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Association of MicroRNA-618 Expression With Altered Frequency and Activation of Plasmacytoid Dendritic Cells in Patients With Systemic Sclerosis

Marzia Rossato,[1] Alsya J. Affandi,[1] Soley Thordardottir,[2] Catharina G. K. Wichers,[1] Marta Cossu,[1] Jasper C. A. Broen,[1] Frederique M. Moret,[1] Lara Bossini-Castillo,[3] Eleni Chouri,[1] Lenny van Bon,[1] Femke Wolters,[1] Wioleta Marut,[1] Maarten van der Kroef,[1] Sandra Silva-Cardoso,[1] Cornelis P. J. Bekker,[1] Harry Dolstra,[2] Jacob M. van Laar,[1] Javier Martin,[4] Joel A. G. van Roon,[1] Kris A. Reedquist,[1] Lorenzo Beretta,[5] and Timothy R. D. J. Radstake[1]

Objective. **Plasmacytoid dendritic cells (PDCs) are a critical source of type I interferons (IFNs) that can contribute to the onset and maintenance of autoimmunity. Molecular mechanisms leading to PDC dysregulation and a persistent type I IFN signature are largely unexplored, especially in patients with systemic sclerosis (SSc), a disease in which PDCs infiltrate fibrotic skin lesions and produce higher levels of IFNα than those in healthy controls. This study was undertaken to investigate potential microRNA (miRNA)–mediated epigenetic mechanisms underlying PDC dysregulation and type I IFN production in SSc.**

Methods. **We performed miRNA expression profiling and validation in highly purified PDCs obtained from the peripheral blood of 3 independent cohorts of healthy controls and SSc patients. Possible functions of miRNA-618 (miR-618) on PDC biology were identified by overexpression in healthy PDCs.**

Results. **Expression of miR-618 was up-regulated in PDCs from SSc patients, including those with early disease who did not present with skin fibrosis. IFN regulatory factor 8, a crucial transcription factor for PDC development and activation, was identified as a target of miR-618. Overexpression of miR-618 reduced the development of PDCs from CD34+ cells in vitro and enhanced their ability to secrete IFNα, mimicking the PDC phenotype observed in SSc patients.**

Conclusion. **Up-regulation of miR-618 suppresses the development of PDCs and increases their ability to secrete IFNα, potentially contributing to the type I IFN signature observed in SSc patients. Considering the importance of PDCs in the pathogenesis of SSc and other diseases characterized by a type I IFN signature, miR-618 potentially represents an important epigenetic target to regulate immune system homeostasis in these conditions.**

Plasmacytoid dendritic cells (PDCs) are a unique subset of DCs that specialize in the secretion of type I interferons (IFNs) upon recognition of microbial single-

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[1]Marzia Rossato, PhD, Alsya J. Affandi, PhD, Catharina G. K. Wichers, BASc, Marta Cossu, MD, Jasper C. A. Broen, MD, PhD, Frederique M. Moret, PhD, Eleni Chouri, MSc, Lenny van Bon, MD, PhD, Femke Wolters, MD, Wioleta Marut, PhD, Maarten van der Kroef, MSc, Sandra Silva-Cardoso, MSc, Cornelis P. J. Bekker, PhD, Jacob M. van Laar, MD, PhD, Joel A. G. van Roon, PhD, Kris A. Reedquist, PhD, Timothy R. D. J. Radstake, MD, PhD: University Medical Center Utrecht, Utrecht, The Netherlands; [2]Soley Thordardottir, MSc, Harry Dolstra, PhD: Radboud University Medical Center, Nijmegen, The Netherlands; [3]Lara Bossini-Castillo, PhD: Consejo Superior de Investigaciones Científicas, Granada, Spain, and Wellcome Trust Sanger Institute, Cambridge, UK; [4]Javier Martin, MD, PhD: Consejo Superior de Investigaciones Científicas, Granada, Spain; [5]Lorenzo Beretta, MD, PhD: Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico di Milano, Milan, Italy.

Address correspondence to Marzia Rossato, PhD, University Medical Centre Utrecht, Heidelberglaan 100, Laboratory of Translational Immunology, Utrecht 3508 AB, The Netherlands. E-mail: marzia.rossato1@gmail.com.

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stranded RNA or double-stranded DNA by Toll-like receptor 7 (TLR-7) and TLR-9, respectively (1). Several observations suggest that PDCs may be the major source of IFNα in autoimmune conditions characterized by increased expression of IFN-responsive genes, substantially contributing to their pathogenesis (2–4). IFNα may promote peripheral tolerance breakdown through the activation of immature myeloid dendritic cells and autoreactive T and B cells, thus leading to autoantibody production (5). Supporting this concept, the depletion of PDCs in murine models of systemic lupus erythematosus (SLE) reduces the activation and expansion of autoreactive immune cells, limits autoantibody production and organ involvement, and leads to decreased transcription of IFN-dependent genes (6–8).

A type I IFN signature has been consistently observed in patients with systemic sclerosis (SSc), a systemic autoimmune disorder characterized by fibrosis of the skin and internal organs, accompanied by vascular and immune dysfunction (9). Aberrant expression of IFN-responsive genes was detected both in the affected skin and in peripheral leukocytes from SSc patients, correlating with disease activity (10). Increased serum levels of IFNα and IFN-induced cytokines positively associate with severe disease manifestations, such as pulmonary arterial hypertension (PAH), lung fibrosis, and digital loss (11). Anti–topoisomerase I antibody–containing SSc sera induce IFNα production from healthy donor leukocytes, particularly from PDCs (11,12). We recently demonstrated that PDCs from SSc patients can infiltrate fibrotic skin and produce high levels of CXCL4, a chemokine associated with progressive fibrosis and PAH and proposed as an SSc biomarker (13). Increased plasma levels of CXCL4 could also be observed in subjects with prefibrotic disease, who also present with the typical type I IFN signature in circulating monocytes (13,14). Despite these findings suggesting a relevant role of PDCs in SSc pathogenesis, the molecular mechanisms leading to persistent PDC dysregulation and the type I IFN response in SSc have not yet been explored.

Epigenetic alterations, including DNA methylation, chromatin marks, and microRNAs (miRNAs), could be critical to the breakdown of tolerance and the development of systemic autoimmune diseases (15). MicroRNAs are single-stranded short noncoding RNAs of 18–23 nucleotides that are able to inhibit gene expression posttranscriptionally. The binding of miRNAs to the 3′-untranslated region (3′-UTR) of protein-coding messenger RNAs (mRNAs) leads to the inhibition of target translation or, to a lesser extent, mRNA degradation. Even if the inhibitory effect of a single miRNA is generally mild (16,17), each miRNA can regulate multiple distinct transcripts and have multiple binding sites on a single mRNA transcript, thus amplifying its impact (18). MicroRNA-mediated regulation has been shown to be crucial in the maintenance of normal homeostatic processes, and the expression of miRNAs is altered in multiple autoimmune conditions, including SSc. The miRNAs miR-21 and miR-29a were reproducibly reported to be dysregulated in SSc skin and fibroblasts, where they act as profibrotic and antifibrotic factors, respectively (19). However, so far no study has addressed the potential involvement of miRNAs in the aberrant behavior of the immune system in SSc patients. In this study we investigated the potential role of miRNAs in the altered maturation and function of PDCs in SSc patients, and identified miR-618 as a possible important contributor to this process.

