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Identification of a microRNA signature in dendritic cell vaccines for cancer immunotherapy

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ABSTRACT

Dendritic cells (DCs) exposed to tumor antigens followed by treatment with T_h1-polarizing differentiation signals have paved the way for the development of DC-based cancer vaccines. Critical parameters for assessment of the optimal functional state of DCs and prediction of the vaccine potency of activated DCs have in the past been based on measurements of differentiation surface markers like HLA-DR, CD80, CD83, CD86, and CCR7 and the level of secreted cytokines like interleukin-12p70. However, the level of these markers does not provide a complete picture of the DC phenotype and may be insufficient for prediction of clinical outcome for DC-based therapy. We therefore looked for additional biomarkers by investigating the differential expression of microRNAs (miRNAs) in mature DCs relative to immature DCs. A microarray-based screening revealed that 12 miRNAs were differentially expressed in the two DC phenotypes. Of these, four miRNAs, hsa-miR-155, hsa-miR-146a, hsa-miR-125a-5p, and hsa-miR-29a, were validated by real-time polymerase chain reaction and northern blotting. The matured DCs from 12 individual donors were divided into two groups of highly and less differentiated DCs, respectively. A pronounced difference at the level of miRNA induction between these two groups was observed, suggesting that quantitative evaluation of selected miRNAs potentially can predict the immunogenicity of DC vaccines.

1. Introduction

Several immunotherapeutic approaches for the treatment of cancer have attracted attention in the past, including the use of dendritic cell (DC)-based cancer vaccines. DCs are antigen-presenting cells and are considered one of the most powerful regulators of the immune system [1]. DC vaccination has been tested in numerous clinical phase I and II trials [2–4] using various experimental conditions for DC retrieval, differentiation, and maturation. Future challenges in the field focus on optimization of DC preparation and enhanced immunogenicity of the applied DC vaccines [3]. Most trials utilize peripheral blood monocytes, which can be obtained from patient blood or leukopheresis products and differentiated to immature DCs using autologous plasma in the presence of growth factors like granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4. Currently, attempts to optimize DC vaccines with the most appropriate phenotype before vaccination are made by assuring that the DCs have a mature phenotype [5], with high expression of CD83 and T-cell costimulatory molecules, such as CD40, CD80, CD86, and CCR7. This is crucial for their ability to migrate to local lymph nodes and induce a $T_h1/CD8^+$ T-cell-driven tumor-specific immune response [6,7]. Furthermore, it is preferable that immunogenic DCs capable of inducing a T_h1 response should also secrete the T_h1-promoting cytokine IL-12p70 and no immunosuppressive cytokines, such as IL-10 [5].

Functionally, immunogenic DCs can be obtained by numerous methods, one of which is DC differentiation. DC differentiation is a process influenced by various types of cytokines and Toll-like receptor ligands, leading to DCs with different phenotypes and capabilities [8]. Moreover, any additional tool that allows a more specific phenotypic characterization of DCs would be beneficial both for *in vitro* characterization of DC maturation and differentiation and, importantly, for quality assurance in DC batches before clinical application. As a result, microRNAs (miRNAs) represent such a new class of molecules that could aid in the characterization of DC phenotypes.

miRNAs are small (20–23 nucleotides) noncoding single-stranded RNA molecules that regulate gene expression in plants, animals, and humans [9]. miRNA molecules act by posttranscriptional control of gene expression through the mechanism of RNA

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interference [10,11]. Recently, the role of miRNAs in the control of the immune system has become evident [12–15]. In particular, miR-155 is believed to be involved in immune functions [16–18]. Studies with miRNA-155$^{KO\text{-}mice}$ demonstrated that these mice were defective in adaptive immunity, with a decreased resistance against a *Salmonella* infection after vaccination [19]. Furthermore, DCs from these mice demonstrated a reduced ability to stimulate antigen-specific proliferation of ovalbumin T-cell receptor transgenic cells [19]. Additionally, miR-155 and miR-146a/b expression was induced in macrophages of both human and mouse origin upon lipopolysaccharide (LPS) stimulation, demonstrating that these miRNAs are involved in the regulation of pathogen-associated molecular pattern signaling [20,21]. Finally, a recent study by Ceppi *et al.* [22] indicated that miR-155 targets the IL-1 pathway in monocyte-derived DCs in a possible negative feedback loop, indicating that the level or prolonged expression of miR-155 could be critical for immune function.

Because miRNAs, and in particular miR-155, seem to be critically involved in immune function, we examined whether specific miRNAs could be linked to the functional state of DCs matured by the classical DC maturation cocktail containing IL-1β, IL-6, tumor necrosis factor (TNF) α, and prostaglandin E_2 (PGE_2) [1].

2. Subjects and methods

2.1. Access to human blood from different donors

Human blood samples in the form of buffy coat from 13 healthy volunteers were supplied from the blood bank of both Gentofte Hospital and Rigshospitalet in Copenhagen, Denmark.

2.2. Preparation of dendritic cells

DCs were prepared according to a modified protocol originally described by Romani *et al.* [23]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated by gradient centrifugation on lymphoprep (Nycomed, Oslo, Norway) and the isolated cells were subjected to plastic adherence for 60 minutes, followed by the removal of nonadherent cells. Adherent cells were cultured in the presence of 700 U/ml hrIL-4 (Gentaur, Brussels, Belgium) and 1,400 U/ml hrGM-CSF (Gentaur), as well as 1.2% autologous plasma, and refreshed after 5 days. For induction of maturation, DCs were stimulated with 10 ng/ml IL-1β (Gentaur), 10 ng/ml IL-6 (Gentaur), 10 ng/ml TNF-α (Gentaur), and 1 μg/ml PGE_2 (Sigma Aldrich) on day 6, and all DCs were harvested on day 7 of culture.

