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miR-146a Is Differentially Expressed by Myeloid Dendritic Cell Subsets and Desensitizes Cells to TLR2-Dependent Activation

Jennifer Jurkin,* Yvonne M. Schichl,† Rene Koeffel,* Thomas Bauer,* Susanne Richter,* Sabine Konradi,* Bernhard Gessbauer,* and Herbert Strobl*

Langerhans cells (LCs) in epithelia and interstitial dendritic cells (intDCs) in adjacent connective tissues represent two closely related myeloid-derived DC subsets that exert specialized functions in the immune system and are of clinical relevance for cell therapy. Both subsets arise from monocyte-committed intermediates in response to tissue-associated microenvironmental signals; however, molecular mechanisms underlying myeloid DC subset specification and function remain poorly defined. Using microarray profiling, we identified microRNA (miRNA) miR-146a to be constitutively expressed at higher levels in human LCs compared with intDCs. Moreover, miR-146a levels were low in monocytes and nondetectable in neutrophil granulocytes. Interestingly, constitutive high miR-146a expression in LCs is induced by the transcription factor PU.1 in response to TGF-β1, a key microenvironmental signal for epidermal LC differentiation. We identified miR-146a as a regulator of monocyte and DC activation but not myeloid/DC subset differentiation. Ectopic miR-146a in monocytes and intDCs interfered with TLR2 downstream signaling and cytokine production, without affecting phenotypic DC maturation. Inversely, silencing of miR-146a in LCs enhanced TLR2-dependent NF-κB signaling. We therefore conclude that high constitutive miR-146a levels are induced by microenvironmental signals in the epidermis and might render LCs less susceptible to inappropriate activation by commensal bacterial TLR2 triggers at body surfaces. The Journal of Immunology, 2010, 184: 4955–4965.

Epidermal Langerhans cells (LCs) form a network of dendritic cells (DCs) in basal/suprabasal layers of the epidermis and are characterized by a unique phenotype (CD207+/CD1a\textsuperscript{bright} CD324+/E-Cadherin+ CD11b\textsuperscript{+}) (1–3). Because of their specific location at body surfaces, LCs are constantly exposed to environmental stimuli. Conversely, interstitial-type DCs (intDCs) are located in adjacent tissues and can be discriminated from LCs phenotypically (CD207− CD1a\textsuperscript{dim} CD324− CD11b\textsuperscript{+}). These DCs constitute a second line of defense against pathogens that have crossed the epithelial barrier. During development both DC subsets originally arise from myeloid progenitor cells via monocyte-committed intermediates in response to specific microenvironmental signals (4). In addition, certain pools of DCs can be replenished by tissue resident cells or monocytes in response to specific microenvironmental signals (2, 4, 5).

MicroRNAs (miRNAs) are a novel class of small noncoding RNA regulators that have been implicated in immune cell development and activation (6, 7). In particular, miRNAs regulate gene expression on a post-transcriptional level either by repressing the translation of their target mRNAs or by inducing mRNA degradation. Many miRNAs are differentially regulated in various hematopoietic lineages and tissues. In addition, some miRNAs have been shown to play important roles as modulators of hematopoietic lineage differentiation. For example, miR-223 is abundantly expressed in granulocytes and positively affects granulopoiesis by targeting NF-κB signaling molecular. miR-424 has been shown to promote monocytogenesis by transcriptional repression of NF-κB resulting in the upregulation of macrophage-CSF receptor (9). In megakaryopoiesis, miR-146a plays a negative role by targeting CXCR4, whereas miR-150 induces megakaryocytes at the expense of erythrocytes by targeting v-myb myeloblastosis viral oncogene homolog (c-Myb) (10). In addition to their role in hematopoiesis, recent studies identified miRNAs as regulators of innate and adaptive immune responses. For example, miR-150 and miR-155 have been implicated in the regulation of T and B cell responses (11–16). Furthermore, miR-181a was shown to modulate TCR signaling thresholds in different stages of T cell development by targeting various phosphatase family members (17). Several studies linked miR-146a and NF-κB signaling within the innate immune system (18–22). These reports were pioneered by a study published by Taganov et al. in 2006 (18), which showed that miR-146a is quickly induced upon activation of monocytes. Considering its confirmed targets, IL-1R-associated kinase 1 (IRAK1) (18) and TNFR-associated factor 6 (TRAF6) (18, 23), miR-146a may act as a negative regulatory feedback mechanism by interfering with TNFR/IL1R and TLR pathways as well as...
miR-146a REGULATES DC SENSITIVITY TO TLR-MEDIATED ACTIVATION

with NF-kB activation (18, 24) and retinoic acid-inducible gene I dependent type I IFN production (25).

Despite multiple reports on a role of miRNAs in the regulation of innate and adaptive immune response, little is known how miRNAs regulate the DC system. The miR-34a and miR-21 regulation of wingless-type mouse mammary tumor virus (MMTV) integration site family, member 1 (WNT1) and jagged 1 (JAG1) was recently shown to be important for proper differentiation of DCs from monocyte precursors (26). Furthermore miR-155 expression was shown to be important for proper DC activation (11) probably via the regulation of suppressor of cytokine signaling 1 protein levels (27). Moreover, miR-155 seems to target PU.1 in DCs leading to downregulation of the C-type lectin CD209 and a concomitant decrease in CD209-mediated pathogen binding (28). However, so far it is unknown whether miRNAs are involved in the differential regulation of DC subset development or function.

