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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 Gesslbauer,* 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 undetectable 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: 000–000.
with NF-κB 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+ monocyte intermediates from CD34+ progenitor cells share many characteristics 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 phenotypically 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 regulator of TLR and NF-κB signaling (18), to be expressed at much higher levels in LCs as compared to any other myelopoietic cell subsets studied, including intDCs. Our data indicate that a gene circuitry TGF-β1-Pu.1-miR-146a operative within the epidermal microenvironment decreases LC sensitivity to TLR2-dependent activation.

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). CD1a+ LCs were enriched from epidermal cell suspensions from healthy individuals undergoing reconstructive surgery as two-step culture system with slight modifications (29). Briefly, CD34+ cells described previously (38). IntDCs were generated using a previously described protocol. Cells in serum-free CellGro DC medium (CellGenix, Freiburg, Germany) supplemented with 20 ng/ml prior to subculturing with lineage specific cytokines. LC cultures were maintained 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 hybridization, data acquisition, and data processing were performed by LC Sciences (Houston, TX). Human miRNA array chip (Chip ID: MRA1001-human) based on the Sanger miBase Release 10.0, was used. Details to the chip as well as the detailed processing 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. Microarray 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.targetscan.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 instructions. Purified RNA was subjected to polyadenylation and cDNA synthesis using the NCodex miRNA First-Strand cDNA Synthesis Kit (Invitrogen) 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 NCodex miRNA First-Strand cDNA Synthesis Kit (Invitrogen) was used as reverse primer. The miRNA isolation and RT-PCR were performed as described (36). The following primers were used: TLR2: forward, 5'-GCT GCC ATT CCT ATT CTG CTG-3'; reverse, 5'-GAC CCT AGG CCA GAG TGT CCT TG-3'; HPRT: forward, 5'-GAC GAC CAG TCA ACA GAG GAC ATP-3'; reverse, 5'-AAC ACT TCG TGG GCT CCT TTT C-3'.

Retroviral vectors, gene transcription, and linolenic acid transfection
The hsa-mir-146a gene was cloned from U937 cell genomic DNA via PCR by using primers containing restriction enzyme linkers that allowed cloning into the murine stem cell virus-GFP retroviral expression vector (forward [XhoI] 5’-ACGCTGCGTCAAAGTCT TCAAAATATGCC-3’ and reverse [HpaI] 5’-GACGTCGAGAAGAATGTTGGATTAG-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 MSCV-LMP vector containing a nonsilencing hairpin (Open BioSystems, Huntsville, AL) served as control vector. PBMN-PU.1-L-GFP was described previously (40). Transfection of packaging cell lines Phoenix-E and Phoenix-GP as well as infection of target cells was performed as previously 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 (Exiqon, Vedbaek, Denmark) using foli Molecule 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 × 10⁷ cells per lane were loaded on 10% SDS-polyacrylamide gels. Resolved proteins were transferred to a polyvinylene-difluoride membrane (Immobilon-P; Millipore, Billerica, MA). Membranes were probed with Abs against phosphorylated p38 MAPK, total p38 MAPK, phosphorylated ERK, total ERK, phosphorylated JNK; total JNK (Cell Signaling Technology, Beverly, MA), phospho-Akt, and Akt (Cell Signaling Technology); total Beclin, U1, CEBPα,
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. 6p < 0.05; 66p < 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
TRA65 (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 was performed as described (41). Murine mAbs of the following specificities were used: FTC- conjugated mAbs specific for CD14 and CD1a (BD Biosciences, San Jose, CA); PE-conjugated mAbs specific for CD207 (ImmuneX, Marseille, France), CD14, CD15, CD83 (BD Biosciences) lectofino (Caltag, An der Grub, Austria), TLR2 [clone TLR2.1 (42) (Biolegend, San Diego, CA)] and isotype control (clone VIAP, kindly provided by O. Majdic, Vienna, Austria); Biotinylated mAbs specific for CD80, CD86 and CD11b (BD Biosciences); Second step reagent was streptavidin-PE (BD Biosciences); Allophycocyanin conjugated Abs for CD1a and CD14 (BD Biosciences) and CD324 (Biolegend). Intracellular staining of lectofino 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 488 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.chib.upenn.edu/tecss/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 0.5–1 mg/ml chromatin fragments. Subsequent ChIP steps were performed according to the protocol from Upstate Biotechnology (Charlotteville, VA). Each ChIP reaction included 2 µg anti-PU.1 (Santa Cruz Biotechnology); anti-Cyp1a1 (Santa Cruz Biotechnology) served as unspecific control. 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.

