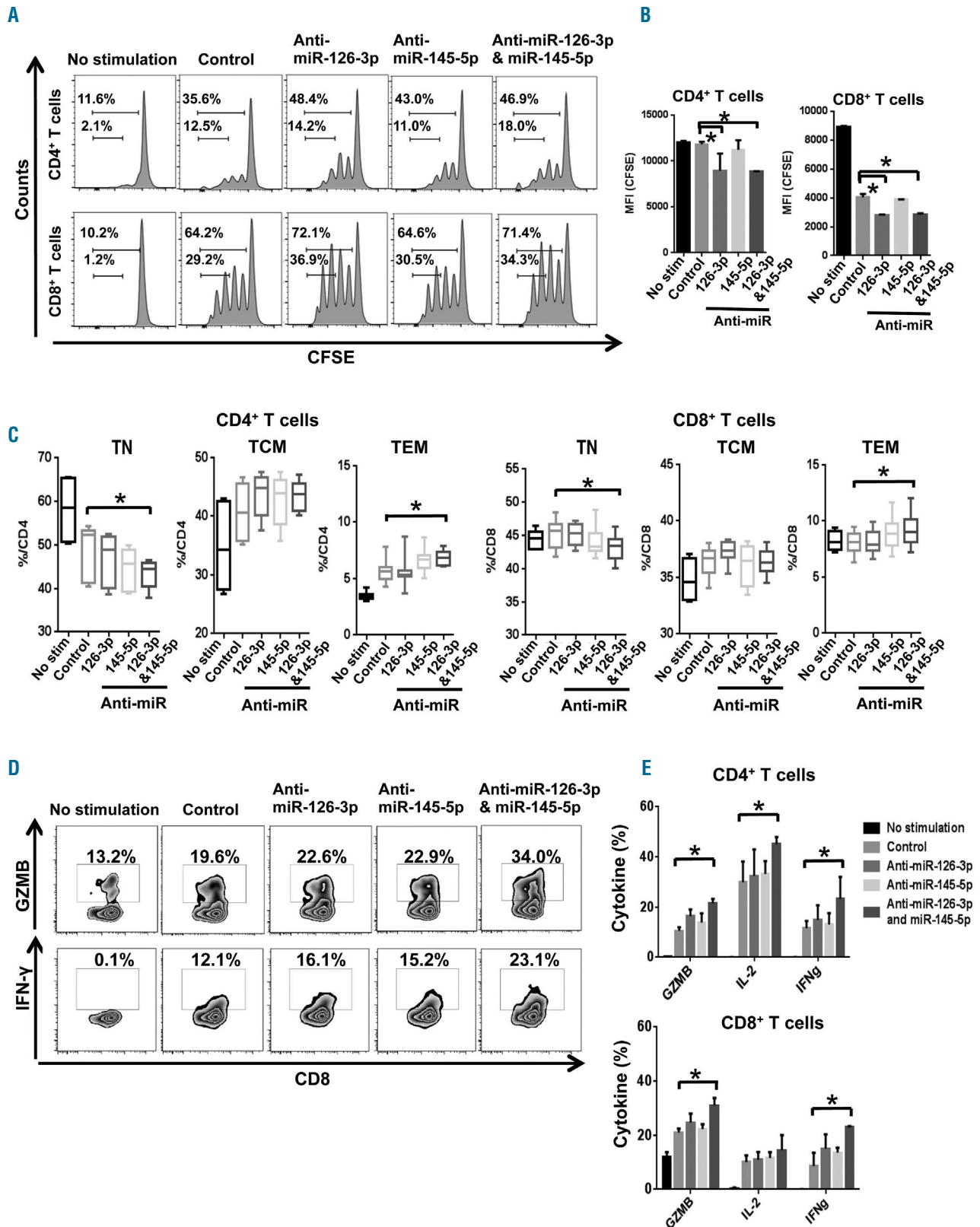
Figure 6. Functional study of miRNA down-regulated in T cells of AA patients. Purified CD4+ and CD8+ T cells from healthy controls were labeled with CFSE and transfected with anti-miR-126-3p and/or anti-miR-145-5p, followed by stimulation with anti-CD3/CD28 beads. (A) Representative histograms of CD4+ and CD8+ T-cell proliferation. (B) Frequency of CD4+ or CD8+ T-cell proliferation was quantified as the median fluorescence intensity (MFI) of CFSE after transfection of control, anti-miR-126-3p, anti-miR-145-5p, or both miRNA. *P<0.05 [one-way analysis of variance (ANOVA)]. (C) Percentages of TN, TCM and TEM were examined in CD4+ or CD8+ T cells transfected with anti-miR-126-3p and/or anti-miR-145-5p 48 h later. *P<0.05 [one-way analysis of variance (ANOVA)]. (D) Representative plots of intracellular GZMB and IFN-γ expression following *in vitro* stimulation with anti-CD3/CD28 beads. (E) Percentages of T cells producing GZMB, IFN-γ, and IL-2 were examined in CD4+ or CD8+ T cells transfected with anti-miR-126-3p and/or anti-miR-145-5p 48 h later. Data are from three independent experiments (means ± SEM). *P<0.05. [two-way analysis of variance (ANOVA)].