PATIENTS AND METHODS

Patients. Peripheral blood samples from patients and sex- and age-matched healthy donors were obtained from the Boston University School of Medicine (Boston, MA; discovery cohort), University Medical Center Utrecht and Radboud University Nijmegen (fibrotic cohort), and IRCCS Policlinico of Milan (nonfibrotic cohort). All patients and healthy donors signed an informed consent form approved by the local institutional review boards prior to participation in the study, as previously described (13). Samples and clinical information were made anonymous immediately after collection. The demographic and clinical characteristics of the patients included in the study are shown in Table 1. SSc patients fulfilled the American College of Rheumatology/European League Against Rheumatism 2013 classification criteria (20) and were categorized according to the extent of skin fibrosis as having limited cutaneous SSc (lcSSc) or diffuse cutaneous SSc (dcSSc) (21). Patients with dcSSc with a disease duration of ≤24 months were classified as having early dcSSc, while those with a longer disease duration were classified as having late dcSSc (13). Patients who fulfilled the classification criteria but did not present with skin fibrosis are referred to as patients with noncutaneous SSc. Finally, we also included patients with Raynaud's phenomenon and positivity for SSc-specific autoantibodies and/or typical nailfold capillaroscopy abnormalities, who are referred to as patients with "early SSc" in ref. 22 or as patients with "undifferentiated connective tissue disease at risk for SSc" in ref. 23.

The fibrotic cohort comprised healthy controls, lcSSc patients, and dcSSc patients, while the nonfibrotic cohort comprised healthy controls and patients with noncutaneous SSc and those with early SSc, who did not have skin involvement. The presence of interstitial lung disease (ILD) was defined as typical involvement of the lung parenchyma >5% on high-resolution computed tomography accompanied by reduced forced vital capacity or diffusing capacity for carbon monoxide <80% of predicted values. PAH was confirmed by catheterization of the right side of the heart.

Table 1. Demographic and clinical characteristics of the controls and SSc patients*

	No.	Age, years	Female, no. (%)	Disease duration, years	ANA positive, no. (%)	ACA positive, no. (%)	Antitopo positive, no. (%)	MRSS	FVC, % predicted	DLco, % predicted
Discovery cohort										
Healthy controls	7	46.5 (35.3–48)	6 (85.7)	NA	NA	NA	NA	NA	NA	NA
Late dcSSc	7	47 (37.5–59.5)	6 (85.7)	4 (3.5–7.5)	6 (85.7)	1 (14.3)	5 (71.4)	18.5 (8.8–23.8)	56 (45.8–70)	47 (40–48.8)
Early dcSSc	5	52.5 (46.3–62.5)	5 (100)	2 (1.25–2)	5 (100)	0 (0)	4 (80)	23 (18–46)	90 (55–92)	56.5 (44.3–76.3)
Fibrotic cohort										
Healthy controls	23	51.5 (42–59.8)	17 (73.9)	NA	NA	NA	NA	NA	NA	NA
lcSSc	25	54 (49–61)	18 (72)	5 (2–14)	23 (92)	12 (48)	4 (16)	4 (1.75–7.5)	105 (88–116)	66 (39.8–82.3)
dcSSc	10	55 (43.8–66)	4 (40)	2 (1.5–4.5)	10 (100)	0 (0)	7 (70)	13 (8.5–21.5)	59 (51–69.3)	42 (29.8–58)
Nonfibrotic cohort										
Healthy controls	11	35 (30–47)	10 (90.9)	NA	NA	NA	NA	NA	NA	NA
Early SSc	23	46 (40.1–66.5)	21 (91.3)	NA	20 (86.9)	12 (52.2)	3 (13.0)	0	108 (101–121)	82 (68–100)
Noncutaneous SSc	12	56 (49.2–66.4)	12 (100)	1 (1–5.5)	12 (100)	11 (91.7)	0 (0)	0	121 (93.5–118.5)	80 (69–98)

* Except where indicated otherwise, values are the median (interquartile range). SSc = systemic sclerosis; ANA = antinuclear antibody; ACA = anticentromere antibody; antitopo = antitopoisomerase antibody; MRSS = modified Rodnan skin thickness score; FVC = forced vital capacity; DLco = diffusing capacity for carbon monoxide; NA = not applicable; dcSSc = diffuse cutaneous SSc; lcSSc = limited cutaneous SSc.

Cell isolation and culture. PDCs and monocytes were isolated by positive selection from peripheral blood mononuclear cells using immunomagnetic labeling (blood dendritic cell antigen 4 [BDCA-4]/neuropilin 1 and CD14 MicroBead kits, respectively) on an AutoMACS Pro column (Miltenyi Biotec). The purity of isolated PDCs (>90%) was assessed by fluorescence-activated cell sorting (FACS) after staining with anti–BDCA-4 and CD123 (data available upon request from the corresponding author). PDCs from selected healthy controls were cultured in RPMI 1640 GlutaMax (Gibco) containing 10% heat-inactivated fetal bovine serum (FBS; BioWest), penicillin/streptomycin (Gibco), and 10 ng/ml interleukin-3 (IL-3) (ImmunoTools).

The CAL-1 human PDC line, established from a blastic natural killer cell lymphoma (24), was grown in complete RPMI 1640 medium (Gibco) supplemented with 2 m*M* L-glutamine, 1 m*M* sodium pyruvate, 10 m*M* HEPES, 1× minimum essential medium, nonessential amino acids (Gibco), and 10% FBS. Cells were cultured for fewer than 3 weeks at a concentration of $<10^6$ cells/ml, by passaging every 2 days. HEK 293 cells were cultured in Dulbecco's modified Eagle's medium (4.5 gm/liter glucose) with GlutaMax (Gibco), supplemented with 10% FBS and 1% penicillin/streptomycin.

CAL-1 and PDC transfection. CAL-1 cells (n = 200,000) or PDCs (n = 100,000) isolated from healthy controls were transfected using Lipofectamine Plus (Invitrogen) for 4 hours with 500 n*M* miR-618 mimic or scrambled miRNA control (Ambion); 100 n*M* Stealth small interfering RNA against IFN regulatory factor 8 (IRF-8) mRNA (assay IDs HSS105169, HSS105170, and HSS105171) or a scrambled control (Life Technologies); or 100 n*M* miRCURY LNA Power microRNA miR-618 inhibitor (anti–miR-618) or control A (anti–scrambled miRNA) (Exiqon). The miRCURY LNA Power microRNA inhibitors block miRNA activity by forming stable complexes with them and do not induce miRNA degradation (https://www.exiqon.com/ls/Documents/Scientific/miRNA-inhibitor-manual.pdf). Cells were allowed to recover overnight in complete medium, and the next day were left untreated or stimulated with 1 μ*M* unmethylated type C CpG-containing oligodeoxyribonucleotide (CpG ODN) (InvivoGen) for 24 hours. Supernatants were harvested for Luminex-based immunoassay, and cells were lysed for RNA extraction or processed for flow cytometric analysis.

