miR-22 Controls *Irf8* mRNA Abundance and Murine Dendritic Cell Development

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Abstract

MicroRNAs (miRNAs) have emerged as critical regulators of many cellular responses, through the action of miRNA-induced silencing complex (miRISC)- or miRNA ribonucleoprotein complex (miRNP)-mediated gene repression. Here we studied the role of miRNAs in the development of dendritic cells (DCs), an important immune cell type that is divided into conventional DC (cDC) and plasmacytoid DC (pDC) subsets. We found that miR-22 was highly expressed in mouse $CD11c^+$ $CD11b^+$ $B220^-$ cDCs compared to pDCs, and was induced in DC progenitor cell cultures with GM-CSF, which stimulate $CD11c^+$ $CD11b^+$ $B220^-$ cDC differentiation. Enforced overexpression of miR-22 during DC development enhanced $CD11c^+$ $CD11b^+$ $B220^-$ cDC generation at the expense of pDCs, while miR-22 knockdown demonstrated opposite effects. Moreover, overexpression and knockdown of miR-22 showed significant effects on the mRNA abundance of *Irf8*, which encodes the transcription factor IRF8 that plays essential roles in DC development. Luciferase reporter assays confirmed that miR-22 binds directly to the 3′UTR of the mouse *Irf8* mRNA. Collectively, these results suggest that miR-22 targets *Irf8* mRNA for posttranscriptional repression and controls DC subset differentiation.

Introduction

MicroRNAs (miRNAs) refer to a large family of short (usually 21–25 nucleotides in length), single-stranded non-coding RNA molecules [1]. They are found in various tissues across different species, and function as a posttranscriptional component of gene regulatory mechanisms in normal and malignant cell development. In vertebrates, most miRNAs are transcribed by RNA polymerase II from introns of protein-coding genes, then undergo sequential cleavages catalyzed by the nuclear RNase III enzymes Drosha and Dicer at primary and precursor miRNA levels [2]. The mature miRNA strand is incorporated into the so-called miRNA-induced silencing complex (miRISC) or miRNA ribonucleoprotein complex (miRNP), each containing an Argonaute protein, which will subsequently bind to the 3′UTR of target mRNAs based on seed nucleotide complementarity [1,3,4]. In mammalian cells, miRNAs repress gene expression mainly by inhibiting translation, or promote mRNA degradation via decapping or deadenylation. In addition, Argonaute 2 in miRISC or miRNP was reported to mediate mRNA silencing by its intrinsic RNA cleavage activity [5,6].

miRNA expression is not only associated with distinct cell lineages, but also related to cellular differentiation/maturation stage. In the hematopoietic system, miRNAs regulate hematopoietic progenitors, T and B lymphocytes, as well as other immune cells [7–10]. For example, miR-155 is involved in homeostasis and function of the immune system, and disruption of miR-155 leads to defective dendritic cell (DC)-mediated antigen presentation, reduced numbers of germinal center B cells and Th2-biased T-cell responses with impaired Th1 and Th17 activation [2,11–14]. By contrast, miR-223 is a negative regulator that is required to sustain granulocyte progenitors and granulopoiesis [15–17]. Experiments using conditional Dicer knockout mice suggest that Dicer-dependent miRNA expression is required for normal lymphocyte development [18–23].

In the current study, our goal was to identify miRNA regulators in DC lineage development. Two distinct DC populations, namely conventional DCs (cDC) and plasmacytoid DCs (pDC), have been classified in human and mouse. cDCs have been further subdivided on the basis of tissue localization, cell surface marker protein expression and function. Both cDC and pDC populations arise from common DC progenitors (CDPs, lin^- $Flt3^+$ $CD115^+$ $CD117^{-/lo}$) in bone marrow, under control of key cytokines and lineage-restricted transcription factors (reviewed in [24] and [25]). Despite accumulating evidence indicating the important roles of miRNAs in hematopoiesis, little is known about their function in controlling DC development. By profiling miRNA expression in mouse pDCs and cDCs, we found that miR-22 is highly enriched in $CD11c^+$ $CD11b^+$ $B220^-$ cDCs and suppressed in pDCs compared to its abundance in DC progenitors. We show that miR-22 binds to the 3′UTR of *Irf8* mRNA, which encodes IRF8, a transcription factor that is essential for pDC, $CD8\alpha^+$ cDC and $CD103^+$ cDCs, but not for $CD11c^+$ $CD11b^+$ $B220^-$ cDCs. miR-22 reduces *Irf8* mRNA

amounts, suggesting posttranscriptional control of *Irf8* that may play a role in mediating DC lineage decisions.

Materials and Methods

Ethics Statement

C57BL/6NCr and congenic CD45.1$^+$ mice were obtained from the National Cancer Institute or the Jackson Laboratory, and maintained in a specific pathogen free (SPF) facility at the University of Texas MD Anderson Cancer Center. All experimental procedures were approved and performed in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines at MD Anderson Cancer Center (Protocol Number: 050205834).

Isolation of DC progenitors, cDCs and pDCs

Murine bone marrow cells were obtained by flushing femurs and tibias with complete culture medium (RPMI supplemented with 10% FCS, 1% antibiotics and β-mercaptoethanol). Spleens were minced through nylon mesh and single cell suspensions were prepared. After lysis of RBCs, cells were stained with a rat anti-mouse lineage marker cocktail, including anti-CD3, CD19, Gr1, Ter-119 and F4/80, followed by staining with anti-rat IgG microbeads (Miltenyi). The lineage marker-positive cells were removed by passing cells through a magnetic column, according to the manufacturer's instructions (MACS). Negatively selected cell samples were further labeled with fluorescently-labeled antibodies against CD3, CD19, Gr1, Ter119, F4/80, Flt3, CD11c, CD11b and B220 (bone marrow) or CD11c, CD4, CD8α$^-$ CD11b, and B220 (spleen) to sort DC progenitors (lineage marker (lin)$^-$ Flt3$^+$), bone marrow pDCs (CD11c$^+$ CD11b$^-$ B220$^+$), splenic pDCs (CD11c$^+$ CD11b$^-$ B220$^+$), splenic CD4$^+$ cDCs (CD11c$^+$ CD11b$^+$ CD4$^+$ CD8α$^-$), splenic CD8α$^+$ cDCs (CD11c$^+$ CD11b$^-$ CD4$^-$ CD8α$^+$) and splenic CD4$^-$ CD8α$^-$ cDCs (CD11c$^+$ CD11b$^+$ CD4$^-$ CD8α$^-$) by FACS, as described previously [26]. All antibodies were purchased from BD or eBioscience.

Retro- and lentiviral constructs

To generate retroviral constructs that co-express miR-22 and GFP, sequences encoding the wild type (wt) or mutant (mut) miR-22 were released by XhoI and EcoRI digestion from pSuper-retroviral plasmids (kindly provided by Dr. Didier Picard, University of Geneva) [27] and inserted into a similarly digested pSuper-retroviral vector containing GFP. The GFP$^+$ lentiviral vector-based anti-miR-22 or scrambled hairpin control, miRZip22 and MiRZip000, respectively, were kindly provided by Dr. Hidetoshi Tahara (Hiroshima University) [28]. Using these viruses, we routinely observed infection rates between 30–50% in Flt3L cultures and 70–90% in GM-CSF cultures, as judged by the frequency of GFP$^+$ cells (data not shown).

