Regulation of the multifaceted functions of human plasmacytoid dendritic cells: a polyphonic policy

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Citation for published version (APA):

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MICRORNA-146A REGULATES SURVIVAL AND MATURATION OF HUMAN PLASMACYTOID DENDRITIC CELLS

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Blood 2013; 122 (17): 3001-3009
ABSTRACT

During microbial infections, plasmacytoid dendritic cells (pDCs) are a main source of type I interferons (IFNs)-α/β. Nucleic acids from microbes are sensed by Toll-like-receptor (TLR)-7/9, which are selectively expressed in pDCs. Activated pDCs also produce pro-inflammatory cytokines and upregulate co-stimulatory molecules. Together this equips pDCs with the ability to prime T, B, and NK cells and conventional (c)-DCs, thereby initiating adaptive immune responses. To avoid deleterious effects to the host, tight regulation of pDC activation is required. Despite numerous data linking aberrant activation of pDCs with autoimmune diseases, little is known about mechanisms controlling pDC activation. Here, we investigated the role of microRNA-146a (miR-146a) in TLR pathway regulation in human pDCs. We show that miR-146a expression was induced upon TLR7/9 signalling. Furthermore, ectopic miR-146a expression effectively impaired TLR-mediated signalling in pDCs as TLR-induced NF-κB activation was reduced. This consequently diminished the production of pro-inflammatory cytokines, and reduced pDC survival. Moreover, miR-146a expressing pDCs had decreased ability to induce CD4^+ T cell proliferation likely due to reduced expression levels of MHCII and co-stimulatory molecules. Taken together, these data unravel the crucial immunomodulatory role of miR-146a in pDCs and may add to our understanding of aberrant responses in autoimmune diseases.
INTRODUCTION

Plasmacytoid dendritic cells (pDCs) form a unique subset within the DC lineage. In contrast to conventional (c)-DCs, pDCs selectively express Toll-like-receptor (TLR)-7 and TLR9, which recognize microbial single-stranded RNA or double-stranded DNA, respectively (reviewed in Liu\(^1\)). TLR activation in pDCs leads to rapid secretion of high amounts of type I Interferons (IFNs), which prevent viral replication and are involved in regulating antigen specific immune responses. In addition, TLR-activated pDCs secrete interleukin-6 (IL-6) and mature in response to autocrine production of the pro-inflammatory cytokine tumor necrosis factor (TNF)-\(\alpha\) thereby upregulating the expression of co-stimulatory molecules, such as CD40, CD80 and CD86, and MHC class II.\(^2\) Collectively, this contributes to activation of T, B and NK cells.\(^3\)

Signal transduction via TLR7 and TLR9 depends on recruitment of the myeloid differentiation primary response gene 88 (MyD88) adaptor molecule in complex with IL-1 receptor-associated kinase (IRAK)-1 and IRAK-4, tumor necrosis factor receptor-associated 6 (TRAF6) and TRAF3, and the transcription factors interferon response factor (IRF)-7 and IRF-5.\(^4\) Furthermore, all TLR signalling pathways culminate in activation of the transcription factor nuclear factor-\(\kappa\)B (NF-\(\kappa\)B), which requires the phosphorylation and degradation of inhibitory \(\kappa\)B (I\(\kappa\)B) proteins triggered by two kinases, I\(\kappa\)B kinase alpha (IKK-\(\alpha\)) and IKK-\(\beta\). Several NF-\(\kappa\)B members have been identified, including RelA (also known as p65), RelB, c-Rel, p52 and p50. Rel proteins can form homo- or heterodimers, of which the most frequently activated form after TLR signalling is the RelA/p50 heterodimer.\(^5\) RelA/p50 dimers are directly responsible for the expression of co-stimulatory molecules, while IRF-5 nuclear translocation, together with NF-\(\kappa\)B and MAPK activation, is crucial for the production of inflammatory cytokines.\(^6\) Conversely, the phosphorylation of IRF-7 leads to its translocation into the nucleus where it initiates type I IFN gene transcription.\(^7,8\)

Unwanted production of IFN-\(\alpha\) has been shown to be involved in the pathogenesis of several human autoimmune diseases, such as systemic lupus erythematosus (SLE),\(^9,10\) Sjögren’s syndrome (SS),\(^11,12\) and psoriasis.\(^13\) In SLE, one of the driving mechanisms of the disease involves uncontrolled and chronic IFN-\(\alpha\) and IL-6 production by activated pDCs, which promote survival and differentiation of auto-reactive B cells into autoantibody-secreting plasma cells.\(^14\) Given the potency of type I IFNs and pro-inflammatory cytokines to activate a wide range of cells of the innate and adaptive immune system, pDC activation needs to be tightly controlled. Therefore, pDCs express an array of surface receptors, such as the C-type lectin blood dendritic cell antigen 2 (BDCA-2), dendritic cell immunoreceptor (DCIR), immunoglobulin-like transcript 7 (ILT7), high-affinity immunoglobulin E receptor (Fc\(\varepsilonRI\)), and natural killer partner 44 (NKP44), which counter-regulate the prominent TLR signalling pathway.\(^15-18\)

