Dissertation

Role of microRNA-424(322)/503 in epidermal Langerhans cell and monocyte-derived dendritic cell differentiation

submitted by

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STATUTORY DECLARATION

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice“.

April 2021 Victoria Zyulina

DISCLOSURES

The major part of this thesis has been published in the research paper (1):

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Armin Zebisch (Division of Hematology, Medical University of Graz, Graz, Austria; Otto Loewi Research Center for Vascular Biology, Immunology and Inflammation, Division of Pharmacology, Medical University of Graz) contributed to the design of the study.

John Easton, Jiyang Yu (Department of Computational Biology, St. Jude Children's Research Hospital, USA) contributed to the design of the study, coordinated the RNA sequencing part of the project.

Jose M. Silva (Department of Pathology, Icahn School of Medicine at Mount Sinai, New York, New York, USA) provided miR-424(322)/503 KO mice, contributed to experimental design and data interpretation, revised the manuscript.

Herbert Strobl (Otto Loewi Research Center, Division of Immunology and Pathophysiology, Medical University of Graz, Austria) initiated the project, provided the funding, contributed to the design of the study, interpreted the data and co-wrote the manuscript.

All co-authors read and approved the final version of the manuscript. The co-authors declare no conflicts of interest and agreed to the re-use of their data in this dissertation.

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ABBREVIATIONS

Ag	Antigen
AGO	Argonaute protein
Ahr	Aryl hydrocarbon receptor
AML	Acute myeloid leukemia
APC	Antigen presenting cell
BATF3	Basic leucine zipper ATF-like transcription factor 3
BM	Bone marrow
BMDC	Bone marrow-derived dendritic cells
BMP7	Bone morphogenetic protein 7
BMDC	Bone-marrow derived dendritic cells
cDC	Classical/ myeloid dendritic cell
CDP	Common dendritic cell progenitor
CD207	Langerin
CLPs	Common lymphoid progenitors
CMPs	Common myeloid progenitors
CCR7	CC-chemokine receptor 7
CTL	Cytotoxic T cells
CyTOF	Mass cytometry
DC	Dendritic cell
DTR	Diphtheria toxin receptor
FLT3L	Fms-like tyrosine kinase 3 ligand
FOXP3	Forkhead box P3
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GMPs	Granulocyte macrophage progenitors
GSEA	Gene set enrichment analysis
G-CSF	Granulocyte-colony stimulating factor
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
IACUC	Institutional Animal Care and Use Committee

ID2	Inhibitor of DNA binding 2
IFN	Interferon
IL 2	Interleukin 2
IRF4	IFN regulatory factor 4
IMQ	Imiquimod
iNOS	Macrophages inducible nitric oxide synthase macrophages
intDCs	Interstitial-type/dermal DCs
KLF4	Kruppel-like factor 4
KD	Knockdown
KO	Knockout
LC	Langerhans Cell
LN	Lymph nodes
LPS	Lipopolysaccharides
M-CSF	Macrophage-colony stimulating factor
Mac	Macrophage
MafB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MDPs	Macrophage/DC progenitors
MHC	Major histocompatibility complex
moDC	Monocyte-derived dendritic cell
miRNA (miR)	MicroRNA
miR-KO	miR-424(322)/503 knockout
moLCs	Monocyte-derived Langerhans cells
MoP	Monocyte progenitor
moMacs	Monocyte-derived macrophages
mRNAs	Messenger RNAs
NFI-A	Nuclear factor 1 A type
NFκB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	Natural killer cells
PAMPs	Pathogen-associated molecular patterns
PAR-CLIP	Photoactivatable ribonucleoside enhanced crosslinking and immunoprecipitation
PBMCs	Peripheral blood mononuclear cells

PGN	Peptidoglycans
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
SLE	Systemic lupus erythematosus
TF	Transcription factor
Th	T helper
TGF-β1	Transforming growth factor beta 1
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
Treg	Regulatory T cells
WT	Wild type
Zbtb46	Zink finger and domain containing

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ZUSAMMENFASSUNG

MicroRNAs sind kleine nicht-kodierende RNAs, die wichtige biologische Prozesse in Immunzellen regulieren und deren Phänotyp und Funktionen definieren. Ein Mangel an Dicer, einem für die microRNA-Verarbeitung kritischen Enzym, in murinen CD11c $^{+}$ -Zellen zeigte eine dysregulierte Entwicklung und Funktion der dendritischen Zellen (DC). Die Bedeutung einzelner microRNAs für den Prozess der DC-Differenzierung ist jedoch noch wenig bekannt. Daher konzentrierten wir unsere Studien auf die molekularen Mechanismen, die durch spezifische microRNAs in der DC-Subset-Spezifikation reguliert werden.

Unsere Gruppe suchte zuvor nach microRNAs, die von menschlichen DC-Untergruppen unterschiedlich exprimiert werden. Wir haben festgestellt, dass miR-424 (322)/503 in entzündungsfördernden, von Monozyten abgeleiteten dendritischen Zellen (moDCs) im Vergleich zu entzündungshemmenden Langerhans-Zellen (LCs) stark hochreguliert ist. Mittels lentiviral vermittelter Gain- oder Loss-of-function-Mutationen haben wir bestätigt, dass miR-424(322) 503 für die Entwicklung von moDC entscheidend ist. Umgekehrt waren LCs von einem miR-424 (322) / 503-Mangel nicht betroffen. Wir haben festgestellt, dass miR-424 (322)/503 für die Differenzierung von moDC erforderlich ist.

Um festzustellen, ob miR-424 auch an molekularen Mechanismen der Differenzierung von moDCs *in vivo* beteiligt ist, haben wir miR-424(322)/503 (miR-KO) -Mäuse einem klinisch relevanten Modell einer Psoriasis-ähnlichen Entzündung unterzogen. Wir beobachteten, dass moDCs-Subgruppen in der Dermis von miR-KO-Mäusen unter entzündlichen Bedingungen im Vergleich zu WT-Mäusen signifikant reduziert sind. DC-Vorläufer im frisch isolierten Knochenmark waren jedoch unter beiden Bedingungen in ähnlichem Umfang vorhanden. Der Prozentsatz an *ex vivo* differenzierten DCs aus dem Knochenmark (BMDC) bei miR-KO-Mäusen war wiederum verringert. Unsere Mausdaten bestätigen damit die Resultate unserer Studien mit menschlichen Zellen, welche zeigten, dass die Differenzierung von moDCs im Gegensatz zu LCs von miR-424 (322) / 503 abhängig ist.

Schließlich charakterisierten wir das Transkriptionsprofil von BMDCs, die aus miR-KO-Mäusen erzeugt wurden, und stellten fest, dass TGF-β-Signaturgene in miR-KO-Zellen hochreguliert sind. In Übereinstimmung damit begünstigt der Verlust von miR-424/503 die TGF-β1-abhängige LC-Differenzierung auf Kosten der moDC-Differenzierung. Daher schlugen wir ein Modell vor, bei dem miR-424(322)/503 als molekularer Schalter fungiert, welcher mittels Modulation der TGF-β-Signalübertragung über die Differenzierung zu

Gunsten von LCs oder moDCs entscheidet. Unsere Ergebnisse untermauern die zentrale Rolle von miR-424(322)/503 bei der Differenzierung von moDCs, sowohl *in vitro* als auch *in vivo*. In der Studie lieferten wir mehrere neue Einblicke in die Mechanismen, die der Differenzierung zweier funktionell unterschiedlicher DC Zelltypen (moDCs und LCs) vom gemeinsamen monozytischen Vorläufer zugrunde liegen.