GM-CSF/IL-4-dependent monocyte-derived DCs (moDCs) generated either from CD14+ monocytes or via CD14+/CD11b+ mono-
cyte intermediates from CD34+ progenitor cells share many char-
acteristics with intDCs (29) and inflammatory dendritic epithelial cells in vivo (30). On the other hand, LCs generated from CD34+
cells under serum-free TGF-β1-dependent conditions phenotypi-
cally resemble epidermal-resident LCs (31, 32). Because these two DC subsets are of considerable interest for clinical cell therapy studies (33), we rationalized that an improved understanding of their development and function is of substantial relevance. In this study, we screened for miRNAs differentially expressed by LCs versus intDCs. We identified miR-146a, a postulated negative reg-
ulation of DC subset development or function.

Materials and Methods

Isolation of cells

Cord-blood samples from healthy donors were collected during healthy full-
term deliveries. Approval was obtained from the Medical University of Vienna institutional review board for these studies. Informed consent was provided in accordance with the Declaration of Helsinki. CD34+ cells were isolated as described (34). CD14+ monocytes were isolated from peripheral blood as described (34). CD14+ LCs were enriched from epidermal cell suspensions from healthy individuals undergoing reconstructive surgery as described (35, 36). LCs were further purified using FACS sorting to obtain purities that exceeded 95%.

Cytokines and reagents

Human stem cell factor (SCF), thrombopoietin, TNF-α, GM-CSF, Flt3, IL-6, IL-1α, macrophage-CSF, and G-CSF were purchased from PeproTech (London, U.K.); TGF-β1; IFN-γ and IL-1β was purchased from R&D Systems (Wiesbaden, Germany); ultrapure LS from Escherichia coli and Pam3CSK4 was purchased from InvivoGen (San Diego, CA); peptidogly-
can (PGN) Staphylococcus aureus was purchased from Sigma-Aldrich (St. Louis, MO).

In vitro culture of CD34+ cord-blood cells

CD34+ cord-blood cells were cultured serum-free for 2 to 3 d under pro-
genitor expansion conditions (Flt3, SCF, and thrombopoietin, each at 50 ng/ml) prior to subculturing with lineage specific cytokines. LC cultures were described before (37). Clusters were purified by 1 g sedimentation as described previously (38). intDCs were generated using a previously described two-step culture system with slight modifications (29). Briefly, CD34+ DCs (1 × 10^6 to 2 × 10^7/ml per well) were cultured in 24-well tissue culture plates in serum-free CellGro DC medium (CellGenix, Freiburg, Germany) supplemented with 10% FCS, GM-CSF (100 ng/ml), SDF (20 ng/ml), Flt3 (50 ng/ml), and TNF-α (2.5 ng/ml) for 3–5 d and subsequently in RPMI 1640 (10% FCS) supplemented with GM-CSF (100 ng/ml) and IL-4 (2.5 ng/ml) for 4–5 d. For the generation of monocytes and granulocytes, expanded progenitor cells were cultured in serum-free X-VIVO 15 medium (BioWhittaker, Walkersville, MD) as described (34). All cultures were supplemented with GlutaMAX (2.5 mM; Life Technologies/Invitrogen, Carlsbad, CA) and penicillin/streptomycin (125 U/ml each).

Cell lines

U937TE cells expressing the ecotropic Moloney murine leukemia virus envelop receptor were described previously (39). Cell lines were main-
tained in RPMI 1640 medium (Sigma-Aldrich) plus 10% FCS. U937TE cells were induced to develop into CD14+CD11b+ monocytes in the presence of 2.5 ng/ml 1,25-VD3 as described (34).

Microarray analysis and miRNA target analysis

Total RNA was isolated from purified intDCs and LCs generated in parallel cultures from three different donors. Total RNA was isolated using the miRNA mini Kit (Qiagen, Hilden, Germany). RNA from the three different donors was pooled. RNA processing, microarray fabrication, array hy-
bridization, data acquisition, and data processing were performed by LC Sciences (Houston, TX). Human miRNA array chip (Chip ID: MRA1001-
human) based on the Sanger miRBase Release 10.0, was used. Details to the chip as well as the detailed process can be found at www.lcsciences.com. Data were adjusted by log2 transformation. Differentially detected signals were accepted as valid when the ratios of the p value were <0.01. Micro-
array data were submitted to the GEO database (www.ncbi.nlm.nih.gov/ geo; accession number GSE20215). Target prediction was performed using the open source program targetscan (www.targets.can.org).

RNA isolation and real-time RT-PCR

Cells were harvested and total RNA was isolated using miRNAeasy Mini Kit (Qiagen) including DNase I treatment according to the manufacturer’s in-
structions. Purified RNA was subjected to polyadenylation and cDNA synthesis using the Ncode miRNA First-Strand cDNA Synthesis Kit (In-
vitrogen) according to the manufacturer’s instructions. Real-time RT-PCR was performed in a Roche LightCycler using Platinum SYBR Green quantitative PCR SuperMix-UDG (Invitrogen). Melting curve analyses were performed. Values were normalized to SS RNA. Forward primers were always identical to the entire mature miRNA or SS RNA sequence. The universal quantitative PCR Primer supplied with the Ncode miRNA First-
Strand cDNA Synthesis Kit (Invitrogen) was used as reverse primer. The miRNA isolation and RT-PCR were performed as described (36). The fol-
lowing primers were used: TLR2: forward, 5'-GCC ACT CCA GGT AGG TCT TG-3'; HPRF: forward, 5'-GAC CAG TCA ACA GGG GAC AT-3'; reverse, 5'-'GATCGTAAAGCAAAATGTTGAGTACAGA-3'. The fragments produced by PCR contained the miRNA precursor (99 nt) flanked on both sides by around 250 nt of genomic sequence, resulting in an insert of 613 nt. The MSCI-LMP vector containing a nonsilencing hairpin (Open Bio-
systems, Huntsville, AL) served as control vector. PBMMN-PU.1-L-1GFP was described previously (40). Transfection of packaging cell lines Phoenix-E and Phoenix-GP as well as infection of target cells was performed as pre-
viously described (41). For miR-146a specific knock-down in LCs, cells were purified by 1 g sedimentation (38), yielding 70–90% purity. Afterward, cells were transfected with 25 pmol miR-146a knock-down or unspecific (miR-159) linolenic acid (LNA) oligos (Exigion, Vedbaek, Denmark) using the Amaxa Nucleofector Device (Cologne, Germany). Cells were replated 24 h before further analyses.

