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Concise report

microRNA downregulation in plasmacytoid dendritic cells in interferon-positive systemic lupus erythematosus and antiphospholipid syndrome

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Abstract

Objective. To investigate miRNA expression in relation to transcriptomic changes in plasmacytoid dendritic cells (pDCs) in SLE and APS. pDCs are major producers of IFNα in SLE and APS, and miRNAs are emerging as regulators of pDC activation.

Methods. miRNA and mRNA expression were measured by OpenArray and RNA-sequencing in pDCs of SLE, SLE + APS (APS secondary to SLE) and primary APS (PAPS) patients. The miRNA profile of patients was compared with the miRNA pattern of TLR7-activated pDCs.

Results. Among 131 miRNAs detected in pDCs, 35, 17 and 21 had a significantly lower level of expression in SLE, SLE + APS and PAPS patients, respectively, as compared with healthy controls (HC). Notably, the miRNA profile did not significantly differ between SLE and APS, but was driven by the presence or absence of an IFN signature. TLR7 stimulation induced a general downregulation of miRNAs, similar to the pattern observed in SLE and APS patients. miR-361-5p, miR-128-3p and miR-181a-2-3p expression was lower in patients with a high IFN signature (false discovery rate <0.05) as compared with patients with a low IFN signature and HCs. Pathway enrichment on the overlap of miRNA targets and upregulated genes from the RNAseq indicated that these miRNAs are involved in pDC activation and apoptosis.

Conclusion. Lower miRNA expression in pDCs is shared between SLE, SLE + APS and PAPS and is related to the IFN signature. As pDCs are the alleged source of the IFN signature in these patients, a better understanding of the molecular mechanisms/pathways leading to pDC dysregulation in SLE and APS might open novel pathways for therapeutic intervention.

Key words: microRNA, plasmacytoid dendritic cell, systemic lupus erythematosus, antiphospholipid syndrome, toll-like receptor, interferon signature, epigenetics

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BASIC SCIENCE

Rheumatology key messages

- Plasmacytoid dendritic cell miRNAs are downregulated in SLE and APS.
- Downregulation of plasmacytoid dendritic cells miRNAs reflects plasmacytoid dendritic cell activation in SLE and APS.
- Reduced expression of miR-361-5p, miR-128-3p and miR-181a-2-3p affects pathways involved in plasmacytoid dendritic cell activation.

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Introduction

SLE is a chronic relapsing autoimmune disease that predominantly affects women of child-bearing age. In SLE, immune complexes are deposited into tissues, including skin, joint and kidneys, leading to tissue inflammation. The pathogenesis of SLE is complex and includes derailments of both the innate and adaptive immune system [1]. aPLs are strongly associated with thrombotic events and pregnancy morbidity among patients with SLE. Approximately

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20% of SLE patients have APS, defined as the persistent presence of aPLs in patients who have experienced at least one thrombotic or obstetric complication [2]. APS also affects patients without an underlying disease and is then termed primary APS (PAPS). SLE and PAPS share many pathologic features, from genetics to perturbations in immune cells [3], although both conditions are rarely studied together.

Both SLE and PAPS are characterized by an IFN signature [4–6], which stands for an increased expression of type I IFN-inducible genes. The IFN signature is detected in ∼75% of SLE (±APS) patients and ∼50% of PAPS patients [5] and is linked to disease activity in SLE [6, 7] and vascular disease in both SLE and PAPS [4]. Plasmacytoid dendritic cells (pDCs) are considered the source of elevated IFNα levels in SLE and APS because immune complexes and aPL induce IFNα production by triggering Toll-like receptor (TLR) 7 in pDCs [8, 9]. However, due to their scarcity in the circulation (<0.5% of leucocytes), little is known about the molecular alterations of pDCs in patients with SLE and APS.

miRNAs are short (18–25 nucleotides) non-coding RNAs that regulate gene expression at a post-transcriptional level by binding to complementary sequences in target genes, resulting in the reduced translation of these target genes. miRNAs fine-tune cellular activation and differentiation, and their dysregulation is emerging as an underlying cause of autoimmunity [10]. The expression of several miRNAs is altered in patients with autoimmune diseases [10, 11]; however, most studies have used bulk peripheral blood cells or plasma and are therefore of limited value in identifying the pathways regulated by altered miRNA expression in a cell-specific manner.

