ROLE OF MiRNA-424 IN DENDRITIC CELL SUBSET SPECIFICATION

Final report

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Abstract

MicroRNAs are small non-coding RNAs, which regulate key biological processes in immune cells and define their phenotype and functions. Deficiency of Dicer, an enzyme critical for microRNA processing, in murine $CD11c^+$ cells revealed dysregulated dendritic cell (DC) development and function. The role of individual microRNAs in these processes remains poorly understood. Therefore, the purpose of this study was to investigate the role and molecular mechanisms of specific microRNAs in DC development.

Our group previously searched for microRNAs that are differentially expressed by human DC subsets. Our data revealed that microRNA-424 is strongly upregulated in pro-inflammatory monocyte –derived dendritic cells (moDCs) in comparison to other myeloid cells. Using lentiviral gain and loss-of-function approach we confirmed that microRNA-424 is critical for moDC development. Conversely, Langerhans dendritic cells (LCs) were unaffected by microRNA-424 knockdown. Given that both DC subsets arise from monocytopoietic precursors, microRNA-424 may participate in important regulatory pathways in DC subset differentiation in human.

To determine whether microR-424 is also involved in molecular mechanisms of moDCs differentiation *in vivo*, we subjected microRNA-424(322) knockout mice to a clinically relevant model of psoriasis-like inflammation. We observed that moDCs subsets are significantly reduced in the dermis of microRNA-424(322) knockout mice under inflammatory conditions in comparison with the WT mice. Similarly, bone marrow - derived DC differentiation *in vitro* was affected in microRNA-424(322) knockout mice. Consequently, our murine data corroborate our findings in human moDC vs LC differentiation that moDCs were selectively dependent on microRNA-424.

Until now, microRNA-424 has been regarded mostly as a tumor suppressive miRNA, and its function in myeloid cells as well as in human $CD34^+$ cells has been poorly understood. Our findings substantiate the pivotal role of microRNA-424 in moDCs differentiation both *in vitro* and *in vivo*. Together these data provide the first evidence that microRNA-424 controls monocyte/macrophage lineage and inflammation.

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List of abbreviations

4-SU 4- thiouridine
AGO Argonaute protein
APC antigen presenting cell
BMDC bone-marrow derived dendritic cells
cDC classical/ myeloid dendritic cell
CDP common dendritic cell progenitor
CMP common myeloid progenitors
DC dendritic cell
FLT3L fms-like tyrosine kinase 3 ligand
FT flow through
GFP green fluorescent protein
GM-CSF granulocyte/macrophage-colony stimulating factor
HPC hematopoietic progenitor cell
IACUC Institutional Animal Care and Use Committee
IMQ imiquimod
INOS macrophages inducible nitric oxide synthase macrophages
intDCs interstitial-type/dermal DCs
KLF4 Kruppel-like factor 4
IP immunoprecipitation
IPA Ingenuity Pathway Analysis
KD knockdown
LC Langerhans Cell
M-CSF macrophage-colony stimulating factor
Mac macrophage
KO knockout
MDPs macrophage/DC progenitors
moDC monocyte-derived dendritic cell
miRNA- microRNA
moLCs monocyte-derived Langerhans cells
MoP monocyte progenitor
moMacs monocyte-derived macrophages
mRNAs messenger RNAs
NFIA nuclear factor 1 A type
NFκB nuclear factor kappa-light-chain-enhancer of activated B cells
PAR-CLIP photoactivatable ribonucleosideenhanced crosslinking and immunoprecipitation
pDC plasmacytoid dendritic cell
pre-DCs precursors of DCs
RBPs RNA-binding proteins
RISC RNA-induced silencing complex
RUNX3 Runt-related transcription factor 3
SCF stem cell factor
TF transcription factor
TGF-β1 transforming growth factor beta 1
TLR Toll-like receptor
TNFα tumor necrosis factor alpha
WT wild type

Introduction

1.1 Dendritic cell subsets

Dendritic cells (DCs) serve as a bridge between the adaptive and innate immune response. They were first described as a group of antigen-presenting cells, which play a crucial role in mediating adaptive Steinman and Cohn in 1973 as adherent cell population prepared from mouse peripheral lymphoid organs (spleen, lymph node, Peyer's patch) with distinct morphological features (1). DC system promotes inflammation in response to pathogens and endogenous signals as well as maintains immune tolerance. DCs in human and in mice are characterized by different location (circulating blood DCs, lymph-node resident DCs, epidermal and dermal DCs), maturation state (steady-state and proinflammatory) and surface markers expression (conventional DCs, plasmacytoid DCs, monocyte-derived DCs, Langerhans cells). In the skin monocyte-derived dendritic cells (moDCs) are frequently recognized as “proinflammatory DCs”, because of their ability to infiltrate dermal layer of the skin upon inflammation. On the other hand, residing in epidermis Langerhans cells (LCs) are participating in maintenance of immune tolerance by interacting with a pool of skin resident T cells.

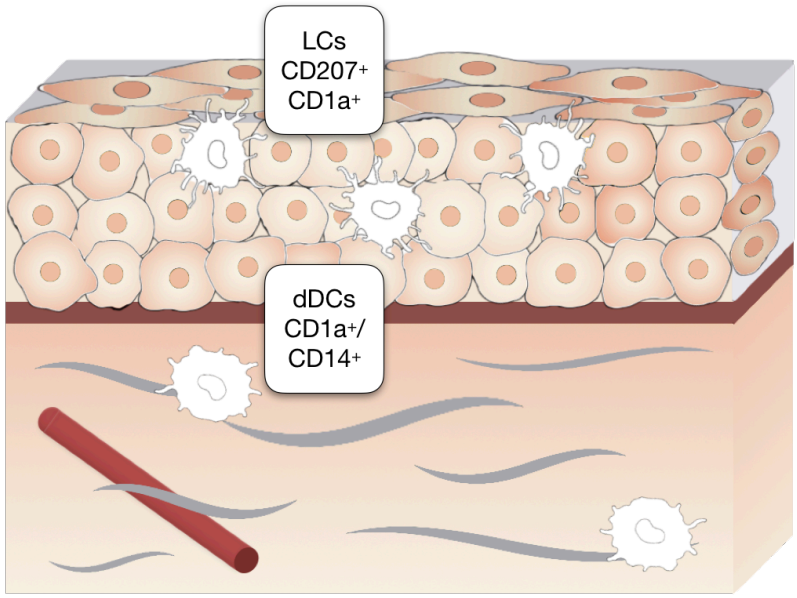

Figure 1. Schematic representation of DCs subset distribution in the human skin and their unique phenotypical marker expression profile.

1.2 Dendritic cell ontogeny

DC lineage originally comprise from hematopoietic stem cells (HSC), which give rise to common myeloid progenitors (CMPs) and then macrophage/DC progenitors (MDPs). In turn MDPs in bone marrow further proliferate into monocytes and common DC precursors (CDPs), which generate circulating precursors of DCs (pre-DCs). It was previously shown that monocytes comprise of heterogeneous population and distinct monocyte sunsets give rise to $INOS^{+}$ inflammatory macrophages and $CD209^{+}$ moDCs (2). Pool of DC populations is maintained by renewal from bone marrow precursors. In contrast to dermal DCs, epidermal LCs are independent on bone marrow progenitors and can be renewed from an embryonic precursor. Therefore, DCs types are more dependent on hematopoietic stem cell precursor than LCs. However, transcriptional mechanism driving monocyte differentiation towards moDCs and inflammatory macrophages still remains poorly understood.

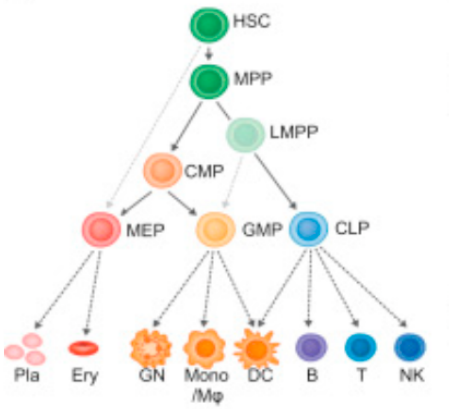

Figure 2. Schematic diagram of the hematopoietic tree. Dashed-arrows indicate omitted intermediate progenitors. Illustration from Paul et al. (2015, Cell, doi.org/10.1016/j.cell.2015.11.013).