ABSTRACT IN ENGLISH

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. However, the implication of individual microRNAs in the process of DC differentiation remains poorly understood. Therefore, we focused our studies on the molecular mechanisms regulated by specific microRNAs in DC subset specification.

Our group previously searched for the microRNAs that are differentially expressed by human DC subsets. We identified that miR-424(322)/503 is strongly upregulated in pro-inflammatory monocyte –derived dendritic cells (moDCs) in comparison to anti-inflammatory Langerhans cells (LCs). Using lentiviral gain- and loss-of-function approach we confirmed that miR-424(322)/503 is critical for moDC development. Conversely, LCs were unaffected by the miR-424(322)/503 deficiency. We identified that miR-424(322)/503 is required for moDC differentiation.

To determine whether miR-424/503 is also involved in molecular mechanisms of moDCs differentiation *in vivo*, we subjected miR-424(322)/503-KO (miR-KO) mice to a clinically relevant model of psoriasis-like skin inflammation. We observed that moDCs subsets were significantly reduced in the dermis of miR-KO mice under inflammatory conditions in comparison with the WT mice. However, DC-precursors in the fresh isolated bone marrow were equally present in both conditions. Similarly, the percentage of *ex vivo* differentiated bone marrow - derived DCs (BMDCs) was diminished in miR-KO mice. Consequently, our murine data corroborate our findings in human moDC vs. LC differentiation that moDCs development was selectively dependent on miR-424(322)/503.

Finally, we characterized the transcriptional profile of BMDCs generated from miR-KO mice and found TGF-β signature genes to be upregulated in miR-KO cells. Consistent with that, loss of miR-424(322)/503 facilitated TGF-β1-dependent LC differentiation at the expense of moDC differentiation. Thereby, we proposed a model where miR-424(322)/503 acts as a molecular switch for LCs vs. moDCs cell fate lineage decision via modulating TGF-β signaling. Our findings substantiate the pivotal role of miR-424(322)/503 in moDCs differentiation both *in vitro* and *in vivo*. In the study we provided several novel insights into the mechanisms underlying differentiation of two functionally different DC subsets (moDCs and LCs) from the common monocytic precursor.

1. INTRODUCTION

1.1 Innate and adaptive immunity

The immune system provides defense against infectious microorganisms and non-infectious foreign substances. All the components of the host immune system including cells and signaling molecules provide a coordinated response upon intrusion of pathogens that is called the immune response (2). Equally important function of the immune system is to avoid self-reactivity by maintaining immune tolerance. Failure in the mechanisms of immune tolerance may lead to reaction against autologous antigens resulting in autoimmune diseases, e.g. Type 1 diabetes, rheumatoid arthritis and multiple sclerosis (3).

Immune responses are mediated by both innate and adaptive immunity. Innate immunity is non-specific and crucial for the first-line host defence because it provides a rapid response within few hours. The main components of innate immunity are external physical barriers, natural killer (NK) cells, macrophages, dendritic cells (DC), granulocytes as well as defensins and complement (4). In contrast to the innate immunity, adaptive immunity is responsible for the later stages of inflammation and it takes several weeks to be triggered. The adaptive immune system is activated in order to specifically recognize the pathogen and build the immunological memory. During evolution adaptive immunity has been based on the immune receptors of T- and B- lymphocytes in the jawed vertebrates (5). The former concept that innate and adaptive immune processes are relatively separated in time has been changed in the last two decades after discovering the strong links between them (6). With the time it became more evident that antigen-presenting DCs as a part of the innate immune system induce activation of the adaptive immune response. Therefore, DC provide an essential link between the adaptive and innate immune system (7-10).

1.2 Immune regulatory role of dendritic cells

Dendritic cells as part of antigen-presenting cells (APCs) capture antigens and mediate both T cell immunity and tolerance (11). Functional properties of DCs differ according to their state of differentiation and maturation. Immature DCs reside in the skin, gut, blood, lymphoid organs and lungs (12-15). In the steady state, DCs are responsible for immune-surveillance and are constantly sampling their microenvironment to detect potential pathogens (16). Moreover,

immature dendritic cells maintain peripheral tolerance by either deletion of T cells or expansion of regulatory T cells (Tregs) (17-20), which in turn regulate other immune responses such as tumor immunity, allergy and graft-versus-host disease (17). When DCs get in contact with bacterial lipopolysaccharide, viral RNA or other inflammatory stimuli they undergo maturation process. Upon maturation, DCs load the internalized antigens on major histocompatibility complex (MHC) molecules (18) and migrate to the lymph nodes or spleen to prime näive T cells (19). This process is accompanied by the upregulation of CD86, CD80, CD40 and CD83 co-stimulatory molecules on DC membrane (20) and production of proinflammatory cytokines.

Mature DCs, depending on the nature of the antigens and the way they have been captured, stimulate different types of T-helper (Th) cells such as Th1, Th2 or Th17 (21). Th1 cells are mediating cellular immunity via production of interleukin 2 (IL-2) and interferon γ (IFN γ), whereas Th2 cells are regulating humoral immunity via IL-4, IL-5 and IL-13 cytokine production. Th17 cells play important role in induction of inflammation by releasing IL-17, IL-21, IL-22. They are known to regulate immune responses against bacteria, viruses and fungi. Th17 cells are implicated in pathological mechanisms of autoimmune diseases such as inflammatory bowel disease, rheumatoid arthritis and multiple sclerosis (22). Tregs have suppressive function on other T cells and maintain homeostasis through secretion of TGF- β1 and IL-10 cytokines (23,24). Mouse experiments including depletion of DCs showed that DCs are selectively required for Treg homeostasis (25,26).

Overall, DCs play an important role in the immune cell regulation in healthy and pathological conditions. Understanding the mechanisms of DC differentiation and function is crucial for modulating the immune response, identifying novel vaccines and deepening our knowledge in the pathology of inflammatory diseases.