A more recently discovered and novel layer of gene regulation is mediated by microRNAs (miRNAs), which are an evolutionary conserved class of endogenous ~19 to 23-nucleotides long non-coding RNAs.\(^19\) They act by repressing gene expression through targeting of the 3'-untranslated region (UTR) of mRNAs resulting in either mRNA degradation or translation inhibition, or a combination of both.\(^20\) This mode of post-transcriptional regulation of gene expression has recently been shown to play a role in modulating the TLR response in
a broad range of human immune cells, including monocytes, macrophages and T cells. Of these miRNAs, miR-146a emerged as a negative master regulator of TLR activation. MiR-146a is a member of the miR-146 miRNA family consisting of two evolutionary conserved miRNA genes; miR-146a and miR-146b. Increased expression of miR-146a has been observed in human monocytes in response to TLR4 stimulation by lipopolysaccharides (LPS) through direct NF-κB-mediated induction. Moreover, miR-146a was shown to act as a key regulator of the TLR-MyD88 pathway by directly targeting IRAK1 and TRAF6 mRNAs, suggesting its role as a “brake on immunity”. In line with this, miR-146a−/− mice suffered from myeloproliferative disorders and LPS hypersensitivity. Furthermore, it has been demonstrated that stimulation of peripheral blood mononuclear cells (PBMCs) from healthy donors with TLR7 and TLR9 agonists induced miR-146a expression and negatively affected type I IFN production, via downregulation of IRF-5 and STAT1. Interestingly, in SLE patients, miR-146a was found to be expressed at lower levels as compared to healthy donors, which might well explain the exacerbated type I IFN production by PBMCs of these patients. Based on the notion that in human blood only pDCs express TLR7 and TLR9 and secrete type I IFNs after stimulation, it may suggest that the function of pDCs is altered by miR-146a, but this has not been formally addressed. It is also not known whether miR-146a controls other functions of pDCs, such as expression of co-stimulatory molecules and production of pro-inflammatory cytokines as well as their survival.

Despite the growing evidence involving microRNA in the regulation of TLR functioning, no extensive studies have been performed on purified human pDCs. Therefore, we aimed at clarifying the role of miR-146a in regulating TLR-induced pDC activation and maturation. We made use of both primary pDCs as well as the pDC cell line CAL-1, which we previously validated as a model to study human pDC activation and maturation. We observed strong induction of miR-146a expression upon activation of primary pDCs using either TLR7 or TLR9 agonists. In addition, we investigated the role of miR-146a using overexpression studies in the CAL-1 pDC cell line. Our results suggest that miR-146a is crucially involved in the survival and TLR-induced maturation of pDCs, which has direct consequences for their ability to induce proliferation and IFN-γ production of CD4+ T cells.

METHODS

Cells and reagents for functional assay
The pDC cell line CAL-1 was cultured in RPMI-1640 medium (Invitrogen) supplemented with 8% FCS, and maintained at 37 °C, 5% CO2. For activation and maturation of pDCs, cells were cultured in Yssel’s medium, supplemented with 2% human serum (Invitrogen). Oligodeoxynucleotides CpG-A (ODN2216), CpG-B (ODN2006), and R848 were purchased from Invivogen and used at 10µg/mL.

Lentiviral constructs and transductions
Overexpression of miR-146a in human cells was done using a vector-based miRNA expression system. Briefly, the ~500 bp fragment corresponding to the miR-146a-genomic region or the
control human telomerase (hTR)-genomic region (a nontranslated-RNA coding for human telomerase RNA\textsuperscript{10}) were cut out from the pMSCV-Blasticidin vector (kind gifts from R. Agami, The Netherlands Cancer Institute, Amsterdam) using BamHI-EcoRI restriction sites, and subcloned into the lentiviral vector using the copepod \textit{Pontellina plumata} GFP as a reporter gene (copGFP), pCDH1-copGFP (System Biosciences).

\textbf{PCR}

Total RNA was extracted using Trizol reagent (Invitrogen). RNA concentration and quality were determined using the Nanodrop spectrophotometer (Thermo Fisher Scientific). MiRNA quantitative real-time RT-PCR (QPCR) was performed using the TaqMan MicroRNA Reverse Transcription Kit with TaqMan MicroRNA Assay primers for human miR-146a (Applied Biosystems, Foster City, CA, USA). Total mRNA was reverse transcribed using the RNA-to-cDNA kit (Roche). cDNA was amplified using an iCycler and SYBRgreen supermix (BioRad) for QPCR, using specific primer sets (supplemental table 1). The levels of miRNA were normalized to RNA U6 controls, whereas mRNA levels were normalized to the 3 housekeeping genes β-Actin, GAPDH, and HPRT.