1.3 Transcriptional control of DC development

Hematopoietic stem cell specification and lineage commitment is a complex process regulated by precise control of multiple transcription factors (3). Some of transcription factors have been known to mediate cell fate decisions on the early stages of dendritic cell development and DC subset specification (4). Previous studies identified that monocyte/ macrophage lineage specific factors such as PU.1 and KLF4 are instructing dendritic cell commitment of myeloid progenitors (5,6). PU.1 is known to be a master transcription factor of cell lineage decisions.

PU.1-deficient murine precursors fail to differentiate into DCs upon stimulation with GM-CSF in vitro. PU.1 plays an essential role in monocyte differentiation by antagonizing pro-granulocyte transcription factors, such as GATA-1, GATA-2 and C/EBPα, and inducing myeloid-specific factors, such as IRF8 and KLF4. Transcription factor KLF4 acts as key switch factor regulating differentiation of monocytes into LCs versus moDCs (7). Runt-related transcription factor 3 (RUNX3) is another important player in lineage specification decisions. Ectopic RUNX3 expression promotes LCs but inhibits moDCs subset differentiation. Downregulation of KLF4 in monocytes transcriptionally repressed RUNX3, therefore allowing LC commitment.

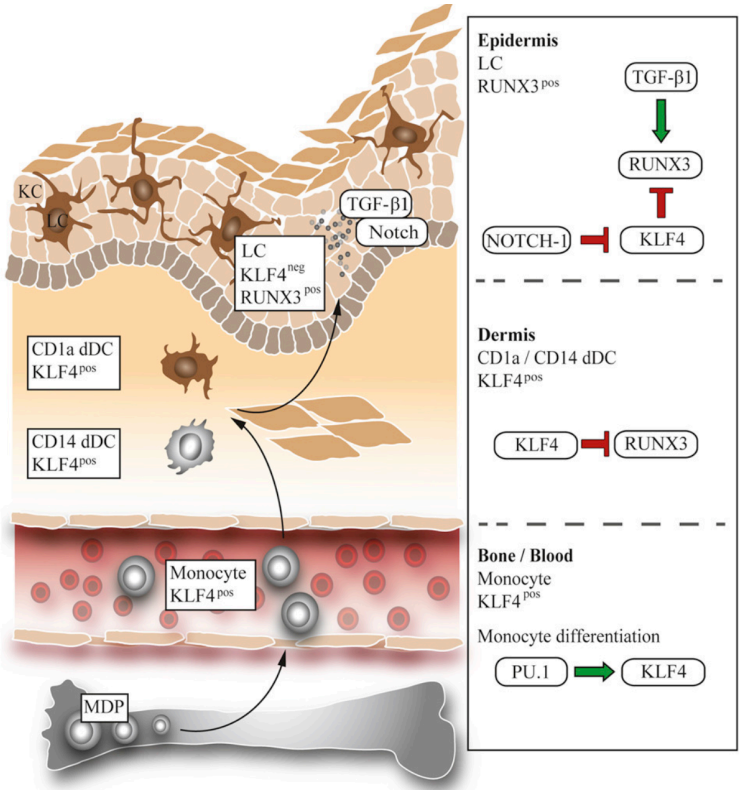

Figure 3. KLF4 and RUNX3 dependent transcriptional regulation of monocyte differentiation. Illustration from Jurkin, Krump et al. (JACI, 2017).

1.4 miRNAs biogenesis and gene silencing

In addition to the role of the primary transcription factors recent studies have demonstrated microRNAs as the important players in hematopoietic lineage development. miRNAs are small non-coding RNAs (19-24 nucleotides), which regulate gene expression on post-transcriptional level. MiRNAs were first discovered in 1993 by Lee et al. in *Caenorhabditis elegans*. Mammalian genome contains more than 2500 miRNAs and most of them are conserved across the species, allowing to use

different model organism for studying mechanisms of miRNA – dependent gene regulation. miRNAs are selectively expressed in different tissues and cells, therefore regulating specific protein profile of specific subpopulations. There is an evidence that miRNAs can also be carried by small extracellular vesicles i.e. exosomes. Alternatively, dysregulation of miRNA expression can lead to pathological processes, such as cancer and systemic inflammation.

To be fully functional miRNAs have to undergo multistep maturation process. In the nucleus miRNAs are initially transcribed a primary RNA and processed by Drosha enzyme. Then precursor-miRNAs are released to the cytoplasm, where they are cleaved by another RNAse III family enzyme Dicer to become mature miRNAs. On the last step miRNA is incorporated to RNA-induced silencing complex (RISC), composed of Argonaute protein (AGO). RISC complex guides recognition and binding of the 3'UTR of messenger RNAs (mRNAs) in order to either inhibit their translation or induce mRNA degradation.

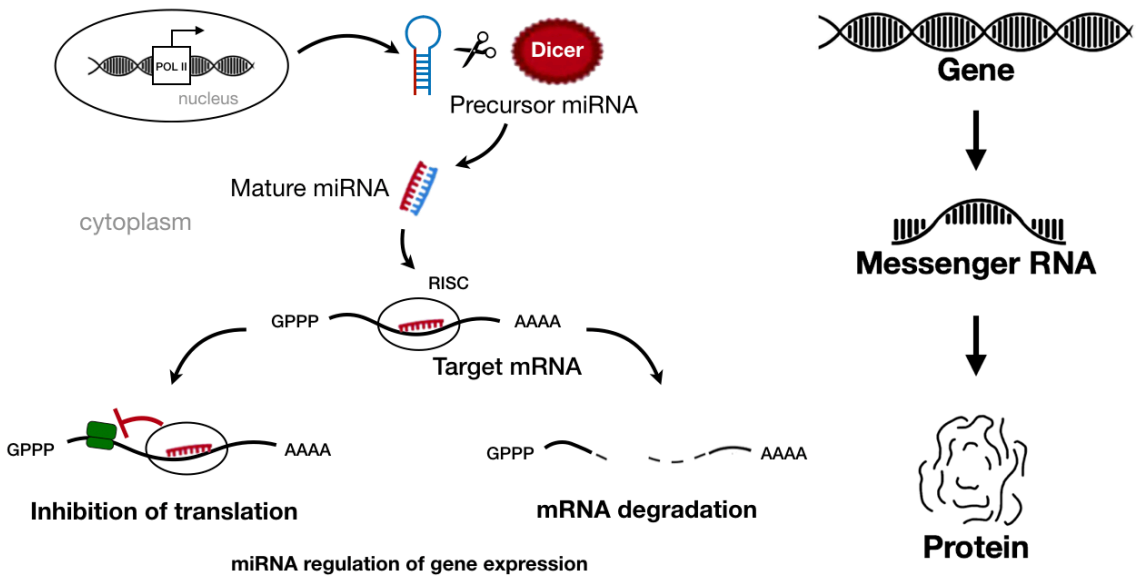

Figure 4. Scheme of miRNA biogenesis and gene silencing mechanism. Adapted from Sachindra et al. Mol Cel. Pahrmacol, 2012.

MiRNAs fine-tune expression of the genes that are likely to be important for maintenance of many cellular functions, such as metabolism, development and differentiation (8). Deletion of Dicer enzyme in mouse DCs, causing a full depletion of miRNAs, results in strongly dysregulated epidermal Langerhans cell (LCs) homeostasis and leads to ablation of LCs with age in mice (9) . Therefore, the main focus in the field is currently on the role of individual miRNAs in various regulatory hematopoietic pathways.

MiR-424 as regulators of dendritic cell differentiation and function MiRNAs are an integral part of regulatory network that orchestrates immune cell differentiation, function and activation. Therefore, the main focus in the field is currently on the role of individual miRNAs in various regulatory hematopoietic pathways. For instance, miR-223, miR-146, miR-155 and miR-125 have already been in-depth studied in the context of myeloid developmental biology (10-14). Some of those miRNAs, such as miR-223 may be regarded as a potential diagnostic marker and therapeutic target against different inflammatory diseases (15). Another miRNA-22, which promotes monocyte/macrophage differentiation, was shown to be downregulated in acute myeloid leukemia patients and has a potential therapeutic value (16). Furthermore miR-34a and miR-21 were shown to inhibit endogenous WNT1 - JAG1 signaling pathway for proper moDCs differentiation (17). Despite multiple studies the transcriptional mechanism underlying moDCs versus LCs differentiation still remains elusive.