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⁺CD324/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 (miR-342-3p, miR-424, miR-21, miR-663, and miR-29a) 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 play a role 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/EBP, 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.1hi cells (Fig. 2D, dot plots). 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 monocyctic 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 mielopoiesis. 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 miRNA 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...
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⁺), 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 TLR 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 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⁺), 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.

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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/ml 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 expression levels in CTRL or miR-146a LNA knock-down probe transfected cells from eight independent experiments (± SEM). F, Immunofluorescence stainings of LCs transfected 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).
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

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

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Cells were activated with the indicated PGN concentrations for 15 min.

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

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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.
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 LC differentiation in a TGF-β1–dependent manner, and that ectopic PU.1 fails to induce the upregulation of costimulatory molecules by LCs (40). These data are consistent with the presented observation in this study that immature epithelial-type LCs express high levels of miR-146a. Interestingly, we showed that PU.1 strongly induces miR-146a promoter activity, whereas C/EBPα repressed it. These observations are in line with the demonstration that C/EBPα versus PU.1 control granulocyte/monocyte versus LC lineage decisions from CD34+ hematopoietic progenitors (43). It is interesting to speculate that undetectable low levels of miR-146a in granulocytes might be due to high C/EBPα and low PU.1 expression. In combination, these observations suggest that miR-146a is directly regulated by transcription factors that control granulomonopoiesis versus LC differentiation of myeloid progenitor cells. Mice deficient in TRAF6 or RelB show perturbations in myelopoiesis (47). Despite decreased TRAF6 expression and reduced NF-κB signaling in miR-146a–transduced cells, lineage differentiation cultures of CD34+ cells failed to reveal alterations in myeloid subset differentiation by miR-146a. Therefore, our experiments argue against a direct regulatory function of miR-146a in human myeloid subset specification. Instead, miR-146a seems to exert a functional role in differentiated LCs in response to microenvironmental TGF-β1. Importantly, preceding studies that used differentially stimulated moDCs found that TGF-β1 impairs activation signaling (50, 51). These observations are in line with the presented model in this study.

We used an in vitro culture system for the generation of well-defined LCs and intDCs. Those defined DC subsets were then analyzed for differences in their miRNA expression profile. To our knowledge, this is the first study that addressed whether DC subsets differ in miRNA expression. Several of the other identified miRNAs (e.g., miR17-92 cluster, miR-181a/b, and miR15) might target signaling processes in DC activation/maturation. It will be interesting to systematically evaluate other identified miRNAs, beyond miR-146a, in future mechanistic studies for their role in DC subset differentiation and function.
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 signaling is hampered by confounding variables (e.g., varying TLR expression levels by these cells). In line with previous data (53, 54), we found TLR2 to be substantially expressed by both DC subsets (Supplemental Fig. 2). Interestingly, we observed weaker NF-κB activation in LCs when comparing both DC subsets for their TLR2 downstream signaling responses (data not shown). It is therefore tempting to speculate that LCs may require prolonged or stronger receptor stimulation for proper NF-κB activation as compared with intDCs. One key finding of our study was that ectopic miR-146a expression severely impaired NF-κB and p38 signaling in monocyctic cells stimulated with PGN. In line with this, similar observations were made when analyzing effects of the TLR2-specific ligand Pam 3CSK4 (data not shown). Several previous studies implicated miR-146a as a negative regulator of TLR signaling (18, 21). These studies showed that miR-146 targets IRAK1 and TRAF6 (18) and that increased levels of miR-146a inhibited LPS-induced IFN-γ and inducible NO synthase production in splenic lymphocytes (21). However, direct functional data in monocyctic cells or DCs, as presented by our study were not reported previously. Because downstream signaling in response to PGN or polyinosinopolycytidylic acid was similarly impaired by ectopic miR-146a in monocyctic cells (data not shown), it is likely that miR-146a is involved in fine-tuning not only of TLR2, but also other TLR responses.

The miR-146a might represent one of several negative-regulatory molecules induced in LCs. Similar to miR-146a, E-cadherin/CD324 expressed in LCs seems to be involved in the negative regulation of LC activation (55, 56). Therefore, several molecular pathways might be involved in desensitizing LCs to inappropriate 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 to- lerogenic functions, it will be important to elucidate inhibitory 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.