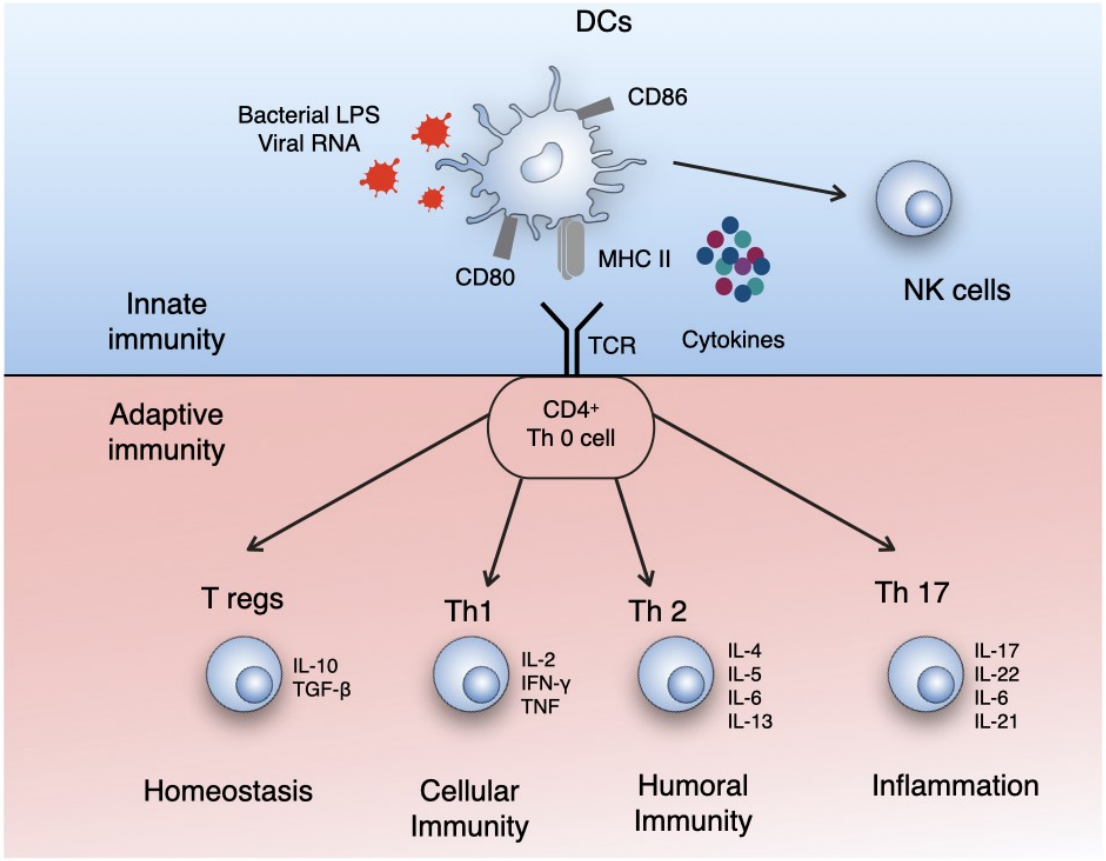

Figure 1. Dendritic cells bridge the innate and adaptive immunity.

The role of DCs in the innate immune system is to get triggered by the different pathogen-associated molecular patterns (PAMPs) and release cytokines that further activate other components of the innate immune system (e.g. NK cells). As part of the adaptive immune system DCs migrate to the secondary lymphoid organs where they induce naïve T cells to differentiate into Tregs, Th1, Th2 or Th17 cells. Each effector T cell type has unique functional features and cytokine profile.

1.3 DC subsets

DCs comprise a heterogeneous group of immune cells with distinct surface marker expression, function, localization (circulating blood DCs, lymphoid DCs, tissue-resident DCs) and their physiological state (steady-state vs. inflammatory). While DCs were studied in both human and mice, current knowledge doesn't allow to use specific markers to fully correlate DC subsets across the species (27) . However, transcriptional profiling of DC subpopulations in human and mice provided a powerful tool for a robust DC classification (28-31). Computational studies suggested that mouse and human plasmacytoid DCs (pDCs) share a lot of signature genes in contrast to the lymph-node resident conventional DCs (cDCs) that have smaller conserved

profiles between human and mouse species (32). Precise definition of DC subsets and their hierarchy is only possible by incorporation of unbiased approaches such as mass cytometry (CyTOF) and single cell RNA sequencing (33-35). Under steady -state condition there are three most abundant DC subpopulations in the skin: epidermal Langerhans cells (LCs), pDCs and dermal cDC1/cDC2. Under inflammatory condition a fourth subpopulation of dermal inflammatory DCs can be distinguished.

1.3.1 Epidermal Langerhans cells

Epidermal LC subset is located in the basal/ suprabasal layers of the epithelia and represents 3-5% of epidermal cells (36). Human epidermal LCs can be identified by the expression of $CD207^+$/langerin, $CD1a^+$, EpCAM and E-cadherin$^+$. Surface expression of E-cadherin mediates LC adhesion to surrounding keratinocytes, but is dispensable for LC maturation, migration and function (37). Langerin expression contributes to the cytoplasmic formation of Birbeck granules- the hallmark organelles of LCs (38,39) - and has frequently been used as a specific marker for LC phenotyping. Inducible ablation of LCs in Langerin- diphtheria toxin receptor (DTR) mice provided a powerful tool for investigation of the LC function (40). These studies let to the discovery that langerin is expressed not only by LCs, but also a distinct subpopulation of Langerin$^+$ dermal DCs (41-44). Epidermal LCs and Langerin$^+$ dermal DCs exhibit differences in CD11b, EpCAM and CD103 expression (36). LCs are constantly monitoring the skin environment for the presence of pathogens and the integrity of the cutaneous barrier (45). In the steady -stage LCs ensure immune tolerance towards self-antigens by inducing Tregs (46). Upon inflammation they migrate to the skin-draining lymph nodes (LNs) to present the processed antigen (Ag) to $CD4^+$ T cells (47,48). After leaving the epidermis, migratory LCs upregulate MHC II, CD40, CD205 and CC-chemokine receptor 7 (CCR7) expression and loose E-cadherin. LCs appear to be more efficient in priming naïve T cells than dermal DCs (49). Additionally, LCs are able to cross-present Ag to $CD8^+$ T cells after langerin-mediated Ag internalization (50,51).

Role of LCs in the pathogenesis of inflammatory diseases remains poorly understood. There is some evidence that LCs have both immunostimulatory and immunosuppressive function, depending on the different pathological conditions (52,53). Glitzner et al. showed that LCs in psoriatic mouse skin play anti-inflammatory role by releasing IL-10 (immunosuppressive cytokine) and decreasing levels of IL-23 (pro-inflammatory cytokine)

(54). Importantly, depletion of LCs during onset of psoriatic inflammation resulted in the aggravation of psoriatic disease (54).

1.3.2 Plasmacytoid DCs

Plasmacytoid DC is a unique DC subset that was initially found in the human lymph nodes. Human pDCs comprise 0.2%–0.8% of peripheral blood mononuclear cells (PBMCs) (55). They are characterized by expression of specific markers BDCA-2 (CD303), BDCA-4 (CD304), CD123 in human peripheral blood and bone marrow (56). pDCs have a well-recognized plasmacytoid morphology: eccentric nucleous, basophilic cytoplasm and acroplasm (57). The key function of pDCs is to produce type I interferons (IFNs) and therefore promote antiviral immunity and other associated effects. *In vivo* studies have shown that pDCs' Toll-like receptor 4 (TLR4), TLR7 and TLR9 activation to recognize viral RNA and DNA within the endosomal compartments (58), whereas cDCs act only via TLR9 and partially TLR7 ligands (59). Activated pDCs induce B cells to differentiate into plasma cells and stimulate NK cells coactivation (60,61). In contrast to cDCs, steady-state pDCs do not have capacity to prime naïve T cells as well as secrete IFN. However, activated pDCs are able to prime T cells and interact with cDC1 cells to induce cross-presentation (62).

The aberrant pDC activation, induced by overexpression of antimicrobial peptides, leads to IFN-driven autoimmunity in psoriasis (63). Defective regulatory loop between pDCs and regulatory B cells contributed to the pathogenesis of systemic lupus erythematosus (SLE) (64). Moreover, RNA seq data have shown that association between IFN production by pDCs and polymorphisms in gene loci related to SLE (65). Implication of pDCs in the pathogenesis of autoimmune diseases and anti-viral response makes them important target for therapeutic strategies.