production in CD4+ T cells ($P<0.05$, Figure 6D,E, *Online Supplementary Table S6*), but no significant difference was found in CD8+ T cells.

Furthermore, we tested whether overexpression of miR-126-3p and/or miR-145-5p in T cells from AA patients had opposite functional effects. Decreased proliferation was observed in both CD4+ and CD8+ T cells co-transfected with miR-126-3p and miR-145-5p, as compared to cells transfected with control miRNA ($P<0.05$, Figure 7A,B). Transfection of miR-126-3p and miR-145-5p decreased IFN-γ production in both CD4+ and CD8+ T cells ($P<0.05$, Figure 7C,D), but GZMB and IL-2 production were not significantly affected.

T cells from a murine model of bone marrow failure show distinct microRNA profiles

MiRNA profiling was performed in T-cell populations from a murine model of minor histocompatibility antigen-mismatched bone marrow failure (*Online Supplementary Figure S5*). Fatal bone marrow failure was induced by infusion of LN cells from B6 mice into C.B10 recipients which were mismatched at multiple minor histocompatibility antigens, including the immunodominant antigen H60. In comparison to mice that received total body irradiation only without LN cell infusion, four recipient mice that received B6 LN cells were alive but had severe neutropenia ($0.21 \pm 0.02 \ 10^9$/L *versus* $0.05 \pm 0.03 \ 10^9$/L, $P<0.05$), throm-
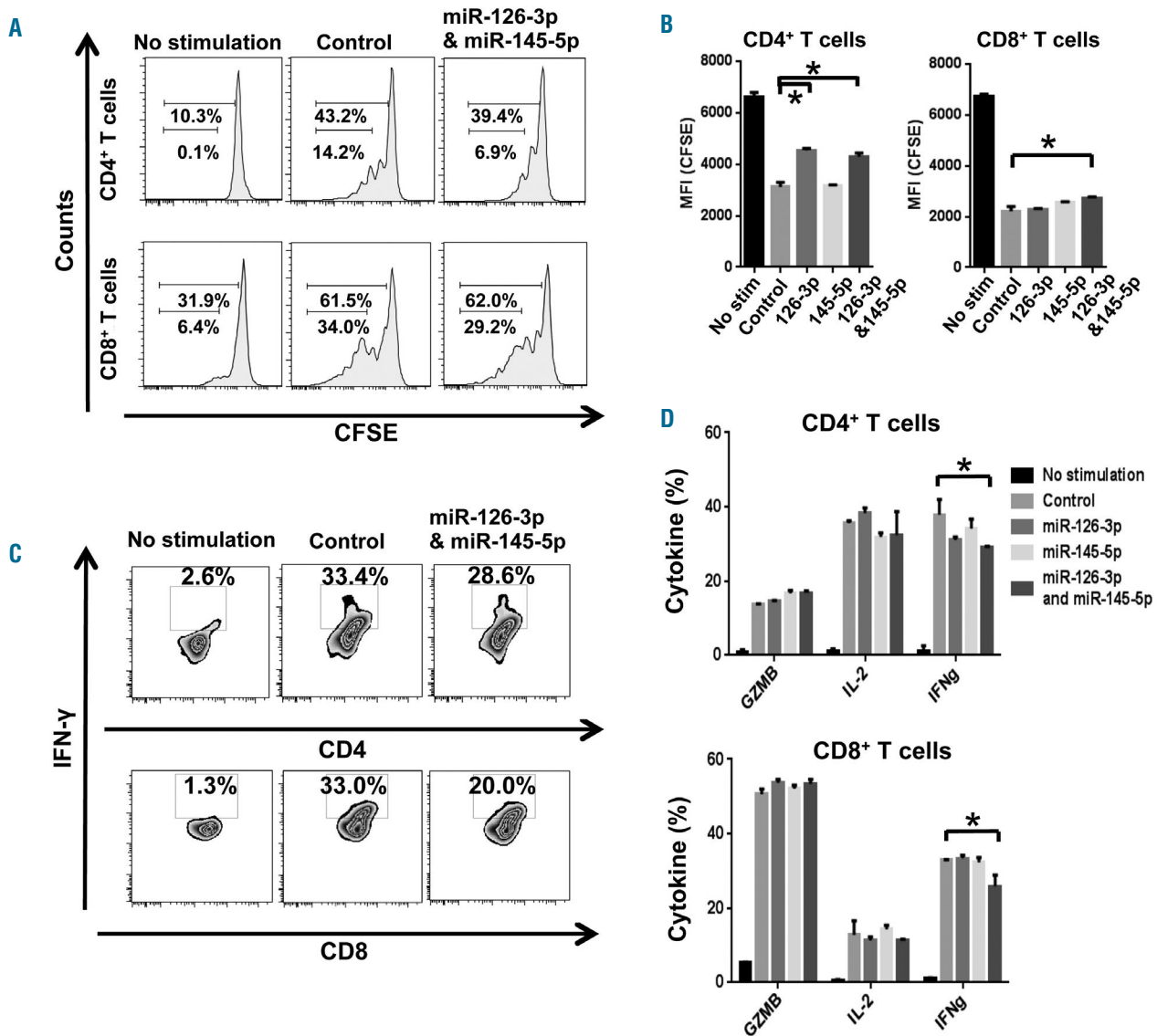