2.3. Flow cytometry

Cells to be analyzed were incubated for 15 minutes with 5% human AB serum (Lonza, Basel, Switzerland) before labeling with fluorescein-conjugated antibodies. For analysis of DCs, the following mouse monoclonal antibodies were used: anti-HLA-D (Tü39, Becton Dickinson [BD], San Jose, CA; recognizes HLA-DR/DQ/DP), anti-CD80 (2D10.4, eBioscience), anti-CD86 (FUN-1, BD), anti-CCR7 (150503, R&D Systems, Abingdon, UK), anti-CD83 (HB15e, BD), and appropriate isotype controls (eBioscience and BD). All labeled cells were analyzed on a FACSCalibur (BD).

2.4. Cytokine enzyme-linked immunosorbent assay

DC culture supernatants were collected and stored at −80°C. Amounts of IL-10, IL-12p70, and IL-23 were measured by standard sandwich enzyme-linked immunosorbent assay using commercially available antibodies and standards according to the manufacturer's protocols (eBioscience).

2.5. miRNA array analysis

DCs were prepared from five different donors as described above. Total RNA was extracted using the Trizol RNA isolation protocol as outlined by the manufacturer (Invitrogen, Paisley, UK). Two micrograms of total RNA was labeled with Hy5 for immature and Hy3 for mature DCs and vice versa using the miRCURY LNA array labeling kit (Exiqon, Vedbaek, Denmark). The labeled RNA samples were mixed before hybridization to the microarray using the miRCURY LNA microRNA array (Exiqon) corresponding to version 8.1 of the Sanger miRBase. The version 8.1 microRNA array contained probes against 474 human miRNAs representing the earliest identified and most abundantly expressed miRNAs in human tissue. Following hybridization and washes in an HS-400-Pro microarray hybridization station (Tecan, Grödig, Austria) according to the manufacturer's instructions, the dried slides were scanned in an ArrayWoRx white-light CCD-based scanner (Applied Precision, Issaquah, WA) at 10-μm resolution. The resulting images were imported into ImaGene 8.0 (BioDiscovery, El Segundo, CA), where spot intensities and background measurements were calculated. To identify differentially expressed miRNAs in mDC compared with imDC, a ratio analysis was conducted using GeneSight-Lite 4.1.6 (BioDiscovery). Ratio values from all experiments were compiled using both Lowess normalization (with a smoothing parameter of 0.2 and linear degree of fitness) and division by mean signal normalization. Prior to statistical analysis, ratio values were $\log_2$ transformed and significance analysis of microarray (SAM) was conducted on the data selecting only the miRNAs capable of fulfilling the criteria of a false discovery rate of 0%.

2.6. Validation of miRNA differential expression using real-time reverse transcription-polymerase chain reaction (RT-PCR)

Differential expression of selected miRNAs was validated using the TaqMan microRNA assays (Applied Biosystems, CA). Single-stranded cDNA was synthesized from 10 ng of total RNA using the looped primers of the TaqMan microRNA assay and the TaqMan microRNA reverse transcription kit (Applied Biosystems). Each cDNA generated was amplified by real-time PCR using the sequence-specific primers from the TaqMan microRNA assay. Real-time PCR was performed using the standard TaqMan microRNA assays protocol on the ABI StepOne Plus real-time PCR machine (Applied Biosystems). The reactions were incubated in a 96-well plate at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Each sample was analyzed in triplicate in up to three independent repetitions. The level of miRNA expression was measured using C_t (threshold cycle) according to the $\Delta\Delta C_t$ method described by Livak and Schmittgen [24]. To normalize the relative abundance of miRNAs, U6 snRNA was used as endogenous control. U6 snRNA was detected using the TaqMan microRNA assay for U6 snRNA. The ΔC_t was calculated by subtracting the C_t of U6 snRNA from the C_t of the respective miRNA. The $\Delta\Delta C_t$ was calculated by subtracting the ΔC_t of the reference sample (immature DC) from the ΔC_t of the mature DC phenotype.

2.7. Validation of differential miRNA expression using northern blot analysis

Further validation of selected miRNAs was conducted using northern blot analysis employing total RNA from a single donor. According to the method of Válóczi *et al.* [25], we used LNA-modified oligonucleotide probes (Exiqon) for the specific hybridization to hsa-miR-155, hsa-miR-146a, hsa-miR-125a-5p, and hsa-miR-29a. Briefly, 10 pmol of probes was 5′-end labeled with [γ-^{32}]P-ATP using T4 polynucleotide kinase. For hybridization we used the ULTRAhyb-Oligo hybridization buffer (Ambion). Prehybridization was conducted at 68°C for 30 minutes, and after the labeled probe was added, hybridization was performed overnight at 42°C. Following hybridization, the membranes were washed in 2× SSC +0.1% sodium dodecyl sulfate for 5 minutes at 42°C and at high stringency in 0.1× SSC +0.1% sodium dodecyl sulfate twice for 5 minutes at 65°C.

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2.8. Criteria for categorizing donors into two groups based on expected immunogenicity

Based on the expression of the two important phenotypical markers for mature DC function, CCR7 and CD83 [26,27], DCs from the 12 donors were divided into two groups: highly differentiated and less differentiated. The highly differentiated group included donors whose DC population was >70% positive for both CCR7 and CD83 (based on single staining and measured individually by flow cytometry), whereas DCs that did not fulfill these criteria were defined as less differentiated.