DC culture, viral transduction and flow cytometry

FACS-purified DC progenitors were cultured in the presence of complete RPMI medium containing Flt3L (100 ng/ml) or GM-CSF (50 ng/ml). In Flt3L cultures, 90% or greater of the CD11c$^+$ CD11b$^-$ B220$^+$ pDCs express Siglec-H [26], while cDCs are a mixture of CD8α$^+$-equivalent and CD8α$^-$ cDCs [29,30]. By contrast, GM-CSF represses pDC production and mainly promotes the generation of CD11c$^+$ CD11b$^+$ B220$^-$ (CD8α$^-$) cDCs [31]. Two or three days later, cultured cells were collected for miRNA analysis by qPCR or subjected to viral transduction. To generate miR-22 overexpressing retroviruses, 293T cells were transfected with pSuper-GFP plasmids encoding wt or mut miR-22, in addition to the viral packaging plasmid pCL-Eco. To prepare anti-miR-22 lentiviruses, 293T cells were transfected with

miRZip000 vector or miRZip22 plasmids, along with the viral packaging plasmids pPACKH1-GAG, pPACKH1-REV and pVSV-G. Virus-containing supernatants were collected at 48 and 72 h and used to infect cultured DC progenitor cells via a spin infection method (2300 rpm, 60 min). Two days following infection, cells were analyzed by flow cytometry using fluorescently-labeled antibodies against CD11c, CD11b, B220, MHC II, CD80 and CD86.

Intravenous injection of DC progenitors

Lin$^-$ Flt3$^+$ DC progenitors were isolated from CD45.1$^+$ congenic mice and cultured with Flt3L or GM-CSF as described (Methods, DC culture section). Two days later, cells were incubated with retroviruses expressing wt or mut miR-22, or with miRZip22 or miRZip000 lentiviruses for 8 hours. Progenitor cells were then washed with PBS, and injected i.v. into C57BL/6 mice (10^5 cells/mouse/100 μl PBS). Recipient mice were sacrificed at d 7, and spleens were removed and analyzed for DC subset differentiation by flow cytometry.

miRNA extraction, miRNA microarray profiling and qPCR analysis

Total RNA and miRNA were purified from freshly isolated DC progenitors, pDCs, cDCs or total bone marrow following culture in Flt3L or GM-CSF, as indicated in the figure legends, using the miRNeasy mini kit (Qiagen). The miRNA array profiling was performed at the Microarray Core Facility at MD Anderson Cancer Center, using Exiqon miRCURY LNA miRNA array 8.1. In addition, cDNAs were reverse transcribed by miScript Reverse Transcriptase and subjected to qPCR using QuantiTect SYBR Green PCR master mix (Qiagen). miRNA expression in qPCR was normalized to the internal control 5SrRNA [32] via the ΔC_T method [33]. The primer sequences for primary miR-22 are: sense 5′-CGAACAGCAGGGTGGATGAT-3′, antisense 5′-GGCA-GAAAGCCTTGGGTTGT-3′.

Dual luciferase reporter assays

The mouse *Irf8* 3′UTR (chromosome 8 genomic coordinates 120757176 to 120756694; encompassing 1521 bp downstream (3′) of the *Irf8* mRNA stop codon) was amplified by PCR with primers containing XbaI sites and cloned into the XbaI site of the PGL3-SV40 vector (Promega) downstream (3′) of the firefly luciferase region. Truncated *Irf8* 3′UTR sequences that lack of one or both predicted miR-22 seed regions were cloned via a similar approach, as indicated in the figure legends. D2SC/1 cells [34] were then transfected with the pGL3 constructs containing full length or truncated *Irf8* 3′UTRs, phRL-TK (encoding *Renilla* luciferase), and plasmids that overexpress or block miR-22, using Lipofectamine 2000 (Invitrogen). Firefly luciferase activity was determined 48 h after transfection, and normalized to the control *Renilla* level, using the Dual-Luciferase Reporter Assay System (Promega). Transcription of the firefly luciferase gene is constitutively controlled by the SV40 promoter, and is affected by the *Irf8* 3′UTR through the binding of miR-22.

Statistical analysis

Data were compared by unpaired two-tailed Student's *t*-test using GraphPad Prism 5 (http://www.graphpad.com); *p* values <0.05 were considered to be statistically significant.

Results

miR-22 is differentially expressed in pDCs and cDCs

To characterize miRNA expression in DCs, we isolated total RNA including miRNA from bone marrow pDCs and splenic CD11c$^+$ CD11b$^+$ B220$^-$ cDCs and hybridized labeled RNAs to Exiqon miRCURY LNA miRNA array 8.1. We also analyzed RNA from lineage-negative, fms-like tyrosine kinase receptor-3 (Flt3)-positive (lin$^-$ Flt3$^+$) DC progenitors in bone marrow to provide a comparison for potential developmental changes in miRNA expression. We profiled miRNA samples in 2 independent experiments and found highly consistent results showing 21 miRNAs that were differentially expressed in pDCs versus CD11c$^+$ CD11b$^+$ B220$^-$ cDCs (Fig. 1A). For example, miR-22, miR-142-3p and miR-142-5p were upregulated in CD11c$^+$ CD11b$^+$ B220$^-$ cDCs and downregulated in pDCs relative to progenitor expression levels, while miR-20a, miR-17-5p and miR-130a showed the reverse pattern. miR-22 showed the most remarkable difference between the two DC subsets (Fig. 1A), and thus we selected it for further analysis. To confirm differential miR-22 expression in DCs and their progenitors, we performed qPCR analysis on RNA samples from cell populations isolated from bone marrow or spleen. We found that miR-22 is highly enriched in all splenic cDC subsets, including CD4$^+$, CD8α^+ and CD4$^-$ CD8α^- cDCs, while being expressed at relatively lower amounts in pDCs from bone marrow or spleen (Fig. 1B). To examine whether miR-22 is regulated during DC development, we cultured lin$^-$ Flt3$^+$ DC progenitors ex vivo with Flt3L or GM-CSF, conditions that promote, respectively, the differentiation of pDCs and cDCs in an approximate 1:1 ratio, or selective cDC differentiation including CD11c$^+$ CD11b$^+$ B220$^-$ cDCs (reviewed in [25]). We found that miR-22 was highly induced in GM-CSF cultures, while slightly repressed in Flt3L cultures (Fig. 1C). These results collectively suggest that miR-22 expression is regulated during DC development, with upregulation in CD11c$^+$ CD11b$^+$ B220$^-$ cDCs and suppression in pDCs compared to DC progenitors.

miR-22 affects DC subset differentiation and maturation

To examine the role of miR-22 in DC development and function, we employed GFP-encoding retro- and lentiviral constructs to enforce its overexpression or knockdown. We confirmed that wt or seed nucleotide mut miR-22 was overexpressed by pSUPER retroviral vectors in Flt3L- or GM-CSF-cultured lin$^-$ Flt3$^+$ DC progenitors, relative to mock-infected controls, using qPCR analysis (Fig. 2A). The enhanced expression of wt and mut miR-22 observed in DC progenitors cultured with GM-CSF versus Flt3L is likely due to higher endogenous miR22 levels in GM-CSF conditions or suppressive effects of Flt3L (Fig. 1C), as indicated by the mock-infected controls. In addition, we found that the miRZip lentiviral vector-based anti-miR-22, which antagonizes miR-22 function by blocking binding to target mRNAs, was expressed in >90% transfected 293 T cells (Fig. 2B), indicating efficient generation of anti-miR-22 lentiviruses.