\textbf{ELISA}

ELISA was done to detect human IL-6 (U-cytech Biosciences, Utrecht, Netherlands) and IFN-β (PBL Interferon source, Piscataway, NJ, USA) proteins in the supernatants of CAL-1 cells according to the manufacturer’s instructions.

\textbf{Flow cytometry}

For analysis, single cell suspensions were stained with fluorescein isothiocyanate (FITC), phycoerythrin (PE), PE-cyanine (Cy7), allophycocyanin (APC), APC-Cy7 coupled anti-human monoclonal antibodies (Abs) targeting the following cell surface markers: CD40, HLA-DR (Biolegend), CD80, CD86, CCR7 or isotype controls (BD Bioscience). For detection of phosphorylated p65 (phospho-S529; phospho-p65) protein, cells were fixed using cytofix/cytoperm buffer, permeabilized in ice-cold methanol and washed with Perm/Wash buffer (BD PharMingen) before incubation with APC-conjugated phospho-p65 Ab (BD biosciences). For apoptosis staining we used AnnexinV-PE, or AnnexinV-APC (BD Bioscience) and 7-AAD viability staining solution (eBioscience). For intracellular cytokine detection, PE-conjugated anti-human-IFN-γ and IFN-α antibodies (BD Biosciences) were used. Samples were analyzed on a LSRII fluorescence-activated cell sorter (FACS) analyzer (BD Bioscience) and analyzed using FlowJo software (TreeStar).

\textbf{Isolation of primary human pDC from tissues}

Peripheral blood of healthy volunteers was used for isolation of pDCs (Sanquin Bloodbank, Amsterdam, The Netherlands). PBMCs were isolated via Ficoll-Hypaque density gradient and negatively selected for CD3/CD14/CD16/CD19/CD20 by immunomagnetic bead selection using anti-FITC beads (Miltenyi Biotec). Postnatal thymic (PNT) tissue was obtained from surgical specimens removed from children up to 3 years of age undergoing open-heart surgery (LUMC,
Leiden, The Netherlands), approved by the Medical Ethical Committee of the Academic Medical Center. Thymocytes were isolated from a Ficoll-Hypaque density gradient. Subsequently, BDCA4⁺ cells were enriched by immunomagnetic bead selection using a BDCA4-cell separation kit (Miltenyi Biotec). CD123⁺CD45RA⁺ or BDCA4⁺ pDCs were sorted by flow cytometry on a FACS Aria (BD Biosciences). Purity was ≥ 99% and confirmed by reanalysis of sorted cells.

**LNA transfection**

Sorted pDCs were transfected using the Neon Transfection system (Invitrogen) with 1 µg of either a FITC-conjugated human-miR-146a locked nucleic acid (LNA) (5’-AACCCATGGAAUTCAGUUCUCA-3’) or control LNA (5’-GUGTAACACGUUTAUACGCCCA-3’) (Ribotask, Odense, Denmark) by electroporation (Microporator, Digital Bio; 1200V, 20ms, 1 pulse).

**Allogeneic T cell stimulation**

Peripheral blood CD4⁺ T cells were enriched by immunomagnetic bead selection using a CD4-cell separation kit (Miltenyi Biotec). CAL-1 cells transduced with miR-146a or hTR control were incubated with or without the TLR7 ligand R848 for 48h, irradiated (60 Gy) and subsequently co-cultured with T cells at a 1:1 ratio for 6 days in Yssel’s medium supplemented with 2% human serum. T cell proliferation was assessed by flow cytometry using the CellTrace-violet proliferation kit. T cells activated with Human T-expander CD3/CD28 beads (Dynabeads, Dynal, Invitrogen) were used as a positive control. For detection of intracellular IFN-γ, CD4⁺ T cells were restimulated with PMA/ionomycin (1 µg/mL each) at day 6 for 6h in the presence of BrefeldinA and stained with anti-IFN-γ PE (BD Pharmingen).

**Statistical analyses**

Data were subjected to two-tailed paired Student’s t-test analysis using Graphpad Prism 5 for Windows (Graphpad software, San Diego, USA) and considered significant when at least \( P < 0.05 \).

**RESULTS**

**MiR-146a expression is induced in human pDCs in response to TLR7 and TLR9 activation**

To study whether miR-146a has a role in pDCs, we first set out to analyze whether ex vivo pDCs expressed miR-146a after stimulation with TLR9 agonists (CpG-A, CpG-B) or TLR7 agonist (R848). After 16h miR-146a levels were measured by QPCR. We observed that both TLR7 and TLR9 engagement induced miR-146a expression as compared to medium cultured pDCs (Figure 1A). The leukemic pDC cell line CAL-1, which represent a valid model to study certain aspects of pDC biology, expressed miR-146a as well upon TLR7 or TLR9 ligation (Figure 1B). Stimulation with IL-3 did not upregulate expression of miR-146a in primary pDCs or CAL-1 cells (Figure 1). Collectively, this suggests that miR-146a may have a role in TLR-induced responses in pDCs.