3. Results

3.1. Characterization of DC phenotypes using FACS analysis and cytokine measurement

Monocytes derived from healthy human donors were differentiated into immature DCs and treated with a classical maturation cocktail containing IL-1β, IL-6, TNF-α, and PGE_2 commonly used for DC vaccines in cancer immunotherapy [1]. Analysis of surface marker expression for human leukocyte antigen (HLA)-D (HLA-DR/DQ/DP), CD80, CD83, CD86, and CCR7 showed an increase in mean fluorescence intensity (MFI) for all five markers (Fig. 1A and B). Additionally, the culture supernatant after 24 hours of incubation with the cocktail was collected and levels of cytokine secretion of IL-10, IL-12p70, and IL-23 were measured in up to 11 donors (Fig. 2). When the average cytokine induction in mature DCs compared with immature DCs of all donors was examined, IL-23 secretion was significantly upregulated ($p < 0.001$, t test), but not that of IL-12p70 or IL-10. However, 9 of 11 individual donors exhibited a modest increase in IL-12p70 (>1.5-fold induction), whereas only 5 of 11 donors showed induction of IL-10 (>1.5-fold).

3.2. Identification of differentially expressed miRNAs using microarray analysis

Five donors (D1–5) were used for the microarray screening. The resulting SAM analysis on the calculated ratio values based on two different normalization methods of the array data revealed that a total of 12 miRNAs were differentially expressed between immature and mature DCs. Only $\log_2$ transformed average ratio values above or below 0.5 and −0.5, respectively, were taken into account. Table 1 shows that five miRNAs were induced in mature DCs, whereas seven miRNAs appeared to be more abundantly expressed in immature DCs. In only two cases, hsa-miR-146a and hsa-miR-155, more than a twofold difference (corresponding to $\log_2$ transformed ratio values above 1 or below −1) was seen between expression levels in immature and mature DCs. Hence, generally moderate changes were observed in the miRNA profiles of DCs that were undergoing a transformation from an immature to a mature phenotype.

3.3. Validation of differential expression of miRNAs in mature DC

Additional donors (D6–13) were enrolled in the validation of selected miRNAs using TaqMan based real-time RT-PCR. We focused our interest on 6 of the 12 identified differentially expressed miRNAs and performed real-time PCR analyses on a total of 12 donors (D2–13).

As shown in Fig. 3, there was a significant ($p < 0.01$, one-class t test) induction of hsa-miR-155, hsa-miR-146a, and hsa-miR-125a-5p in mature DCs compared with the expression level in immature DCs. Although a trend toward induction of hsa-miR-29a in mature DCs was observed, the level of induction was not statistically significant. In the case of hsa-miR-27a and hsa-miR-623, we were not able to verify the observed differential expression in mature DCs.

A single donor (D12) was selected for further validation using northern blot analysis. Fig. 4 shows the results of the northern analyses for hsa-miR-155, hsa-miR-146, hsa-miR-125a-5p, and hsa-miR-29a. There was a clear induction of all four tested miRNAs using both northern blotting and real-time PCR analysis in mature DCs compared with the immature DCs, thus showing a good correlation between the levels of induction measured by these methods.

3.4. miRNA markers to distinguish between highly and less differentiated DCs according to their expression of CD83/CCR7

Criteria to control the quality of DCs used for immunotherapy currently include the percentage of positive cells in the population for expression of CD83 and CCR7 [26,27]. Based on the expression of these markers in the matured phenotypes, we divided the 12 donors into a group of highly differentiated DCs ($n = 6$: D2, D3, D4, D5, D9, D12), which all showed expression of CD83 and CCR7 in more than 70% of the cell population, and a less differentiated phenotype ($n = 6$: D6, D7, D8, D10, D11, D13), which did not fulfill these requirements (Table 2).

We examined the average level of induction of all maturation markers (HLA-D, CD80, CD83, CD86, CCR7, IL-12p70, and IL-23) in the highly and less differentiated donor groups. The levels of surface maturation markers were determined by FACS and expressed as a fold increase in MFI compared with their corresponding immature DC phenotype (Fig. 5A). Similarly, the levels of secreted IL-12p70 and IL-23 were compared between mature DCs and the corresponding immature DCs and expressed as a fold increase for the highly differentiated and the less differentiated DC groups.

Statistical evaluation using a two-sided, unpaired t test indicated significantly higher CCR7 MFI values in the highly differentiated group than in the less differentiated group, compared with their corresponding immature phenotypes ($p < 0.001$). IL-23 secretion was also significantly higher in the highly differentiated group than in the less differentiated group ($p < 0.043$; Fig. 5A), whereas

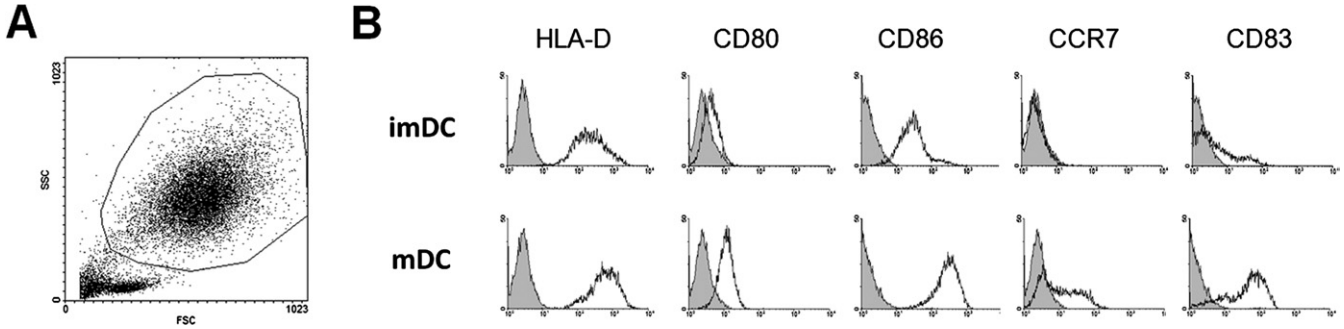

Fig. 1. Characterization of cell surface markers of immature and mature DC. DCs were generated in the presence of GM-CSF/IL-4 for 8 days, and the cells were collected and examined for their surface antigen expression by flow cytometry. (A) Forward scatter/side scatter dot plot used for DC gating. (B) Shaded histograms show isotype-matched controls, and open histograms show the staining with antibodies for indicated markers of immature (imDC) and mature (mDC) DCs, respectively. The data are one representative of 10 individual experiments.