Western blot analysis

Cells were directly lysed in 1× SDS-loading dye at 95°C for 5 min. For Western blot analysis, lysates of 1–2 × 10^6 cells per lane were loaded on 10% SDS-polyacrylamide gels. Resolved proteins were transferred to a polyvinylene-difluoride membrane (Immobilon-P; Millipore,Billericia, MA). Membranes were probed with Abs against phosphorylated p38 MAPK; total p38 MAPK; phosphorylated ERK; total ERK, phosphory-
lated JNK; total JNK (Cell Signaling Technology, Beverly, MA); phospho-
rylated IκBα (Cell Signaling Technology); total IκBα, PU.1, C/EBPα,
FIGURE 1. Identification of miRNAs inversely expressed by LCs versus intDCs. 
A, Schematic overview on in vitro generation of LCs and intDCs from CD34+ granulocyte/monocyte progenitor cells. B, Histograms represent gated CD1a+ cells analyzed for the indicated molecules. C, Microarray analysis of miRNA expression in purified LCs and intDCs. FACS diagrams depict phenotypic characteristics and sort-window settings. The scatter plot shows background-subtracted raw intensities for each probe on both channels for Cy3-labeled LC and Cy5-labeled intDC samples. Each dot represents one miRNA probe. The differentially expressed (p < 0.01) miRNAs are indicated in red. D, Bar representation of differentially expressed miRNAs as log2 transformed difference between signal values of LCs versus intDCs. E, Real-time RT-PCR validation of candidate differentially expressed miRNAs in LCs versus intDCs. Experiments were performed in duplicates. Bars represent mean values of two to six independent donors (as indicated) (± SEM). F, Real-time RT-PCR against miR-146a. Sorted in vitro generated LCs, intDCs, monocytes, and granulocytes either unstimulated or stimulated (50 ng/ml IFN-γ, 20 ng/ml IL-1β, and 2 μg/ml LPS) for 48 h were analyzed for miR-146a expression levels. Values represent the mean fold induction (± SEM) observed in four independent donors. *p < 0.05; ***p < 0.001. G, Real-time RT-PCR analysis of miR-146a expression in ex vivo isolated cells. LCs and keratinocytes were isolated from human epidermis. Monocytes were purified from peripheral blood. Bars represent the mean of two independent donors (± SD).
FIGURE 2. Constitutive miR-146a in LCs is controlled by the transcription factor PU.1. A, Schematic representation of the miR-146a promoter. PU.1 and C/EBPα sites are indicated in gray. Binding sites of primers used for ChIP experiments are marked using arrows. B, Sorted cell fractions of primary myeloid/DC subsets as well as U937 cells analyzed for PU.1 and C/EBPα by Western blotting (LCs; intDCs; Mo, monocytes; G, granulocytes). C, Western blot against PU.1 protein levels of CD34+ cells cultured 6 TGF-β1 for 72 h under LC promoting conditions. D, FACS analysis of CD34+ cells cultured 6 TGF-β1 for 72 h under LC promoting conditions. Diagrams represent CD207 versus PU.1 or isotype stainings. Histograms represent total PU.1 levels of myeloid progenitors in LC promoting cultures at 0 h (filled histogram), 72 h without TGF-β1 (gray line), and 72 h with TGF-β1 treatment (black line). Dotted line represents isotype control. E–G, MiR-146a promoter luciferase reporter assays in 293T cells. MiR-146a promoter-luc and indicated expression plasmids were cotransfected and luciferase reporter assays were performed. The promoter construct was previously described (22); promoter sequences are depicted schematically in A. Luciferase values were normalized to cotransfected β-Gal and are represented as mean fold induction. Error bars represent
TRAF6 (Santa Cruz Biotechnology, Santa Cruz, CA), or β-actin (Sigma-Aldrich, Vienna, Austria), followed by HRP-conjugated goat anti-rabbit or rabbit anti-goat IgG Abs (Pierce Biotechnology, Rockford, IL). Detection was performed with the chemiluminescent substrate SuperSignal WestPico or WestDura (Pierce Biotechnology).

Flow cytometry
Flow cytometry staining and analysis were performed as described (41). Murine mAbs of the following specificities were used: FITC-conjugated mAbs specific for CD14 and CD1a (BD Biosciences, San Jose, CA); PE-conjugated mAbs specific for CD207 (Immunecheck, Marseille, France), CD14, CD15, CD83 (BD Biosciences) lactoferrin (Caltag, An der Grub, Austria), TLR2 [clone TLR2.1 (42) (Biologend, San Diego, CA)] and isotype control (clone VLA-1, kindly provided by O. Majdic, Vienna, Austria); Biotinylated mAbs specific for CD80, CD86 and CD11b (BD Biosciences); Second step reagent was streptavidin (SA)-PerCP (BD Biosciences); Allophycocyanin conjugated Abs for CD1a and CD14 (BD Biosciences) and CD324 (Biologend). Intracellular staining of lactoferrin was described previously (34). For intracellular staining of PU.1, cells were fixed with 2% paraformaldehyde for 10 min at 37°C and were made permeable with 90% methanol. Afterward, cells were stained with anti-PU.1 (Santa Cruz Biotechnology) or isotype control (Cell Signaling Technology) and indicated markers. Second step reagent was Alexa 647 labeled goat anti-rabbit IgG (Invitrogen, Molecular Probes, Eugene, OR).