**Figure 1: MiR-146a is upregulated upon activation of pDCs.** (A) Freshly isolated pDCs or (B) CAL-1 cells were activated with the TLR9 agonists CpG-A or CpG-B, the TLR7 agonist R848 (each 10µg/mL), IL-3 (10 ng/mL) or cultured in medium alone for 16h. The relative expression of the mature form of miR-146a was assessed by QPCR using specific TaqMan primers and the QPCR TaqMan kit. MiR-146a levels were normalized to the level of the small nuclear RNA U6, and the medium control condition was set to 1. Data are shown as means ± SD of independent pDC donors (CpG-A, n = 5; CpG-B, n = 4; R848, n = 5; IL-3, n = 2) or independent experiments using CAL-1 cells (CpG-A and CpG-B, n = 3; R848 and IL-3, n = 4). *P < 0.05.

### MiR-146a Impairs Expression of Components in the NF-κB Signaling Pathway

To further characterize the role of miR-146a in pDCs, we transduced CAL-1 cells with a lentiviral vector expressing either miR-146a or hTR as a control. The vector also allows expression of GFP to trace transduced cells by flow cytometry. GFP+ cells were sorted and western blot analysis was performed to assess the protein levels of IRAK1, which was shown to be a direct miR-146a target.22 MiR-146a overexpression resulted in 25% decreased IRAK1 levels as compared to hTR control transduced cells (Supplementary Figure 1). In line with these results, we observed reduced nuclear phospho-p65 (RelA) levels in miR-146a transduced CAL-1 cells as compared to hTR control transduced cells (Supplementary Figure 1). In line with these results, we observed reduced nuclear phospho-p65 (RelA) levels in miR-146a transduced CAL-1 cells as compared to hTR control transduced cells after stimulation with CpG-B (n=4, *P=0.014) or R848 (n=4, **P=0.004) (Figure 2). This shows that miR-146a inhibits NF-κB activation in response to TLR engagement in pDCs, possibly through targeting of components in the NF-κB pathway, such as IRAK1.

### MiR-146a Overexpression Induces Apoptosis in CAL-1 Cells

The anti-apoptotic gene BCL2-A1 is a direct target of Spi-B involved in human pDC development and survival.27 In addition, NF-κB directly induces BCL2-A1 expression.28 This prompted us to investigate the role of miR-146a in pDC survival. CAL-1 cells, which depend on constitutive NF-κB activity for their survival (Supplementary Figure 2), were transduced with lentiviral vectors marked with GFP to overexpress miR-146a or hTR as a control. The absolute number of GFP+ cells as measured by flow cytometry was reduced when miR-146a transduced CAL-1 cells were cultured over time as compared to control transduced CAL-1 cells (Figure 3A). Furthermore, the percentages of apoptotic cells as assessed by flow cytometry after AnnexinV and 7-AAD staining...
was on average 1.8-fold higher when CAL-1 cells were cultured after transduction with miR-146a as compared to hTR control (Figure 3B-C; n=3, *P<0.05). A similar increase in percentage of apoptotic miR-146a transduced CAL-1 cells was notable at several time points analyzed (Supplementary Figure 3). Consistent with this, BCL2-A1 mRNA levels were significantly lower in GFP-sorted CAL-1 cells in which miR-146a was overexpressed as compared with hTR control cells (Figure 3C; n=4, **P<0.01). Thus, our data provide evidence for a specific role of miR-146a in the induction of apoptosis in pDCs, which may correlate with downregulation of BCL2-A1 levels.

**MiR-146a affects pDC activation and maturation after TLR stimulation**

To substantiate the role of miR-146a in CAL-1 cells we activated the cells with the TLR7 agonist R848 or the TLR9 agonist CpG-B. Sorted GFP* cells were activated with R848 for 4h, and analyzed...
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pDC survival and function is controlled by mir-146a