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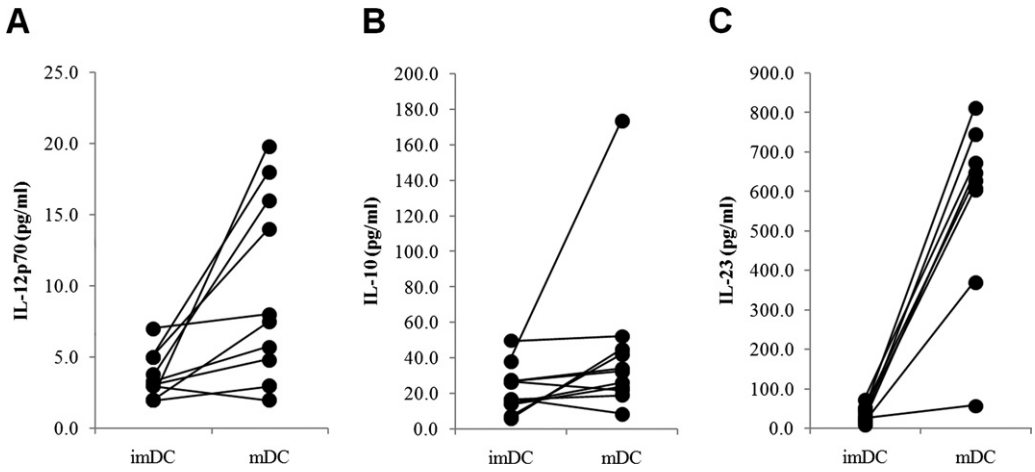

Fig. 2. Secretion of IL-10, IL-12p70, and IL-23 from immature and mature DC. Cell culture supernatant of day 7 DCs treated for the last 24 hours without (immature DC [imDC]) or with (mature DC [mDC]) the maturation cocktail was collected, and the secreted amount of (A) IL-12p70 ($n = 11$), (B) IL-10 ($n = 11$), and (C) IL-23 ($n = 9$) was determined by enzyme-linked immunosorbent assay. Each symbol (closed circle) indicates the concentration of secreted cytokine of each individual donor, and corresponding measurements are connected with a line.

none of the other markers were significantly different between the two differentiated groups of DCs.

Similarly, the miRNA induction levels of the highly and the less differentiated DC donor groups were determined by averaging the real-time RT-PCR results for each group of donors expressed as $-\Delta\Delta$Ct values corresponding to the $\log_2$ transformed fold increase relative to the corresponding immature DCs (Fig. 5B). Four miRNAs, hsa-miR-155, hsa-miR-146a, hsa-miR-125a-5p, and hsa-miR-29a, were shown by a one-class *t* test to be significantly induced (*i.e.,* significantly different from 0 in the group of donors representing highly differentiated DCs; $p < 0.0004$, $p < 0.002$, $p < 0.0007$, and $p < 0.009$, respectively; Fig. 5B). In the group of DCs from donors with less differentiated DCs, only hsa-miR-155 and hsa-miR-146a were still significantly induced ($p < 0.0006$ and $p < 0.00005$, respectively; Fig. 5B). The induction of hsa-miR-155 in the highly differentiated group of DCs was significantly higher than that of the less differentiated group ($p < 0.0006$, Student's *t* test; Fig. 5B).

These data indicated that the level of DC differentiation/maturation potentially could be predicted by measuring the level of induction of the maturation marker CCR7 and the secreted cytokine IL-23, both of which were significantly increased in highly differentiated DCs compared with less differentiated DCs. Differences in the miRNA expression pattern in mature DCs compared with immature DCs showed that induced levels of hsa-miR-155, hsa-miR-146a, hsa-miR-125a-5p, and hsa-miR-29a, and in particular, hsa-miR-155 could distinguish donors giving rise to highly differentiated DCs from those giving rise to less differentiated ones. As an example, the expression of hsa-miR-155 reached an average 8-fold increase in the highly differentiated mature DCs compared with immature DCs, whereas the less differentiated mature DCs reached only an average 1.7-fold increase compared with immature DCs.

3.5. Correlation between miRNA and known markers

Finally, we tested whether the level of traditional differentiation markers correlated positively or negatively with the induction of any of the four miRNAs using the Pearson's product moment correlation. Of all the possible correlations tested (seven different markers or cytokines against four different miRNAs), significant correlations (expressed as $\log_2$ transformed fold induction in the differentiated DCs vs their immature counterpart) were reported

Table 1
List of statistically significant differentially expressed miRNAs in mature versus immature DCs determined by SAM using two different normalization algorithms (global Lowess and divide-by-mean-density)

MicroRNA	$\log_2$ transformed average ratio value (mature/immature) (global Lowess)	$\log_2$ transformed average ratio value (mature/immature) (divide-by-mean-density)
hsa-miR-21*	− 0.72 ± 0.79	−0.82 ± 0.84
hsa-miR-27a	0.70 ± 0.28	0.69 ± 0.30
hsa-miR-29a	0.91 ± 0.47	0.96 ± 0.60
hsa-miR-125a-5p	0.76 ± 0.52	0.88 ± 0.54
hsa-miR-129-1	−0.70 ± 0.57	−0.87 ± 0.62
hsa-miR-146a	1.46 ± 0.81	1.64 ± 0.93
hsa-miR-155	2.78 ± 1.52	2.49 ± 0.81
hsa-miR-185*	−0.76 ± 0.64	−0.83 ± 0.53
hsa-miR-202	−0.44 ± 0.24	−0.63 ± 0.45
hsa-miR-623	−0.55 ± 0.41	−0.92 ± 0.78
hsa-miR-671-5p	−0.65 ± 0.46	−1.08 ± 0.91
hsa-miR-744	−0.74 ± 0.63	−1.09 ± 0.84

Average ratio values ± standard deviations are indicated.