IRF-8 intracellular staining. After staining with fixable viability dye e450 (eBioscience) and fluorochrome-conjugated antibodies against CD303 (Miltenyi Biotec) and CD123 (BioLegend), PDCs or CAL-1 cells were fixed and permeabilized using FoxP3/Transcription Factor Staining Buffer Set (eBioscience). Cells were labeled with anti–IRF-8 antibody (eBioscience) or mouse IgG isotype control (eBioscience), and data were acquired on an LSRFortessa (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Generation of DCs from CD34+ hematopoietic stem cells. CD34+ stem cells (89–97% pure) were isolated from the granulocyte colony-stimulating factor–mobilized peripheral blood of healthy donors using anti-CD34 immunomagnetic CliniMACS beads (Miltenyi Biotec). Generation of CD34-derived DCs was performed as previously described with minor adjustments (25). Briefly, CD34+ cells were cultured in Cellgro DC medium (CellGenix) supplemented with 2% human serum (PAA Laboratories), 1 μ*M* StemRegenin 1 (Cellagen Technology), 50 μg/ml vitamin C (Centrafarm), and 100 ng/ml of the following cytokines: thrombopoietin, stem cell factor, and Flt-3L (ImmunoTools). Medium was refreshed every 2–3 days and generation of DCs was evaluated by surface staining with antibodies against BDCA-4 (BioLegend), CD123 (BD Biosciences), HLA–DR (Beckman Coulter), and CD14 (Beckman Coulter). Cells were acquired on a Gallios flow cytometer (Beckman Coulter) and analyzed with FlowJo software (Tree Star).

Viral transduction of CD34-derived cell culture. HEK 293T cells were transfected with lentiviral packaging plasmids (pMD2G and psPAX2) and pEZX-MR03-GFP encoding for pre–miR-618 or a pre–scrambled miRNA (GeneCopoeia) using Lipofectamine (Invitrogen). Medium was changed 24 hours later. Lentiviral particles were collected 48 hours after transfection and concentrated by ultracentrifugation. CD34-derived cells (n = 500,000) in CD34+ DC culture medium were transduced on day 3 in 35-mm–diameter petri dishes (Corning) precoated with 1.25 μg/cm² RetroNectin (Takara), by adding 1 ml of viral particles. Seventy-two hours after transduction, transduced cells were isolated by FACS

using a FACSAria sorter (BD Biosciences) on the basis of GFP expression and cultured for an additional 7 days in CD34+ DC culture medium.

RNA extraction. Total RNA, including the small RNA fraction, was isolated from cell lysates using an AllPrep Universal kit (Qiagen), according to the manufacturer's instructions. RNA from whole blood was obtained using a PAXgene Blood RNA system (Qiagen) following the manufacturer's recommendations. RNA was quantified with a Qubit RNA kit (Life Technologies), and RNA integrity was evaluated using an Agilent Bioanalyzer.

Profiling of miRNA. Mature miRNAs were amplified from 100 ng of total RNA with the Illumina human miRNA profiling panel kit version 1, which contains primers for 470 annotated human miRNAs. The resulting amplicons were hybridized to a 96-sample universal probe capture array, and fluorescent signals were detected by confocal laser scanning. All steps were performed according to the Illumina instruction manual. Intensity data were processed using Beadstudio software. Data filtering and normalization were performed using GenePattern (Broad Institute). Differentially expressed miRNAs were identified by applying comparative marker selection analysis and 10,000 permutations (random seed: 779948241) to achieve correction for multiple testing. The miRNA profiling data have been submitted to the Gene Expression Omnibus with accession no. GSE100867.

Analysis of miRNA and gene expression. Expression analysis of individual miRNAs was performed using TaqMan miRNA Human Assays (Life Technologies), using 10 ng of total RNA. Complementary DNA specific to miRNA was measured with a miRNA-specific TaqMan assay on a QuantStudio 12k Flex system, using TaqMan Fast Advanced Master Mix (Life Technologies). The miRNA expression values were calculated according to the comparative threshold cycle method (26), using ubiquitous and stably expressed let-7a and RNU44 as endogenous controls. Let-7a was used to normalize the expression of miRNAs in the real-time quantitative polymerase chain reaction (qPCR) analysis performed in the patient cohorts, while RNU44 was used to normalize data in the in vitro experiments. The mean value of control samples was set to 1, and the fold change in miR-618 expression in patients or treated cells versus controls was calculated.

Expression of protein-coding genes was analyzed by real-time qPCR using a 3-ng RNA equivalent after retrotranscription with an iScript reverse transcriptase kit (Bio-Rad). Reactions were conducted using SYBR Select Master Mix with 500 n*M* specific primer pairs on a QuantStudio 12k Flex system. Real-time qPCR data were normalized to the expression of GUSB and analyzed as described for miRNAs. A list of all primers and TaqMan probes used is available upon request from the corresponding author.

Luciferase assay. Luciferase assays were performed on 10,000 HT0180 cells transfected with 100 ng LightSwitch 3′-UTR–specific or control reporter vectors in the presence of 50 n*M* miR-618 mimic or a non-targeting scrambled miRNA for 24 hours. Luciferase activity was measured on a luminometer after the addition of LightSwitch Luciferase Assay Reagent and expressed as the percentage of the luciferase signal observed with the miR-618 mimic compared to the non-targeting control.

Luminex analysis. Measurement of soluble IFNα, IL-6, tumor necrosis factor (TNF), and CXCL4 was performed on cell-free supernatants diluted 1:50, using a multiplex immunoassay based on Luminex xMAP technology at the MultiPlex Core Facility of the Laboratory of Translational Immunology, University Medical Center Utrecht (27). Acquisition was performed with a Bio-Rad FlexMap 3D system using xPonent software version 4.2. Concentration values were calculated using Bio-Plex Manager software version 6.1.1.

Statistical analysis. The nonparametric Mann-Whitney and Kruskal-Wallis tests were applied to compare 2 groups or multiple groups, respectively, unless otherwise stated. The correlation analysis was computed using Spearman's rho. All analyses were performed using GraphPad Prism 6.0 software. *P* values less than 0.05 were considered significant.

RESULTS

Up-regulation of miR-618 expression in PDCs from SSc patients with overt cutaneous fibrosis. To identify potential dysregulation of miRNA expression in PDCs from patients with SSc, miRNA profiling was performed in PDCs isolated from the peripheral blood of patients with the most severe form of cutaneous fibrosis, namely dcSSc, with a disease duration of $\leq$2 years (n = 5) or >2 years (n = 7) (discovery cohort) (Table 1). Of the 472 miRNAs measured, 42 miRNAs were differentially expressed in the entire group of dcSSc patients compared to healthy controls (Supplementary Table 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40163/abstract). Among these, 21 miRNAs were also significantly up-regulated when patients with early dcSSc were considered as a separate group (Supplementary Table 1).