The first systematic study of miRNAs that a differentially expressed by moDCs/intDCs and LCs was carried out by Jurkin et al (18). Among forty-six miRNAs in the screen miR-424 was among the strongest inversely expressed miRNA, showing higher levels of moDCs versus LCs expression. A growing body of literature has previously examined the role of miRNA-424 in cancer cell proliferation (19) and epithelial-mesenchymal transition (20) and found that miR-424 acts as a tumor suppressor in different types of cancer (21-24). However, mir-424 is increasingly becoming an important player in hematopoietic lineage differentiation. Upon PU.1 activation miR-424 promotes monocyte/macrophage differentiation through repression of NFI-A pathway (25,26). Another study showed that predicted targets of miR-424 might be involved in TGF-β, MAPK, JAK-STAT, p53 signaling pathways as well as in acute myeloid leukemia and antigen processing and presentation pathways (27). To investigate whether miR-424 regulated dendritic cell subset specification and cell intrinsic mechanisms, we performed a range of *in vitro* and *in vivo* experiments.

1.5 Imiquimod-induced psoriasis model for studying proinflammatory immune cell types *in vivo*

Imiquimod- induced psoriasis model was initially described by van der Fits et al. at 2009 (28) and is widely used for studying psoriasis in murine models. Psoriasis is a chronic inflammatory skin disease, which is characterized by skin infiltration of neutrophils, monocytes and different T cell subpopulations. Pathogenesis of psoriasis includes upregulation of proinflammatory cytokines such as

IL-17A, IL-23, IL-22 and TNF alfa. All those cytokines together with activation of keratinocytes induce a strong inflammation and dermatitis.

The imiquimod (IMQ) also known as Aldara cream is frequently used in the clinical practice for topical treatment of basal cell carcinoma. Since IMQ is inducing local and systemic inflammation through activation of toll-like receptor 7/8 (TLR 7/8), daily application of IMQ to the mouse skin can induce psoriasis-like lesions in mice.

According to initial protocol commercially available 5% IMQ cream is applied to shaved mouse back or ears for approximately 5-7 consequent days. A growing body of literature shows that this model has advantages and disadvantages (as described by Hawkers et al. and others), that are very important to consider before planning the experiments.

Advantages of IMQ- model for studying psoriasis-like inflammation:

1. IMQ-model is very easy to set, because it doesn't need special training or conditions in the lab, such as using extremely clean rooms or going for a special diet.
2. Topical application of the drug resulting in skin swelling and lesions helps the researchers to visually control the efficiency of IMQ-treatment.
3. IMQ-model induces inflammation relatively quickly. First signs of inflammation occur already after 2-3 days of treatment. They include ear swelling, erythema, weight loss. It allows researchers to generate a large amount of data, which is also very easy to reproduce.
4. Because of the topical way of IMQ application, mice relatively less suffer from systemic effects of treatment. It is also important when it comes to ethical approval of studies and helps to avoid unnecessary stress for animals.
5. 5% IMQ cream is cheap and available in most of the clinical dermatological departments/pharmacies.

Disadvantages of IMQ- model for studying psoriasis-like inflammation:

1. The mechanism of IMQ action is poorly understood. Although IMQ induces primarily TLR 7/8, some studies also showed that Aldara vehicle can activate keratinocytes directly. Therefore, IMQ-model requires set up of proper experimental controls.
2. Topical application isn't always beneficial way of treatment because of mouse grooming behavior. Inflammation can result in significant excoriation and thickening of the epidermis, as well as increased scratching by animals. As a result, mouse behavior can significantly

change towards increased grooming and physical activity, which might in turn influence experimental results.

3. Accurate application of 5% Aldara cream to mouse ears need a proper restrain of the animal. Also, epidermal thickness measurement by Caliper device needs some practice and gives a lot of variations depending on the investigator's experience. Therefore, it's very important to do blind measurements of epidermis and ear thickness if it's technically possible.
4. IMQ-induced inflammation might be different among different mouse strains. Most often used strains have C57BL/6 and BALB/c background, which have their own limitations. The IMQ - related effects on other background haven't been described yet.
5. IMQ-model only partially recapitulate human psoriasis. IMQ-induced skin lesions showed a different cytokine profile of IL-10 and IL17A in comparison to patient's psoriatic lesions (29). On top of that, IMQ-induced inflammation resembles acute, localized picture of psoriasis flare up, but it's absolutely dispensable for studying chronic inflammation, which is more often seen in patients.

Taken together, IMQ-induced psoriasis model closely resembles human acute stage of psoriasis and produces erythema, swelling and acanthosis similar to human skin. Inflammatory infiltration, which occurs after IMQ treatment, consists of increased number of neutrophils, DCs, T cells and monocytes. IL-23 and IL-17 signaling are both critically important for the pathogenesis of this IMQ-model and therefore makes it suitable for studying of potential antipsoriasis drugs in mice.

1.6 Role of FVB background of the mice

All the experiments were carried out in the miR-424 knockout and WT mice, which were generated with FVB background. During performing the IMQ experiment, it was noticed that those mice generally get less ear swelling upon IMQ treatment than C57BL/6 mice (data not shown). Therefore, more information about the background of the mice might be needed to fully understand the mechanisms of immune cells differentiation. As it was previously described in the literature, the inbred FVB strain presents enhanced sensitivity to tumorigenesis and is favored for cancer-related studies. To further investigate the role of this miRNA cluster in carcinogenesis, Jose M Silva's lab crossed C57BL/6/miR-424(322)/503−/− model with wild-type FVB mice for more than six generations to produce a >98.5% clean FVB/miR-424(322)/503−/− background. Next, they in detail investigated the mammary epithelia of >1-yr-old FVB wild-type, miR-424(322)/503+/−, and miR-424(322)/503−/− virgin females by carmine red staining and H&E staining. As it was described in the

paper (24), while none of the wild-type animals presented mammary epithelial abnormalities, all of the miR-424(322)/503−/− females displayed enlargement of the terminal ductal lobular units and 50% of them had detectable microscopic invasive carcinomas. Heterozygous miR-424(322)/503+/− mice showed the intermediate phenotype. Ki-67 immunohistochemistry revealed that these lesions presented a higher proliferative index than the mammary epithelia of wild-type animals. Because the miR-424(322)/503 cluster has a known role in post-lactational mammary involution and it was previously shown that pregnancy promotes accumulation of abnormalities in the mammary epithelium of knockout animals, they next investigated the development of breast carcinomas in parous miR-424(322)/503−/− females. Carmine red staining and H&E staining of mammary glands from >1-yr-old parous mice that passed through one round of pregnancy between the ages of 4 and 7 months showed a very enlarged mammary epithelium in miR-424(322)/503−/−animals, with a large majority of the animals (73%) presenting microscopic invasive carcinomas. Although the majority of the invasive lesions was microscopic, palpable tumors developed in a small fraction of the animals (two out of 40). Overall, these data demonstrated that loss of miR-424(322)/503 induces mammary epithelial tumorigenesis that is promoted by pregnancy. Although these animals presented large tumors when they reached 12 months and later on, all the mice for IMQ experiments were 8- 16 weeks old and didn't have any signs of tumorigenesis. It allowed us to successfully incorporate FVB miR-424(322) −/− mice into our experimental settings.