Flow cytometric analysis was performed using a LSRII instrument (BD Biosciences) and the CellQuest Pro software (BD Biosciences). For FACS sorting the BD FACSAria flow cytometer (BD Biosciences) was used.

Promoter analysis and chromatin immunoprecipitation
Putative binding sites for transcription factors were identified using TESS (www.cbil.upenn.edu/tess/index.html). For chromatin immunoprecipitation (ChIP) 5 × 10⁶ cells were treated with 0.6% formaldehyde in PBS for 2.5 min at 37°C, followed by the addition of glycine (final concentration, 0.25 M). After washing with PBS, cells were lysed on ice and sonicated to obtain 0.5- to 1 kb sheared chromatin fragments. Subsequent ChIP steps were performed according to the protocol from Upstate Biotechnology (Charlottesville, VA). Each ChIP reaction included 2 µg anti-PU.1 (Santa Cruz Biotechnology); anti-Cyp1al (Santa Cruz Biotechnology) served as unspecific control. The presence of DNA target sequences was assessed by PCR. PCR products were resolved by 2% agarose gel electrophoresis and quantified using a Lumiprin imager analyzer and the Lumianalysis software (Roche, Basel, Switzerland). Signal intensities at input dilutions of 1:5 were compared with Ab precipitates and unspecific controls. MiR-146a promoter specific primers were 5'-CGCCTGACGACATTCTTC-3' (forward) and 5'-ACGCTAACCTCT-CTGGAAAATC-3' (reverse).

Cytokine measurement
Cells were seeded (5 × 10⁶ to 1 × 10⁷/500 µl) in 48-well plates, and supernatants were collected 48 h later as described (39). Quantification of TNF-α, IL-12 p40 was performed using the Luminex system (Austin, TX).

Subcellular analysis of p65
Cells were washed and fixed with Fix and Perm (An der Grub, Kaumberg, Austria). They were put on an adhesion slides (Marienfeld, Lauda-Königshofen, Germany), fixed with acetone and methanol and made permeable with 0.1% Triton X-100. Afterward, slides were blocked with 2% BSA/0.1% Tween20 in PBS and incubated with anti-p65 Ab (Santa Cruz Biotechnology), followed by incubation with goat anti-rabbit Alexa Fluor 546 (Invitrogen, Molecular Probes). DAPI (1 µg/ml) was added for 10 min room temperature. Cells were mounted with Dako Fluorescent mounting media (Dako UK, Cambridgeshire, U.K.). Pictures were taken on a Nikon Eclipse 80i microscope with a MuTech MV2500 camera using the Lucia G Software for image acquisition.

Luciferase assays
293T cells were grown in 24-well plates and transfected with the pGL3basic-miR-146a-promoter Luciferase construct (kindly provided by K. Pichler, Erlangen, Germany) and empty vector control or the indicated expression plasmids using the calcium phosphate method. PBMM-PU.1-IRES-GFP was described previously (40). GC-C/EBSpo-GFP was a kind gift from A. Iwama (Tsukuba, Japan) (43). Luciferase values were normalized to cotransfected β-galactosidase.

Statistical analysis
Statistical analysis was performed using the paired, 2-tailed Student t test; p values of <0.05 were considered significant.

Results
Identification of miRNAs inversely expressed by LCs versus intDCs
To identify differentially expressed miRNAs in DC subsets, we performed a miRNA screen. Therefore, we generated LCs (37) or intDCs (29) from human CD34+ cord blood hematopoietic progenitor cells as described (Fig. 1A). The well-established immunophenotype of LCs (CD1a⁺CD11b⁻CD207/E-Cadherin⁺) and intDCs (CD1a⁺CD11b⁺CD207⁻CD324⁻) was confirmed by FACS (Fig. 1B). These two DC subsets were purified (Fig. 1C, upper panel) and submitted to differential miRNA profiling (Fig. 1C, lower panel). Forty-six miRNAs were identified to be >2-fold differentially expressed by LCs versus intDCs (Fig. 1C, 1D). Among these, several upregulated (miR-146a, miR-92, miR-19b, and miR-181a) and downregulated miRNAs (miR342-3p, miR424, miR21, miR663, and miR29a) were validated by real-time RT-PCR (Fig. 1E) and analyzed for their potential targets using a common bioinformatic algorithm (Materials and Methods). miR-146a was of particular interest for further analysis, because evidence from previous studies indicated a regulatory function for this miRNA in TLR signaling (18).

miR-146a is expressed at high levels in nonactivated LCs
It has recently been described that miR-146a is rapidly induced in monocytes upon activation (18). Based on this finding together with the fact that miR-146a targets TRAF6 and IRAK1, it was proposed that miR-146a is involved in a negative-regulatory feedback loop securing monocyte deactivation after stimulation (18). In contrast, we identified high levels of miR-146a in unstimulated LCs (Fig. 1E, 1F). Therefore, we further compared the magnitude of miR-146a expression in LCs to the expression in other myeloid cell subsets, including activated monocytes. Among all cell types studied, LCs clearly expressed highest levels of miR-146a (~6-fold more than intDCs, and 50-fold more than monocytes, Fig. 1F). Interestingly, miR-146a was virtually undetectable in granulocytes (Fig. 1F). In response to stimulation with IFN-γ, IL-1β, and LPS, monocytes strongly upregulated miR-146a expression (~50-fold). Importantly, miR-146a expression levels in stimulated monocytes did not exceed those observed in unstimulated LCs (Fig. 1F). Therefore, we identified miR-146a to be constitutively expressed at high levels in immature LCs. Moreover, in contrast to monocytes that strongly upregulate miR-146a upon stimulation, activated LCs did not show enhanced miR-146a expression levels (Fig. 1F). This indicates that miR-146a may participate in different regulatory pathways in LCs versus monocytes. In line with this, real-time RT-PCR analysis revealed substantially higher miR-146a RNA levels in ex vivo isolated LCs compared with peripheral blood monocytes or ex vivo isolated keratinocytes (Fig. 1G).
Constitutive miR-146a in LCs is controlled by the transcription factor PU.1