Independent miRNA-specific analysis was performed using a second unrelated cohort (the fibrotic cohort) comprising SSc patients (n = 35) and healthy controls (n = 23). Seven miRNAs ranked in the top quartile were selected for replication (Figure 1A). This analysis confirmed that miR-618 was consistently up-regulated in PDCs from SSc patients compared to their healthy counterparts (fold change 3.85; $P < 0.0001$), and miR-618 expression was similar in lcSSc (fold change versus healthy controls 3.37; $P = 0.002$) and dcSSc (fold change versus healthy controls 4.87; $P = 0.003$) (Figures 1B and C). Of note, the majority of patients included in the dcSSc group (7 of 10) had a disease duration of $\leq$2 years, and they showed higher miR-618 expression than healthy controls. In addition, miR-618 expression levels correlated well with the presence of the SSc-specific complication ILD (fold change 2.37; $P = 0.034$) (Figure 1D) but not significantly with PAH (fold change 2.11; $P = 0.120$) (Figure 1E). No correlation with other clinical parameters was observed. The expression of the other 5 miRNAs was not replicated (data available upon request from the corresponding author), and

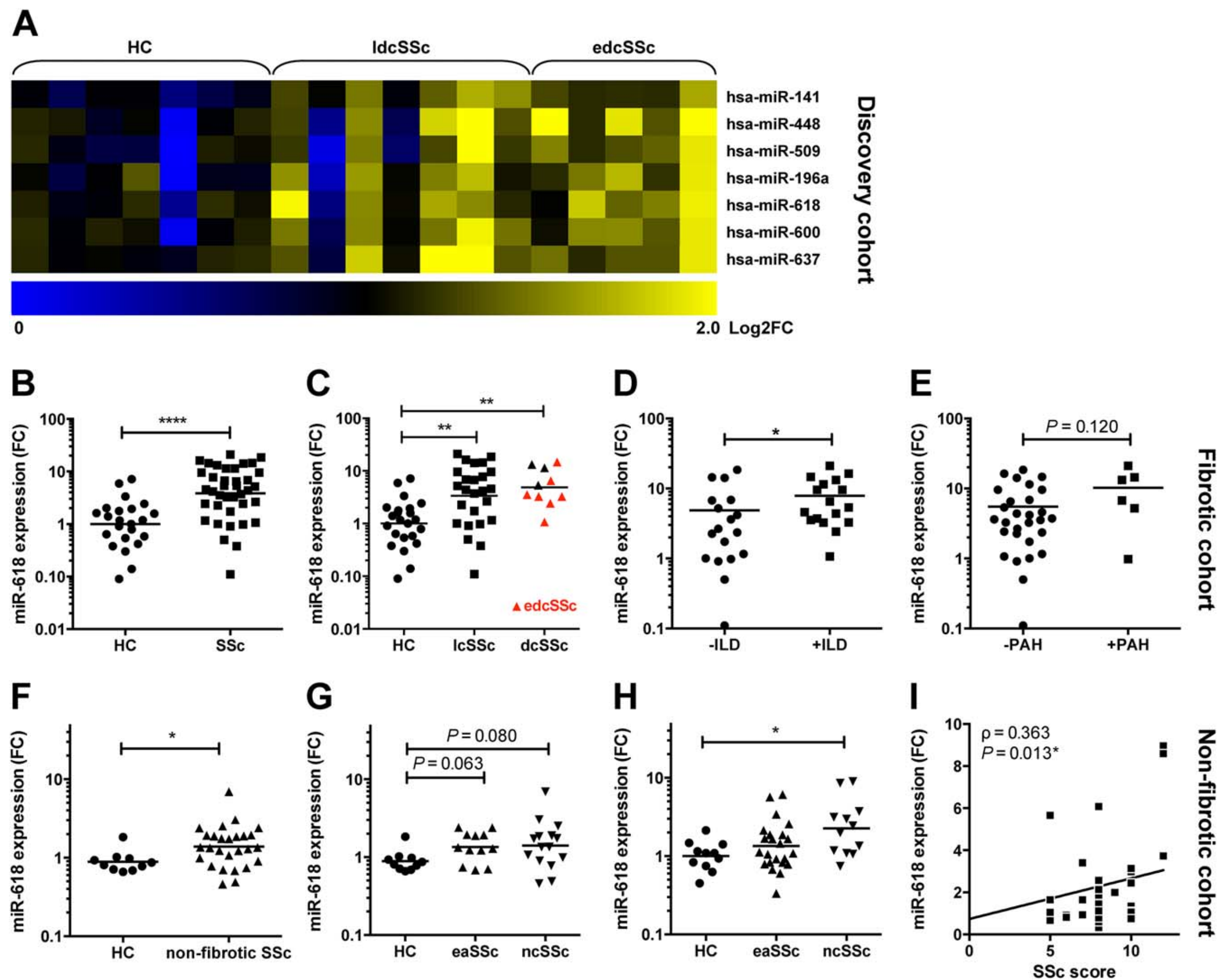

Figure 1. Up-regulation of microRNA-618 (miR-618) expression in plasmacytoid dendritic cells (PDCs) from patients with systemic sclerosis (SSc). **A,** Heatmap showing the relative expression of 7 miRNAs that were significantly up-regulated in PDCs from patients with late diffuse cutaneous SSc (ldcSSc; disease duration >2 years) and patients with early dcSSc (edcSSc; disease duration ≤2 years) compared to PDCs from healthy controls (HC), out of a total of 470 miRNAs examined, in the discovery cohort. **B** and **C,** Expression of miR-618, measured by real-time quantitative polymerase chain reaction, in PDCs from patients in the fibrotic cohort as a whole (**B**) and patients in the fibrotic cohort divided into those with limited cutaneous SSc (lcSSc) and those with dcSSc (**C**). **D** and **E,** Expression of miR-618 in patients who did not have interstitial lung disease (ILD) and those who did have ILD (**D**) and in patients who did not have pulmonary arterial hypertension (PAH) and those who did have PAH (**E**). **F** and **G,** Expression of miR-618 in PDCs from patients in the nonfibrotic cohort as a whole (**F**) and patients in the nonfibrotic cohort divided into those with early SSc (eaSSc) and those with noncutaneous SSc (ncSSc) (**G**). One healthy control was removed from analysis after application of the robust regression and outlier removal method. **H,** Expression of miR-618 in whole blood samples from the same donors as in **G**. **I,** Correlation of miR-618 levels in whole blood samples from patients in the nonfibrotic cohort with the European League Against Rheumatism/American College of Rheumatology 2013 SSc classification score. Correlation was determined using Spearman's rho. In **B–H,** symbols represent individual subjects; horizontal lines show the geometric mean fold change (FC) in expression relative to healthy controls. $* = P < 0.05$; $** = P < 0.01$; $**** = P < 0.0001$, by nonparametric Mann-Whitney test in **B, D, E,** and **F** and by Kruskal-Wallis test followed by Dunn's correction for multiple comparisons in **C, G,** and **H**.

hence only miR-618 was included in further functional analysis.

Up-regulation of miR-618 expression in SSc patients without cutaneous fibrosis. Since miR-618 expression was consistently up-regulated in dcSSc patients at an early disease stage, we further examined its levels in PDCs from subjects with nonfibrotic SSc features in a third cohort comprising 35 patients and 11 healthy controls, i.e., the nonfibrotic cohort. This cohort comprised patients with noncutaneous SSc and those