Recent evidence suggests an important role for miRNAs in regulating pDC activation [12, 13] and type I IFN responses [10]. Here we performed miRNA profiling and RNA sequencing (RNAseq) on purified pDCs from patients with SLE, SLE + APS and PAPS in order to identify changes in miRNA expression and their target genes. Our results support an increased activation state of pDCs in SLE and APS, as reflected by a reduced expression of miRNAs in patients with high IFN signatures.

Methods

pDC isolation

pDCs were isolated from consecutive patients with SLE ($n = 20$), SLE + APS ($n = 10$, APS secondary to SLE) and PAPS ($n = 10$) and healthy controls (HCs, $n = 12$; supplementary Table S1, available at *Rheumatology* online) from peripheral blood mononuclear cells by positive selection using the BDCA4 isolation kit, following the manufacturer's instruction (Miltenyi). Classification criteria were used to classify patients as SLE, SLE + APS or PAPS [2, 14]. None of the patients had clinical evidence of an ongoing infection at the time of enrolment. All participants signed informed consent prior to the donation of blood, and approval by the ethical committee from the University Medical Centre Utrecht was obtained.

The purity of isolated pDCs was assessed by flow cytometry using CD123 and BDCA2 fluorochrome-labelled antibodies. The average purity was 92% (±3) and did not differ between patients and controls.

miRNA profiling and RNAseq

RNA was extracted from isolated pDCs [using the Allprep universal kit (Qiagen) according to the manufacturer's instructions], and 10 ng was reverse transcribed using the miRNA multiplex reverse transcriptase primer pools (either v2.1 for pool A or v3.0 for pool B) and the TaqMan miRNA reverse transcription kit (Life Technologies), allowing the detection of 758 miRNAs, including housekeeping snRNA. Reverse transcriptase products were pre-amplified using the Megaplex PreAmp Primer pools A and B in the presence of the TaqMan PreAmp Master Mix (Life Technologies), while using the following thermal cycler conditions: 10 min, 95°C; 2 min, 55°C; 2 min, 72°C and 16 cycles of 15 s, 95°C and 4 min, 60°C, and one single cycle of 10 min, 96°C. The miRNA Open Array profiling was performed on the amplified cDNA, diluted 1:40, with $0.1 \times$ TE buffer (pH 8.0) and subsequently 1:2 with TaqMan Open Array MasterMix on the QuantStudio 12 K Flex Real-Time PCR System (Life Technologies).

miRNA profiling data was analysed using the Thermofisher Cloud (www.thermofisher.com). miRNA expression was normalized to the mean expression of RNU44 and RNU48. Normalized expression values ($\Delta\Delta$Crt) were used for statistical analysis. miRNAs with Crt values >27 were set at 27. Only miRNAs with <10% of missing values were included for further analysis. The original data from the pDC miRNA profiling is available in supplementary Table S2, available at *Rheumatology* online. A publicly available miRNA profile (GSE21160) generated using the same experimental approach in pDCs activated by TLR7 agonist R837 (imiquimod) was reanalysed using the same criteria.

RNAseq was performed on the same pDCs used for miRNA profiling. RNAseq libraries were generated with the TruSeq RNA Library Prep Kit (Illumina) and sequenced on an Illumina HiSeq 4000, generating ∼20 million 100 bp paired-end reads. The sample qualities were assessed by FastQC, and the sequencing reads were aligned to the human genome (GRCh38 build 79) using STAR aligner. Gene expression data for the annotated genes was generated using HTSeq-count. The variance-stabilizing transformation normalized expression values (normalized read counts) were calculated using R-package DESeq2. Type I IFN scores were calculated from normalized read counts as previously described [5]. Patients were stratified by the presence (IFN-high) or absence (IFN-low) of a type I IFN signature by setting a threshold at the highest of the HCs.