For IFN-β and IL-6 mRNA levels by QPCR. Notably, ectopic expression of mir-146a resulted in a 4-fold decrease in the expression of IFN-β mRNA and a 5-fold decrease in IL-6 mRNA levels as compared to TLR7 activated hTR-CAL-1 cells (Figure 4A; n=2, *P<0.05). In line with this, IFN-β and IL-6 protein levels as measured by ELISA were reduced in the supernatants of miR-146a CAL-1 cells as compared to control cells (Supplementary Figure 4). It was unexpected that IL-6 and IFN-β levels in miR-146a CAL-1 cells cultured in medium are increased as compared to hTR-CAL-1 cells, but as yet this remains unexplained. Furthermore, flow cytometric analysis revealed that miR-146a CAL-1 cells after electronic gating on GFP+ cells were significantly impaired to upregulate CD40, CD80, CD86, HLA-DR and CCR7 after TLR7 stimulation as compared to control cells (Figure 4B-C, n=4-6, *P<0.05, **P<0.01). A similar reduction in expression of CD40 and CD80 was observed after TLR9 stimulation,
Figure 4: Ectopic miR-146a expression affects pDC activation and maturation. (A) QPCR analysis of IL-6 and IFN-β mRNA levels in CAL-1 cells transduced with hTR control or with miR-146a cultured in medium or activated with TLR7 ligand R848 (10 µg/mL) for 4h. Shown are mean values ± SD of two independent experiments. The values for hTR transduced cells cultured in medium are set to 1. * P < 0.05. (B) Flow cytometric analysis of CAL-1 cells after transduction with hTR control (grey line) or miR-146a (black line). Cells were activated overnight with the TLR7 ligand R848 and stained for expression of CD40, CD80, CD86, HLA-DR, and CCR7. The filled grey histograms represent isotype control antibody stained cells. RCN, relative cell number. (C) Cells were analyzed as in (B). Statistical analysis of the relative mean fluorescence intensities (MFIs) ± SD of 4-6 different experiments. MFIs of proteins expressed on hTR control cells are set to 1. * P < 0.05, ** P < 0.01. (D) Primary pDCs isolated from blood were transfected with FITC-conjugated LNA-miR146a or LNA-control followed by activation with CpG-A (10µg/mL) or medium only for 18h. Cells were analyzed by flow cytometry after staining with an anti-IFN-α antibody or isotype control antibody. Shown are the mean percentages ± SD of IFN-α expressing pDCs of 2 independent experiments.
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pDC survival and function is controlled by miR-146a

though no difference in CD86 and HLA-DR expression and no consistent effect on CCR7 expression was observed between miR-146a cells and control cells (Supplementary Figure 5).

To enforce our findings in a more physiological relevant manner, we silenced miR-146a activity using a locked nucleic acid (LNA) that specifically binds miR-146a. We transfected primary human pDCs with a FITC-conjugated LNA-miR-146a or LNA-control miRNA. After stimulation with the TLR9 agonist CpG-A, a known inducer of type I IFNs, we observed a 2.2-fold increase in the percentage of IFN-α expressing pDCs when LNA-miR-146a was transfected as compared to LNA-control miRNA (Figure 4D). Collectively, these data support the notion that miR-146a controls the TLR-induced upregulation of co-stimulatory molecules, MHCII and pro-inflammatory cytokines in pDCs.

**CAL-1 cells ectopically expressing miR-146a are impaired in inducing alloreactive T cell proliferation**

Since maturation of miR-146a transduced CAL-1 after TLR7 stimulation was impaired as compared to hTR-control transduced cells, we investigated their ability to activate CD4⁺ T cells. Therefore in vitro allogeneic T cell stimulation assays using transduced CAL-1 cells were performed. Sorted GFP⁺ CAL-1 cells were pre-activated for 48h with R848, irradiated to avoid further cell growth, and co-cultured with resting allogeneic CD4⁺ T cells for 6 days. T cell proliferation was assessed by measuring the loss of the CellTrace Violet membrane dye upon cell division as detected by flow cytometry (Figure 5A). We included 7-AAD in the staining to measure cell death. We observed that TLR7 pre-activated CAL-1 cells induced T cell proliferation and prevented T cell death as compared to medium cultured CAL-1 cells (Figure 5A, upper panels). Allogeneic T cell proliferation induced by TLR7-activated CAL-1 cells was reduced compared to polyclonal stimulation using anti-CD3/CD28 beads (left panel). Strikingly, miR-146a CAL-1 cells pre-activated by the TLR7 agonist were less capable to induce T cell proliferation (Figure 5A: 7.6%) as compared to activated hTR CAL-1 cells (Figure 5A: 20%). Like non-activated hTR transduced CAL-1 cells, also non-activated miR-146a transduced CAL-1 cells were unable to induce T cell proliferation. When analyzing several independent donors (n=6) we observed a low, but significant reduction in T cell stimulatory capacity of miR-146a CAL-1 cells as compared to hTR CAL-1 cells (Figure 5B; n=6, * P<0.01). To assess whether CD4⁺ T cells were affected in their capacity to produce cytokines, we analysed intracellular IFN-γ levels by flow cytometry upon restimulation of the T cells after 6 days (Figure 5C). We observed a reduced percentage of IFN-γ producing CD4⁺ T cells after co-culture with miR-146a CAL-1 cells as compared to control CAL-1 cells (n=3, * P<0.05). Taken together, these results suggest that ectopic expression of miR-146 impairs efficient TLR-induced maturation of pDCs resulting in compromised ability to activate CD4⁺ T cells.