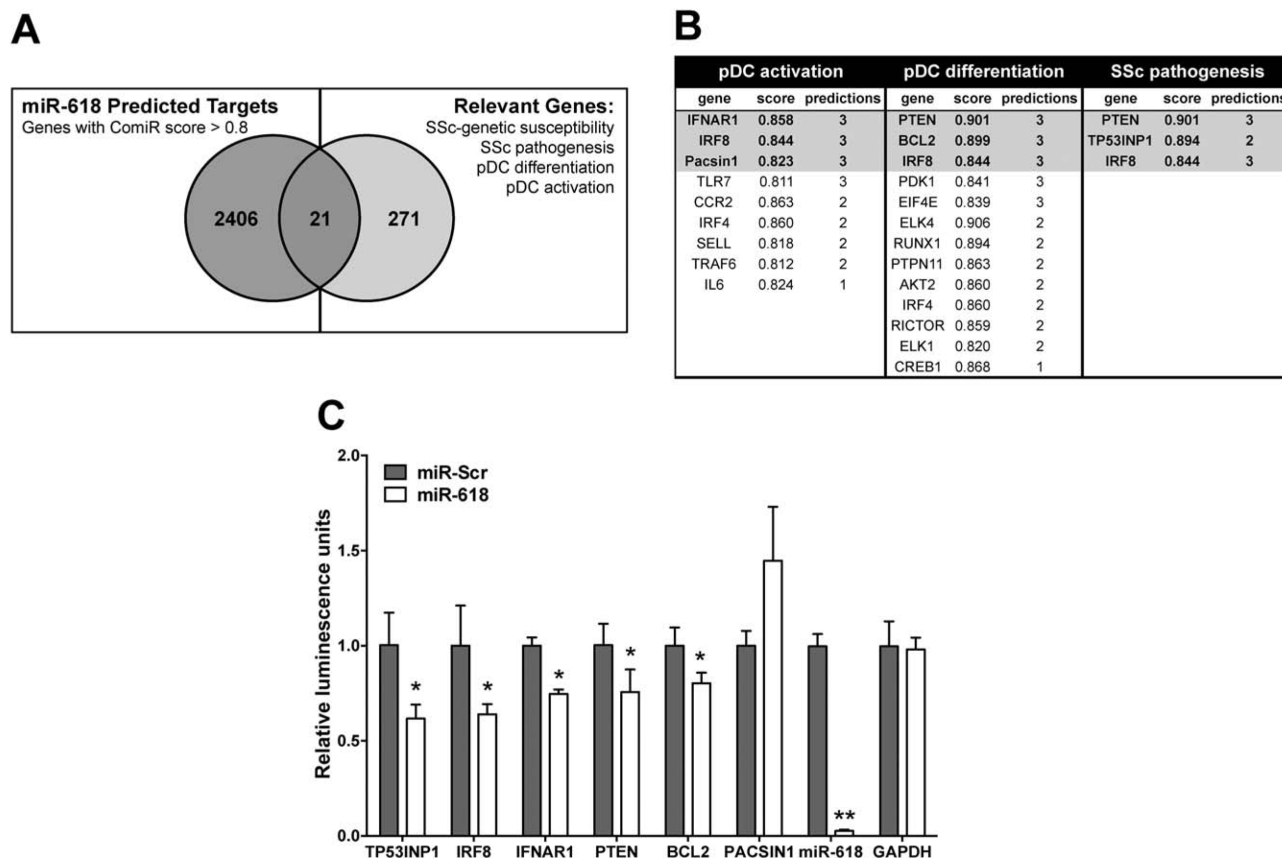

Figure 2. Targeting of multiple genes important for plasmacytoid dendritic cell (PDC) biology and systemic sclerosis (SSc) pathogenesis by microRNA-618 (miR-618). **A,** Venn diagram showing the overlap of miR-618 target genes as predicted by ComiR, according to the algorithms miRanda, PITA, TargetScan, and mirSVR, and a list of relevant genes for SSc PDCs compiled on the basis of literature searches. **B,** Genes selected from the intersection of the Venn diagram in **A,** listed according to the number of prediction algorithms identifying the interaction with miR-618 and the ComiR prediction score. Shading indicates the top-ranking genes for each category, i.e., those with the highest ComiR scores and predicted by all algorithms considered. **C,** Response of the 6 best-ranking genes in **B** to treatment with miR-618. The 3′-untranslated regions of the 6 best-ranking genes were cloned downstream of the *Renilla* luciferase gene and cotransfected into HT0180 cells with a miR-618 mimic or a non-targeting miRNA control (scrambled miRNA [miR-Scr]). Luciferase assays were performed, and the response to miR-618 treatment was expressed as relative luminescence units comparing miR-618 to the average of the scrambled miRNA–treated samples, set to 1. The miR-618 construct was used as a positive control carrying a sequence perfectly complementary to miR-618, while the GAPDH construct was used as negative control given that this gene does not contain any predicted binding site for miR-618. Bars show the mean ± SD of 3 experiments. $* = P < 0.05$; $** = P < 0.01$, by *t*-test.

with early SSc, who do not have skin involvement (Table 1). The expression of miR-618 was significantly higher in nonfibrotic patients as a whole (fold change versus healthy controls 1.56; $P = 0.016$) (Figure 1F), while it showed a trend toward up-regulation when the 2 groups were considered independently (Figure 1G). These results demonstrate that miR-618 is up-regulated in SSc from the earliest nonfibrotic stages of the disease. Similar to what was observed in PDCs, the expression of miR-618 in the whole blood of the same patients showed a similar trend and correlated well with the SSc classification score (20) (Figures 1H and I). In contrast, no variations in miR-618 levels were detected in monocytes from any SSc patient groups, indicating that miR-618 up-regulation could be cell specific (data available upon request from the corresponding author).

Targeting of multiple genes implicated in PDC regulation and SSc pathogenesis by miR-618. To gain insight into the biologic relevance of miR-618 up-regulation in the PDCs of SSc patients, a list of ~2,500 genes with high likelihood to be potential miR-618 targets (data available upon request from the corresponding author), with a prediction score of >0.8 according to ComiR (http://www.benoslab.pitt.edu/comir) [28]), was compared to a group of ~300 genes linked to SSc pathogenesis, SSc genetic susceptibility, PDC activation, and