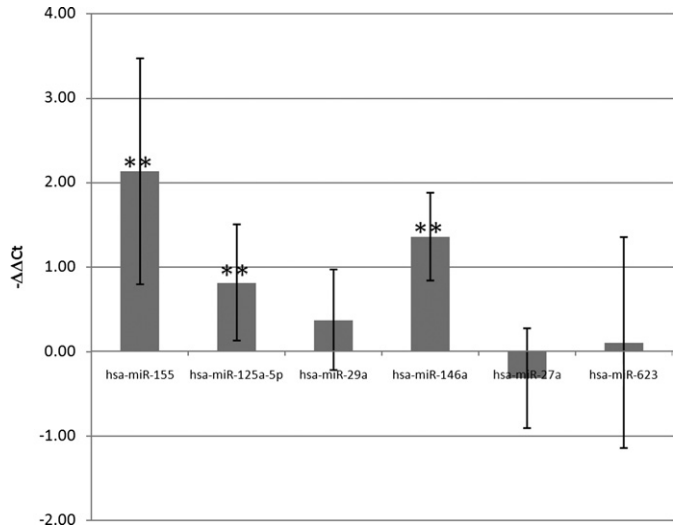

Fig. 3. Real-time PCR validation of differential expression of miRNAs in mature versus immature DC. The columns represent average $-\Delta\Delta C_t$ values of 12 different donors (D2-13) that indicate the relative expression of level of a given miRNA in mature DC compared with the respective immature DC level. As calibrator in the analysis, U6 snRNA were used. Significant differential expression of miRNAs in mature DC versus immature DC is indicated by asterisks: $^{**}p < 0.01$, one-class *t* test.

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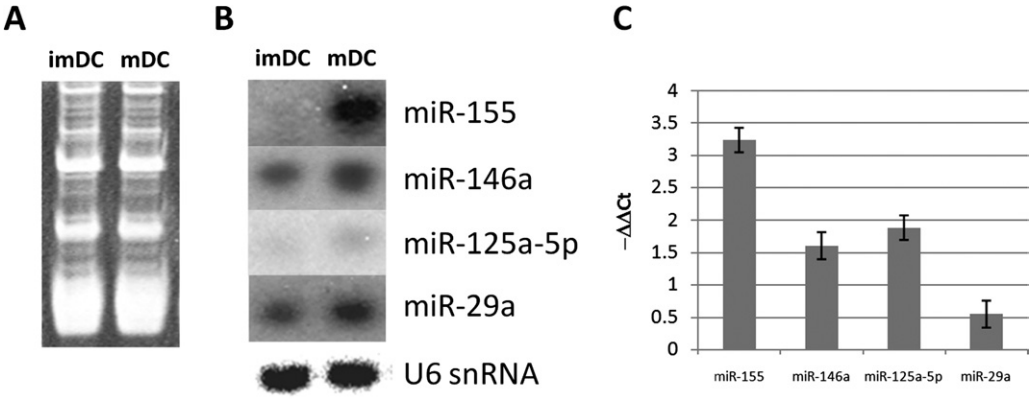

Fig. 4. Validation of differential expression of microRNAs in a single donor (D12) using northern blotting and real-time PCR. (A) Total RNA loaded on the 15% polyacrylamide gel (imDC, immature DC; mDC, mature DC), (B) northern blotting showing the mature form of the respective miRNAs and the U6 snRNA, which has been used as a normalization reference in real-time PCR, and (C) results of real-time PCR analysis showing the $-\Delta\Delta C_t$ values using U6 snRNA as calibrator and immature DC as reference.

between CCR7 and hsa-miR-155 ($r_p = 0.85$, $p < 0.01$; Fig. 6A) and between IL-23 secretion and hsa-miR-155 ($r_p = 0.72$, $p < 0.05$; Fig. 6B). A borderline significant correlation was observed between secretion of IL-12p70 and hsa-miR-155 expression ($r_p = 0.59$, $p < 0.05$; data not shown).

4. Discussion

We have demonstrated that DCs undergoing differentiation induced by treatment with a maturation cocktail, commonly used to obtain immunogenic DCs for cancer vaccine immunotherapy, change their miRNA expression pattern. Of 12 miRNAs, which were initially detected by microarray screening to be differentially expressed during the DC maturation process, 3 miRNAs (hsa-miR-155, hsa-miR-146a, and hsa-miR-125a-5p) were validated by real-time PCR to be significantly differentially expressed in mature DCs compared with immature DCs. Furthermore, when the DCs of the 12 donors in the validation study were divided into two groups based on the percentage of receptor-positive cells of CD83 and CCR7 (>70% CD83 and >70% CCR7), it was found that hsa-miR-29a was significantly induced in the group of highly differentiated mature DCs.

The level of DC differentiation correlates with their immunogenicity [28]. The ability to predict the immunogenicity of a given DC-based vaccine before administration will most likely improve the success of a given immunotherapeutic intervention. We have demonstrated that the level of hsa-miR-155 induction in particular was significantly higher in a group of highly differentiated mature DCs compared with a group of predicted less differentiated DCs. Similarly, the significant upregulation of hsa-miR-29a, although moderate, was only evident in the group of highly differentiated mature DCs. In addition, hsa-miR-146a and hsa-miR-125a-5p had slightly higher induction levels in the highly differentiated DCs compared with the less differentiated mature DCs. Hence, by performing up to four parallel simple quantitative real-time PCR analyses, an evaluation could potentially be conducted to assess the immunogenicity of a DC-based vaccine before administration. Clearly, this needs to be further verified (*e.g.,* by comparing the ability of DCs belonging to either group of the DCs to elicit an antitumor CTL response).