Figure 7. Overexpression of miR-126-3p and miR-145-5p in T cells from AA patients decreases T-cell proliferation and inhibits IFN-γ production. Purified CD4+ and CD8+ T cells from AA patients were labeled with CFSE and transfected with miR-126-3p and/or miR-145-5p, followed by stimulation with anti-CD3/CD28 beads. (A) Representative histograms of CD4+ and CD8+ T-cell proliferation. (B) Frequency of CD4+ or CD8+ T-cell proliferation was quantified as the median fluorescence intensity (MFI) of CFSE from dividing cells, after transfection of control, miR-126-3p, miR-145-5p, or both miRNA. *$P<0.05$ [one-way analysis of variance (ANOVA)]. (C) Representative plots of intracellular IFN-γ expression following *in vitro* stimulation with anti-CD3/CD28 beads. (D) Percentages of T cells producing GZMB, IFN-γ, and IL-2 were examined in CD4+ or CD8+ T cells transfected with miR-126-3p and/or miR-145-5p 48 h later. Data are from three independent experiments (means ± SEM). *$P<0.05$. [two-way analysis of variance (ANOVA)].

bocytopenia (126.0 ± 13.4 10^9/L *versus* 73.8 ± 11.8 10^9/L, *P*<0.05), and reduction in total bone marrow cells (23.4 ± 0.7× 10^6 cells/mL *versus* 7.0 ± 0.3 × 10^6 cells/mL, *P*<0.05) (*Online Supplementary Figure S5*). Bone marrow cells from the four surviving mice were sorted into CD4^{+} and CD8^{+} T cells by flow cytometry, followed by miRNA expression profiling of individual T-cell populations. Unexpectedly, 22 miRNA, including miR-126a-3p and miR-145a-5p, were increased in CD4^{+} and CD8^{+} T cells in the bone marrow failure model mice (>2 FC, *P*<0.05). MiR-155-5p expression, which is known to be up-regulated in the major antigen MHC-mismatched GVHD model mice,[23] was increased (>2 FC, *P*<0.05) both in CD4^{+} and CD8^{+} T cells, whereas 32 miRNA and 29 miRNA were decreased in CD4^{+} or CD8^{+} T cells, respectively (*Online Supplementary Figure S5*). Both CD4^{+} and CD8^{+} T cells had reduced levels of expression (>2 FC, *P*<0.05) of miR-142-3p, miR-142-5p, and miR-29a-3p which have been reported to be miRNA down-regulated in allo-reactive T cells using AGO-CLIP-ChIP procedures and mRNA CLIP-ChIP profiles.[24]