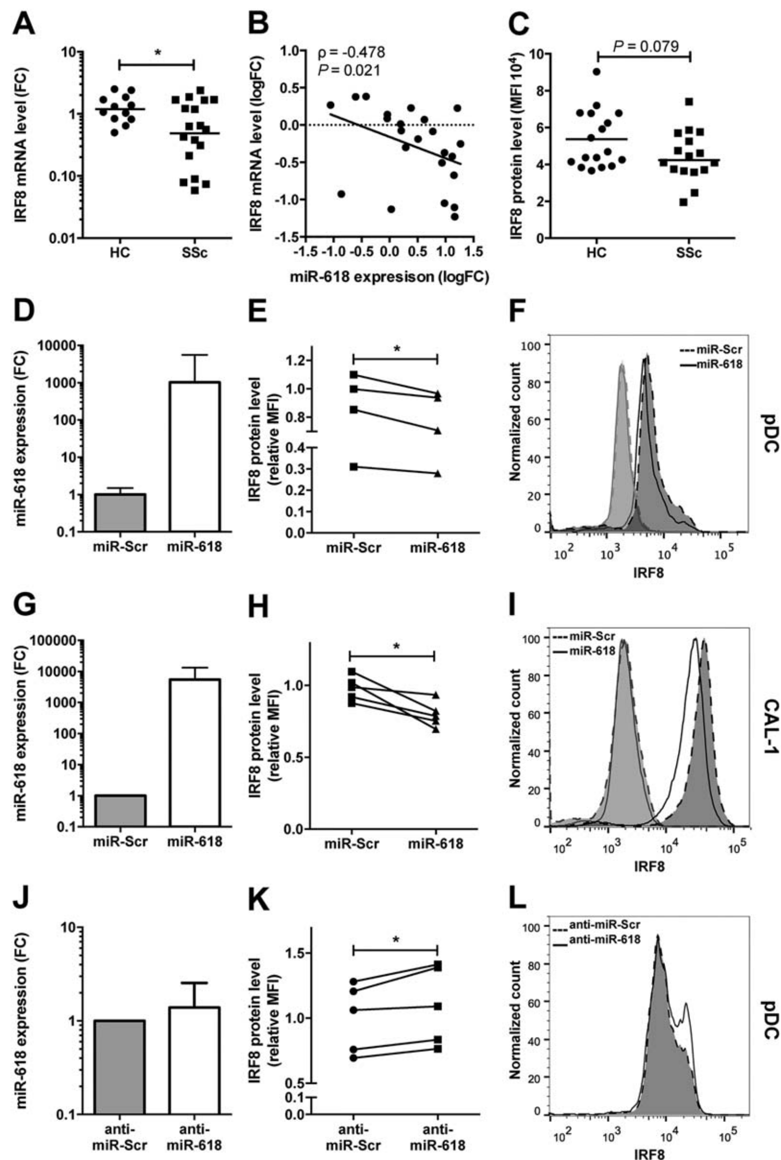

Figure 3. MicroRNA-618 (miR-618) targets interferon regulatory factor 8 (IRF-8) in plasmacytoid dendritic cells (PDCs). **A** and **C**, Expression of IRF-8 mRNA (**A**) and protein (**C**) in PDCs from healthy controls (HC; n = 12) and patients with systemic sclerosis (SSc; n = 18) in the fibrotic cohort, analyzed by real-time quantitative polymerase chain reaction (qPCR). Symbols represent individual subjects; horizontal lines show the geometric mean. **B,** Correlation of IRF-8 mRNA expression with miR-618 expression. Correlation was determined using Spearman's rho. **D–L,** Expression of miR-618 (**D, G,** and **J**), expression of IRF-8 (**E, H,** and **K**), and representative overlays of flow cytometric analysis (**F, I,** and **L**) in PDCs from healthy donors (**D–F**), CAL-1 cells (**G–I**), and PDCs from SSc patients (**J–L**). Cells were transfected with miR-618 or scrambled miRNA (miR-Scr), or with anti–miR-618 or anti–scrambled miRNA as indicated. After 24 hours, the expression of miR-618 was evaluated by real-time qPCR; anti–miR-618 is not expected to modify miR-618 expression. In parallel, a duplicate sample was stained intracellularly with anti–IRF-8 antibody or an isotype control, and the expression of IRF-8 was analyzed by flow cytometry. The median mean fluorescence intensity (MFI) of each sample was expressed relative to the nontransfected sample, set to 1. Gray and black histograms represent isotype and IRF-8 staining, respectively. In **D**, **G**, and **J**, bars show the median and range. ∗ = $P < 0.05$ by Mann-Whitney test in **A,** by *t*-test in **C,** and by *t*-test for paired observations in **E, H,** and **K**. FC = fold change.

PDC differentiation (data available upon request from the corresponding author) (1,9,15,19,29–33). This comparison led to the identification of 34 possible SSc- and PDC-relevant miR-618 target candidates, of which 21 genes are expressed in the human immune system (GeneCards; http://www.genecards.org/) (Figures 2A and B). To experimentally verify whether these genes are actively regulated by miR-618, we performed