The fact that hsa-miR-146a did not seem to be related to DC differentiation/maturation levels was in line with the data from our previous study [29], where the elevation of hsa-miR-146a was also observed in functionally regulatory DCs obtained by treatment with 1,25-dihydroxyvitamin D_3 and, subsequently, the maturation cocktail.

Interestingly, in a very recent study three of the four validated upregulated miRNAs (miR-155, miR-146a, and miR-125a-5p) were found to be upregulated in mouse peritoneal macrophages during infection with vesicular stomatis virus [30], thus indicating a similar coordinated stimulation of miRNA-expression as we observed in cocktail-induced DCs. These authors provided supporting evidence that miR-146a directly controlled Toll-like receptor and cytokine signaling through the NF-κB pathway by targeting TRAF6 and IRAK1, previously shown by Taganov *et al.* [20], and now also IRAK2. MicroRNA miR-146a is thus a key regulator in the immune system and its upregulation in response to viruses, LPS, and maturation cocktails as used in this study most likely represents a very early and fundamental property of the innate immune response.

We found that expression of CCR7 and secretion of IL-23 were significantly different between the groups of highly and less differentiated mature DCs and that the induction of CCR7, and IL-23 in particular, in the individual donors correlated positively with the induction of hsa-miR-155 ($p < 0.01$ and $p < 0.05$, respectively). This positive correlation could indicate that hsa-miR-155 targets a negative regulator of CCR7 and potentially one or both of the IL-23 subunits, p19 and/or p40. To further validate the relationship among miR-155, CCR7, and IL-23, knockdown studies of miR-155 should be performed to examine whether CCR7 expression and IL-23 secretion are inhibited with a subsequent impairment of DC maturation and function. Similarly, *in vivo* studies are needed to determine whether different induction levels of miR-155, corre-

Table 2
List of donors used in the study separated on the basis of the percentage of positive cells for expression of CCR7 and CD83, respectively, in the mature phenotype

	% CCR7-positive cells	% CD83-positive cells
Highly differentiated DCs		
Donor 2	86.9	73.8
Donor 3	89.9	84.2
Donor 4	83.2	97.3
Donor 5	97.4	90.7
Donor 9	98.4	84.1
Donor 12	92.6	83.0
Less differentiated DCs		
Donor 6	36.0	17.6
Donor 7	82.2	38.0
Donor 8	64.2	13.7
Donor 10	67.8	39.8
Donor 11	64.4	23.4
Donor 13	90.6	5.3

Donors with highly differentiated mature DCs are defined as having expression of both CCR7 and CD83 in more than 70% of the cell population. Donors with less differentiated mature DCs are defined as having either CCR7- or CD83-positive cells below 70%.

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Fig. 5. Marker profiles of donors categorized into two groups representing donors with less and highly differentiated mature DCs. (A) Diagram showing the average induction level of surface markers or secreted cytokines for the respective group. For each marker the fold induction was calculated by dividing either the MFI value or the concentration of secreted cytokines of each matured DC with the corresponding values of the respective immature DC of each donor. For each group of donors the average fold induction was subsequently calculated and depicted. Asterisks indicate whether the difference between the two groups was statistically significant using a two-side, unpaired *t* test ($^{**}p < 0.01$ and $^{*}p < 0.05$). (B) The results of real-time PCR validation of miRNAs showing the average miRNA induction level of all donors in each group. Asterisks indicate the level of statistical significance with which the value was different from 0 (one-class *t* test) or whether there was a statistical significant (Student's *t* test) difference between the induction level between the two groups of donors ($^{**}p < 0.01$ and $^{*}p < 0.05$).

sponding to less or highly differentiated DCs, will show different functional immune effects. However, these studies were beyond the scope of the present study.

Several validated human targets for miR-155 have been published, including BACH1, CUTL1, and CEBPB [31]. In addition, Ceppi *et al.* recently showed that an additional 20 genes were upregulated after knocking down hsa-miR-155 in human monocyte-derived DCs [22]. In particular, the TAB2 gene was further substantiated to be a direct target of hsa-miR-155 in this study. TAB2 acts as a multifunctional signaling molecule facilitating, for example, p38 MAPK activation. The authors proposed a model wherein high levels of hsa-miR-155 induced by LPS silenced expression of IL-1β and other proinflammatory cytokines like IL-23-p19. In our study we measured the accumulated IL-23 and IL-12p70 over a 24-hour period after cocktail addition, whereas the hsa-miR-155 induction level was determined as an end-point measurement at the 24-hour time point relative to the level in untreated immature DCs. Thus, our experimental setup did not allow us to observe any potential negative feedback mechanism of excessive levels of hsa-miR-155 after 24 hours of treatment at the level of secreted IL-23 or IL-12p70 in the culture medium.

However, the level of induction of hsa-miR-155 determined by Ceppi *et al.* reached 50-fold after 16 hours of LPS stimulation, whereas we only observed an 8-fold induction of hsa-miR-155 after 24 hours of cocktail-stimulation. With the assumption that the levels of hsa-miR-155 in the non-differentiated immature DCs in these two separate studies were equal, this could indicate that the highly differentiated mature DCs in our study had not reached the late phase of DC maturation, where, according to Ceppi *et al.*, the DCs would begin to inhibit and silence inflammatory responses. Hence, the study by Ceppi *et al.* combined with our present results could be extremely important for future development of potent immunogenic DC-based vaccines. Apparently, the level of hsa-miR-155 is important for the ability of DCs to induce a potent immune response. This is a consideration where extremely high levels of hsa-miR-155 might induce DC exhaustion by downregulation of inflammatory cytokines like IL-1β. In this respect, DCs might induce a more potent antitumor response in the patient if the DCs are injected before hsa-miR-155 expression peaks or, alternatively, by controlling levels of hsa-miR-155 to remain at a level where the immunogenic response is constitutively high [22].