To investigate whether miR-22 controls pDC and/or cDC differentiation, we cultured lin$^-$ Flt3$^+$ DC progenitors in Flt3L or GM-CSF for 2 days, infected cells with miR-22 overexpression or knockdown viruses, and analyzed the development of CD11c$^+$ CD11b$^-$B220$^+$ pDCs and CD11c$^+$ CD11b$^+$ B220$^-$ cDCs within the GFP$^+$ population. We found that overexpression of wt miR-22 enhanced the production of CD11c$^+$ CD11b$^+$ B220$^-$ cDCs and inhibited generation of pDCs in response to Flt3L, as evidenced by corresponding changes in the frequency (Fig. 2C upper panels) and absolute number (Fig. 2F) of each DC subset. A similar trend was observed in GM-CSF cultures (Fig. 2D upper panels; 2F);

however, the changes in cDC numbers did not reach statistical significance, which is most likely due to the predominant role of GM-CSF in driving cDC production and repressing pDCs [31]. By contrast, when miR-22 was blocked by miRZip22, pDC development was enhanced and CD11c$^+$ CD11b$^+$ B220$^-$ cDC generation was suppressed in both Flt3L and GM-CSF cultures (Fig 2C–D lower panels; 2F). To determine whether miR-22 regulates DC development in vivo, Flt3L- or GM-CSF-cultured CD45.1$^+$ DC progenitors were infected with miR-22-overexpression or knockdown viruses, or the appropriate controls, then transferred into CD45.2$^+$ recipient mice. For these assays, we chose the cytokine conditions that displayed the optimal differences in DC subset development ex vivo (Fig. 2C and D). Six days after adoptive transfer, spleens were collected and analyzed for CD45.1$^+$ CD11c$^+$ DC development. Consistent with the ex vivo results, overexpression of miR-22 in Flt3L-stimulated DC progenitors significantly enhanced CD11c$^+$ CD11b$^+$ B220$^-$ cDC production while repressing pDCs (Fig. 2E upper panel). Moreover, miR-22 knockdown in progenitors exposed to GM-CSF had the opposite effects (Fig. 2E lower panel). Collectively, these data suggest that miR-22 influences DC subset development.

Since miR-22 was predominantly expressed in CD11c$^+$ CD11b$^+$ B220$^-$ cDCs compared to pDCs (Fig. 1), we examined its function in cDCs derived from GM-CSF cultures. We used GFP-encoding miR-22 overexpression or knockdown vectors to manipulate miR-22 expression, and analyzed MHC class II (MHC II), CD80 and CD86 expression on GFP$^+$ CD11c$^+$ CD11b$^+$ B220$^-$ cDCs. We found that miR-22 overexpression in cDCs upregulated MHC II expression, relative to MHC II amounts observed with mut miR-22 overexpression (Fig. 3, upper panels). We also observed a modest increase in CD80 and CD86 expression upon miR-22 overexpression (Fig. 3, upper panels). By contrast, we found that miR-22 knockdown suppressed surface presentation of MHC II, CD80 and CD86 compared to cells infected with the miRZip000 control vector (Fig. 3, lower panels). Importantly, since we used comparisons with cells infected with mut or empty vector in these assays, our approach ruled out the possibility that changes in MHC II, CD80 and CD86 were related to viral infection. Thus, our results suggest that miR-22 directly or indirectly enhances cell surface expression of MHC II and costimulatory molecules in CD11c$^+$ CD11b$^+$ B220$^-$ cDCs.

miR-22 affects Irf8 mRNA through direct binding to its 3′UTR

Individual miRNAs can target hundreds of mRNAs through complementarity of the seed region nucleotides [1,3,4]. To identify potential targets of miR-22, we performed an algorithm-based prediction using two widely utilized software programs (Target Scan and MiRanda). Among more than 2000 potential target genes (not shown), we selected Irf8 and Batf3 for further study, as these two genes encode transcription factors critical for DC development and function [35–43]. We found that the 3′ UTR of Irf8 mRNA (~1500 bp) contains 2 putative miR-22 binding sites, while the 3′UTR of Batf3 (~400 bp) contains 1 potential binding site (Fig. 4A). To assess whether Irf8 and Batf3 are targets of miR-22, we quantified their mRNA amounts in lin$^-$ Flt3$^+$ DC progenitors following enforced overexpression of wt or mut miR-22, or in response to miRZip22-mediated miR-22 knockdown. We observed a dramatic (~10-fold) reduction of Irf8 mRNA amounts upon miR-22 overexpression in lin$^-$ Flt3$^+$ DC progenitors cultured with Flt3L (Fig. 4B). By contrast, we found that Irf8 was expressed at lower amounts in lin$^-$ Flt3$^+$ DC progenitors cultured with GM-CSF compared to Flt3L, and miR-22 overexpression did not appear to affect Irf8 expression in GM-
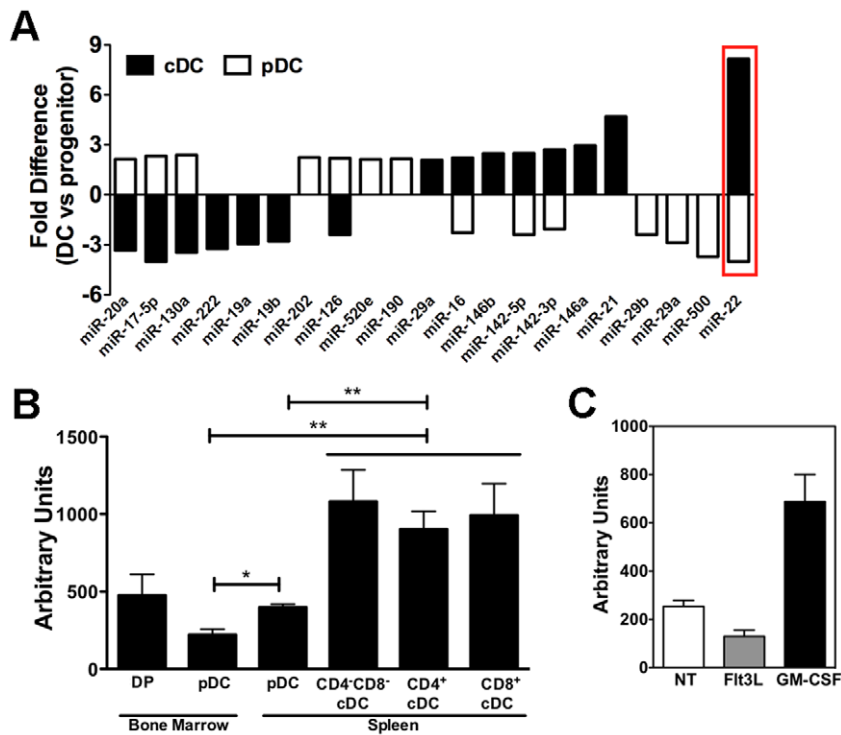
Figure 1. miRNA profiling in pDCs and cDCs. A. Total RNA, including miRNA, was isolated from FACS-purified bone marrow lin⁻ Flt3⁺ progenitors, bone marrow pDCs and splenic CD11c⁺ CD11b⁺ B220⁻ cDCs and analyzed by miRNA array. miRNA expression in pDCs and cDCs was normalized to expression in bone marrow lin⁻ Flt3⁺ progenitors, and presented as mean ± SD of 2 independent experiments. **B.** miR-22 expression in FACS-purified lin⁻ Flt3⁺ DC progenitors (DP), pDCs or individual cDC subsets, isolated from bone marrow or spleen as indicated, was analyzed by qPCR. **C.** lin⁻ Flt3⁺ DC progenitors were cultured in the presence of Flt3L or GM-CSF for 72 h; cells were analyzed for miR-22 expression by qPCR. **B–C.** Data are normalized to 5SrRNA, and shown as mean ± SD of 3 independent experiments.
doi:10.1371/journal.pone.0052341.g001