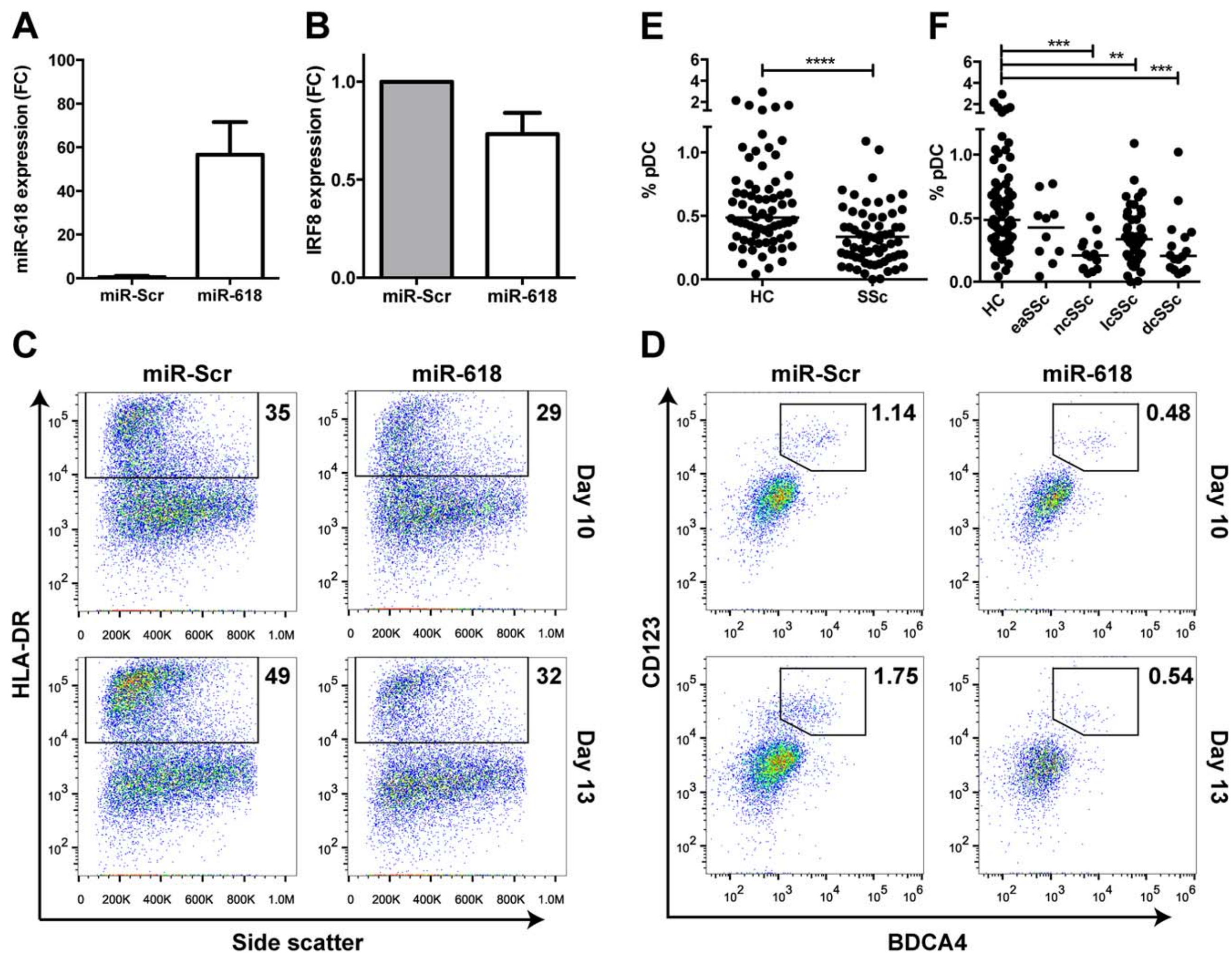

Figure 4. Inhibition of plasmacytoid dendritic cell (PDC) development by up-regulation of microRNA-618 (miR-618). CD34+ hematopoietic progenitor cells were retrovirally transduced to generate stem cell precursors stably overexpressing miR-618 or a scrambled miRNA control (miR-Scr), and cultured in vitro to obtain CD123+BDCA4+ PDCs. **A** and **B,** Levels of miR-618 (**A**) and interferon regulatory factor 8 (IRF-8) mRNA (**B**), measured by real-time quantitative polymerase chain reaction 3 days after transduction. Bars show the median and range. **C** and **D,** Frequency of HLA–DR+ cells (**C**) and PDCs (**D**) on day 10 and day 13 of culture, determined by flow cytometric analysis using anti–HLA–DR, anti-BDCA4, and anti-CD123 monoclonal antibodies. Plots show a representative experiment of 2 independently performed experiments with similar results. Values are the percentage of cells. **E** and **F,** Percentage of PDCs circulating in the peripheral blood of healthy controls (HC) and systemic sclerosis (SSc) patients as a whole (**E**) and in the peripheral blood of healthy controls and SSc patients divided into those with early SSc (eaSSc), noncutaneous SSc (ncSSc), limited cutaneous SSc (lcSSc), and diffuse cutaneous SSc (dcSSc) (**F**). The percentage of PDCs was determined by flow cytometric analysis after staining the peripheral blood mononuclear cell fraction using anti–HLA–DR, anti-CD123, and anti-CD303 monoclonal antibodies. PDCs were decreased in the circulation of SSc patients. Symbols represent individual subjects; horizontal lines show the median percentage of PDCs of total mononuclear cells. ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$, by Kruskal-Wallis test followed by Dunn's correction for multiple comparisons. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.40163/abstract.

luciferase assays by cotransfecting a miR-618 mimic or a scrambled miRNA control together with reporter constructs carrying the 3′-UTRs of the 6 best-ranking genes. When compared to scrambled miRNA–transfected cells, the presence of miR-618 significantly inhibited the luciferase activity of 5 of the 6 genes tested, thus demonstrating that these genes, namely *TP53INP1*, *IRF8*, *IFNAR1*, *PTEN*, and *BCL2*, contain a functional binding site for miR-618 in their 3′-UTR (Figure 2C). Overall, these data show that miR-618 can regulate genes implicated in PDC activation and differentiation, possibly contributing to the dysregulation of these cells in SSc.

IRF-8 is an endogenous target of miR-618 in PDCs. In order to identify the strongest miR-618 target in the context of PDCs from SSc patients, the expression of the 5 validated targets of miR-618 was analyzed in

PDCs isolated from SSc patients and healthy controls in the fibrotic cohort. In PDCs from SSc patients, mRNA for IRF-8 was significantly lower than in healthy controls (fold change 0.4; $P = 0.047$) and was inversely correlated with the expression of miR-618 ($\rho = -0.478$, $P = 0.021$) (Figures 3A and B). In contrast, the other genes tested were not consistently modulated in SSc, and their expression did not correlate with that of miR-618 (data available upon request from the corresponding author). Consistent with differences observed in *IRF8* mRNA expression levels, IRF-8 protein expression showed a trend toward down-regulation in PDCs from SSc patients (Figure 3C). To verify whether IRF-8 is an endogenous target of miR-618 in PDCs, the miR-618 mimic was transfected into primary PDCs from healthy donors. The efficient overexpression of miR-618 in PDCs (Figure 3D) resulted in the inhibition of IRF-8 protein expression compared to cells transfected with a non-targeting miRNA ($P = 0.044$) (Figures 3E and F). Similar results were obtained by performing the same experiment in the human PDC line CAL-1 ($P = 0.023$), confirming the ability of miR-618 to inhibit IRF-8 expression in PDCs (Figures 3G–I). In contrast, inhibition of miR-618 in PDCs isolated from SSc patients partially normalized IRF-8 expression levels (Figures 3J–L).

Modulation of PDC development and activation by the up-regulation of miR-618. IRF-8 governs the differentiation of DC subsets and is essential for PDC development (34). To determine whether miR-618 affects PDC differentiation by targeting IRF-8 activity, we overexpressed miR-618 in an in vitro model of PDC development (25). Consistent with the findings described above, IRF-8 expression was lower when miR-618 was overexpressed in this model (Figures 4A and B). Furthermore, the number of HLA–DR+ cells and BDCA4+CD123+ PDCs generated from stem cell precursors overexpressing miR-618 was reduced on both day 10 and day 13 of culture (Figures 4C and D). These experiments suggest that the inhibition of IRF-8 mediated by miR-618 is functional and does influence the development of PDCs. Consistent with this finding, we observed that the number of circulating PDCs was significantly reduced in the peripheral blood of all SSc patient groups except the early SSc group (Figures 4E and F). These results suggest that the up-regulation of miR-618 may contribute to the reduced presence of this cell type in the circulation of SSc patients.

IRF-8 and other possible targets of miR-618 constitute important molecular mediators of cytokine release upon PDC stimulation (34). To examine this, PDCs isolated from healthy individuals were transfected with either miR-618 mimic or a non-targeting scrambled miRNA control and activated in vitro with the synthetic

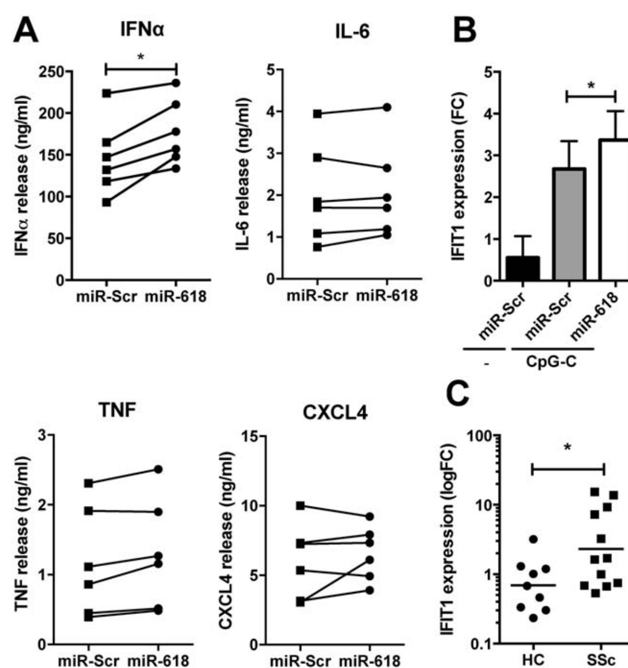

Figure 5. Up-regulation of microRNA-618 (miR-618) enhances interferon-α (IFNα) release by plasmacytoid dendritic cells (PDCs). **A,** Release of IFNα, interleukin-6 (IL-6), tumor necrosis factor (TNF), and CXCL4 by PDCs from healthy controls (HC) that were transfected with miR-618 or scrambled miRNA (miR-Scr), rested overnight, and stimulated for 24 hours with 1 μM type C CpG-containing oligodeoxyribonucleotide (CpG ODN). Release was measured in cell-free supernatants using Luminex assays. **B,** Expression of *IFIT1* by RNA isolated from PDCs from healthy donors that were transfected with miR-618 or scrambled miRNA, rested overnight, and stimulated for 24 hours with 1 μM type C CpG ODN. Bars show the geometric mean ± SEM of 8 experiments. **C,** Relative levels of *IFIT1* mRNA in PDCs isolated from healthy controls and systemic sclerosis (SSc) patients in the fibrotic cohort. Symbols represent individual subjects; horizontal lines show the geometric mean. * = $P < 0.05$ by nonparametric Wilcoxon test for paired samples in **B** and by Mann-Whitney test in **C**. FC = fold change.