Data handling and statistics

miRWALKm2.0 (http://zmf.umm.uni-heidelberg.de/apps/zmf/mirwalk2/) was used to search Targetscan and miRANDA for potential target genes of selected miRNAs. Pathway enrichment (based on the KEGG database) was performed on the intersect of the miRNA predicted targets and upregulated

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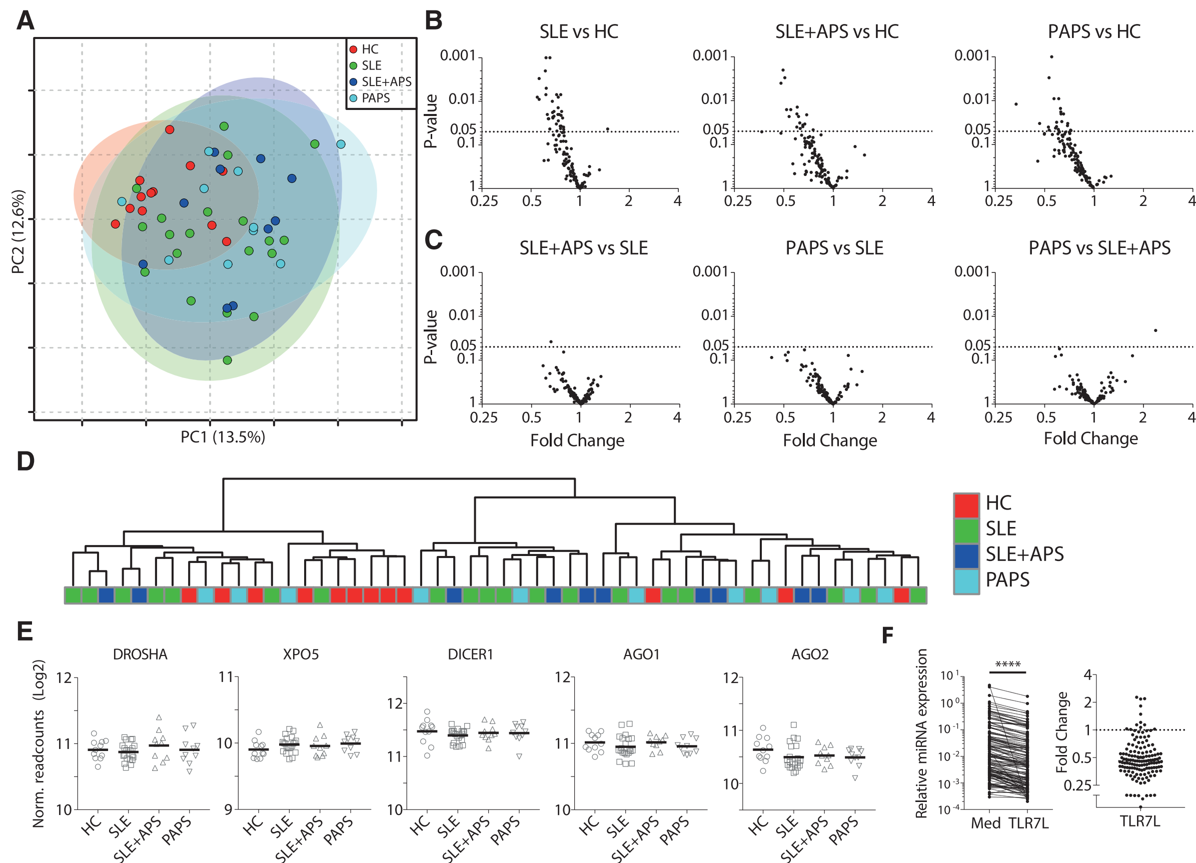
FIG. 1 Downregulation of pDC miRNAs in SLE, SLE + APS and PAPS