We next investigated how miR-146a expression levels are induced/maintained in LCs. In silico analyses revealed two PU.1 and two C/EBPα binding sites in the miR-146a promoter (Fig. 2A). Interestingly, these two transcription factors are critically involved in the control of LC subset specification from myeloid progenitors (43). We and others previously showed that ectopic PU.1 strongly promotes LC differentiation (36, 40, 43). Similarly, ectopic expression of A-C/EBPα, a dominant negative molecule, which interferes with various C/EBP members, was shown to promote LC differentiation at the expense of granulomonocytic cells in cultures of human CD34+ cells (43). Consistent with these data, we found LCs to lack C/EBPα, and to express high levels of PU.1 (Fig. 2B). In comparison, intDCs express substantially lower PU.1 levels than LCs (Fig. 2B). The differentiation of CD207+ LCs is strictly dependent on TGF-β1 (32, 37, 44, 45). Furthermore, it was shown that TGF-β1 induces PU.1 during LC differentiation (40) and that ectopic PU.1 promotes LC differentiation (40, 43). In line with this, PU.1 levels were substantially higher in cultures supplemented with LC-inducing cytokines plus TGF-β1 than in parallel cultures in which TGF-β1 was omitted (Fig. 2C, 2D, histogram). Furthermore, single-cell flow cytometric analysis for intracellular PU.1 versus CD207 confirmed that TGF-β1–dependent CD207+ cells segregate among PU.1+ cells (Fig. 2D, dot blots). Next, we studied miR-146a promoter regulation using the promoter fragment depicted in Fig. 2A. In luciferase assays, PU.1 transfected 293T cells showed >20-fold upregulation of miR-146a promoter activity compared to empty vector control (Fig. 2E). Conversely, cotransfection of C/EBPα resulted in downregulation of luciferase activity (Fig. 2F). It has been reported that C/EBPα can interfere with PU.1-mediated promoter induction by heterodimerization (46). In line with this, we found that cotransfection of C/EBPα completely abrogated PU.1-mediated induction of the miR-146a promoter (Fig. 2G). Together, these data indicate that C/EBPα represents a negative regulator of miR-146a expression, which is in line with its negative role in LC differentiation. In subsequent experiments using U937 monocytic cells, we found PU.1 to be endogenously bound to the miR-146a promoter (Fig. 2H, upper lane) of these cells. Furthermore, ectopic expression of PU.1 in this cell line leads to increased binding of PU.1 on the miRNA promoter (Fig. 2H, lower lane), which was accompanied by >70-fold induction of miR-146a expression levels compared with control cells (Fig. 2I). These data indicate that high PU.1 levels in LCs might be involved in the establishment of high miR-146a expression levels in these cells. In support for this, we found high levels of PU.1 endogenously bound to the miR-146a promoter in LCs (Fig. 2J).

Ectopic expression of miR-146a does not influence myelopoiesis or DC subset differentiation

We found that miR-146a levels are higher in LCs than in intDCs or unstimulated monocytes (Fig. 1F). Furthermore, miR-146a was undetectable in granulocytes (Fig. 1F). Because NF-κB and TRAF6 were shown to regulate myelopoiesis and DC subset differentiation (41, 47, 48) we analyzed whether ectopic miR-146a might influence myelopoiesis. Thus, we generated a retroviral expression construct for miR-146a by inserting the genomic sequence of miR-146a ± 250 bp flanking regions into the retroviral vector backbone murine stem cell virus-GFP (Fig. 3A). To validate the functionality of the mRNA construct, U937 cells were transduced with miR-146a or control vector and subsequently sorted for GFP+ cells (Fig. 3B). As expected, miR-146a expression levels were much higher in miR-146a transduced GFP+ cells compared with GFP+ cells transduced with non-silencing hairpin cells (Fig. 3C). Furthermore, miR-146a transduced cells showed diminished expression of the miR-146a target TRAF6 (Fig. 3D), confirming functionality of the produced miR-146a hairpin. To analyze the influence of miR-146a expression on DC subset generation and myelopoiesis, CD34+ cells were

FIGURE 3. Ectopic expression of miR-146a does not influence DC subset generation. A, The genomic DNA region encoding miR-146a ± 250 bp flanking region was inserted into a retroviral backbone 5′ of a PGK-driven GFP cassette. B–D, Ectopic miR-146a expression in U937 monocytic cells. GFP+ cells were sorted prior to analyses. B, Sort windows for control (CTRL) and miR-146a transduced cells. C, Real-time RT-PCR analysis against miR-146a of control (CTRL) or miR-146a–transduced U937 cells. Real-time RT-PCR was performed in duplicates. Bars represent mean (± SEM). D, Western blot of control (CTRL) or miR-146a–transduced U937 cells against TRAF6. E and F, Human CD34+ progenitor cells were transduced with vectors encoding miR-146a or control hairpin (CTRL) under progenitor expansion conditions. After 48 h transduction, cells were subcultured in LC, intDC, monocyte, or granulocyte cultures (Materials and Methods). Percentages of phenotypically defined cell subsets among GFP+ cells were determined; CD207+/CD11c+ (LCs), CD11b+/CD1a+ (intDCs), CD11b+/CD14+ (monocytes), and lactoferrin+ (granulocytes). Bars in E represent mean percentages (± SEM) of indicated cell subsets observed in four independent experiments. FACS diagrams in F represent gated GFP+ cells analyzed for indicated marker molecules.
transduced with these vectors, followed by induction of myeloid subset differentiation using specific cytokine combinations (Materials and Methods). GFP+ cells from control or miR-146a-transduced cultures showed similar percentages of LCs (CD207+CD1a+), intDCs (CD11b+CD1a+), monocytes (CD14+CD11b+), and granulocytes (lactoferrin+) (Fig. 3E, 3F), demonstrating that ectopic expression of miR-146a does not interfere with DC subset differentiation or myelopoiesis.