CSF conditions (Fig. 4B). Consistent with this, we observed that miRZip22-mediated miR-22 knockdown enhanced *Irf8* mRNA levels in GM-CSF-cultured progenitors in comparison to miR-Zip000 control, while miR-22 knockdown had only modest effects upon the already high amounts of *Irf8* mRNA in Flt3L cultures (Fig. 4C). miR-22 overexpression or knockdown did not appear to affect *Batf3* mRNA, with the exception of increased *Batf3* mRNA amounts in GM-CSF cultures with miR-22-overexpression (Fig. 4C). These data indicate that miR-22 specifically reduces *Irf8* mRNA abundance, potentially via enhanced mRNA degradation.

To address whether *Irf8* mRNA is a direct target of miR-22, we performed luciferase reporter assays in a cDC cell line, D2SC/1 [34]. For these experiments we generated a pGL3 reporter construct that contains the full length *Irf8* 3′ UTR downstream of the open reading frame of firefly luciferase (Fig. 5A and data not shown). We also generated 2 truncated *Irf8* 3′ UTR constructs, which lack one or both of the putative miR-22-binding sites, to better evaluate the function of these regions (Fig. 5A). The firefly luciferase activity of all three constructs is controlled by the SV40 promoter, and the addition of *Irf8* 3′UTR with or without the miR-22 seed region(s) allows us to monitor the changes in reporter activity fine-tuned by miR-22 binding. We found that wt miR-22 inhibited reporter activity of the construct containing the full length *Irf8* 3′UTR, relative to effects of the mut miR-22 on reporter activity (Fig. 5B). Consistently, knockdown of miR-22 function by miRZip22 induced an approximate 2-fold increase in the activity of the full length *Irf8* 3′UTR reporter compared to effects of the miRZip000 control on reporter function (Fig. 5C).

These results suggest that miR-22 negatively regulates transcription of the firefly luciferase reporter gene in the presence of the *Irf8* 3′UTR. Moreover, we demonstrated that miR-22-binding sites in the *Irf8* 3′UTR are required for miR-22-mediated repression of reporter activity, as the truncated *Irf8* 3′UTR reporter constructs that lack one or both miR-22-binding sites were either partially or completely refractory to miR-22-mediated regulation in both overexpression and knockdown settings (Fig. 5B, 5C). These results collectively indicate that miR-22 interacts with miR-22 seed regions in the *Irf8* 3′UTR and mediates *Irf8* mRNA repression.

Discussion

Despite the fact that murine pDCs and cDCs originate from a common DC progenitor, they require distinct growth factors and molecular cues for their lineage specification and development, which results in the acquisition of their divergent morphology and function. We report here that miR-22 enhances the generation of CD11c⁺ CD11b⁺ B220⁻ cDCs from DC progenitors in vivo and in vitro and stimulates the mature phenotype of this subset, consistent with abundant miR-22 amounts observed in CD11c⁺ CD11b⁺ B220⁻ cDCs relative to pDCs or DC progenitors. Furthermore, we found that miR-22 directly regulates *Irf8* mRNA amounts in DCs, potentially via targeting *Irf8* mRNA for destruction. To our knowledge, this is the first study demonstrating the role of miR-22 in DC development, suggesting miR-22 involvement in normal hematopoiesis and cell differentiation.