Aim of the study

MicroRNAs regulate key biological processes in immune cells, defining their phenotypic and functional characteristics. Deficiency of Dicer, an enzyme critical for miRNA processing, causes aberrations in epidermal LC homeostasis and reduces their Ag presentation capacities to $CD4^+$ T cells. Our group previously performed miRNA profiling of two human dendritic cell subsets, i.e. LCs and monocyte-derived interstitial-type/dermal DCs (intDCs). Approximately 20 miRNAs were found to be differentially expressed by these two subsets. MiR-424 was among the strongest inversely expressed miRNAs in this screen, showing higher levels of moDCs versus LCs expression. Using lentiviral shRNA knockdown, we then silenced individual miRNAs from this screen in human hematopoietic stem cell progenitor cells undergoing cytokine-driven LC versus moDC differentiation. We found that miR-424 knock-down interferes with moDC but not LC differentiation. As our data were derived from an *in vitro* differentiation model, we now searched for potential collaborators to validate these data in a mouse model. In mice moDCs occur in certain tissues in the steady-state (e.g. skin, gut) and a monocyte-derived DC pathway prevails during inflammation. Therefore, we aimed to extend our studies on the skin inflammation on miR-424(322)/503 -/- mice in Jose M. Silva lab. We initially assessed the frequency distribution of DC subsets in miR-424(322)/503 -/- mice during steady-state and inflammation. Phenotyping of immune cells was carried out by flow cytometry and histology. Our immediate study endpoint was to see whether moDC subsets are diminished in this type of mice. Subsequently we are planning to use human and murine models to characterize in detail downstream mechanisms underlying miR-424 function in the DC system. The aim of our work is to broaden current knowledge of the role of microRNA-424 in monocyte-derived dendritic cell differentiation during normal hematopoiesis and inflammation. Preliminary results so far have been very encouraging and further experiments will employ microRNA-424(322) knockout mice to characterize in detail targets and downstream mechanisms underlying microRNA-424 function in the immune system.

Materials and methods

2.1 Purification and generation of mouse bone marrow-derived dendritic cells

Bone marrow-derived LCs and moDCs were generated *in vitro* from mouse bone marrow (BM) precursors. In brief, femur and tibiae were removed from 8-12-week-old mice and. Bone marrow cells were flushed out with ice-cold PBS, washed and incubated in 1 ml Red Blood Cell Lysing Buffer (Hybri-Max, Sigma-Aldrich) for 1 minute. After washing cells were plated in 24 tissue culture plate (0,5 x 10^6 /ml per well) in DMEM supplemented with 1% penicillin/streptomycin (Lonza #CC-4136), 10% FCS and 1% MEM non-essential amino acids solution (ThermoFisher) under lineage specific cytokine condition for LCs (20 ng/ml GM-CSF, 1 ng/ml TGF-β1) and moDCs (20 ng/ml GM-CSF). On day 3, the medium was changed and cells were harvested on day 7.

2.2 Cytokines and reagents

Cytokines tumor necrosis factor α (TNFα), thrombopoietin, stem cell factor (SCF), Fms-like tyrosine kinase 3 ligand (FLT3-L), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin-4 (IL-4) were purchased from PeproTech, UK. Human recombinant transforming growth factor- β1 (TGF-β1) was purchased from R&D, USA.

2.3 Flow cytometry

All flow cytometry experiments were performed on LSRII and LSR Fortessa flow cytometers (BD Biosciences, USA). The data was analyzed using FlowJo software (Tree Star, Inc.USA). For FACS sorting the FACS Aria instrument (BD Biosciences) was used. Human cells were harvested, resuspended in 40 μm of staining buffer and stained on ice for at least 1 hour. Fc receptors were blocked by incubating for 15 minutes on ice before staining with antibodies.

Mouse ears were harvested and separated for ventral and dorsal sheets. The sheets were incubated for 1 hour at 37 °C in 0.8% Trypsin solution. Then epidermal and dermal sheets were digested separately as previously described (30). Single cell suspension was incubated for 1:1000 diluted purified anti-mouse CD16/32 (Biolegend) for at least 20 min on ice before adding antibodies. Dead cells were excluded by using Zombie NIR viability dye (Biolegend).

2.4 RNA-isolation and real time PCR

The extraction of RNA was performed using miRNA easy Mini Kit (Qiagen) with DNase I treatment according to the manufacturer protocol. RT-PCR and qPCR were performed using TaqMan™ MicroRNA Assay. Probes for TaqMan™ MicroRNA Assay (hsa-miR-424-3p, hsa-miR-424-5p, U6 snRNA control for human samples and Sno-202 for mouse samples) were purchased from ThermoFisher. All the steps were performed according to manufacturer's instructions.

2.5 Experimental animals and treatment

Animal maintenance and experiments were performed under the Institutional Animal Care and Use Committee (IACUC) guidelines. Protocol number is IACUC-2018-0008. Experiments were performed with miR-424(322) −/− mice with homogeneous FvB/NJ background. For genotyping hear samples were incubated in 250 μL of Direct PCR reagent and 20 mg/mL Proteinase K (New England Biolabs, P8102) for 1 h at 55°C. PCR was performed using FastStart polymerase (Roche, 047384200010 under the following conditions: predenaturation for 5 min at 94°C, 35× (denaturing for 30 sec at 94°C, annealing for 30 sec at 55°C, and elongation for 30 sec at 72°C), and final elongation for 7 min at 72°C.

Mice 8-14 weeks old were topical applied of 62 mg of 5% Aldara Imiquimod cream (IMQ) as previously was determined as the most optimal dose to induce ear inflammation (31). Mice were treated with IMQ daily at the same time during 1 week. Ear thickness was measured daily using electronic measurement device C1X018 (Kroeplin, GmbH).

2.6 mRNA sequencing and data analysis

Cells for total RNA sequencing were collected after extraction from mouse bone marrow and 24h after addition of GM-SCF and frozen in lysis buffer until RNA extraction. Total RNA was isolated using either RNeasy Micro Kit (Qiagen) or miRVana isolation kit (Ambion). RNA quality was checked at Bioanalyser before sequencing.

2.7 AGO2 immunoprecipitation and PAR-CLIP

BMDC were obtained as described in 2.2. of approximately 100 x 10^6 cells per condition and PAR-CLIP was performed as described previously (32-34) with several minor modifications. Briefly, cells were treated with 50 μM 4-thiouridine (T4509; Sigma) overnight and cross-linked on ice at 150 mJ/cm^2 365 nm UV. Then cells were lysed with lysis buffer (2.5 mM HEPES (pH 7), 50 mM NaCl, 10% glycerol, 1% Triton X-100, proteinase inhibitor Roche 04693159001, 0.2 mM dithiothreitol, and 1 U/μl RNAse OUT (10777-019; Invitrogen). Then samples were digested with 5 U/μl RNase-T1 (EN0541; Fermentas) at 22°C for 15 min. After pre-clearing immunoprecipitation with 20 ug anti-AGO2 antibody (H00027161-M01; Abnova) and protein A/G magnetic beads (88802, Pierce, ThermoFisher Scientific) was performed overnight. Next day samples were washed twice with washing buffer 1 (50 mM Tris (pH 7.5), 150 mM NaCl, 0.1% NP-40, and 1 mM EDTA) at 4°C for 30 min and once with washing buffer 2 (50 mM Tris [pH 7.5], 500 mM NaCl, and 0.1% NP-40) at 4°C for 30 min. Samples were resuspended in washing buffer 1. One part of the sample was prepared for western blot and another part was incubated with proteinase K (P8102; New England BioLabs) for 1 h at 50°C with further total RNA extraction as described before.

2.8 Statistical analysis

Statistical analysis was carried out using GraphPad Prism software. Student's t-test was used for comparing differences between two groups. Multiple groups were subjected to analysis of variance (ANOVA) analysis. P values <0.05 were considered statistically significant.

Results

3.1 Characterization of immune cell distribution in miR-424 (322 knockout mice upon skin inflammation

Previously we assessed the frequency distribution of moDC in the skin during steady-state and inflammation using miR-424 knockout vs WT mice. Phenotyping of immune cells was carried out by flow cytometry analysis of dermal part of the mouse ears after treatment with Imiquimod for 6 days. We observed that moDCs subset is significantly reduced in the dermis of miR-424(322) knockout mice under inflammatory conditions in comparison with WT mice although there was no such an effect in the steady-state. Our finding corroborated with the previous results generated in our human moDCs in vitro differentiation model. Furthermore, we analyzed if other immune cell subsets present in the epidermis and dermis are impaired. In fact, there was no significant changes in the frequencies of monocytes, $CD103^+$ and $CD11b^+$ cDCs. Interestingly, number of macrophages was decreased upon IMQ treatment in miR-424 knockout mice in comparison to WT mice, although steady -state didn't show any significant changes. This confirms previous observations that miR-424 regulates monocyte/macrophage differentiation via translational repression of NFI-A. Consistently, LCs frequency both in epidermis or lymph nodes of IMQ-treated mice were not impaired. We also didn't notice any changes in LC activation in the presence or absence of miR-424.