1.3.3 Conventional DCs

Conventional/classical DCs play a central role in initiating T cell immunity and include two major subsets cDC1s and cDC2s. In contrast to cDC1 that cross-prime $CD8^+$ T cells and induction of T helper 1 (Th1) cells, cDC2 are known to induce Th1, Th2 and Th17 polarization (66-68). The mouse cDC1 and cDC2 cells are most likely to be equivalent to human $CD141^+$ DCs and $CD1c^+$ DCs. Human CD141(BDCA-3) $^+$ population has been found in the blood and lymphoid tissues (69) . It has been characterized by the expression of signature markers XCR1,

NECL2, CADM1, TLR3 and C-type lectin-like receptor Clec9a (70-72). In mice, dermal cDC1s express CD24, CD103, XCR1 and CD207, while lymphoid-resident cDC1s express CD8α. cDC1s depend on *Irf*8 expression during differentiation and produce IL-12 cytokine (73,74). $CD141^+$ DCs upon skin inflammation upregulate CCR7 and migrate to the skin-draining LNs (75).

Human CD1c (BDCA-1) $^+$ arise via the network of transcription factors including involving IFN regulatory factor 4 (IRF4) (76). Notch2 signaling is required for cDC2 development and efficiency of Ag presentation as well as activation of $CD4^+$ T cells (77). $CD1c^+$ DCs population is the abundant DC subset in human blood, tissues and lymphoid organs. It has a specific phenotypic characteristic such as HLA-DR, CD11b, CD11c, FCER1, CD172 (SIRPα) expression (78) in human and CD11b, CD172 (SIRPα) in mice. Recent study by Zhang et al. showed that human $CD11c^+$ DCs, similar to equivalent murine cDC2s, can be separated into $CD5^{low}$ and $CD5^{high}$ functionally different subpopulations (79). Briefly, $CD5^{high}$ cells had stronger migration capacity and more potentially induced T cell proliferation than $CD5^{low}$ population (79).

Therefore, cDC2 in blood and lymphoid organs has heterogeneous phenotype and gene expression profile. Moreover, cDC2s are specialized on the priming $CD4^+$ T cell proliferation, whether cDC1s promote Ag cross-presentation to $CD8^+$ T cells. The previous research has shown that LN-resident cDCs are a powerful tool for initiating immunity by protein vaccines (80). Given the ability of cDC1s to additionally induce cytotoxic T cells (CTL), they became attractive targets for CTL vaccines *in vivo* (81).

1.3.4 Inflammatory DCs

In the inflammatory microenvironment a distinct DC population -inflammatory DCs (infDCs)- can be identified. Human infDCs, but not inflammatory macrophages, have shown the capacity to induce Th17 cell differentiation, Th1 and Th2 cell-mediated responses (82-84). InfDCs originate from blood $Ly6C^{high}$ monocytes that are recruited to the site of inflammation (85) and, therefore, they are also called monocyte-derived DCs (moDCs). Due to the common origin, it is very important to distinguish $CD1a^+$, $CD11b^+$, $CD209^+$ moDCs and monocyte-derived macrophages (moMacs). Recent studies identified that moDC differentiation is controlled by aryl hydrocarbon receptor (AhR) and IRF4 transcription factors, while moMacs exhibited MAFB transcriptomic signature (86,87) . Chemokine receptor CCR2 is important

molecule in the egress of the monocytes from the BM, but it's dispensable for recruiting the monocytes from blood to the inflamed tissues (86). In turn, moDCs upregulate CCR7 receptor to enable their migration to the draining lymph nodes upon inflammation (87,88).

In humans, moDCs have been found in small intestine mucosa und lungs both in the steady-state and inflammation (89,90). Although identification of moDC subset in the normal skin can be problematic due to the low cell number, psoriatic skin displays 30-fold increase in the number of infDCs that play important role in the pathogenesis (91). Moreover, they critically contribute to the psoriasis by releasing pro-inflammatory cytokines (mainly, TNF-α IL-1β) and require CCR6 expression (92). In psoriatic dermis an additional subset of infDCs exhibiting proinflammatory effect by iNOS (inducible nitric oxide synthase) and TNF-α (tumor necrosis factor-alfa) production, was termed as tumor necrosis factor and inducible nitric oxide synthase-producing DCs (Tip-DCs) (93). Tip-DC exhibit $CD11c^{+}CD1c^{-}CD141^{-}$ phenotype with ectopic CD14, CD163 and CD209 expression. The main function of Tip-DCs is to stimulate the differentiation and activation of Th17 cells (94). Human Tip-DCs can also be generated *in vitro* from monocytic precursors of healthy donors as well as psoriatic patients (95).

Inflammatory dendritic epidermal cells (IDECs) were first described as a distinct population of moDCs in the skin of atopic dermatitis patients (96). These cells have a unique surface marker profile including HLA-DR, CD1a, CD11b, CD11c, CD36, CD206, FcεRI and immunoglobulin E expression. FcεRI-activated IDECs release IL-12 and IL-18, which contribute to switch of Th2 response towards Th1 type response (97,98).

Taken together, moDCs are important contributors of the inflammation and their phenotype depends on the nature of inflammatory stimuli and microenvironmental conditions. However, the interrelationships between distinct moDCs subsets (Tip-DCs, IDECs) are still poorly understood as well as the molecular mechanisms underlying their differentiation from the blood $Ly6C^{high}$ monocytes.

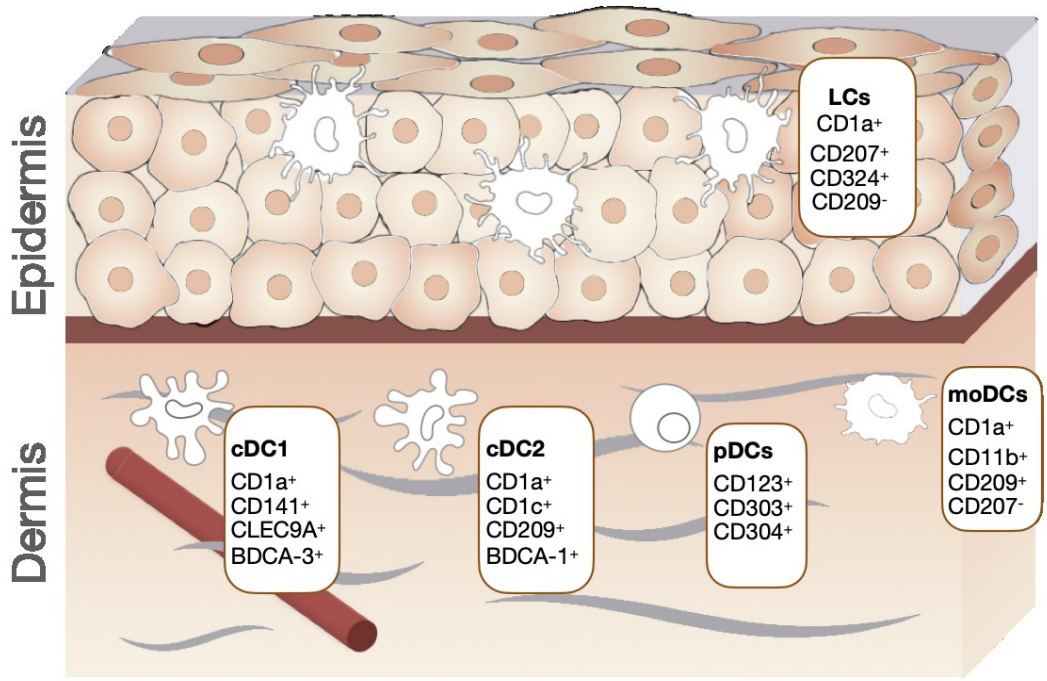

Figure 2. DC subsets in the human skin.