Ectopic miR-146a interferes with PGN-induced downstream signaling in monocyctic cells

Although previous studies suggested that miR-146a inhibits TLR signaling in monocyctic cells (18), functional data were not presented. To analyze whether high levels of miR-146a can interfere with TLRs signaling responses at a molecular level, we ectopically expressed miR-146a in monocyctic cells and performed TLR stimulation experiments. In these experiments, U937 cells were transduced with miR-146a or a control vector and subsequently sorted for GFP+ cells. Thereafter, cells were stimulated to differentiate into a homogenous population of CD14hiCD11b+ monocytes (i.e., U937-Mo) in response to 1,25-VD3 as described previously (34). On stimulation, miR-146a–transduced U937-Mo cells consistently showed dose dependently diminished downstream signaling in response to PGN relative to control-transduced cells (Fig. 4A). Specifically, miR-146a transduced cells displayed lower levels of IKKα phosphorylation and degradation, which indicates impaired NF-κB signaling (Fig. 4A). In addition, they showed decreased p-p38 levels (Fig. 4A). Conversely, p-ERK remained largely unaffected (Fig. 4A). To exclude the possibility that diminished downstream signaling might be due to miR-146a mediated TLR2 downregulation, we analyzed TLR2 expression by miR-146a transduced U937 cells and intDCs. TLR2 expression levels were not reduced in cells expressing miR-146 (miR-146a, GFP+) compared with cells transduced with a nonsilencing control

**FIGURE 4.** Ectopic miR-146a expression interferes with PGN downstream signaling responses in monocytes and intDCs. A. Ectopic miR-146a expression in U937 monocyctic cells. GFP+ cells were sorted prior to analyses. Control and miR-146a–transduced U937 cells were differentiated in the presence of 1,25-VD3 into monocyte-like cells, activated for 15 min with the indicated concentrations of PGN and analyzed for the expression of the indicated proteins by Western blot. B and C. Ectopic miR-146a expression in intDCs. Human CD34+ progenitor cells were transduced with vectors encoding miR-146a or control hairpin (CTRL) under progenitor expansion conditions. Forty-eight hours after transduction, cells were cultured under intDC instructing conditions. Cells were FACs sorted on day 10 for GFP+CD1a+CD11b+ cells. B. Representative sort windows (upper panel). Real-time RT-PCR analysis of control (CTRL) versus miR-146a–transduced cells (lower panel). Bars represent mean relative miR-146a levels of three independent experiments (±SEM). C. Sorted cells were activated for 15 min with graded amounts of PGN and protein extracts were analyzed for the indicated signaling proteins by Western blot. D. Immunofluorescence stainings of miR-146a or control hairpin (CTRL) transduced intDCs. CTRL or miR-146a transduced CD34+ cells were differentiated under intDC instructive conditions and sorted for GFP versus CD1a/CD11b as described in B. Sorted cells were activated for 1 h with 0, 1, 10, or 50 μg/ml PGN and stained for p65/Alexa 546 (red) and DAPI (blue). Representative stainings of cells activated with 1 μg PGN are shown in the left panel. Arrows indicate cells that show nuclear p65 expression. White bars correspond to 10 μm. Bar diagrams on the right represent mean percentages (±SEM) of cells that display p65 in the nucleus after stimulation with the indicated concentrations of PGN (n=3). E. MiR-146a specific knock-down in LCs. LC clusters were purified by 1 g sedimentation yielding a 70–90% pure population of CD1a+CD207+ cells (upper panel). Cells were then electroporated with 25 pmol miR-146a knock-down or miR-159 (CTRL) specific LNA probes. Knock-down efficiencies were determined using real-time RT-PCR analysis (lower panel). Bars represent mean miR-146a fluorescence levels in CTRL or miR-146a LNA knock-down probe transected cells from eight independent experiments (±SEM). F. Immunofluorescence stainings of LCs transected with miR-146a knock-down or miR-159 (CTRL) LNA probes. LCs were purified and transfected as described in E. After 24 h transfection, cells were activated with 0, 1, 10, or 50 μg/ml PGN and stained for p65/Alexa 456 (red) and DAPI (blue); percentages of cells showing p65 nuclear localization were determined. Representative stainings for cells activated with 1 μg/ml PGN are shown in the upper panel. White bars represent 10 μm. Arrows indicate cells that show nuclear p65 localization. Bar diagrams in the lower panel show mean percentages of cells (±SEM) that display nuclear p65 expression at the indicated PGN concentrations (n=3)
vector (CTRL, GFP+) (Supplemental Fig. 1). Therefore, ectopic miR-146a expression in monocytic cells and intDCs interferes with TLR2 downstream signaling processes without diminishing TLR2 expression.