**DISCUSSION**

In the present study, we highlight the immunomodulatory role of miR-146a during human pDC activation and maturation induced by TLR engagement. We show that miR-146a expression was induced in primary pDCs upon TLR7 and TLR9 triggering. Furthermore, ectopic expression
Figure 5: MiR-146a inhibits CD4$^+$ T cell proliferation induced by TLR7 pre-activated CAL-1 cells. (A) Flow cytometric analysis of T cell proliferation induced by CAL-1 cells transduced with hTR control RNA or with miR-146a expressing vectors that were pre-activated for 48h with or without TLR7 agonist R848 (10µg/ml). Pre-activated CAL-1 cells were co-cultured together with freshly isolated allogeneic CD4$^+$ T cells (ratio CAL-1:CD4$^+$ T cells = 1:1) after labeling with the CellTrace violet membrane dye. After 6 days, T cells were analyzed for expression of CellTrace violet and 7-AAD in CD3$^+$ T cells. Percentages of CellTrace-violet$^+$7-AAD$^-$CD3$^+$ cells represent T cells that proliferated and are alive (lower left quadrant). CD4$^+$ T cells activated with anti-CD3/CD28 beads are shown as a positive control for proliferation (grey histogram) as compared with CD4$^+$ T cells cultured only with medium (white histogram). Shown is one representative experiment out of 3. Numbers in plots represent percentages of cells that fall within the indicated quadrant. (B) Cells were analyzed as in (A). Statistical analysis of CD4$^+$ T cell proliferation of 3 independent experiments. Only the mean percentages ± SD of CellTrace-violet$^+$7-AAD$^-$CD3$^+$ T cells are depicted. * P < 0.05. (C) After 6 days of co-culture, CD4$^+$ T cells were restimulated with PMA/ionomycin for 6 hours in the presence of Brefeldin A and analyzed by flow cytometry for IFN-γ expression. Shown are the mean percentages (of a triplo) of IFN-γ$^+$ T cells of one representative out of 3 independent experiments. Error bars indicate SD of a triplo measurement.
of miR-146a in the CAL-1 pDC model cell line downregulated expression of IRAK1, enforcing earlier findings that the transcript encoding this protein is a *bona fide* target of miR-146a. Overexpression of miR-146a increased the level of apoptosis induction in CAL-1 cells, which correlated with lower BCL2-A1 mRNA levels, as well as impaired the expression of co-stimulatory molecules and pro-inflammatory cytokines in response to TLR ligation. Consistent with this, miR-146a transduced CAL-1 cells after activation with the TLR7 agonist R848 were hampered to induce optimal T cell expansion in an allogeneic setting as compared to control transduced cells. It has been demonstrated that miR-146a was induced upon TLR2, TLR4, or TLR5 signalling in the human acute monocytic leukemia cell line THP. While miR-146a expression was not affected after ligation of TLR3, TLR7, and TLR9 in this cell line, others reported that miR-146a expression was induced in PBMCs upon TLR7 and TLR9 triggering. It is currently unclear how to interpret the differential TLR7/9 responsiveness, but it may be attributed to the fact that in one study a monocytic cell line was used and in the other case primary PBMCs. Our results here confirm and extend the findings in primary cells as we demonstrated that miR-146a expression is induced in freshly isolated pDCs both in response to the TLR7 ligand R848 as well as the TLR9 ligands CpG-A and B. Our results are consistent with the notion that in human peripheral blood only pDCs selectively express TLR7 and TLR9. To more extensively analyze the role of miR-146a in pDCs we used the leukemic pDC cell line CAL-1. Recently, we reported that this cell line has many phenotypical and functional overlapping characteristics compared to primary pDCs, including their responsiveness to TLR7 and TLR9 ligation, which validates the use of CAL-1 cells to study the role of miRNAs in gene regulation in pDCs.

In line with earlier observations that IRAK1, which is a key adaptor molecule in the TLR signalling cascade required for activation of NF-kB, is a target of miR-146a, we show that IRAK1 levels were reduced in miR-146a CAL-1 cells. As expected, this correlated with decreased levels of phospho-p65 induced by activation of both TLR7 and TLR9 signalling pathways. Moreover, this correlated with reduced levels of BCL2-A1, co-stimulatory molecules and cytokines, which all depend on NF-kB activity in pDCs.