(**A**) Principal component analysis of all miRNAs expressed in pDCs of SLE, SLE+APS and PAPS patients and HCs. (**B**) Volcanoplots of miRNA expression in SLE, SLE+APS and PAPS patients as compared with HCs or (**C**) among patients. (**D**) Hierarchical clustering of pDC miRNAs. (**E**) Expression of genes related to the biogenesis and transport of miRNA in pDCs as assessed by RNAseq. (**F**) Changes in the expression in purified pDCs upon stimulation by the TLR7 agonist R837. Relative miRNA expression was calculated as 2^{-dCq}, fold change was compared with medium control. ****$P < 0.0001$.

genes from the RNAseq, using TopFunn (https://toppgene.cchmc.org/enrichment.jsp). Pathways related to specific infections were excluded.

Two-sided statistical tests were conducted with an α level of 0.05. Differences between two groups were tested using Welch's *t* test. Correction for multiple testing was performed by determining the false discovery rate according to the Benjamini–Hochberg procedure at a *q*-value of 0.05.

Results

Global downregulation of pDC microRNAs in SLE and APS patients

Out of 755 analysed miRNAs, 131 miRNAs were expressed in pDCs. Neither principal component analysis (Fig. 1A) nor hierarchical clustering (Fig. 1D) of all expressed miRNAs clustered SLE patients as distinct from APS patients, suggesting that these groups do not have strongly differential miRNA patterns. When comparing miRNA expression in SLE, SLE + APS and PAPS patients with that in HCs, respectively, 36, 17 and 21 miRNA were found differentially expressed (Fig. 1B; supplementary Table S1, available at *Rheumatology* online). Remarkably, with the exception of 1 miRNA (miR-222), all differentially expressed miRNAs had a lower expression in patients as compared with that in HCs, as evident from the left-skewed volcano plots of these comparisons (Fig. 1B); whereas the miRNA profiles of patients with SLE, SLE + APS and PAPS did not show strong differences (Fig. 1C). The miRNA expression in pDCs is therefore similarly affected in SLE and APS.

TLR7 signalling induced a downregulation of pDC microRNA expression

Several proteins are involved in the formation, transportation and stabilization of miRNAs, including the nucleases DICER and DROSHA, which cut pre-miRNA transcripts into mature miRNAs, exportin-5 (XPO5) [which exports the miRNA out of the nucleus] and argonaute proteins