**Ectopic miR-146a expression influences DC sensitivity to PGN-induced activation**

The above quantitative RT-PCR data revealed that intDCs express ~6-fold lower miR-146a levels than LCs (Fig. 1F). We hypothesized that high levels of miR-146a in LCs might increase their resistance to TLR-dependent activation. Therefore, we analyzed whether reconstituting miR-146a in intDCs to levels observed in LCs desensitizes cells to TLR ligand-induced activation. In these experiments, we transduced CD34+ cells as above with miR-146a or control vectors (Fig. 3A) and induced them to differentiate to intDCs. GFP+ cells showing intDC phenotype (CD11b+CD1a+) were FACs purified (Fig. 4B, dot blots). Real-time RT-PCR analysis revealed a mean 10-fold overexpression of miR-146a in miR-146a–transduced intDCs relative to control (Fig. 4B, bar diagram). To test if elevated miR-146a can interfere with TLR-dependent downstream signaling in intDCs, GFP+CD1a+CD11b+ cells were stimulated with different concentrations of PGN. Indeed, ectopic miR-146a expression rendered intDCs less responsive to PGN (Fig. 4C, 4D). Specifically, miR-146a transduced intDCs showed decreased levels of p-IκBα and p-p38 whereas p-ERK levels remained equivalent for miR-146a versus control (Fig. 4C). Furthermore, miR-146a transduced cells showed consistently (n = 3) lower percentages of nuclear p65 positive cells at low concentrations of PGN (1 µg/ml) (Fig. 4D, Table I). Conversely, when we used the same single cell-based assay for NF-κB activation in cluster-purified LCs in which miR-146a was silenced by miR-146–specific LNA knock-down oligos, we observed the opposite effect (Fig. 4E, 4F). Specifically, transfection of LCs with miR-146a antisense oligos (yielding knock-down efficiencies of ~50–70%; Fig. 4E) resulted in increased percentages of nuclear p65 positive cells relative to control (Fig. 4F, Table II). As a control for these experiments miR-159, a miRNA with no homology to any known miRNA or mRNA sequences in human was used.

**Ectopic miR-146a in intDCs interferes with TLR2-dependent cytokine production but not with the upregulation of costimulatory molecules**

To investigate functional consequences of ectopic miR-146a expression in intDCs, we analyzed the upregulation of costimulatory molecules as well as cytokine production. For this purpose miR-146a overexpressing intDCs obtained by cell sorting were stimulated for 1 h with Pam3CSK4 or PGN and upregulation of costimulatory molecules as well as cytokine production was analyzed after 48 h. Interestingly, although we did not observe significant changes in the upregulation of the costimulatory molecules CD80, CD83, and CD86 (Fig. 5A), miR-146a transduced intDCs consistently showed decreased production of IL-12p40 and TNF-α relative to control-transduced cells (Fig. 5B). A hypothetical model is shown in Fig. 6.

**Discussion**

In this study, we demonstrated that miR-146a is expressed at high levels in epidermal LC-type DCs. Our data suggest that epidermal LCs use miR-146a at steady-state to increase their activation thresholds to environmental TLR2 ligands. Unlike all other myeloid cell subsets, including intDCs, monocytes, and granulocytes, LCs abundantly expressed miR-146a. Moreover, the identified constitutive high miR-146a expression pattern in unstimulated LCs is in sharp contrast to its previously described regulation in lymphocytes (19, 20, 22) and monocytes (18). In the latter, miR-146a is induced in response to inflammatory signals and constitutes a negative feed-back loop involved in desensitizing monocytes to activation signals (18). Conversely, we demonstrate that miR-146a is expressed in unstimulated LCs already at levels equivalent to those detected in activated monocytes. Furthermore, in marked contrast to monocytes, miR-146a is not upregulated upon LC activation. Our data moreover suggest that constitutive expression of miR-146a is an intrinsic property of LCs that is mediated by TGF-β1–induced PU.1 expression. We showed in this study that PU.1 binds to the miR-146a promoter in LCs and positively regulates miR-146a expression. TGF-β1 is a critical epidermal microenvironmental signal for LC differentiation and function (44, 45). Conversely, intDCs develop in GM-CSF plus IL-4 supplemented cultures independently of TGF-β1 (49) and these cells show lower

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**Table I. Percentages of nuclear p65 positive cells among miR-146a– and CTRL-transduced intDCs**

<table>
<thead>
<tr>
<th></th>
<th>0 µg/ml PGN</th>
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<td></td>
<td>CTRL+a</td>
<td>miR-146a+b</td>
<td>CTRL</td>
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<tr>
<td>Experiment 1</td>
<td>25%</td>
<td>5%</td>
<td>36%</td>
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<td>Experiment 2</td>
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<td>24%</td>
<td>2%</td>
<td>31%</td>
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Cells were activated with the indicated PGN concentrations for 15 min.

*aNonspecific miR-159 LNA-transfected LCs.

*bThe miR-146a LNA knock-down probe transfected LCs.

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**Table II. Percentages of nuclear p65 positive cells among miR-146a knock-down and CTRL LNA probe transfected LCs**

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<thead>
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<th></th>
<th>0 µg/ml PGN</th>
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<td>miR-146a+b</td>
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<tr>
<td>Experiment 1</td>
<td>8%</td>
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<td>4%</td>
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<td>Experiment 3</td>
<td>7%</td>
<td>22%</td>
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Cells were activated with the indicated PGN concentrations for 15 min.

*aNonspecific miR-159 LNA-transfected LCs.

*bThe miR-146a LNA knock-down probe transfected LCs.

*Percentage of cells that show nuclear expression of p65.
miR-146a expression levels. Therefore, constitutive high miR-146a expression in response to microenvironmental TGF-β1 may represent a novel mechanism that desensitizes LCs to inappropriate TLR signaling at epithelial surfaces (see model in Fig. 6).

We previously described that TGF-β1–induced LC differentiation of myeloid progenitors is accompanied by PU.1 upregulation. Moreover, we observed that ectopic PU.1 promotes TGF-β1–dependent LC differentiation (40). High PU.1 levels in LCs lead to direct transcriptional induction of the miR-146a promoter and sustained high expression levels of miR-146a in LCs. Elevated levels of miR-146a in LCs increase their resistance to TLR2-induced activation by targeting key downstream signaling molecules. Decreased signaling strength downstream of the receptor results in diminished signal output and therefore weak activation response (i.e., IL-12 and TNF-α production).