An important role of miR-146a in regulating myeloid and lymphoid development, growth/proliferation, and survival of leukocytes in mice was demonstrated in miR-146a−/− mice. These miR-146a−/− mice suffered from severe splenomegaly, which was mostly due to increased myeloid proliferation in comparison to wild type (WT) animals. Interestingly, several studies in humans reported that the levels of miR-146a were reduced in various acute myeloid leukemias (AMLs) as compared to healthy CD34+ progenitor cells. When enforcing expression of miR-146a in these AML cell lines, this resulted in a significant block in cell proliferation and in the induction of apoptosis, thereby implicating this miRNA in the pathogenesis of AML. The mechanism underlying these observations remained unaddressed. Interestingly, our data demonstrated that overexpression of miR-146a in CAL-1 cells induced apoptosis as well, and correlated with reduced levels of BCL2-A1 as compared to control transduced cells. While we speculated that miR-146a may control cell survival through direct targeting of this anti-apoptotic gene, using the *in silico* prediction software TargetScan (release 6.2, June 2012, www.targetscan.org) no miR-146a binding sites in the 3′-UTR of the BCL2-A1 mRNA were found (data not shown). We also
studied whether the transcript of Spi-B is targeted by miR-146a, since Spi-B is highly expressed in pDCs and directly regulates BCL2-A1 gene expression.27 Despite the fact that the 3′-UTR of Spi-B mRNA is predicted to contain miR-146a binding sites, however, we were unable to observe a consistent reduction in Spi-B protein levels in miR-146a CAL-1 cells (JJK, unpublished findings). This, together with the notion that bona fide targets of miR-146a are TRAF6 and IRAK1,22 suggests that in pDCs, and possibly also AML, cell survival by miR-146a is more likely to be regulated at the level of NF-κB activation instead of its target genes.

Excessive or inappropriate immune activation can be deleterious for the organism, requiring the need for various molecular mechanisms ensuring tight control of the immune response. A significant number of studies have linked alteration of miR-146a expression with initiation, development, and severity of several autoimmune diseases. Studies in mice showed that the serum of aged miR-146a−/− mice contained on average a 60-fold higher titer of auto-antibodies directed against double-stranded DNA as compared to WT animals.23 This, together with the observation that these mice develop a pathological condition strongly suggests the involvement of miR-146a in preventing autoimmune disease. In humans, miR-146a expression levels in PBMCs isolated from SLE patients were found to be decreased as compared to healthy control individuals.25 Furthermore, low miRNA-146a levels were correlated with increased severity of the disease. Given the accepted role of pDCs in the pathogenesis of SLE via production of elevated levels of type I IFNs, it has been speculated that downregulation of miR-146a in these cells may be one of the leading mechanisms driving their uncontrolled activation. This notion is enforced by our results showing that miR-146a controls the levels of type I IFNs in pDCs. Conversely, contrasting data were reported on patients suffering from SS and rheumatoid arthritis (RA), two autoimmune diseases in which pDCs have been implicated as well.11,12,36 These studies showed that miR-146a was overexpressed in PBMCs and synovial fluid, as compared to healthy control individuals. It is noteworthy that the levels of miR-146a in RA and SS patients have only been analyzed in total PBMCs and not in individual cell types, including pDCs. As pDCs represent only a minor population of total PBMCs, a putative downregulation of miR-146a specifically in pDCs of these patients may have remained unnoticed. Moreover, miR-146a levels are increased in CD4+ T cells from RA patients, and since the number of CD4+ T cells in blood is much higher compared to pDCs, this more likely reflects the observed difference when analyzing total PBMCs. Further, elevated miR-146a levels in total PBMCs may be the consequence of chronic inflammation leading to constitutive NF-κB activation. Hence, it is important to perform expression profiling of miRNAs, but also of mRNAs, in purified leukocyte subsets in order to correlate the level of expression to their putative role in a disease setting.

Previously it was demonstrated that T cells from lupus patients exhibited a marked increase in proliferation as assessed by Ki67 staining.41 Hence, T cells in these patients may have received substantial activation signals in vivo. The molecular mechanisms underlying excessive T cell activation in SLE have not been fully understood, but may be attributed to abnormal CTLA-4 expression in lupus responder T cells or aberrant signalling downstream of the T cell receptor.42 Here, we provide evidence that miR-146a regulates the maturation status of pDCs, thereby impairing their capacity to stimulate proliferation of T cells. Hence, it is reasonable to assume
that underexpression of miR-146a in pDCs, as observed in lupus,\textsuperscript{25} leads to increased levels of co-stimulatory molecules on pDCs that will enhance T cell proliferation. Therefore, reduced levels of miR-146a in lupus pDCs may not only drive the overproduction of type I IFNs, but in addition may increase the expansion of lupus T cells. These T cells may subsequently contribute in the generation of auto-antibody producing B cells.

Collectively, our results further clarify the crucial role of miR-146a as a brake of the TLR-induced activation and maturation specifically in pDCs. Given the growing evidence indicating that pDCs are key players in the initiation and/or maintenance of autoimmune diseases, our work enforces that therapies aimed at targeting the miR-146a-dependent regulation of immunity and inflammation in pDCs has potential for future interventions.