Numerous miRNAs have been shown to regulate immune cell development and function, such as miR155, miR-223 and miR-181 (reviewed in [7,44,45]). These miRNAs exert their roles by
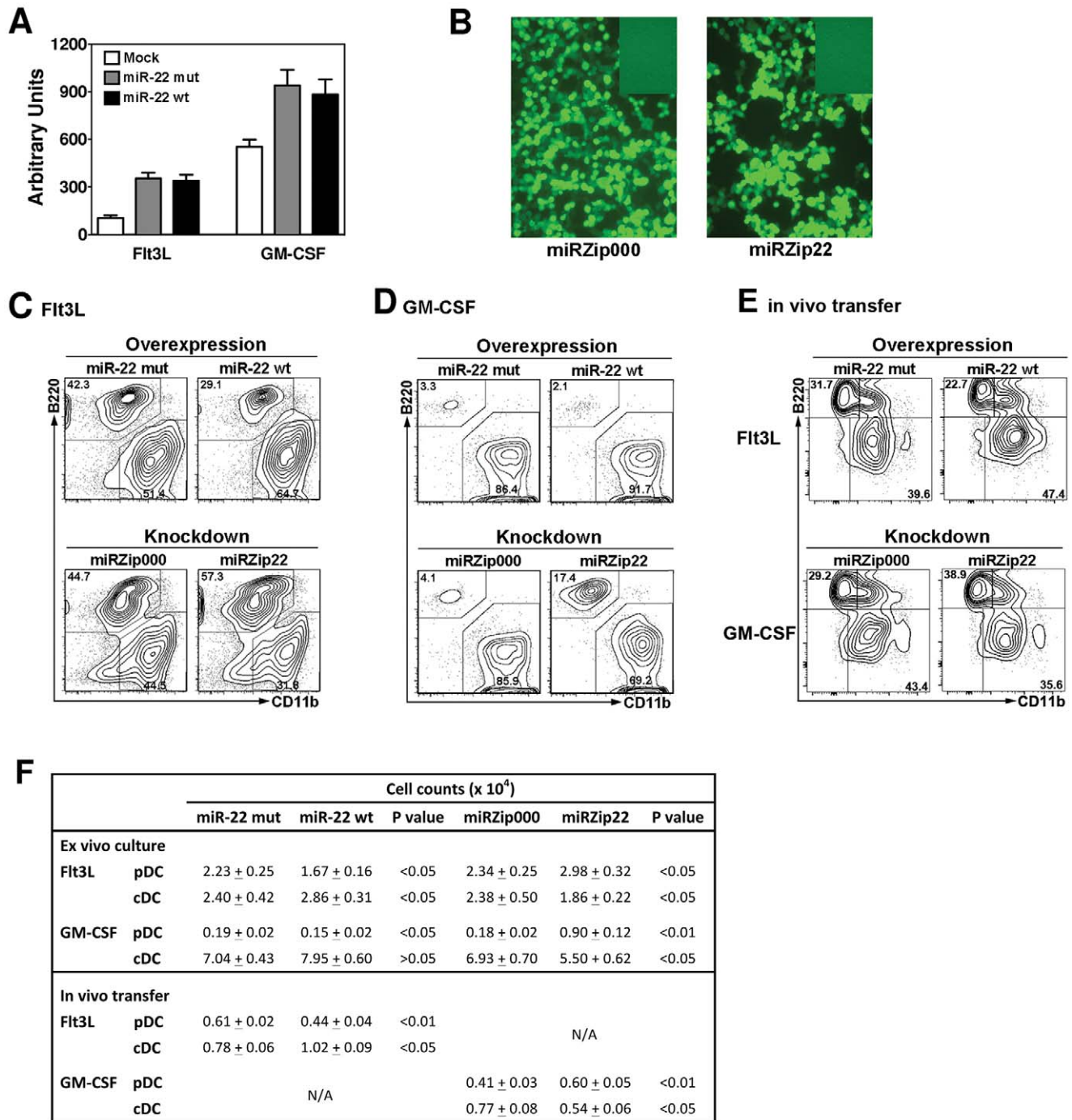
Figure 2. miR-22 facilitates cDC generation while suppressing pDC development. A. lin$^-$ Flt3$^+$ DC progenitors were cultured with Flt3L or GM-CSF for 2 days, and infected with retroviruses expressing wt or mut miR-22 or with empty vector (mock), as indicated. Two days after infection, cells were collected and analyzed for miR-22 expression by qPCR. Data were normalized to 5SrRNA, and shown as mean $\pm$ SD of 3 independent experiments. **B.** 293T cells were transfected with miR-22-knockdown plasmid miRZip22 or miRZip000 control vector, together with pPACK packaging plasmids. GFP expression was analyzed by fluorescence microscopy 2 days following transfection. The small rectangle on the right upper corner shows the uninfected control. **C–D.** 10^5 lin$^-$ Flt3$^+$ DC progenitors were cultured with Flt3L (**C**) or GM-CSF (**D**). Two days later, cells were infected with retroviruses expressing wt or mut miR-22 (overexpression), or with miRZip22 or miRZip000 lentiviruses (knockdown). Two days after infection, cells were collected and analyzed for DC generation by FACS. Representative flow data within the gated GFP$^+$ CD11c$^+$ population from 3 independent experiments are shown in **C–D**. **E.** 10^5 lin$^-$ Flt3$^+$ DC progenitors from CD45.1$^+$ congenic mice were cultured with Flt3L (upper panel) or GM-CSF (lower panel) for 2 d, followed by viral infection to overexpress or knockdown miR-22 as described in **C–D**. Eight hours after infection, cells were injected i.v. into C57BL/6 mice (CD45.2$^+$). Six days later, spleens were collected and analyzed for DC populations by FACS. Representative flow data are shown within the gated CD45.1$^+$ CD11c$^{+/lo}$ cells. **F.** The absolute number of pDCs and cDCs from the in vitro (**C, D**) or in vivo (**E**) assays were determined by enumeration. The mean $\pm$ SD of absolute DC numbers (3–5 experiments/group) are shown.
doi:10.1371/journal.pone.0052341.g002
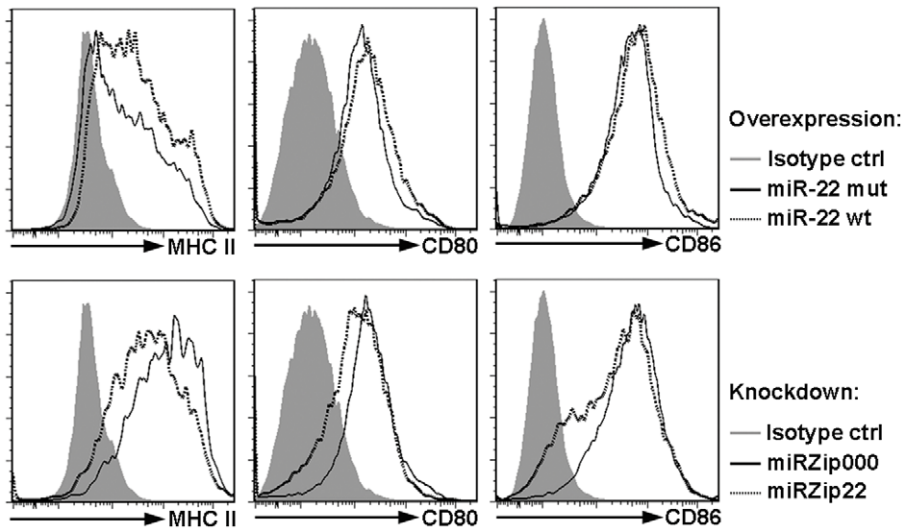
Figure 3. miR-22 promotes MHC and costimulatory molecule expression on cDCs. Total bone marrow cells were cultured with Flt3L or GM-CSF for 3 days, followed by infection with retroviruses expressing wild type (wt) or mutant (mut) miR-22. Two days after infection, GFP⁺ cells were gated and analyzed for DC markers by flow cytometry. MHC II, CD80 and CD86 expression on GFP⁺ CD11c⁺ CD11b⁺ B220⁻ cDCs is shown. Results represent 3 independent experiments.
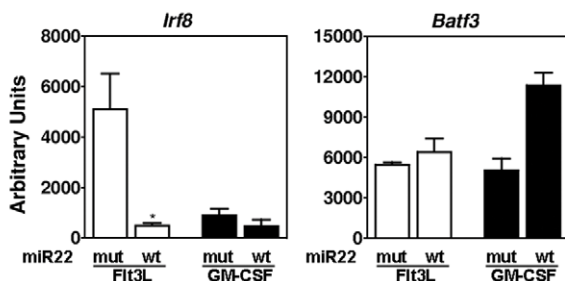doi:10.1371/journal.pone.0052341.g003

fine-tuning the expression levels of critical transcription factors and genes involved in mature cell activities. While *Batf3* and *Stat5* were identified in our software analyses as putative miR-22 targets, we were unable to obtain evidence for their direct regulation by miR-22. By contrast, here we show that miR-22 targets murine *Irf8*

mRNA directly through a complementary seed region in the *Irf8* 3′UTR. We have previously demonstrated that *Irf8* is inducible by the DC growth factor Flt3L, whereas the cytokines GM-CSF and IFN-α directly repress or stimulate *Irf8* transcription via the transcriptional regulators STAT5 or STAT1, respectively [31,33].
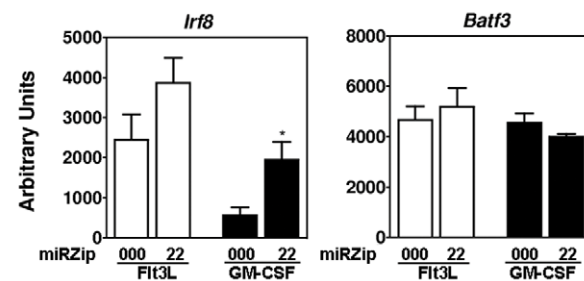