Epidermis, the outer layer of the skin, is formed by the multiple keratinocyte that provide a physical barrier to the pathogens. Langerhans cells (LCs) reside in the basal/ suprabasal layers of the epidermis. Different DC subsets coexist in the dermis: conventional DC1 (cDC1), cDC2, plasmacytoid DCs (pDCs), monocyte-derived DCs (moDCs). DCs subsets can be distinguished based on their surface marker expression both in the steady-state and upon inflammation.

1.3.5 Murine homologs of human DCs

Human DC studies over the last decades substantially broadened our consideration of the phenotype and function of various DC subsets mostly extracted from the human peripheral blood and lymphoid organs. However, the major progress in the understanding of DC development has been done *in vivo*. Due to a wide range of disease models and availability of the genetically modified animals, the *in vivo* validation of human studies helps to deep our understanding of regulation of DC lineage commitment. Murine and human DC populations often have different phenotype, which makes cross-species DC comparison very challenging. Therefore, identification of mouse DC counterparts requires unbiased high dimensional single-cell approach (CyTOF) and designing precise gating strategies for cell purification. Many relevant studies addressed the heterogeneity of DCs populations in different species by

comparing DC transcriptome profiles (reviewed in (99,100)). For instance, recent study identified using CyTOF murine pDC subpopulation that is equivalent to the recently described human AXL^{+}DCs (101-103). Due to the similarities in their gene expression profile and capacity to promote allogeneic T cell proliferation, they were termed conserved population transitional DCs (tDCs) in both species (104). Similar to human pDCs, murine pDCs are also critically dependent on FLT3 signaling. It has been shown that selective deletion of Flt3 ligand in the mouse lymphoid tissue diminishes the pDCs population (105,106).

The analog of human CD141^{+}cDCs is murine CD103^{+}CD11b^{-} DC subset, which shares its function with CD8^{+}cDCs in lymphoid tissues (107-109). Similar to human cDC1s, the murine CD103^{+} DCs are superior in the antigen cross-presentation to T cells. Consistently, the homologs of human CD1c^{+}cDCs correspond to CD11b^{+}CD103^{-} murine DCs. Previous studies have shown the functional heterogeneity among CD103^{+}CD11b^{-} DC and CD11b^{+}CD103^{-} DCs. Particularly, CD11b^{+}CD103^{-} DCs were able to induce Th2 response in the small intestine unlike CD103^{+}CD11b^{-}DCs that rather induce Th2 cells in the murine colon (110). Interestingly, the commitment to the CD11b^{+}CD103^{-} DCs and CD103^{+}CD11b^{-} cDCs in mice occurs already on the early stages of differentiation in the BM (111) and is regulated, respectively, by either IRF4 or IRF8 TFs (112).

Human and murine LCs have major differences in Langerin expression, which for a long time has been known as a unique surface marker for LCs. Bigley V. et al. identified mouse dermal langerin^{+} DCs that are different to LCs and closely related to CD1c^{+} DCs (113). Additionally, murine LCs express monocyte-macrophage markers such as CD11, F4/80 and lack CX_3CR1 expression (36). Murine MHCII^{++} LCs together with moDCs are increased in the murine inflamed skin and virtually absent in the steady-state (92). Topical application of Imiquimod (IMQ), a TLR 7/8 agonist, leads to induction of IL-23-mediated psoriatic-like inflammation in the mouse skin. Singh et al. identified Ly6C^{high} and Ly6C^{low} populations of moDCs in the inflamed murine dermis, using the combination of MHCII, CD11b, MerTK, CD64 markers (92). Although the functional difference between Ly6C^{high} and Ly6C^{low} populations remains unclear, broadly defined moDC population have shown the ability to mediate IL-23-induced inflammation via secretion of IL-1β and TNF cytokines.

Taken together, mouse and human dendritic cell populations can be distinguished by different phenotypic characteristics and these differences need to be taken into considerations for translating mouse DC studies. Although all DC subsets in mice have corresponding

counterparts in human, unbiased transcriptome analysis and identification of unique surface markers is needed for proper inter-species comparison of DC nomenclature.

Table 1. Phenotype differences of mouse and human DCs.

Cell type	Mouse surface markers	Human surface markers
LC	Langerin$^+$, F4/80$^+$, CD11b$^+$, CD11c$^+$, CD11b$^+$, CD205$^+$, CD103$^-$, EpCAM$^+$, E-Cadherin	Langerin$^+$, EpCAM$^+$, E-Cadherin$^+$, CD1a$^+$, MHCII$^+$, SIRPα$^+$
pDC	CD11c$^+$, B220$^+$, Grl$^+$, Syglec-H$^+$, Bst2$^+$, Ly6C$^+$, SIRPα$^+$,CD14$^+$, CD123$^+$	CD303$^+$, CD304$^+$, CD123$^+$, CD14$^+$, SIRPα$^+$
cDC1	CD103$^+$, CD8a$^+$, Langerin$^+$, Clec9a$^+$, CD205$^+$, CD24$^+$, XCR1$^+$	CD141$^+$, Necl2$^+$, Clec9a$^+$, XCR1$^+$
cDC2	CD11b$^+$, CD103$^-$, CD4$^+$, SIRPα$^+$	CD1c$^+$, CD1a$^+$, CD1b$^+$, SIRPα$^+$, XCR1$^-$
moDC	MHCII$^+$, CD11b$^+$, Ly6C^{high}, Ly6C^{low}, MerTK$^-$, CD64$^-$	MHCII$^+$, CD11b$^+$, CD1a$^+$, CD209$^+$

1.4 DC development

1.4.1 DC progenitors

DCs are derived in the bone marrow, where they undergo several steps of intermediate DC-progenitors until becoming mature DCs that comprise 0.5-1.5% of PBMCs. Previous studies have identified common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs) as early committed DC-progenitors in the bone marrow (114,115). In turn, CMPs give rise to the granulocyte macrophage progenitors (GMPs), which further differentiate into macrophage DC progenitors (MDPs). Fogg et. al identified MPDs as Lin$^-$ c-kit$^+$ CX3 CR1$^+$ shared progenitor population for DC and macrophages (116). The commitment of MDPs diverges into monocytes (also called monocytic progenitors or MoPs) and common DC

progenitors (CDPs) (117,118), however, the transcriptional mechanism of divergence at the stage of MDPs remains unclear.

Initially $Ly6C^+$ monocytes were considered as a main precursor of inflammatory DCs in mice (119). Menezes et al. addressed the heterogeneity of monocytes in mice and suggested that all $MHCII^+$ $CD172a\ (SIRPa)^+$ $CD115^+$ $Ly6C^+$ monocytes could be further subdivided into 3 subpopulations: $CD11c^-Flt3^-$ (R1), $CD11c^-Flt3^+$ (R2) and $CD11c^+Flt3^+$ (R3) (120). According to this study R1 population is a precursor for $INOS^+$ macrophages, R2 population is able to differentiate into moDCs upon GM-CSF stimulation and R3 population phenotypically corresponds to pre-DCs (120).