The importance of appropriately balancing the physiologic levels of miR-155 expression was also seen by O'Connell *et al.,* who noted that myeloproliferative disorders could be caused either by overexpression of miR-155 or specific knock-down of the inositol phosphatase SHIP1, a primary target of miR-155 [32]. Furthermore, miR-155 has also been shown to be overexpressed in myeloid leukemias [33]. Taken together, these observations point toward a fine-tuned balance of miR-155 expression in control of inflammatory responses and the onset of myeloproliferative disorders and leukemias. The delicate balance of miRNA expression and its quantitative impact on gene expression will in the future require tunable and inducible expression systems to study in more detail the gain and loss of function of miRNAs, either alone or in combination with other miRNAs.

In our study we observed only a modest induction of IL-12p70 in the mature DCs upon stimulation with maturation cocktail. However, this was not surprising given that the maturation cocktail contains PGE_2, which suppresses the ability of DCs to induce IL-12p70 [34]. DCs in this study were, however, not defective in the production of IL-12p70 because upon further stimulation with LPS, significant levels of IL-12p70 were secreted from the differentiated DCs (data not shown).

The fact that LPS-treated DCs induced high levels of hsa-miR-155, leading to activation of a negative feedback mechanism that potentially decreases immunogenicity, could also indicate that the use of TLR agonists in cocktails for DC vaccine applications should be considered carefully, and the immunogenic potential should be analyzed in detail after cocktail addition so as not to apply exhausted DCs to the patients.

Improvements of the phenotypical and functional characterization of DCs intended for vaccination applications are important to accomplish. Currently, used markers like HLA-D, CCR7, CD80, CD83, CD86, and IL-12p70 should preferably be supplemented with other types of markers that more accurately characterize the function and fate of DCs once administered *in vivo*, such as their viability, migratory potential, sustained immunogenicity, and overall functional stability.

Our study suggests that analyses of new biomarkers, such as miRNA hsa-miR-155, could be included in future quality control of the differentiation/maturation status of DC batches for vaccine use. The ability to design a validated and simple quantitative real-time PCR assay that only requires minute amounts of cells will enable a fast and relatively inexpensive assessment of the induction level of hsa-miR-155 of each batch of matured DCs. However, the use of miRNA profiles as suggested in the present study is still in its infancy, and further validation of our findings is required to apply such markers in clinical settings. The perspective is that accumulated knowledge of the function of specific miRNAs (*e.g.,* in relation to the lifespan, migration, and immunogenicity of DCs intended for vaccine applications) may lead to the inclusion of miRNA-based DC

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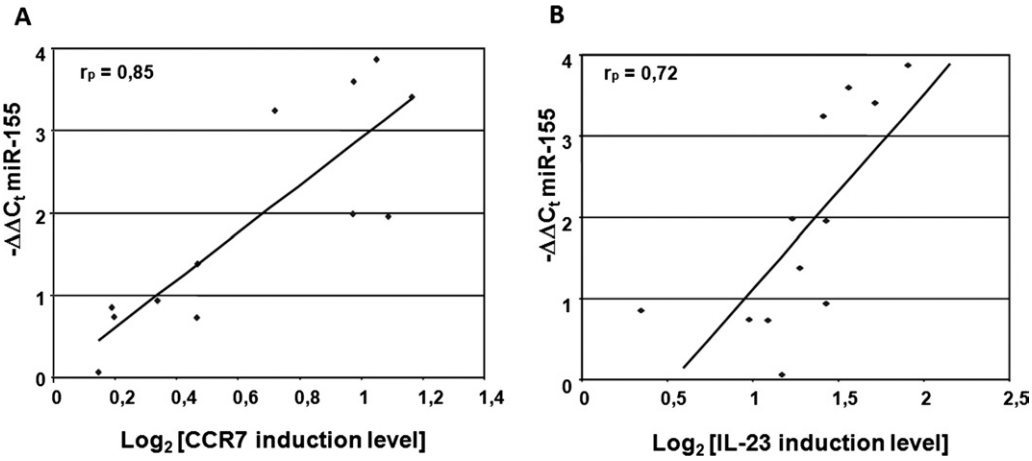

Fig. 6. Correlation among miR-155, CCR-7, and IL-23 induction levels. Scatter diagram of the correlation between the fold of induction in the mature phenotype compared with the immature phenotype of the marker CCR7 (A) and the cytokine secretion of IL-23 (B) with fold of miR-155 induction levels, respectively. Pearson's correlation coefficient (r_p) is shown and used for the calculation of significance using a two-sided t test. The CCR7-miR-155 correlation was significant with $p < 0.01$, and the IL-23-miR-155 correlation was significant with $p < 0.05$ ($n = 12$).

markers as tools for quality control of DC vaccines in future clinical trials that will ensure better patient survival.