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FIGURE 5. Ectopic miR-146a in intDCs interferes with PGN-induced cytokine production but not with upregulation of costimulatory molecules. A, Control hairpin (CTRL) or miR-146a–transduced CD34+ cells were subcultures under intDC specific conditions for 10 d. Cells were activated for 1 h with the designated concentrations of PGN and analyzed after 48 h for the expression of costimulatory molecules. Gated GFP+CD1a+CD11b+ cells are analyzed for their expression of CD80, CD86, and CD83. Lines represent cells after activation with the indicated concentrations of PGN; filled histograms correspond to unstimulated cells. Numbers depicted in histograms represent mean fluorescence intensities. B, Control hairpin (CTRL) or miR-146a–transduced CD34+ cells were differentiated under intDC instructive conditions and sorted as described in A. Cells were activated with the indicated concentrations of Pam3CSK4 for 1 h. Activated cells were replated and analyzed after 48 h for cytokine production. Three independent donor experiments are compared.

FIGURE 6. Hypothetical model of miR-146a function in LC-like DCs. TGF-β1 induces LC commitment of monocyte/LC progenitor cells accompanied by PU.1 upregulation. Enforced PU.1 promotes TGF-β1–dependent LC differentiation (40). High PU.1 levels in LCs lead to direct transcriptional induction of the miR-146a promoter and sustained high expression levels of miR-146a in LCs. Elevated levels of miR-146a in LCs increase their resistance to TLR2-induced activation by targeting key downstream signaling molecules. Decreased signaling strength downstream of the receptor results in diminished signal output and therefore weak activation response (i.e., IL-12 and TNF-α production).
Our study not only found miR-146a to be differentially expressed by moDC subsets, but also demonstrates that miR-146a regulates TLR signaling in these cells. Specifically, our data implicate miR-146a as a modulator of TLR2 signaling in myeloid DC subsets. First, retroviral gain of function analysis showed that reconstituting miR-146a levels in intDCs to levels approximating those constitutively observed in LCs is sufficient to impair PGN-induced downstream signaling and TLR2-mediated cytokine production, without affecting TLR2 expression itself. Second, the inverse experiment, namely, silencing of miR-146a in LCs, led to enhanced PGN-dependent NF-κB activation in these cells. Our single-cell–based subcellular analysis confirmed increased p65 nuclear localization in LCs in response to miR-146a silencing. With regard to the latter, it was not possible to assess LC functions after prolonged culture, due to technical reasons. Future studies using novel techniques, for example, the retroviral/lentiviral “spoon” method (52), will be required to further study miR-146a function in LCs. Nevertheless, the presented data indicate that miR-146a desensitizes LCs to TLR2-dependent activation. Because TLR2 is potentially triggered by commensal Gram-positive bacteria at epithelial surfaces, it is interesting to speculate that high miR-146a expression in this epithelial DC subset protects them from inappropriate activation by such stimuli at epithelial surfaces.

Whether decreased expression of miR-146a in intDCs is functionally related to their increased capacity to produce TNF-α and IL-12 relative to LCs as reported previously (29) remains to be analyzed. A side-by-side comparison of both DC subsets for TLR activation at epithelial surfaces. Mechanisms that functionally distinguish epithelial LC-type DCs from other DC subsets are only beginning to be understood (57). In light of LCs potential tolerogenic functions, it will be important to elucidate inhibitory pathways or mechanisms operative in these cells in further detail. Moreover, in vivo targeting of miR-146a with novel approaches [e.g., LNA-mediated miRNA silencing or enhancement (58)] might open new possibilities to alter DC functions in future immunopharmacological or cell therapy studies.

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Disclosures
The authors have no financial conflicts of interest.

References


Jurkin et al. “miR-146a is differentially expressed by myeloid dendritic cell subsets and desensitizes cells to TLR2-dependent activation.”

Supplementary data

Supplementary Figure 1. Ectopic miR-146a expression fails to impair TLR2 expression. Cells were transduced with vectors encoding miR-146a or control hairpin (CTRL) (vector graphics, see Figure 3A). A-B, Control and miR-146a transduced U937 cells were differentiated in the presence of 1,25-VD3 into monocyte-like cells and analyzed for TLR2 mRNA expression levels by real time RT-PCR (A) or TLR2 surface expression by FACS (B). B, filled grey histogram: isotype control antibody; dashed line: miR-146a transduced cells; black line: control vector transduced cells. C, Ectopic miR-146a expression in intDCs. Human CD34⁺ progenitor cells were transduced under progenitor expansion conditions. 48 h post transduction, cells were cultured under intDC instructing conditions. On day 10 GFP⁺CD1a⁺ cells were gated and analyzed for TLR2 expression. Filled grey histogram: isotype control antibody; dashed line: miR-146a transduced cells; black line: control vector transduced cells.

Supplementary Figure 2. TLR2 expression by in vitro generated LCs and intDCs. A, TLR2 mRNA expression in LCs and intDCs. LCs and intDCs were generated from CD34⁺ cells using specific cytokine combinations (see Materials and Methods and Figure 1A). CD1a⁺CD207⁻CD11b⁻ LCs and CD1a⁺CD207⁻CD11b⁺intDCs were FACS-sorted and analyzed for TLR2 expression using RT-PCR. B, TLR2 surface expression on LCs and intDCs. Histogram overlays show CD1a⁺ gated cells. Filled grey histograms represent isotype controls; black lines represent TLR2.
Supplementary Figure 1
### Supplementary Figure 2

#### A

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#### B

- **CD1a gated**
  - **LCs**
  - **intDCs**
- **TLR2**
  - `- TLR2`
  - `- CTRL`