Murine CDPs were identified as Lin^- $c\text{-}kit^{int}$ $Flt3^+$ $Csf\text{-}1R^+$ population that has a capacity to further differentiate via the step of pre-cDCs into the lymphoid/nonlymphoid tissue cDCs or directly to pDCs (121). A number of studies have shown that most of CDPs pre-committed on the early stages of differentiation and pre-cDC1/ pre-cDC2 populations could be identified in the bone marrow (111,122). In contrast, pDCs represent a distinct branch in the differentiation that is separate from conventional myeloid cells. Pre-cDCs migrate with the blood flow to the peripheral lymphoid organs, where they differentiate terminally into cDC1 and cDC2. In human, distinct circulating precursor (hpre-cDC) of $CD1c^+$ and $CD141^+$ DC has recently been identified in the cord and peripheral blood, bone marrow and peripheral lymphoid organs (123). Hpre-cDCs could be further differentiated into mature pDCs and cDCs using *in vitro* differentiation model, indicating that they are indeed multipotent (123,124).

In contrast to the other DCs, LCs have a distinct origin and two alternative pathways for differentiation. On the one hand, mouse LCs develop from the yolk sack precursors and fetal liver monocytes on the early stages of embryogenesis (125,126). Human LCs were identified already at the 8-10 week of prenatal life and most likely derive from fetal hematopoiesis (125). At 18-24 week of fetus development human LCs constitute a fully formed APC (127,128). LCs self-renew locally in the steady-state condition, constantly proliferating *in situ* from tissue precursors (129,130). On the other hand, LC pool can be maintained by LC differentiation from blood $Gr\text{-}1^{hi}$ monocytes upon inflammatory conditions *in vivo* (131). There is an evidence that in the absence of monocytes LC pool can be reconstituted by myeloid - committed precursors (132), suggesting the existence of a second-wave long-term LCs. Very recently, alternative pathway of LC differentiation from $CD1c^+$ blood DCs *in vivo* has been revealed (133). Moreover, $CD1c^+$ DCs are able to differentiate into LCs-like cells *in vitro*

(134,135), providing additional approach for generating LC differentiation. However, the transcriptomic comparison of $CD1c^+$ derived LCs and freshly isolated skin LCs is needed to address the relevance of these studies.

1.4.2 Cytokine control of DC differentiation

DC lineage differentiation and homeostasis are regulated by different hematopoietic cytokines. FLT3L, GM-CSF, IL-4 and TGF- β1 cytokines are essential at different stages of DC differentiation process from the BM precursors.

FLT3L

The Fms-related tyrosine kinase 3-ligand (FLT3L) is a crucial regulator of DC commitment that is expressed by the early myeloid and lymphoid progenitors (106,136,137). *In vitro* BM cultures supplemented with FLT3L generate a large number of pDCs and cDCs (138). FLT3L acts as a growth factor for hematopoietic progenitors *in vivo* and its deletion leads to the loss of pDCs and cDCs in LNs and spleen (139). LCs, granulocytes and monocytes are not affected by the loss of FLT3L signaling. However, FLT3L in combination with TGF-β1 substantially promotes *in vitro* LC differentiation (140). FLT3L overexpression or injection expanded the numbers of cDCs and pDCs in the blood and lymphoid tissue (141,142). Interestingly, $CD1c^+$ DCs and their precursors also exhibited elevated numbers in respond to increased FLT3L levels (123). FLT3L mutations in 30% of cases are associated with the onset of acute myeloid leukemia (AML) (143) and were reported to be an important prognostic factor for AML patients (144).

GM-CSF

Granulocyte-macrophage colony stimulating factor (GM-CSF) was initially identified as an *in vitro* mediator of proliferation and differentiation in granulocytes and macrophages (145,146). Further studies suggested the key role of GM-CSF in DC differentiation from the hematopoietic progenitors and monocytes (147,148). GM-CSF deficient mice did not show any defect in the spleen and LN cDCs development (149), however, they exhibited a dramatic reduction in the cDCs number in the peripheral non-lymphoid tissues (150). Although the role of GM-CSF in DC function is not well- characterized as in DC development, the range of studies have shown that GM-CSF mediates $CD8^+$ DC function (reviewed in (151)). A number of studies revealed

that elevated GM-CSF levels are associated with autoimmune and inflammatory diseases, such as multiple sclerosis and rheumatoid arthritis (152). The reason is that GM-SCF regulates Th17 autoimmune response and, therefore, exhibit pro-inflammatory properties. Blockage of GM-CSF pathway by a monoclonal antibody against GM-CSF receptor (mavrilimumab) appears to be a very promising therapeutic approach in the treatment of rheumatoid arthritis (153-155).

IL-4

IL-4 cytokine is frequently used for *in vitro* DC differentiation in conjunction with GM-CSF (156). Initially IL-4 seemed to prevent overgrow of macrophages, but recent study has shown that addition of IL-4 to BMDC cultures limits but does not prevent macrophage generation (157). Combination of IL-4 with both GM-CSF and TNF-α impaired DC maturation and function *in vitro* (158). Secretion of IL-4 is a feature of Th2 cells, however, it can also be released by basophils, NK cells, mast cells and eosinophils (159).

TGF- β1

Transforming growth factor (TGF- β1) is a pleiotropic cytokine, which is implicated in immune cell regulation and function (160). Initially TGF-β1 has been known as a key cytokine instructing LC differentiation in mice and human (161-163) . This statement was supported by the fact that TGF-β full knockout mice exhibit absolute deficiency of LCs (164). *In vitro*, addition of TGF-β1 to $CD34^+$ hematopoietic stem cells is essential for LC generation (165). $CD14^+$ monocytes can also acquire LC-phenotype *in vitro* upon stimulation with TGF-β1/GM-CSF/Delta-1 cytokine cocktail (161). However, TGF-β1 is not only required for the proper LC differentiation, but also maintains LC and memory T cell residence in the epidermis (163). It has been shown that TGF-β1 is secreted by LCs and keratinocytes in autocrine/paracrine manner and the autocrine TGF-β1 is crucial for epidermal residence of LCs (162,166). Interestingly, conditional TGF-β1 knockout under DC-specific promoter leads to spontaneous LC maturation and increased their migratory capacity (166). Canonical TGF-β1 signaling pathway involves TGF β type I receptor (TGF-β R1) and downstream activation of ALK 5 and SMAD2/3 proteins. In the absence of canonical TGF- β1-signaling, LC differentiation can be induced by bone morphogenetic protein 7 (BMP7) via ALK3 (also known as BMPR1a) and SMAD 1/5/8 co-activation. BMP7 signaling is known to modulate inflammatory DC in the

psoriatic skin (167,168). *In vitro,* TGF-β1 can utilize ALK-3 dependent pathway for TGF-β1-induced LC differentiation (169).

However, recent studies identified that TGF-β1 signaling is not only restricted to LC differentiation and function. TGF-β1 instructs differentiation of common DC progenitors into cDCs and suppresses pDCs development (170). In addition to that, TGF-β1 drives $Ly6C^{hi}$ monocyte differentiation into macrophages in the murine intestinal mucosa (171). TGF-β1 is also known to mediate $CD4^{+}CD25^{-}$ naïve T cell conversion to $CD4^{+}CD25^{+}$ Tregs via induction of Foxp3 expression *in vivo* (172). Further studies using transgenic mouse models highlighted the role of TGF-β1 signaling in maintaining of the immunotolerance. Mice with Cre-mediated DC-specific deletion of *Tgfbr2*, which leads to the loss of TGF-β1 signaling, exhibited autoimmune multiorgan inflammation (173). On the other hand, increased levels of TGF-β1 positively correlated with the recovery from autoimmune diseases due to its multiple suppressive effects (174). Depending on the microenvironmental stimuli, TGF-β1 can either suppress adaptive immune system via Tregs induction or activate adaptive responses (for instance, through Th17 stimulation) (175). Therefore, further studies are needed to find out which mechanisms are involved TGF-β1-dependant modulatory effects in the immune system.