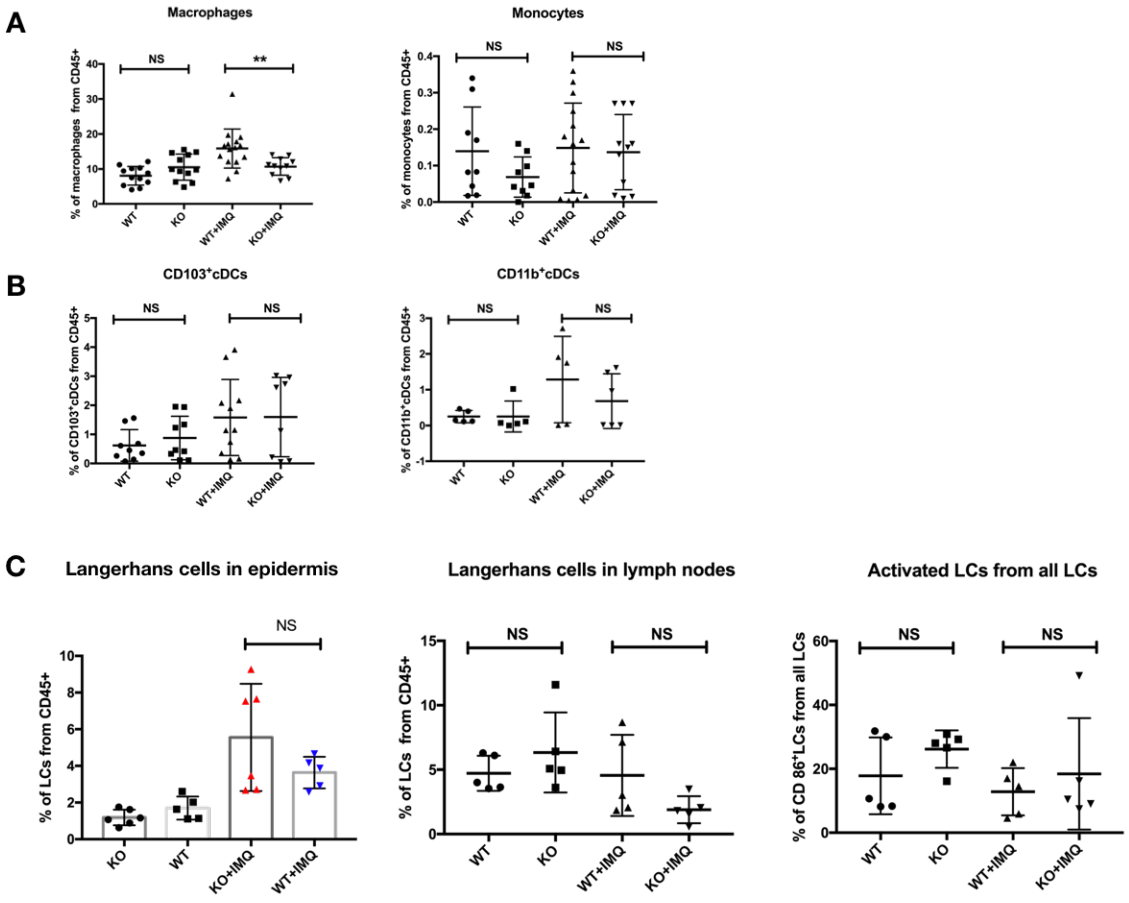

Figure 5. Characterization of immune cells distribution in miR-424(322) knockout mice vs WT mice upon skin inflammation. (A) and (B) FACS plots represent frequency of monocytes, macrophages and cDCs isolated from mouse dermis upon Imiquimod induced inflammation. (C) Percentages of phenotypically defined LCs in epidermis of mouse ears and regional lymph nodes. Data is shown in 6 mice per group, * P<0.05; ** P<0.005; *** P<0.0005.

3.2 MiR-424 presence doesn't interfere with the number of monocytic precursors in mouse bone marrow

For further study of miR-424 effect on moDCs differentiation, we assessed the frequency of monocytic precursors in the bone marrow of miR-424 knockout and WT mice. Menezes et al.(2) showed that Ly6C$^+$ monocytes in the mouse bone marrow are heterogeneous and they can be divided into 3 subpopulations with a distinct potential to generate INOS$^+$ macrophages (R1), inflammatory moDCs upon GM-CSF exposure (R2) or FLT3 dependent DCs (R3). To investigate whether the number of distinct precursors is impaired in miR-424 knockout mice, we quantified precursors in fresh isolated bone marrow using FACS (Fig.6 A). Phenotyping of different subsets showed that Flt3$^+$CD11c$^-$ (R2)

population which corresponds to moDCs precursors is similarly present in miR-424 knockout and WT mice (Fig 6 B, C). Accordingly, FLT3l^{-} CD11c^{-} (R1) and FLT3l^{+} CD11c^{+}(R3) subsets were also not impaired. We conclude that miR-424 doesn't influence the number of precursors in vivo and acts as a regulator later during GM-CSF induced moDCs differentiation.

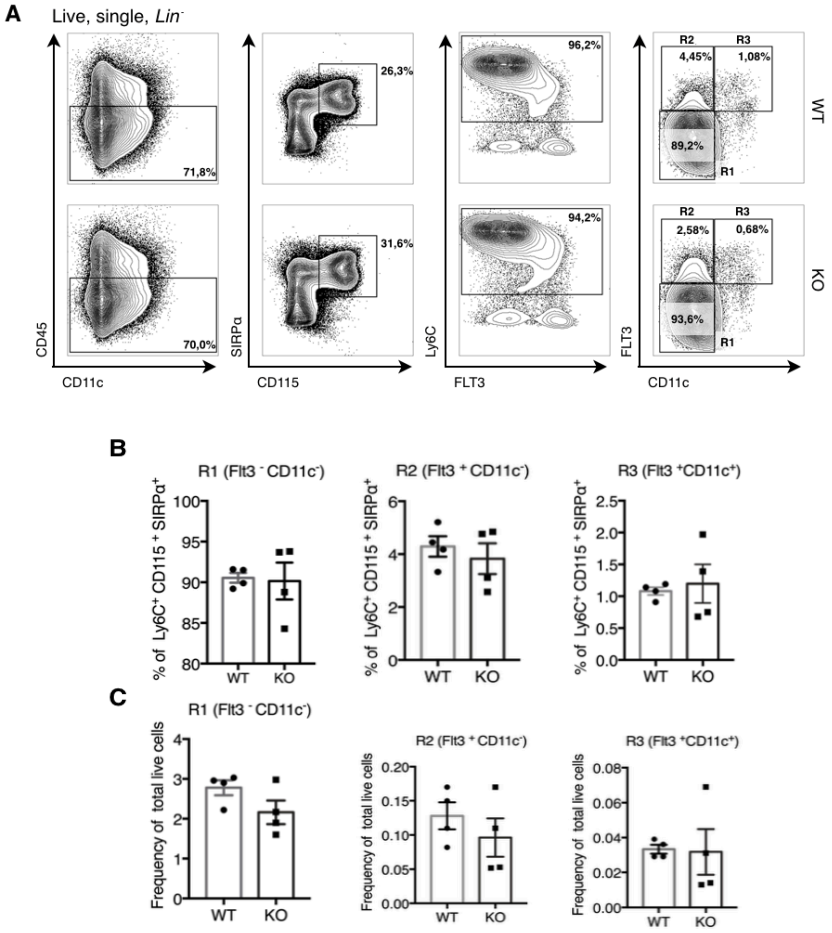

Figure 6. MiR-424 doesn't interfere with the number of monocytic precursors in mouse bone marrow. (A) Flow cytometry analysis of fresh isolated mouse bone marrow for dendritic cell precursors. (B) Graph depicts frequency of R1 (precursors for iNOS macrophages), R2 (moDCs precursors), R3 (pre-DC precursors) from Ly6C^{+}CD115^{+}SIRPα^{+} population. (C) Graph shows frequency of R1 (precursors for iNOS macrophages), R2 (moDCs precursors), R3 (pre-DC precursors) from total live cells.