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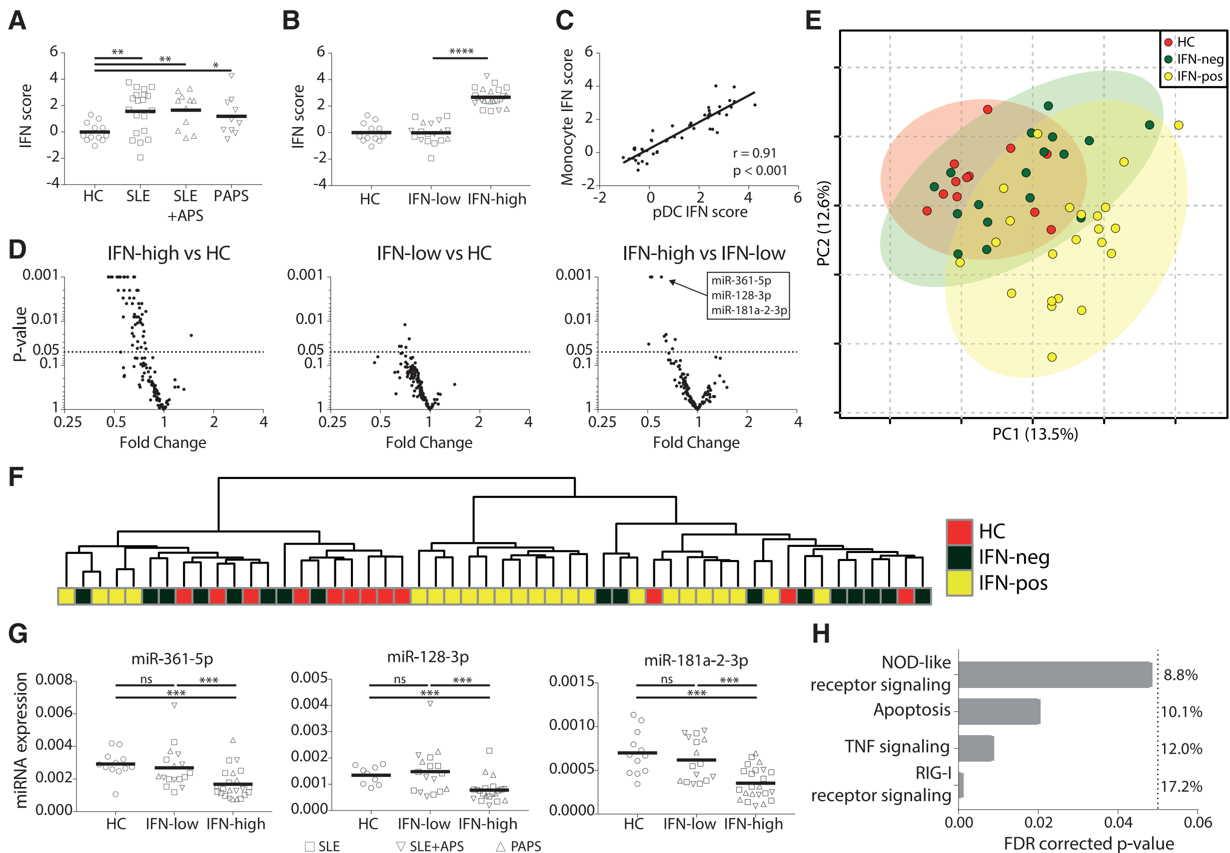
FIG. 2 Downregulation of pDC miRNAs is associated with a pDC IFN signature



(**A**) IFN scores of purified pDCs in patients stratified by clinical diagnosis or (**B**) IFN status. (**C**) Correlation of IFN scores between monocytes and pDCs. (**D**) Volcanoplots of pDC miRNA expression in patients stratified by IFN status. (**E** and **F**) Principal component analysis and hierarchical clustering of patients based on all miRNA stratified by IFN status. (**G**) Expression (2^{-dCq}) of the three most significant miRNAs between patients with or without an IFN signature. (**H**) Pathway enrichment of predicted target genes of miR-361-5p, miR-128-3p and miR-181a-2-3p and upregulated genes in pDCs of patients with high IFN signatures. Percentages indicate the number of affected genes over the total genes annotated in the pathway. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS: non-significant.

(AGO1/AGO2) [which form the RNA-induced silencing complex]. RNAseq on the same pDC samples that were used for miRNA profiling, however, did not show significant differences in the expression of these genes (Fig. 1E). The global miRNA downregulation in pDCs of SLE and APS seemed, therefore, not due to alterations in the miRNA machinery. In contrast, activation of pDCs by TLR7 agonists induced a global downregulation of miRNA, to a similar extent as that seen in the patient samples (mean fold-change 0.45, Fig. 1F), suggesting that the downregulation of miRNA in pDCs in SLE and APS reflects pDC activation.

pDC miRNA expression was related to the type I IFN signature in pDCs

RNAseq revealed increased type I IFN scores in pDCs of SLE, SLE + APS and PAPS, which was strongest in the patients with SLE and SLE + APS (Fig. 2A). Of the patient samples, 58% ($n = 23$) were classified as IFN-high (Fig. 2B). The IFN scores in pDCs strongly correlated with the IFN scores we previously reported for monocytes of the same samples [5] (Fig. 2C).