1.5 microRNAs

MicroRNAs (miRNAs) are small non-coding RNAs that play important role in regulation of many developmental and cellular processes by modulating gene expression at post-transcriptional level. MiRNAs are evolutionary conserved across the species. They are expressed in a tissue- or cell-specific manner in mammals and control over 50 percent of all protein -coding genes (176). Previously non-coding RNAs were recognized as a “junk DNA” in a human genome, but nowadays this paradigm has shifted towards far more complex approach, where non-coding RNAs fine-tune gene expression, thereby functionally regulating thousands of target genes. Under physiological conditions miRNAs have been proven to regulate cell differentiation, tissue homeostasis, apoptosis and cell proliferation. miRNAs constitute an abundant class of small RNAs and their dysregulation is frequently implicated in the mechanism of human diseases, such as cancer (177,178), cardiovascular and metabolic disorders (179-181). Secreted miRNAs can be packed in macrovesicles and transferred by biological liquids to other tissues, which allows them to regulate the long-distance cell-cell signaling. A number of studies have shown that circulating exosomal miRNAs in the liquid

biopsies can be used as biomarkers of breast cancer, non-small-cell lung cancer and colon cancer (182-184). Recently, some progress has been made in development of miRNA therapeutics (antisense oligonucleotides, mimics, miRNA sponges) and discovery of miRNA delivery tools (exosomes, viral vectors, nanoparticles) (185).

1.5.1 miRNA biogenesis and function

miRNAs comprise 19-25 nucleotide single-stranded RNAs. Initially transcribed by RNA polymerase as longer RNAs in the nucleus, precursor miRNAs (70-100 nucleotides) undergo sequential cleavage by Drosha enzyme (Figure 3). MiRNA genes can be transcribed either independently for each miRNA or into single polycistronic transcripts (186). Then precursor miRNAs (pre-miRNA) are exported to the cytoplasm by Exportin 5. In the cytoplasm pre-miRNAs are processed by Dicer (second RNase III nuclease), resulting in the miRNA duplex and, further, into single -stranded mature miRNA. Subsequently, the mature miRNA incorporates to the RNA-induced silencing complex (RISC) in association with Argonaute proteins (AGO). Within the RISC complex miRNA "seed" sequence binds to 3'untranslated regions (3'-UTRs) of target mRNAs, thereby inducing either inhibition of mRNA translation or mRNA degradation (Figure 3). Depending on the degree of complementarity, one miRNA can silence hundreds to thousands target genes, thereby interfering with the downstream protein expression in the signaling pathways. miRNAs with the same "seed" sequence are grouped into miRNA families and share sets of target genes. Multiple miRNAs can co-target same mRNA and, therefore, increase their potential to enhance repressive effect in a cell (187,188). On the other hand, single miRNA can increase its impact by targeting multiple components in a signaling cascade (189). Moreover, miRNAs can counteract with the other miRNAs providing an autoregulatory feedback loop and downstream control of miRNA biogenesis. MiRNA biogenesis is subjected to a sophisticated regulation by number of transcription factors binding to miRNA gene promotor; at the level of Drosha and Dicer processing; by RNA editing and methylation; by AGO availability (190,191). Advancements in computational biology allow to identify list of predicted mRNA gene targets that can be experimentally verified in particular cells or tissues. However, complexity of miRNA biogenesis and target regulation suggests that miRNAs broadly act on multiple targets at different time points rather than have specific effect on a few specific targets.

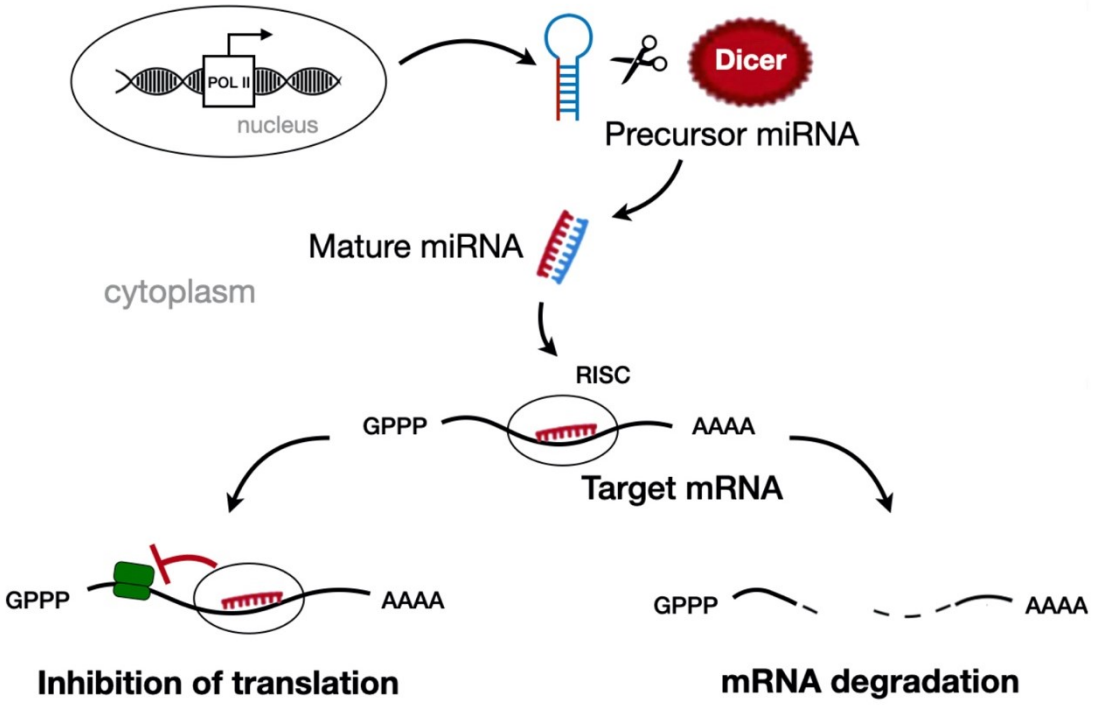

Figure 3. The mechanism of miRNA-regulated gene silencing.

miRNA genes are transcribed in the nucleus into single or polycistronic transcript. Pre-miRNA is transferred in the cytoplasm, where it undergoes cleavage by Dicer enzyme to produce miRNA duplex and mature miRNA. After mature miRNA is incorporated in the RNA-induced silencing complex (RISC), it binds to 3'untranslated regions (3'-UTRs) of target mRNAs. Therefore, miRNAs inhibit protein synthesis by either inhibition of mRNA translation or target mRNA degradation.