3.3 MiR-424 is upregulated on the early stages of BMDC differentiation

Furthermore, we measured miR-424 expression at different time points during moDCs differentiation *in vitro*. To do that we extracted bone marrow from WT mice and induced it into differentiation of

GM-SCF bone-marrow dendritic cells. Relative expression analysis of miR-424 showed that the levels of miR-424 were increased at the early staged of differentiation and reached its peak at day 2. Therefore, we picked day 0 and day 2 time points and performed RNA-seq differential gene expression analysis (data not shown).

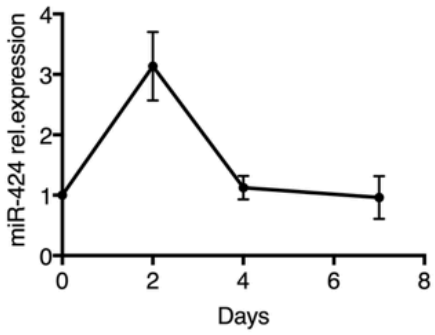

Figure 7. MiR-424 is upregulated on the early stages of BMDC differentiation. Graph shows endogenous expression of miR-424 in BMDC cultures on the indicated time points.

3.4 PAR-CLIP analysis for identification of miR-424 targets

To investigate miR-424 targets we performed PAR-CLIP (photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation). The idea of this method is to crosslink RNAs to interacting RNA-binding proteins (RBPs) ultraviolet light of 365 nm using pretreatment of the cells with 4-thiouridine (4-SU). After that the RBPs were immunoprecipitated using anti-Ago2 antibody and mouse IgG antibody as a control.

To do that we had to optimize the protocols for PAR -Clip, which were previously used in the lab and adapt them from Llobet-Navas et al., 2014; Hafner et al., 2010; Lu et al. 2014 to our BMDC cultures.

Final version of the PAR-CLIP protocol used for AGO2 enrichment

1. Expand cells to approx. 100-400 x 10^6 cell in 10 cm tissue culture plates. Pretreat cells with 50uM of 4-thiouridine overnight, wash with PBS, remove PBS completely and UV-crosslink uncovered at 150mJ/cm2 at 365nm UV on ice. Scrape cells off with a rubber policeman in 1PBS per plate, transfer to 50 ml tube and centrifuge for 5min ,500g, 4 degree. 100 x 10^6 cells should come up as 1ml of wet pellet.

2. For BMDCs harvest BMDC by incubation in PBS on ice for 10 min, wash twice with PBS and reconstitute in lysis buffer [2.5mM Hepes pH7, 50mM NaCl, 10% glycerol, 1% Triton x-100, proteinase inhibitor (Roche #04693159001), 0.2mM DTT and 1U/uL RNAseOUT (Invitrogen #10777-019)]. Incubate in ice 10 min and clear lysate by centrifugation 13000g, 15 min, 4ºC.
3. Perform mild (5U/uL) RNase-T1 (Fermentas #EN0541) digestion at 22ºC for 15 minutes in water bath (temperature and duration are critical), cool on ice for 5 min. Collect the input!
4. Prepare Pierce magnetic beads: take 40 ul beads, wash them twice with 280 ul lysis buffer (don't vortex). Resuspend in 40 uL lysis buffer and add 5 uL to lysate for preclearing. Keep 45 minutes- 1h at 4 degree rotating. Collect the beads with magnet and proceed to IP.
5. Immunoprecipitation: Add lysate + 10uG of anti-AGO2 antibody+ beads and incubate overnight at 4ºC.
6. Collect the flow through! Wash samples twice with washing buffer 1 (50mM Tris pH7.5, 150mM NaCl, 0.1% NP-40 and 1mM EDTA) at 4ºC for 30 minutes.
7. Washed beads twice with washing buffer 2 (50mM Tris pH7.5, 500mM NaCl and 0.1% NP-40) at 4ºC for 30 minutes.
8. Resuspend in 100-200 uL washing buffer 1. Take small aliquote for WB (add 2x loading dye to the tube and heat the sample to 96 degree for 10 minutes, magnetically separate the beads and take out the supernatant contains protein). Treat rest with 2 uL proteinase K (New England Biolabs #P8102) per 100 ul for 1 hour at 50ºC.

Finally, extract was lysed and RNA extracted using the Qiagen miRNA isolation Kit according to the instructions provided. Target-mRNA can be quantified by real-time PCR using specific primers.

As shown on the Figure 8, AGO2 protein was enriched after immunoprecipitation in bone-marrow derived dendritic cells, there was no Ago2 enrichment in mouse IgG condition, which served as a control. mRNAs were extracted from both immunoprecipitated WT and KO samples was send for mapping and gene enrichment analysis.

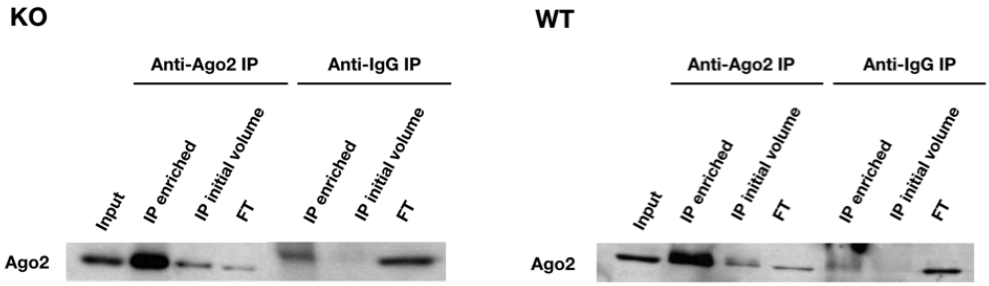

Figure 8. CLIP analysis for identification of miR-424 targets. Western blot analysis for AGO2 to determine the AGO2 immunoprecipitation in BMDCs generated from KO and WT mice. Mouse IgG was used as a negative control. IP stood for immunoprecipitation; FT stood for flow through.

3.5 Bioinformatically predicted promotor binding sites and possible targets of miR-424

Bioinformatic analysis was carried out to find potential transcription factor binding sites on the miR-424 promotor. Based on bioinformatical analysis we identified sequenced-based motifs for binding transcription factors. TFbind software (http://tfbind.hgc.jp) showed a list of 221 potential transcription factors, as well as transcription factor affinity prediction web tool (http://trap.molgen.mpg.de/) identified 67 potential transcription factors, which could theoretically bind to miR-424 promotor. According to the literature review we sorted out which candidates could be involved in myeloid cell development and came up with a short-list of transcription factors summarized in Table 1. Ingenuity Pathway Analysis (IPA) by Qiagen revealed a regulatory network of upstream and downstream regulators of miR-424.