Discussion

Rossi *et al.* performed miRNA profiling of 17 highly purified human lymphocyte subsets and identified miRNA signatures that were distinct among various subsets and different from those of mouse lymphocytes.[25] In our study, we used flow cytometry-sorted CD4^{+} and CD8^{+} T cells to identify novel genes involved in the regulation of immune responses of T cells in AA. To characterize miRNA signatures in T-cell populations purified from AA patients, we employed the miScript miRNA PCR Array platform, because it is considered appropriate in terms of reproducibility, sensitivity, accuracy, specificity, and concordance of differential expression.[26] Applying these strategies and qPCR validation, we found that miR-145-5p and miR-126-3p were specifically down-regulated in CD4^{+} and CD8^{+} T cells in AA patients, appeared to regulate key cellular functions that contribute to aberrant T-cell activation, and identified *MYC/PIK3R2* as targets of these miRNA. Knockdown of miR-126-3p and miR-145-5p promoted proliferation and differentiation from TN toward the TEM phenotype in both CD4^{+} and CD8^{+} T cells. Moreover, increased GZMB and IFN-γ production was also observed in CD4^{+} and CD8^{+} T cells, indicating enhanced effector differentiation and functionality of CD4^{+} and CD8^{+} T cells as a result of reduced activity of the two miRNA. These results are consistent with previous reports describing increased GZMB and IFN-γ expression in CD8^{+} T cells of AA patients.[2,27]

We speculated that transfusion might affect the miRNA signatures in T cells as a result of allogenic antigen exposure in AA patients.[28] We, therefore, also examined miRNA profiles of T cells from age-matched healthy donors and patients with transfusion-dependent SCD and low-risk MDS for comparison with AA. In addition, we compared the miRNA signatures in CD4^{+} and CD8^{+} T cells in SCD patients before and 1 week after red blood cell transfusion, but miRNA signatures did not change after transfusion (*data not shown*). Low-risk MDS patients are characterized by progressive pancytopenia and may need transfusion of red blood cells and platelets, which are also required in AA. Distinguishing between AA and MDS is

often difficult.[29] We found differences in miRNA signatures in T cells between AA and MDS. Previous studies have described the association of miRNA expression with MDS subtypes and disease outcome, clinical implications of miRNA in MDS, and miRNA deregulation in mouse MDS model systems.[30] Reduced expression levels of miR-378a-3p, miR-143-3p, miR-143-5p, miR-145-5p, and miR-146a-5p have been reported in MDS with del(5q).[31,32] Knockdown of miR-145 and miR-146a together or enforced expression of *TRAF6* in mouse hematopoietic stem and progenitor cells resulted in thrombocytosis, mild neutropenia, and megakaryocytic dysplasia.[31] However, these reports mostly focused on miRNA expression in hematopoietic stem and progenitor cells and not in immune cells. Similarly, some studies of miRNA profiles in SCD patients used erythrocytes[33] or platelets,[34] but not T-cell populations. Thus, we have clarified the miRNA signatures in T cells in low-risk MDS and SCD patients. In addition, we also found reduced miR-199a-5p expression in CD4^{+} and CD8^{+} T cells from patients not only with AA but also with MDS and SCD, and decreased miR-223-3p expression in MDS. It seems likely that miR-199a-5p and miR-223-3p do not reflect specific immunological features of AA and that reduced levels of these two miRNA may be non-specific.

The three miRNA (miR-126-3p, miR-145-5p, and miR-223-3p) which were seen to be down-regulated in CD4^{+} T, CD8^{+} T, and CD19^{+} B cells in AA patients, were shown to be regulators of hematopoiesis in previous studies. MiR-145-5p is abundant in hematopoietic stem and progenitor cells and participates in megakaryopoiesis by activating the innate immunity target, *TIRAP* (Toll-interleukin-1 receptor domain-containing adaptor protein).[31] MiR-145-5p regulates multiple gene targets, such as *MYC*,[35] which regulates various processes involved in cell proliferation, survival, differentiation, and metabolic changes in T cells.[36] MiR-126-3p is expressed in CD34^{+} human cord blood and mobilized peripheral blood cells[37] and modulates primitive erythropoiesis by non-hematopoietic Vcam-1^{+} cells.[38] Reduction in miR-126-3p induces expansion without hematopoietic stem cell exhaustion.[39] MiR-126-3p regulates multiple gene targets, including PIK3R2 and other immune response genes.[40] In T-cell biology, silencing miR-126-3p increased expression of its target *PIK3R2* and altered activation of the *PI3K/Akt* pathway, responsible for reduced induction and suppressive function of regulatory T cells.[41] In AA patients, regulatory T cells are decreased in number and functionally abnormal.[42] Down-regulation of miR-126-3p in CD4^{+} T cells might partly explain the reduced induction and suppressive function of regulatory T cells in AA. MiR-223-3p reduces the commitment of erythroid progenitors and has a crucial role in granulocyte progenitor proliferation and function, and its expression is activated in neutrophils.[43] Thus, miR-126-3p, miR-145-5p, and miR-223-3p are involved in various stages of hematopoietic cell regulation.