Figure 4. miR-22 regulates *Irf8* mRNA abundance. A. Summary of potential miR-22 target sites in the 3′-UTR of *Irf8* and *Batf3* mRNAs. **B–C.** lin⁻ Flt3⁺ progenitors were cultured for 3 days with Flt3L or GM-CSF, as indicated. Cells were infected with retroviral vectors expressing wt or mut miR-22 (**B**) or lentiviral vectors expressing control (miRZip000) or anti-miR-22 (miRZip22) as indicated (**C**). Two days after infection, the relative expression of *Irf8* and *Batf3* mRNA was determined by qPCR and normalized to 5S rRNA amounts. Data are shown as mean ± SD of 3 independent experiments.
doi:10.1371/journal.pone.0052341.g004
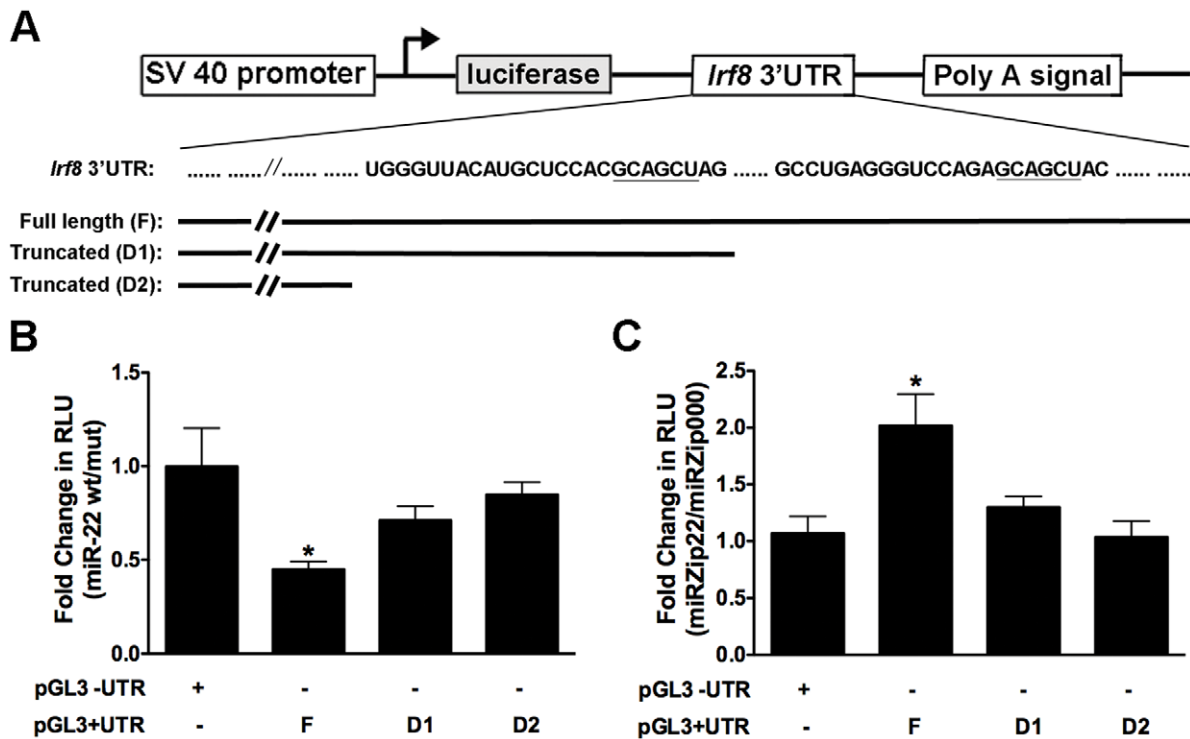
Figure 5. miR-22 targets the 3′UTR of *Irf8*. A. Schematic diagram of the pGL3 luciferase reporter constructs containing full length or truncated versions of the 3′-UTR of *Irf8* mRNA. **B–C.** D2SC/1 cells were transfected using Lipofectamine 2000 with pGL3 constructs described in **A**, phRL-TK, and miR-22 overexpression (**B**) or knockdown (**C**) plasmids. The ratio of *firefly: Renilla* luciferase light units (RLU) was determined 48 h after transfection using the Dual-Luciferase Reporter Assay System, and the fold change in RLU was calculated as indicated on y-axis. Results are presented as mean ± SD of 3 independent experiments.
doi:10.1371/journal.pone.0052341.g005

The GM-CSF-STAT5- and miR-22-mediated regulatory pathways may work cooperatively at transcriptional and post-transcriptional levels, respectively, to precisely control the amount of *Irf8* mRNA expression during DC subset differentiation. By contrast, human *IRF8* 3′UTR does not appear to contain a miR-22 seed region and miR-22 is not differentially expressed in human pDCs and cDCs (data not shown). However, inspection of the human *IRF8* 3′UTR revealed potential target sites for multiple miRNAs, including miR-130a, miR-19a and miR-19b, which are differentially regulated in mouse pDCs and cDCs (results herein). These, and other miRNAs that are specifically regulated in humans, may be involved in controlling human *IRF8* expression in analogous but independent regulatory pathways from the miR-22-mediated mechanism described herein.

IRF8 is a transcription factor that plays an essential role in controlling the development and cytokine secretion of several murine DC subsets, including pDCs, lymphoid CD8α^+ cDCs and non-lymphoid CD103$^+$ DCs [37,38,40,42,46,47]. The role of IRF8 in DC development is conserved in humans as point mutations (T80A, K108E) in the IRF8 DNA-binding domain are found in individuals with immunodeficiency accompanied by impaired DC subset production [36]. By contrast, IRF8 is dispensable for homeostatic production of murine CD11c$^+$ CD11b$^+$ B220$^-$ cDCs, a population that mainly contains CD8α^- cDCs [31,38]. Interestingly, we did not observe a significant difference in miR-22 amounts in splenic CD4$^+$, CD8α^+ or CD4$^-$ CD8α^- cDC subsets, although these populations have differential requirements for IRF8 [35,38]. These results suggest that the developmental effects of miR-22, potentially via

Irf8 regulation, may occur at pre-cDC population prior to cDC subset diversification.

miR-22 was thought to be a ubiquitously expressed miRNA [48], yet we show here that miR-22 is highly enriched in murine CD11c$^+$ CD11b$^+$ B220$^-$ cDCs in comparison to pDCs, suggesting important regulation of miR-22 expression occurs in the hematopoietic system. This distinct expression pattern is consistent with miR-22 function in DC lineage differentiation, as our overexpression and knockdown of miR-22 supports the idea that miR22 promotes CD11c$^+$ CD11b$^+$ B220$^-$ cDC production and inhibits pDC development. In addition, we found that miR-22 modestly enhances MHC class II and costimulatory molecule expression in CD11c$^+$ CD11b$^+$ B220$^-$ cDCs, suggesting it also affects genes that are involved cDC antigen presentation, a major function for the cDC subsets. miR-22 has been previously implicated in regulating histone modifications [49,50], as well as tumorigenesis by controlling tumor cell proliferation, migration/ invasion and apoptosis [51–54]. More importantly, miR-22 has been characterized in multiple signaling pathways that play critical roles in hematopoiesis and cellular function, including NFκB [55], PTEN/AKT [56,57] and estrogen receptor [58] responses. These pathways are also involved in DC development and function. For examples, PTEN was identified as a negative regulator for CD8α^+ and CD103$^+$ DCs downstream of Flt3 signaling [59], while NFκB and estrogen receptor are critical for signals elicited by pattern recognition receptors expressed on DCs [60,61]. As one miRNA may have hundreds of target genes, the regulatory function of miR-22 in these signaling cascades may contribute to its effects on DC development and function.