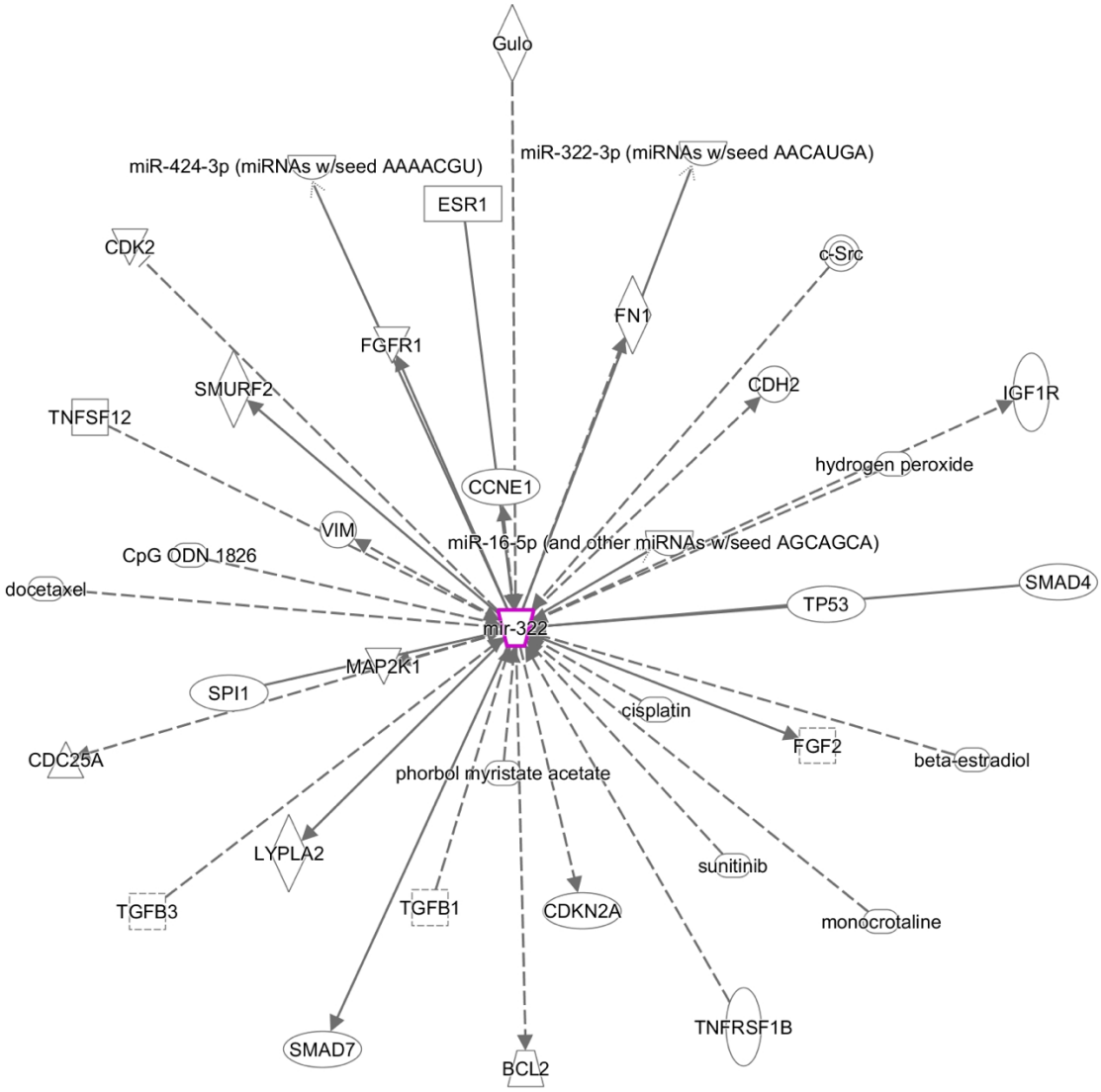

Figure 9. miR-424 targets and transcription factors regulatory network that were predicted by bioinformatic analysis. Dotted line without arrows represents upstream regulators, arrows depict downstream targets of miR-424.

Discussion

4.1 Evaluation of immune cell phenotyping in the mouse skin

Dendritic cells are implicated in the pathogenesis of many inflammatory diseases such as psoriasis, lupus, inflammatory bowel disease. One of the most important features of DCs is their ability to differentiate into immunogenic DCs upon viral or bacterial stimuli and therefore prime T cells. Although DCs from different tissues sometimes share phenotypical und functional characteristics, identifying the direct analogue of human DCs in mice can be very tricky. In this study, we identified different dendritic cell subsets and tried to combine both in vivo and in vitro approach to study dendritic cell subset specification.

To understand the mechanism, which are driving moDCs differentiation, we had to induce psoriasis-like inflammation in the mouse skin. Therefore, we applied TLR7 receptor agonist IMQ to activate IL-23 mediated pathways leading to psoriasis-like skin lesions. Interestingly, miR-424 KO mice showed less systemic inflammation as well as less ear swelling than WT controls. We wondered if this effect is mediated by the lack of moDCs, which are contributing to inflammatory effects. Our data showed that there is a decrease in the number of moDCs in the dermis of miR-424 KO mice in comparison to WT mice. Moreover, our further data supported the finding that moDCs are selectively diminished in the skin of miR-424 KO mice.

Here we describe the distribution of closely related dendritic cell subsets in the mouse dermis phenotyped by flow cytometry. In mouse, LCs comprise only 3-5 % of all cells in the epidermis and in contrast to human LCs, murine LCs were shown to additionally express monocyte/ macrophage markers such as CD11b or F4/80. Our study confirmed previous observations in vitro that the number of phenotypically defined LCs in the epidermis of miR-424 KO mice isn't impaired. Similarly, the activation status of LCs didn't show any significant changes between the groups. Then, we checked if miRNA-424 expression can be involved in the ability of LCs migrate draining lymph nodes upon inflammation. Frequency of steady-state and activated LCs in the draining lymph nodes were also not impaired. Given the fact that LCs distribution showed no significant changes in miR-424 KO and WT mice upon inflammation, we propose miR-424 to be a potential regulator of moDCs but not LCs differentiation.

Mouse cDCs are represented by subset of $CD103^+$ $CD11b^-$ and $CD103^-$ $CD11b^+$ cDCs, which has a common precursor with lymphoid $CD8^+$ cDCs. Those two populations have a different transcription and phenotypic characteristics: $CD103^+$ $CD11b^-$ cDCs lack macrophage-associated markers such as CD11b, CD115, CD172a, F4/80, CX_3CR1. $CD103^+$ $CD11b^-$ mostly populate

connective tissues and comprise only 20-30% of total cDCs population, making them very hard to find in the skin. Nevertheless, our results showed $CD103^+$ $CD11b^-$ cDCs are equally present in the dermis of miR-424 KO and WT mice. Unlike $CD103^+$ $CD11b^-$ cDCs the population of $CD103^-$ $CD11b^+$ cDCs is more abandonly present in the mouse tissues. In lamina propria $CD103^-$ $CD11b^+$ cDCs arise from both cDC-precursor and monocytes, therefore different markers might be used to identify them in different tissues. Most of the time the population of $CD103^-$ $CD11b^+$ cDCs comprise 70-80% of all cDCs and predominantly consists of tissue cDCs and macrophages. To identify the number of macrophages we used the combination of Mer tyrosine kinase (MerTK) and CD64 markers, because recent studies revealed that CD64 can be used to specifically distinguish moDCs or macrophages from cDCs. Our data showed that the number of macrophages in the dermis of miR-424 KO mice is significantly decreased in comparison to WT controls, which support human *in vitro* finding that miR-424 corroborates monocyte/macrophage differentiation through NFI-A signaling pathway (26) . Interestingly, $Ly6C^+$ monocytes were also not impaired in the dermis of miR-424 KO mice. Despite this fact, we can't exclude that the monocytic precursor of moDCs cells aren't diminished in the mouse bone marrow and therefore we set up a separate experiment to assess the frequency of different precursor subsets in the mouse bone marrow.

4.2 Evaluation of DC precursors in the mouse bone marrow

In human $CD14^+$monocytes were identified as the immediate precursors of moDCs, monocyte-derived macrophages (moMacs) and monocyte-derived LCs (moLCs). Subsequent studies in mice revealed that $Ly6C^+$ monocytes are heterogeneous and can also be converted to $Ly6C^-$ monocytes in the blood stream. In contrast to $Ly6C^+$ monocytes, $Ly6C^-$ monocytes don't express CCR2 and upregulate transcription factor NR4A1 as well as major histocompatibility complex II (MHC II). Upon inflammation $Ly6C^+$ monocytes start to acquire $iNOS^+$ macrophage and moDCs signatures. Previous studies showed that phagocytic precursors in the mouse bone marrow can be divided into three subpopulations according to their expression of CD135 (Flt3) and CD115 (CSF1R) markers. By pregating on $MHCII^-$ $CD172a\ (SIRP\alpha)^+$ $CD115^+$ $Ly6C^+$ described by Menezes *et al.* we could subsequently distinguish the following populations in the bone marrow of KO and WT mice: $CD11c^-$ $Flt3^-$ (R1), $CD11c^-$ $Flt3^+$ (R2) and $CD11c^+$ $Flt3^+$(R3). Analysis of their transcriptional profile showed that R1 monocytes give rise to $iNOS^+$ macrophages upon inflammatory stimuli, R2 monocytes generate moDCs and R3 subset corresponds to pre-DCs. Interestingly, that R2 precursors exhibited unique $CD209a^+PDL2^+$ moDCs phenotype and adoptive transfer experiments showed that none of

other precursors could transdifferentiate into R2 population upon GM-CSF stimulation both *in vivo* and *in vitro*.