Individual autoimmune diseases display unique miRNA signatures; miR-155 and miR-146a dysregulation is commonly observed.[44] Unlike miR-155, which acts to potentiate the immune response, miR-146a is a negative regulator of the immune response, especially through inhibition of Toll-like receptor signaling.[45] There were no significant changes in these two miRNA in T cells from AA patients, suggesting that miRNA signatures in AA are distinct from those in other immune-mediated diseases. However, there

is some commonality in miRNA dysregulation in other autoimmune diseases. For example, decreased expression of miR-145-5p was detected in T cells from patients with myasthenia gravis[46] and systemic lupus erythematosus.[47] MiR-223-3p is overexpressed in T cells from patients with rheumatoid arthritis,[48] indicating the similarity and difference of miRNA expression in different type of diseases.

We examined miRNA signatures in T cells from mice used as a model of minor histocompatibility antigen-mismatched bone marrow failure. The discrepancy in T-cell miRNA signatures between human AA and the murine bone marrow failure models may be due to the difference of species and/or mouse bone marrow failure models based on minor-histocompatibility antigen-mismatched graft-versus-host disease reactions, given the fact that the dysregulations of miRNA in our model overlapped with those in previous reports.[23,24] We speculate that the mouse models based on MHC mismatching at least in part share miRNA signatures associated with allo-reactivity.

Our study had some limitations, such as the small number of patients' samples and limited number of analyzed miRNA and target genes. We may have missed important miRNA dysregulation and target gene changes that might have been identified using global miRNA and mRNA arrays. MiR-126-3p and miR-145-4p are known to be down-regulated in B-cell malignancies, such as chronic lymphocytic leukemia and diffuse large B-cell lymphoma, respectively.[49,50] Moreover, autoantibodies are frequently detected in patients with AA.[51,52] Perhaps dysregulations of B cells is related to aberrant B-cell activation. Previous research in patients with systemic lupus erythematosus demonstrated overlapping signatures in T- and B-cell-expressed miRNA which are differentially expressed.[53] Our data showed rather modest miRNA expression change. However, miRNA profiling studies often show that subtle changes in miRNA expression, such as a 1.5-fold difference, can have a significant impact on the biology of the cell.[54] The exact mechanisms by which these four miRNA were down-regulated in AA are unclear although *in silico* analysis suggested possible mechanisms by which transcription factors might affect the expression of the four miRNA (*Online Supplementary Figure S6*). We could not show the dysregulation of these miRNA was related to antigen specificity, as the autoantigens remain elusive in AA. We could not examine the miRNA expression in self-reactive T cells because of limited cell numbers. However, this miRNA expression pattern was more likely to repre-sent an early molecular event rather than a signature for auto-reactive T cells, as auto-reactive T cells are usually very infrequent.[55,56] Our data strongly suggest that aberrant miRNA expression is predominantly disease-driven, especially as restoration of the expression levels of miRNA was observed after successful immunosuppressive therapy.

Indeed, normalization of expression of miRNA after immunosuppressive therapy suggests that down-regulation of miRNA may contribute to the immunopathophysiology of AA. Additional studies in large cohorts of patients would be required to define whether miRNA are useful as disease biomarkers and as potential targets for therapeutics. In conclusion, we have provided evidence of altered expression of miRNA in CD4+ and CD8+ T cells in AA, underscoring the importance of this novel class of genes in the regulation of immune responses and pathogenesis of autoimmunity. Our work describes previously unknown potential regulatory roles of the miRNA 145-5p and 126-3p in T-cell activation in AA, in which *MYC* and *PIK3R2* are the respective targets of these miRNA. We suggest that dysregulated miR-145-5p and miR-126-3p promote T-cell proliferation and increase GZMB and IFN-γ production. More importantly, overexpression of these miRNA inhibited the proliferation and cytokine production of CD4+ and CD8+ T cells of AA patients. Targeting or employing miRNA mimics might be novel molecular therapeutic approaches in AA. Understanding these novel pathways may provide new therapeutic strategies and novel mechanistic insights into the epigenetic control of human T-cell effector responses.

Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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