Upon stratification of the patients into those with or without an IFN signature, miRNA expression was more strongly reduced in IFN-high patients, compared with in the IFN-low patients (Fig. 2D). Consequently, principal component analysis and hierarchical clustering on all miRNA showed a better separation of patients on the basis of their IFN status with respect to their clinical disease (Fig. 2E and F). Three miRNAs (miR-361-5p, miR-128-3p and miR-181-2-3p) were expressed at lower levels ($P < 0.001$, false discovery rate < 0.05) in IFN-high patients, compared with those without an IFN signature and HCs, and were significantly correlated with the IFN score ($r = -0.44$, -0.45 and -0.48, respectively, $P < 0.001$) (Fig. 2G). These miRNAs were also downregulated upon TLR7 ligation, whereas in SLE only the expression of mir-361-5p was significantly lower in patients with

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high disease activity (SLEDAI $\geqslant 4$, $n = 12$) as compared with patients with low disease activity (SLEDAI <4, $n = 18$, $P = 0.03$; data not shown).

Altered miRNA expression affected key signalling pathways involved in pDC activation

One miRNA may regulate the expression of several hundreds of genes. By integrating miRNA and mRNA expression data, we investigated the potential pathways affected by altered miRNA expression in pDCs. An intersection of the predicted target genes of miR-361-5p, miR-128-3p and miR-181-2-3p and the upregulated genes from IFN-high patients revealed 680 genes in common. Pathway enrichment on these genes revealed that these miRNAs are regulating the expression of genes involved in pDC activation (retinoic acid-inducible gene I, RIG-I receptor and nucleotide-binding oligomerization domain-like receptors and tumor necrosis factor signalling) and apoptosis (Fig. 2H).

Discussion

Here we studied pDCs directly isolated from the peripheral blood of patients with SLE and APS and for the first time analysed both their miRNome and transcriptome. Strikingly, the overall miRNA expression was lower in both SLE and APS patients *vs* controls, which was most pronounced in patients with a high type I IFN signature. TLR7 activation induced a similar profile of downregulated miRNA expression in pDCs. Therefore, this indicates that the profile of miRNA expression in pDCs of SLE and APS patients is a reflection of pDC activation *in vivo* in these patients.

A reduced expression of miRNAs has previously been reported in neutrophils of SLE and APS patients [15]. This reduced miRNA expression in neutrophils was shown to be due to the decreased expression of the miRNA machinery in these cells [15]. In pDCs we did not see differential expression of these genes. As TLR7 signalling is driving the production of IFNα by pDCs in SLE and APS [8, 9], we assessed changes in miRNA expression after TLR7 activation and observed that miRNA expression in pDCs is highly sensitive to TLR7 stimulation, resulting in the downregulation of the majority (92%) of miRNAs. Thus, the mechanism behind miRNA downregulation in neutrophils and pDCs of SLE and APS patients is cell-specific.

Two previous studies on miRNA expression in monocytes and neutrophils of SLE and PAPS patients reported no differences in miRNA expression between patients with either SLE or PAPS [15, 16]. Similarly, we observed no major differences in miRNA expression between SLE, SLE + APS and PAPS in pDCs. The dysregulation of pDCs in terms of miRNA expression is therefore another shared immunologic abnormality between patients with SLE and APS [3], while the presence or absence of an IFN signature, rather than having SLE or APS, is defining miRNA expression in pDCs.

miR-361-5p, miR 128-3p and miR-181a-2-3p have not been previously linked to the pathogenesis of SLE or APS [10]. These miRNAs are specifically reduced in pDCs of patients with high IFN signatures. The expression of the targets of these miRNAs in pDCs of the same patients suggests that these miRNAs are regulating the expression of genes involved in key signalling pathways participating in pDC activation, such as RIG-I signalling as well as pDC apoptosis.

As pDCs are the alleged source of the type I IFN signature, we believe that a better understanding of the factors (including miRNAs) underlying the dysregulation of pDCs in SLE and APS could yield novel insights into the pathogenesis of SLE and APS. Our data suggest that aberrances in miRNA expression may be key mediators in regulating pDC activity and the immunopathology of SLE and APS.

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Supplementary data

Supplementary data are available at *Rheumatology* online.

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