While previous studies suggested that miR-424 plays an important role in promoting the monocytic differentiation in U937 human leukemia cell line by targeting CDX2, we wondered if the lack of moDCs in miR-424 KO mice might be a consequence of the lack of monocytic precursors but not the direct effect of impaired moDCs differentiation. Therefore, we accessed a frequency of distribution of those monocytic precursors in the fresh isolated bone marrow of WT versus KO mice and we noticed that all tree subsets were equally present in both groups. Next, we checked endogenous levels of miR-424 in WT BMDCs cultures, generated by adding GM-CSF. Our data indicates that miR-424 is upregulated on day 2 of differentiation of BMDCs, suggesting that miR-424 acts as a regulator of moDCs on the early stages of differentiation but it doesn't interfere with the moDCs precursors in the mouse bone marrow.

4.3 Potential transcription regulators of miR-424

The previous studies of the role of miR-424 in dendritic cell differentiation were limited *to in vitro* models and need closer *in vivo* examination. In order to further study molecular mechanisms regulating miR-424 dependent moDCs differentiation, we performed a screening for transcription factors with potential binding sites at miR-424 promotor. Bioinformatical analysis showed more than 200 potential upstream regulators of miR-424, which we narrowed down to a list of few transcription factors (summarized in Table 1), that might be involved in DCs differentiation. One of the candidates was *RUNX3*, which is a downstream target of *KLF4* and promotes LCs but inhibits moDCs subset differentiation. Another candidate *TFAP2C* is known to be highly expressed in B cells, pDCs and endothelial cells according to published single -cell RNA sequencing data and its knockout leads to fetal death in mice. Transcription factor *EVT1* plays role in endothelial cells and its overexpression using lentiviruses leads to formation of multipotent $CD34^{+}CD45^{+}$ hematopoietic progenitors. *ETV 7* is known to be an accelerator of tumor onset and a part of mTOR signaling pathway. Interestingly, *TCF4* was previously shown to be a functional regulator of macrophages. Previous studies revealed that proto-oncogene protein Wnt1 regulated CD36 expression through interaction with PPAR-γ and *TCF4* in macrophages. Upregulation of CD36 further promoted recruitment of of β-catenin and PPAR-γ to the CD36 promoter. Other studies revealed that *TCF4* is typically expressed by mucosal DCs and that pro-inflammatory DCs express unique phenotype of $CD103^{+}$ $RALDH2^{+}$ $TCF4^{+}$ DCs. Moreover, *TCF4* expression in progenitors played a key role in lineage specification between pDCs and cDCs and was

highly upregulated in pDCs. Given all this information, we expect TCF4 to be a positive upstream regulator of miR-424 in moDCs and further experiments are needed to study the role of *TCF4* in the mechanisms of miR-424 regulation. Transcription factor *EBF1* is essential for lineage specification in early B cell development and isn't reposted in any dendritic cell related studies. Finally, the *HOXA* genes were reported to be a part of biologic networks that connect oncogenesis, thymic differentiation pathways, and ectopic gene expression. An intergenic transcriptional activity that is located between the human *HOXA1* and *HOXA2* genes, shows myeloid-specific expression, and is up-regulated during granulocytic differentiation. In addition, sustained expression of *HOXA3* in vivo can modulate bone-marrow derived cell behavior in the wound microenvironment by changing the balance of endothelial progenitor and inflammatory cell recruitment. Taken together, *HOXA2* might be a potential negative regulator of miR-424 promotor.

4.4 Conclusions and outlook

Until now, miR-424 has been regarded mostly as a tumor suppressive miRNA, and its function in myeloid cells as well as in human $CD34^+$ cells has been poorly understood. Our findings substantiate the pivotal role of miR-424 in moDCs differentiation both *in vitro* and *in vivo.* The key finding of our study was the observation that miR-424 knockdown in $CD34^+$ cord blood progenitor cells abrogates moDCs lineage differentiation, although LCs development is not impaired. These conclusions were subsequently validated in a clinically relevant model of psoriasis-like inflammation in mice, showing that miR-424 knockout mice revealed less inflammatory response. This effect might be explained by the diminished dermal moDC subset, which contributes to inflammation through production of inflammatory cytokines. Interestingly, other immune cell subsets in the skin of miR-424 KO mice weren't impaired.

Thus, miR-424 impairs differentiation capacity $CD34^+$ cells on the early stages of differentiation and might regulate critical pathways that are activated during inflammation. In light with the previous study, addressing the heterogeneity of bone marrow mononuclear phagocyte precursor, we checked if miR-424 is mediating cell differentiation pathways already on the stage of PU.1 $^{hi}Flt3^+$ $MHCII^+$ precursors. Our data didn't show any significant changes in the precursors of $iNOS^+$ macrophages, moDCs or pre-DCs in miR-424 KO vs WT mice. Therefore, miR-424 rather impacts the GM-SCF- induced moDCs differentiation early during cell differentiation from HSC and doesn't interfere with monocytic bone marrow progenitors.

Regarding the upstream mechanisms of miR-424 regulation, the additional information from total RNA sequencing of BMDCs would be useful to access the differences in gene expression between miR-424 KO vs WT mice. Further experiments with knockdown and overexpression of certain transcription factors, which are upregulated in WT mice and downregulated in miR-424 KO mice, could give more information on the transcription regulation of miR-424. For the downstream analysis of the miR-424 targets, we performed PAR-CLIP experiments by pooling down AGO2 protein with mRNAs. The difference in the mRNAs, which are involved in the RISC complex of WT and miR-424 KO mice will indicate which molecules are specifically targeted by miR-424 in BMDCs.

Given all this information, we are going to perform gene ontology (GO) analysis to identify cellular processes that are regulated by miRNA-424 mediated gene regulation. One of the benefits of GO analysis is that it classifies genes to three categories: molecular function (indicates molecular interaction of the gene), biological processes (integrating the gene in the larger cellular and physiological processes) and cellular component (describes the location on the cell and where the gene product executes its function). All together it will provide us the insight into the mechanisms controlling moDCs lineage specification beneficial for our understanding of these general concepts governing hematopoiesis.

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Table 1. List of potentially interesting transcription factors binding to miR-424 promotor

Transcription factor family and name	**Role**
RUNX3, RUNX2	KLF4, TGF beta regulation
TFAP2C:ONECUT2	Involved in development and morphigenesis KO leads to fetal death
ETV7:TBX21	Interacts with mTOR in the cytoplasm Accelerates tumor onset
ETV2:NHLH1	Master regulator of hematoendothelial lineage Promotes stem cell differentiation
SNAI2, TCF3, TCF4	Involved in EMT Master regulator of organogenesis and wound healing
EBF1	Linked to activation of B cell lineage program and Loss of alternative lineage commitment Can restore the ability of PU.1 deficient progenitor to differentiate into B cells
TEAD4:HOXA2	HOXA cluster has myeloid specific expression
TEAD4:HOXA3	Upregulated in granulocytic differentiation

Table 2. List of flow cytometry antibodies used for the experiment

Company	Antibodies(anti-)	Clone	Fluorochrome
Biolegend	CD3	17A2	Pacific blue
	CD11b	M1/70	PE
	CD11c	418	PerCP
	CD19	1D3	PerCP-Cy5
	CD45	30-F11	FITC
	CD86	GL-1	PE-Cy7
	CD103	twoE7	APC
	CD207	4c7	APC
	EpCAM CD326	G 8.8	BV605
	Live/dead dye	Zombie NIR	Far red
	Ly6C	HK 1.4	PE-Cy7
	MHC II/ I-A/I-E	M5/114.15.2	Pacific blue
	TCRαβ	H57-597	PE-Cy7
	TCRγδ	GL3	PE
	CD117	2B8	AF488
	CD3e	145-2C11	AF488
	Ly-6G	1A8	AF488
	CD115(CSF-1R)	AFS98	APC-Cy7
	CD172a(SIRPα)	P84	PE
R&D	CD64	290322	PerCP
	MerTK	108928	AF700
BD Biosciences	CD1a	HI149	Pacific blue
	CD80	L307.4	BV605
	CD86	2331(FUN-1)	PE-Cy7
Beckman Coulter	CD207	DCGM4	PE
Biolegend	CD11b	ICRF44	PE-Cy7
eBoiscience	CD209	eB-h209	APC

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