ACKNOWLEDGMENTS

We thank Pr. R. Agami for providing us with miRNA expressing vectors (Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands). We thank Berend Hooibrink and Toni van Capel for maintaining the FACS facility. We acknowledge Prof.dr. Hazekamp, and staff from the LUMC (Leiden, The Netherlands) for providing human thymus tissue. We thank Dr. R. Schotte for critically reading the manuscript and Femke Muller for technical assistance. JJK is supported through a personal VIDI grant to BB (Dutch Science Foundation, no. 917.66.310). LCMJ and CHU are supported by a grant from the National Institutes of Health (AI 080564, University of California Los Angeles, Center for AIDS Research).

AUTHORSHIP

Contributions: J.J.K. designed research, performed experiments, analyzed data, and wrote the manuscript; L.C.M.J. designed research, performed experiments, analyzed data, and wrote the manuscript, M.L., A.I., K.I.B. M.N. E.W.T-K performed experiments and analyzed data; E.C.dJ., C.H.U. analyzed data; B.B. designed research, analyzed data and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES


Supplementary Figure 1: Ectopic expression of miR-146a in CAL-1 cells decreases IRAK1 protein levels. Western blot analysis of miR-146a transduced CAL-1 or hTR-control cells. Cells were lysed in NP-40 lysis buffer, and protein levels of IRAK1 were analyzed using an anti-IRAK1 specific antibody. Protein levels of β-actin were measured as loading controls. Relative IRAK1 protein levels (normalized to β-actin protein levels) are shown. Protein levels were quantified by using the BioRad QuantityOne software.

Supplementary Figure 2: The NF-κB inhibitor Bay 11-7082 induces cell death in CAL-1 cells. CAL-1 cells were incubated with the NF-κB inhibitor Bay 11-7082 at indicated concentrations for 11 hours. Plotted are the percentages of apoptotic cells as measured by flow cytometric analysis of AnnexinV+ cells.

Supplementary Figure 3: Increased apoptosis induction in miR-146a CAL-1 cells as compared to control CAL-1 cells. CAL-1 cells were transduced with hTR control RNA or miR-146a. Two days after transduction, GFP+ cells were sorted and cultured in medium. On the days indicated, cells were stained with AnnexinV and 7-AAD and apoptosis was analyzed by flow cytometry. Shown is the relative fold difference in the percentage ± SD of early apoptotic (AnnexinV+ 7-AAD−) CAL-1 cells that were transduced with miR-146a (open bar) as compared to control CAL-1 cells (which is set at 1, dotted line). Values are based on 2 independent experiments.
Supplementary Figure 4: Ectopic miR-146a expression impairs TLR-induced cytokine secretion in CAL-1 cells. IL-6 and IFN-β were measured by ELISA in the supernatants of CAL-1 cells that were transduced with hTR control (white bar) or miR-146a (black bar). Two days after transduction, GFP+ cells were sorted and cultured in medium or activated with (A) the TLR7 ligand R848 (10 µg/mL) or (B, C) the TLR9 ligand CpG-B (10 µg/mL) for 18h. Shown are the mean concentrations of cytokines (ng/mL ± SD) of one representative duplo out of 3 independent experiments. nd, not detectable.

Supplementary Figure 5: Ectopic miR-146a expression impairs pDC maturation. Flow cytometric analysis of CAL-1 cells after transduction with hTR control (white bar) or miR-146a (black bar). Cells were activated overnight with the TLR9 ligand CpG-B (10 µg/mL) and stained for expression of CD40, CD80, CD86, HLA-DR, and CCR7. Shown are the relative mean fluorescence intensities (MFIs) ± SD of 4-8 independent experiments. MFIs of proteins expressed on hTR control cells are set to 1. * P < 0.05, ** P < 0.01.
Supplementary Figure 6: Silencing of miR-146a expression in TLR7-activated CAL-1 cells enhances their alloreactive potential to stimulate T cell proliferation. Flow cytometric analysis of T cell proliferation induced by CAL-1 cells transfected with control LNA or miR-146a LNA that were pre-activated for 48h with or without TLR7 agonist R848 (10µg/ml). Pre-activated CAL-1 cells were co-cultured together with freshly isolated allogeneic CD4+ T cells (ratio CAL-1:CD4+ T cells = 1:1) after labeling with the CellTrace violet membrane dye. After 6 days, T cells were analyzed for expression of CellTrace violet and 7-AAD in CD3+ T cells. Percentages of CellTrace-violet7-AAD CD3+ cells represent T cells that proliferated and are alive (lower left quadrant). Shown is one representative experiment out of 2. Numbers in plots represent percentages of cells that fall within the indicated quadrant.

Supplementary Table 1: Primer sequences. Primer pairs were tested for specificity by melting curve analysis and gel electrophoresis. Primer efficiencies were determined by template dilution and were highly similar.

<table>
<thead>
<tr>
<th>Primers for PCR or QPCR</th>
<th>Forward (5'-3')</th>
<th>Reverse (5'-3')</th>
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<tr>
<td>IFN-β1</td>
<td>GAGCTACAACCTTGCTTGATCC</td>
<td>CAAGCCCTCCCATTCAATTGC</td>
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