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References

[1] Jonuleit H, Kühn U, Müller G, Steinbrink K, Paraqnik L, Schmitt E, et al. Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur J Immunol 1997;27:3135–42.
[2] Steinman RM, Banchereau J. Taking dendritic cells into medicine. Nature 2008; 449:419–26.
[3] Erdmann M, Schuler-Thurner B. Dendritic cell vaccines in metastasized malignant melanoma. G Ital Dermatol Venereol 2008;143:235–50.
[4] Engell-Noerregaard L, Hansen TH, Andersen MH, Thor Straten P, Svane IM. Review of clinical studies on dendritic cell-based vaccination of patients with malignant melanoma: assessment of correlation between clinical response and vaccine parameters. Cancer Immunol Immunother 2009;58:1–14.
[5] Figdor CG, de Vries IJ, Lesterhuis WJ, Melief CJ. Dendritic cell immunotherapy: mapping the way. Nat Med 2004;10:475–80.
[6] Dieu MC, Vanbervliet B, Vicari A, Bridon JM, Oldham E, Ait-Yahia S, et al. Selective recruitment of immature and mature dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. J Exp Med 1998;188:373–86.
[7] Hirao M, Onai N, Hiroishi K, Watkin SC, Matsushima K, Robbins PD, et al. CC chemokine receptor-7 on dendritic cells is induced after interaction of with apoptotic tumor cells: critical role in migration from the tumor site to draining lymph nodes. Cancer Res 2000;60:2209–17.
[8] Shortman K, Naik SH. Steady-state and inflammatory dendritic-cell development. Nat Rev Immunol 2007;7:19–30.
[9] Bartel DP. MicroRNAs: genomics, Biogenesis, mechanism, and function. Cell 2004;116:281–97.
[10] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC, et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998;391:806–11.
[11] Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. Science 2002;297:2056–60.
[12] Lodish HF, Zhou B, Liu G, Chen CZ. Micromanagement of the immune system by microRNAs. Nat Rev Immunol 2008;8:120–30.
[13] Tili E, Michaille J-J, Calin GA. Expression and function of microRNAs in immune cells during normal or disease state. Int J Med Sci 2008;5:73–9.
[14] Hoefig KP, Heissmeyer V. MicroRNAs grow up in the immune system. Curr Opin Immunol 2008;20:281–7.
[15] Pedersen I, David M. MicroRNAs in the immune response. Cytokines 2008;43: 391–4.
[16] Tili E, Michaille J-J, Cimino A, Costinean S, Dumitru CD, Adair B, et al. Modulation of Mir-155 and Mir-125b levels following lipopolysaccharide/TNF-α stimulation and their possible roles in regulating the response to endotoxin shock. J Immunol 2007;179:5082–9.
[17] Vigorito E, Perks KL, Aberu-Goodger C, Bunting S, Xiang Z, Kohlhaas S, et al. MicroRNA-155 regulates the generation of immunoglobulin class-swithched plasma cells. Immunity 2007;27:847–59.
[18] Turner M, Vigorito E. Regulation of B- and T-cell differentiation by a single microRNA. Biochem Soc Trans 2008;36:531–3.
[19] Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, Soond DR, et al. Requirement of *bic/microRNA-155* for normal immune function. Science 2007; 316:608–11.
[20] Taganov KD, Boldin MP, Chang K-J, Baltimore D. NF-$\kappa\beta$-dependent induction of microRNA Mir-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci USA 2006;103:12481–6.
[21] O'Connell RM, Taganov KD, Boldin MP, Cheng G, Baltimore D. MicroRNA-155 is induced during the macrophage inflammatory response. Proc Natl Acad Sci U S A 2007;104:1604–9.
[22] Ceppi M, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, Santos MA, et al. MicroRNA-155 modulates the interleukin-1 signalling pathway in activated human monocyte-derived dendritic cells. Proc Natl Acad Sci U S A 2009;106: 2735–40.
[23] Romani N, Gruner S, Brang D, Kämpgen E, Lenz A, Trockenbacher B, et al. Proliferating dendritic cell progenitors in human blood. J Exp Med 1994;180: 83–93.
[24] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2 $(-\Delta\Delta C(T))$ method. Methods 2001;25:402–8.
[25] Válóczi A, Hornyik C, Varga N, Burgyán J, Kauppinen S, Havelda Z, et al. Sensitive and specific detection of microRNAs by northern blot analysis using LNA-modified oligonucleotide probes. Nucleic Acids Res 2004;32:e175.
[26] Sánchez-Sánchez N, Riol-Blanco L, Rodriguez-Fenández JL. The multiple personalities of the chemokine receptor CCR7 in dendritic cells. J Immunol 2006; 176:5153–9.
[27] Czerniecki BJ, Cohen PA, Faries M, Xu S, Roros JG, Bedrosian I, et al. Diverse functional activity of $CD83^+$ monocyte-derived dendritic cells and the implications for cancer vaccines. Crit Rev Immunol 2001;21:157–78.
[28] Steinman RM. Some interfaces of dendritic cell biology. APMIS 2003;111:675–97.
[29] Pedersen AW, Holmstrøm K, Jensen SS, Fuchs D, Rasmussen S, Kvistborg P, et al. Phenotypic and functional markers 1α,25-dihydroxyvitamin D_3 treated dendritic cells. Clin Exp Immunol 2009;157:48–59.
[30] Hou J, Wang P, Lin L, Liu X, Ma F, An H, et al. MicroRNA-146a feedback inhibits RIG-I IFN production in macrophages by targeting TRAF6, IRAK1 and IRAK2. J Immunol 2009;183:2150–8.
[31] O'Connell RM, Rao DS, Chaudhuri AA, Boldin MP, Taganov KD, Nicoll J, et al. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. J Exp Med 2008;205:585–94.
[32] O'Connell RM, Chaudhuri AA, Rao DS, Baltimore D. Inositol phosphatase SHIP1 is a primary target of Mir-155. Proc Natl Acad Sci USA 2009;106:7113–8.
[33] Garzon R, Garofalo M, Martelli MP, Briesewitz R, Wang L, Fernandez-Cymering C, et al. Distinctive microRNA signature of acute myeloid leukemia bearing cytoplasmic mutated nucleophosmin. Proc Natl Acad Sci USA 2008;105:3945–50.
[34] van der Pouw Kraan TC, van Lier RA, Aarden LA. PGE_2 and the immune response. A central role for prostaglandin E_2 in downregulating the inflammatory immune response. J Exp Med 1995;181:775–9.

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