In addition to our results that identify miR-22 as a negative regulator of the DC transcription factor IRF8 by controlling *Irf8* mRNA abundance, recent studies by others have shown that miR21, miR34a, miR-221 and miR-222 are differentially expressed in pDCs and cDCs, and play a role in DC differentiation via inhibitory functions on *Jag1*, *Wnt1*, and possibly the pDC master regulator *Tcf4* (E2–2), respectively [62–66]. Among these, Kuipers *et al.* reported that miR-222 is expressed at 2.5-fold higher levels in cDCs versus pDCs purified from bone marrow cell cultures supplemented with Flt3L and SCF [62]. By contrast, our miRNA array profiling data revealed that miR-222 is expressed at lower levels in freshly isolated splenic cDCs in comparison to bone marrow DC progenitors or pDCs (Fig. 1A). This discrepancy may reflect the regulation of miRNA expression by cytokines, the different cell purification strategies used, or possible differences between microRNA array and miRNA measurements. Nonetheless, the available evidence suggests miRNAs serve as another layer of gene regulation that exerts profound influence on DC subset specification. In future work, it will be important to understand how miRNAs are regulated within DCs as well as the effects they exert on DC development and function. This information may prove valuable in redirecting DCs for use in clinical applications.

Author Contributions

Conceived and designed the experiments: HSL. Performed the experiments: HSL NG NS. Analyzed the data: HSL SSW YJL. Contributed reagents/materials/analysis tools: HSL SSW NS YJL. Wrote the paper: HSL SSW.

References

1. Berezikov E (2011) Evolution of microRNA diversity and regulation in animals. Nature reviews Genetics 12: 846–860.
2. Rodriguez A, Vigorito E, Clare S, Warren MV, Couttet P, et al. (2007) Requirement of bic/microRNA-155 for normal immune function. Science 316: 608–611.
3. Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, et al. (2002) miRNPs: a novel class of ribonucleoproteins containing numerous microRNAs. Genes & development 16: 720–728.
4. Dai R, Ahmed SA (2011) MicroRNA, a new paradigm for understanding immunoregulation, inflammation, and autoimmune diseases. Translational research: the journal of laboratory and clinical medicine 157: 163–179.
5. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, et al. (2004) Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Molecular cell 15: 185–197.
6. Hutvagner G, Simard MJ (2008) Argonaute proteins: key players in RNA silencing. Nature reviews Molecular cell biology 9: 22–32.
7. Baltimore D, Boldin MP, O'Connell RM, Rao DS, Taganov KD (2008) MicroRNAs: new regulators of immune cell development and function. Nature immunology 9: 839–845.
8. Malumbres R, Sarosiek KA, Cubedo E, Ruiz JW, Jiang X, et al. (2009) Differentiation stage-specific expression of microRNAs in B lymphocytes and diffuse large B-cell lymphomas. Blood 113: 3754–3764.
9. Neilson JR, Zheng GX, Burge CB, Sharp PA (2007) Dynamic regulation of miRNA expression in ordered stages of cellular development. Genes & development 21: 578–589.
10. Wu H, Neilson JR, Kumar P, Manocha M, Shankar P, et al. (2007) miRNA profiling of naive, effector and memory CD8 T cells. PLoS One 2: e1020.
11. Vigorito E, Perks KL, Abreu-Goodger C, Bunting S, Xiang Z, et al. (2007) microRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. Immunity 27: 847–859.
12. Thai TH, Calado DP, Casola S, Ansel KM, Xiao C, et al. (2007) Regulation of the germinal center response by microRNA-155. Science 316: 604–608.
13. O'Connell RM, Kahn D, Gibson WS, Round JL, Scholz RL, et al. (2010) MicroRNA-155 promotes autoimmune inflammation by enhancing inflammatory T cell development. Immunity 33: 607–619.
14. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, et al. (2009) Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. Immunity 30: 80–91.
15. Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, et al. (2005) A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. Cell 123: 819–831.
16. Fukao T, Fukuda Y, Kiga K, Sharif J, Hino K, et al. (2007) An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. Cell 129: 617–631.
17. Johnnidis JB, Harris MH, Wheeler RT, Stehling-Sun S, Lam MH, et al. (2008) Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. Nature 451: 1125–1129.
18. Muljo SA, Ansel KM, Kanellopoulou C, Livingston DM, Rao A, et al. (2005) Aberrant T cell differentiation in the absence of Dicer. The Journal of experimental medicine 202: 261–269.
19. Kanellopoulou C, Muljo SA, Kung AL, Ganesan S, Drapkin R, et al. (2005) Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes & development 19: 489–501.
20. Cobb BS, Hertweck A, Smith J, O'Connor E, Graf D, et al. (2006) A role for Dicer in immune regulation. The Journal of experimental medicine 203: 2519–2527.
21. Liston A, Lu LF, O'Carroll D, Tarakhovsky A, Rudensky AY (2008) Dicer-dependent microRNA pathway safeguards regulatory T cell function. The Journal of experimental medicine 205: 1993–2004.
22. Zhou L, Seo KH, He HZ, Pacholczyk R, Meng DM, et al. (2009) Tie2cre-induced inactivation of the miRNA-processing enzyme Dicer disrupts invariant NKT cell development. Proceedings of the National Academy of Sciences of the United States of America 106: 10266–10271.
23. Koralov SB, Muljo SA, Galler GR, Krek A, Chakraborty T, et al. (2008) Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. Cell 132: 860–874.
24. Watowich SS, Liu YJ (2010) Mechanisms regulating dendritic cell specification and development. Immunol Rev 238: 76–92.
25. Li HS, Watowich SS (2012) A STATus report on DC development. J Leukoc Biol 92: 445–459.
26. Li HS, Yang CY, Nallaparaju KC, Zhang H, Liu YJ, et al. (2012) The signal transducers STAT5 and STAT3 control expression of Id2 and E2-2 during dendritic cell development. Blood: (in press).
27. Pandey DP, Picard D (2009) miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor alpha mRNA. Mol Cell Biol 29: 3783–3790.
28. Xu D, Takeshita F, Hino Y, Fukunaga S, Kudo Y, et al. (2011) miR-22 represses cancer progression by inducing cellular senescence. J Cell Biol 193: 409–424.
29. Naik SH, O'Keeffe M, Proietto A, Shortman HH, Wu L (2010) CD8+, CD8−, and plasmacytoid dendritic cell generation in vitro using flt3 ligand. Methods Mol Biol 595: 167–176.
30. Naik SH, Proietto AI, Wilson NS, Dakic A, Schnorrer P, et al. (2005) Cutting edge: generation of splenic CD8+ and CD8− dendritic cell equivalents in Fms-like tyrosine kinase 3 ligand bone marrow cultures. J Immunol 174: 6592–6597.
31. Esashi E, Wang YH, Perng O, Qin XF, Liu YJ, et al. (2008) The signal transducer STAT5 inhibits plasmacytoid dendritic cell development by suppressing transcription factor IRF8. Immunity 28: 509–520.
32. Mishima T, Mizuguchi Y, Kawahigashi Y, Takizawa T, Takizawa T (2007) RT-PCR-based analysis of microRNA (miR-1 and -124) expression in mouse CNS. Brain Res 1131: 37–43.
33. Li HS, Gelbard A, Martinez GJ, Esashi E, Zhang H, et al. (2011) Cell-intrinsic role for IFN-alpha-STAT1 signals in regulating murine Peyer's patch plasmacytoid dendritic cells and conditioning an inflammatory response. Blood 118: 3879–3889.
34. Paglia P, Girolomoni G, Robbiati F, Granucci F, Ricciardi-Castagnoli P (1993) Immortalized dendritic cell line fully competent in antigen presentation initiates primary T cell responses in vivo. J Exp Med 178: 1893–1901.
35. Aliberti J, Schulz O, Pennington DJ, Tsujimura H, Reis e Sousa C, et al. (2003) Essential role for ICSBP in the in vivo development of murine CD8alpha + dendritic cells. Blood 101: 305–310.
36. Hambleton S, Salem S, Bustamante J, Bigley V, Boisson-Dupuis S, et al. (2011) IRF8 mutations and human dendritic-cell immunodeficiency. N Engl J Med 365: 127–138.
37. Schiavoni G, Mattei F, Borghi P, Sestili P, Venditti M, et al. (2004) ICSBP is critically involved in the normal development and trafficking of Langerhans cells and dermal dendritic cells. Blood 103: 2221–2228.
38. Schiavoni G, Mattei F, Sestili P, Borghi P, Venditti M, et al. (2002) ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. J Exp Med 196: 1415–1425.
39. Tailor P, Tamura T, Morse HC 3rd, Ozato K (2008) The BXH2 mutation in IRF8 differentially impairs dendritic cell subset development in the mouse. Blood 111: 1942–1945.
40. Tsujimura H, Tamura T, Ozato K (2003) Cutting edge: IFN consensus sequence binding protein/IFN regulatory factor 8 drives the development of type I IFN-producing plasmacytoid dendritic cells. J Immunol 170: 1131–1135.
41. Bar-On L, Birnberg T, Lewis KL, Edelson BT, Bruder D, et al. (2010) CX3CR1+ CD8alpha+ dendritic cells are a steady-state population related to plasmacytoid dendritic cells. Proc Natl Acad Sci U S A 107: 14745–14750.
42. Edelson BT, Kc W, Juang R, Kohyama M, Benoit LA, et al. (2010) Peripheral CD103+ dendritic cells form a unified subset developmentally related to CD8alpha+ conventional dendritic cells. J Exp Med 207: 823–836.

43. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, et al. (2008) Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity. Science 322: 1097–1100.

44. Carissimi C, Fulci V, Macino G (2009) MicroRNAs: novel regulators of immunity. Autoimmunity reviews 8: 520–524.

45. Belver L, Papavasiliou FN, Ramiro AR (2011) MicroRNA control of lymphocyte differentiation and function. Current opinion in immunology 23: 368–373.

46. Ginhoux F, Liu K, Helft J, Bogunovic M, Greter M, et al. (2009) The origin and development of nonlymphoid tissue CD103+ DCs. J Exp Med 206: 3115–3130.

47. Tamura T, Tailor P, Yamaoka K, Kong HJ, Tsujimura H, et al. (2005) IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. J Immunol 174: 2573–2581.

48. Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, et al. (2007) A mammalian microRNA expression atlas based on small RNA library sequencing. Cell 129: 1401–1414.

49. Li X, Liu J, Zhou R, Huang S, Chen XM (2010) Gene silencing of MIR22 in acute lymphoblastic leukaemia involves histone modifications independent of promoter DNA methylation. British journal of haematology 148: 69–79.

50. Huang S, Wang S, Bian C, Yang Z, Zhou H, et al. (2012) Upregulation of miR-22 Promotes Osteogenic Differentiation and Inhibits Adipogenic Differentiation of Human Adipose Tissue-Derived Mesenchymal Stem Cells by Repressing HDAC6 Protein Expression. Stem Cells Dev 21: 2531–2540.

51. Xiong J, Du Q, Liang Z (2010) Tumor-suppressive microRNA-22 inhibits the transcription of E-box-containing c-Myc target genes by silencing c-Myc binding protein. Oncogene 29: 4980–4988.

52. Li J, Liang S, Jin H, Xu C, Ma D, et al. (2012) Tiam1, negatively regulated by miR-22, miR-183 and miR-31, is involved in migration, invasion and viability of ovarian cancer cells. Oncology reports 27: 1835–1842.

53. Xu D, Takeshita F, Hino Y, Fukunaga S, Kudo Y, et al. (2011) miR-22 represses cancer progression by inducing cellular senescence. The Journal of cell biology 193: 409–424.

54. Tsuchiya N, Izumiya M, Ogata-Kawata H, Okamoto K, Fujiwara Y, et al. (2011) Tumor suppressor miR-22 determines p53-dependent cellular fate through post-transcriptional regulation of p21. Cancer research 71: 4628–4639.

55. Takata A, Otsuka M, Kojima K, Yoshikawa T, Kishikawa T, et al. (2011) MicroRNA-22 and microRNA-140 suppress NF-kappaB activity by regulating the expression of NF-kappaB coactivators. Biochemical and biophysical research communications 411: 826–831.

56. Bar N, Dikstein R (2010) miR-22 forms a regulatory loop in PTEN/AKT pathway and modulates signaling kinetics. PLoS One 5: e10859.

57. Tan G, Shi Y, Wu ZH (2012) MicroRNA-22 promotes cell survival upon UV radiation by repressing PTEN. Biochemical and biophysical research communications 417: 546–551.

58. Pandey DP, Picard D (2009) miR-22 inhibits estrogen signaling by directly targeting the estrogen receptor alpha mRNA. Molecular and cellular biology 29: 3783–3790.

59. Sathaliyawala T, O'Gorman WE, Greter M, Bogunovic M, Konjufca V, et al. (2010) Mammalian target of rapamycin controls dendritic cell development downstream of Flt3 ligand signaling. Immunity 33: 597–606.

60. Zanoni I, Granucci F (2010) Regulation of antigen uptake, migration, and lifespan of dendritic cell by Toll-like receptors. Journal of molecular medicine 88: 873–880.

61. Seillet C, Laffont S, Tremollieres F, Rouquie N, Ribot C, et al. (2012) The TLR-mediated response of plasmacytoid dendritic cells is positively regulated by estradiol in vivo through cell-intrinsic estrogen receptor alpha signaling. Blood 119: 454–464.

62. Kuipers H, Schnorfeil FM, Brocker T (2010) Differentially expressed microRNAs regulate plasmacytoid vs. conventional dendritic cell development. Mol Immunol 48: 333–340.

63. Hashimi ST, Fulcher JA, Chang MH, Gov L, Wang S, et al. (2009) MicroRNA profiling identifies miR-34a and miR-21 and their target genes JAG1 and WNT1 in the coordinate regulation of dendritic cell differentiation. Blood 114: 404–414.

64. Cisse B, Caton ML, Lehner M, Maeda T, Scheu S, et al. (2008) Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development. Cell 135: 37–48.

65. Ghosh HS, Cisse B, Bunin A, Lewis KL, Reizis B (2010) Continuous expression of the transcription factor e2-2 maintains the cell fate of mature plasmacytoid dendritic cells. Immunity 33: 905–916.

66. Nagasawa M, Schmidlin H, Hazekamp MG, Schotte R, Blom B (2008) Development of human plasmacytoid dendritic cells depends on the combined action of the basic helix-loop-helix factor E2-2 and the Ets factor Spi-B. Eur J Immunol 38: 2389–2400.