TLR-9 ligand type C CpG ODN dsDNA. Compared to those transfected with mimic controls, PDCs overexpressing miR-618 secreted higher levels of IFNα but not IL-6, TNF, or CXCL4 (Figure 5A). The increased release of IFNα observed upon miR-618 overexpression did not occur via IRF-8 inhibition, since silencing of IRF-8 in PDCs led to strong inhibition of IFNα production (Supplementary Figure 1, available on the *Arthritis & Rheumatology* web site at http://onlinelibrary.wiley.com/doi/10.1002/art.40163/abstract). Consistently, inhibition of IRF-8 also blocked the release of IL-6 and TNF (Supplementary Figure 1), while up-regulation of miR-618 did not affect these cytokines (Figure 5A).

The release of greater amounts of IFNα by PDCs overexpressing miR-618 was concomitant with

an increased expression of mRNA encoding IFIT1 ($P = 0.042$), an IFN-responsive gene (Figure 5B). Similarly, we found that PDCs from SSc patients had higher *IFIT1* expression than those from healthy controls (fold change 3.3; $P = 0.03$) (Figure 5C), consistent with previous findings demonstrating the presence of a type I IFN signature in the circulation and skin of SSc patients (14,35). Consistent with these observations, previous results by our group obtained using the same culture conditions demonstrated that PDCs isolated from SSc patients produce higher amounts of IFNα in response to TLR-9 stimulation as compared to healthy cells (13). Overall, these results demonstrate that the up-regulation of miR-618 in PDCs can also influence their activation in terms of IFNα release and may therefore contribute to the type I IFN signature present in SSc patients.

DISCUSSION

PDCs constitute a critical source of type I IFNs that can contribute to the onset and perpetuation of autoimmunity. However, the molecular mechanisms that initiate and sustain dysregulation of PDCs are largely unexplored, especially in SSc. Similar to what has been observed in other autoimmune diseases characterized by a type I IFN signature, such as SLE and primary Sjögren's syndrome (36–39), we show here that PDCs are decreased in the circulation of SSc patients and exhibit up-regulated expression of IFN-responsive genes. Using miRNA expression profiling and validation in multiple cohorts, we demonstrated that expression of one miRNA, miR-618, is increased in the PDCs of SSc patients and mediates regulatory mechanisms that may contribute to the derailment of PDCs in this disease. According to prediction analysis and reporter assays, miR-618 targets genes involved in PDC differentiation/activation or previously associated with SSc pathogenesis. Among those tested, IRF-8 was validated as the strongest miR-618 target, as its expression was inversely correlated with miR-618 levels in SSc PDCs and inhibited upon ectopic overexpression of miR-618.

Underlying the importance of IRF-8 targeting by miR-618 is the fact that 3 distinct polymorphisms associated with SSc susceptibility are located downstream of the genomic region encoding for IRF-8 (40–42). However, we have verified that the action of miR-618 on IRF-8 expression is independent of the presence of any SSc risk allele within the *IFR8* genomic region, since none of the previously identified SSc-associated single-nucleotide polymorphisms or their proxies were located inside the binding sites of miR-618 or within the 3′-UTR of IRF-8 (data available upon request from the corresponding author). That both genetic and epigenetic factors independently contribute to IRF-8 dysregulation underscores the importance of this IFN-responsive transcription factor in the pathogenic processes underlying SSc development.

IRF-8 is a crucial transcription factor in both PDC development and PDC activation (34). IRF-8–deficient mice show profound defects in the DC compartment, since they lack both CD11c+CD8a+ DCs and PDCs (43–47). In humans, mutations in IRF-8 lead to either a total absence of or a strong decrease in circulating DCs, resulting in severe immunodeficiency in the affected patients (48). Consistent with the capacity of miR-618 to suppress IRF-8 expression, miR-618 also reduces the development of PDCs from CD34+ cells. Along with the migration of PDCs into the affected tissues previously demonstrated in SSc (13), the up-regulation of miR-618 may therefore favor the decrease in circulating PDCs observed in SSc patients.

Besides its role in cell differentiation, IRF-8 is activated upon TLR stimulation and, in association with other transcription factors, activates or represses the expression of specific genes including numerous cytokines and costimulatory molecules (34). Macrophages and DCs from IRF-8–deficient mice remain immature and have impaired responses to TLR activation. However, the role of IRF-8 in PDC activation is still a subject of controversy (49), as IRF-8 was reported to inhibit cytokine release upon TLR-9 stimulation in the human PDC line CAL-1 (50), while it has also been shown to favor type I IFN release by murine PDCs (51). This is the first study to demonstrate that the silencing of IRF-8 impairs the activation of primary human PDCs, resulting in decreased IFNα secretion by PDCs. Therefore, the higher release of soluble IFNα observed upon miR-618 overexpression in primary human PDCs is likely mediated by additional miR-618 targets other than IRF-8. It is possible that the up-regulated expression of miR-618 in PDCs from SSc patients also results in increased IFNα release in vivo, thus contributing to the establishment of the type I IFN signature observed in SSc patients (14,35,52). This hypothesis would be consistent with our previous finding that PDCs isolated from SSc patients produce a greater amount of IFNα in response to TLR-9 stimulation than healthy cells (13). In contrast, we did not observe any relevant impact of miR-618 expression on the release of CXCL4, the cytokine abundantly released by PDCs from SSc patients (13).

Whether the immune system plays a primary role in SSc initiation and/or disease maintenance remains an open question. However, increasing

evidence demonstrates that autoimmunity is not a mere consequence of tissue damage, but also is a prominent factor that can contribute to SSc pathogenesis. Supporting this concept, we recently demonstrated that the type I IFN signature in blood monocytes from SSc patients is present not only in patients with established SSc but also in patients with early SSc without any signs of fibrosis (14). Similarly, in this study we show that miR-618 is incrementally up-regulated in patients with early SSc (fold change versus healthy controls 1.35), in SSc patients without fibrosis (fold change 1.41), and in subjects with overt fibrosis (fold change 3.85), including those with dcSSc with a disease duration of $\leq$2 years (fold change 3.68). Considering that these patients could progress to a more severe phenotype and develop cutaneous and/or internal organ fibrosis over time, the up-regulation of miR-618 expression may represent one of the multiple molecular aberrances occurring during SSc evolution. Consistently, higher levels of miR-618 are associated with the presence of ILD, a severe clinical complication resulting from the development of visceral fibrosis (53).

Consistent with the recent demonstrations of a notable role of PDCs in the development of systemic autoimmunity in mouse models of SLE (6–8), the data presented here support the concept that molecular aberrances occurring early in the PDCs of patients with SSc could impact the entire immune system, thus inducing autoimmunity and ultimately favoring the onset of SSc and its progression. While in vivo studies are necessary to prove the role of miR-618 in SSc development, this miRNA may represent a potential novel epigenetic target for restoring immune system homeostasis in SSc and in other diseases characterized by a type I IFN signature.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Rossato had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Rossato, Dolstra, van Laar, Martin, van Roon, Reedquist, Radstake.

Acquisition of data. Rossato, Affandi, Thordardottir, Wichers, Cossu, Chouri, van Bon, Wolters, Marut, van der Kroef, Silva-Cardoso, Bekker, Beretta.

Analysis and interpretation of data. Rossato, Affandi, Wichers, Broen, Moret, Bossini-Castillo.

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