1.5.2 Role of miRNAs in DC differentiation and function

The role of miRNAs in hematopoiesis has been studied from different angles, mainly underlining DC development aspects from hematopoietic stem cells/monocytic progenitors and DC activation. One of the earliest studies identified 33 miRNAs expressed in human $CD34^+$ cells and functionally characterized miR-155 as a suppressor of myelopoiesis (192). Then, miR-125, miR-29 and miR-126 were found to be relevant for hematopoietic stem cell (HSC) differentiation (193,194). Notably, $CD34^+$ HSCs extracted from different origins (peripheral blood, cord blood and BM) exhibited differences in miRNA expression profile (195). There is a large group of miRNAs regulating maintenance and self-renewal capacity of HSCs, in particular miR-22, miR-99, miR-29a, miR-127-3p and miR-126 (196-199). For instance, miR-125b has been shown to promote survival and expansion of HSCs by targeting Bcl2 modifying factor and Krueppel-like factor 13 (200). Surprisingly, miR-125a alone was able to increase

the number of HSCs *in vivo* by 8-fold. Genome-wide gene expression study identified that miR-125a together with miR-99 and let-7e comprise an evolutionary conserved cluster expressed in long-term HSCs (201). Dysregulation of specific miRNAs contributing to hematopoietic cell differentiation frequently leads to hematopoietic malignancies. One recent study by Mammoli et al. showed that miR-130a expression in $CD34^+$ HSC is necessary for normal monocyte differentiation, and its disfunction might underlie AML (202). In addition, AML cells secrete miR-4532 to repress normal hematopoiesis through STAT3 signaling pathway (203). Master regulator PU.1 is known to transactivate miR-424, which promotes monocyte/macrophage differentiation through repression of NFI-A pathway (204,205). Another study has shown that predicted targets of miR-424 might be involved in TGF-β, MAPK, JAK-STAT, p53 signaling pathways as well as in AML and antigen presentation pathways (206).

During differentiation of HSCs miRNA profiles undergo significant changes and influence cell fate decisions. Rajasekhar et al. identified 19 upregulated and 27 downregulated miRNAs in monocytes and granulocytes once they were differentiated from CMPs (207). Among them, miR-125b and miR-10a showed highest correlation and were associated with increased lineage commitment. Several miRNAs have been shown to influence FLT3-driven DC-differentiation. For instance, in mice miR-22 regulates cDC and pDC specification by *Irf8* downregulation (208). Another miR-222 and miR-221 were able to modulate cDC/pDCs ratio by their differential expression (209). Additionally, miR-34a and miR-21 were shown to inhibit endogenous WNT1 - JAG1 signaling in DC differentiation from monocytic precursors (210). Other studies identified miR-211 and miR-155 regulation of human DC development via targeting p27kip1, KPC1 and SOCS-1 (211). Jurkin et al. analyzed miRNA expression profiles of moDCs and LCs generated *in vitro* from HSC precursor by microarray (212). Deep sequencing performed on mouse BM-derived HSCs, immature and mature DCs, regulatory DCs identified almost 400 miRNAs to be differentially expressed during DC differentiation (213). Thus, there is a great difference in miRNA expression profiles between HSC progenitors and DCs as well as DC subsets.

In addition to their role in hematopoiesis, many studies identified miRNAs that are regulating maturation and function of DCs. One of them has shown that miR-146a expression impaired TLR 7/9 signaling in pDCs via nuclear factor-κB (NF-κB) activation (214). Interestingly, similar study was performed on human LCs, which express miR-146 at higher

levels compared to moDCs. In LCs, silencing of miR-146a was shown to promote TLR2-mediated NF-κB pathway (212). Comparing of miRNA expression in immature, activated and tolerogenic DCs showed that miR-7, miR-9, miR-155 and miR-182 were upregulated upon maturation in DCs (215). Moreover miR-17, miR-133b, miR-203 and miR-23b cluster were solely expressed by tolerogenic DCs, suggesting that miRNA expression is directly linked to DC type (215). Indeed, miR-155 regulates IL-12 and IL-1β cytokine production and expression of MHC II and DC-SIGN (216-218). Induction of miR-155 in DCs stimulated T cell proliferation as well as secretion of IFN-γ and IL-2 (219). Dueck et al. proposed a miRNA hierarchy model regulated by miR-155 in DCs and macrophages (220). In contrast to pro-inflammatory miR-155, there is also miR-146 that has shown the opposite anti-inflammatory effect in DCs. miR-146 family includes miR-146a and miR146b that are both modulate DC apoptosis through TRAF6/IRAK1-NF-κB axis regulation (221). Besides that, miR-146 acts as a negative regulator of TLR-mediated response by direct targeting TRAF6 and IRAK1 proteins (222). Promotor analysis has shown that NF-κB acts as an upstream regulator of miR-146 (222). In addition, miR-21 also negatively regulates inflammatory response by decreasing IL-6 and IL-10 production (223). Another recent study identified that miR-181a sustained ERK-MAPK pathway and thereby regulated DC-SIGN expression and activation of moDCs (224).

Emerging data have identified an important contribution of individual miRNAs to DC differentiation, maturation and shaping the immune responses. Deletion of Dicer, critically important enzyme for miRNA processing, resulted in apoptosis and ablation of LCs *in vivo* (225), highlighting the crucial role of miRNAs in homeostasis and function of DCs. However, more mechanistical studies are needed to address the effects of miRNAs and their network in DC lineage commitment.

2. AIM OF THE STUDY

Dendritic cells play an essential role in initiation of adaptive and innate immune responses. Unlike other immune cells, DCs recognize the pathogens, become mature and migrate to the lymph nodes, where they present the Ag to T cells. DC specification and lineage commitment is a complex process regulated by the interplay between transcription factors and microRNAs. MicroRNAs fine-tune expression of the genes involved in many cellular functions, such as metabolism, development and differentiation. Deficiency of mature miRNAs in DCs is known to cause the loss of certain DC populations in mice and reduce their antigen presentation capacity. Despite the crucial role of miRNA in DCs, the implication of individual microRNAs in these processes remains poorly understood.

Our group previously identified microRNAs that are differentially expressed by human moDC and LC subsets. Almost fifty miRNAs were found to be differentially expressed by these two subsets. MiR-424/503 was among the strongest inversely expressed miRNAs in this screen, showing higher expression levels in moDCs versus LCs. A growing body of literature has previously examined the role of miR-424(322)/503 in cancer cell proliferation and epithelial-mesenchymal transition and found that miR-424(322)/503 acts as a tumour suppressor in different types of cancer. However, very little is known about the regulatory role of miR-424(322)/503 in the immune cells.

Recent study has shown that miR-424 promoted human monocyte/macrophage differentiation and this effect was mediated by NFI- A. Additionally, miR-424 promotor has a binding site for transcription factor PU.1, which is known as a master regulator of myeloid cell differentiation. In ovarian cancer, miR-424(322)/503 has been shown to regulate chemoresistance by activating the T-cell immune response. However, the role of miR-424(322)/503 in moDC and LCs differentiation is still to be elucidated.

In our work we aimed to study the contribution of miR-424(322)/503 in the mechanisms underlying cell fate choices of LCs and moDCs both *in vitro* and *in vivo*. We performed loss-of-function studies as well as a computational analysis to better characterize the potential targets and physiological function of miR-424(322)/503 in DC subset specification.

3. MATERIALS AND METHODS

3.1 Cytokines and reagents

The reagents and cytokines used for experiments are listed in Table 2.

Table 2. Cytokines and reagents.

Recombinant human GM-CSF	Peprotech	Cat# 300-03
Recombinant human TNF-α	Peprotech	Cat# 300-01A
Recombinant human SCF	Peprotech	Cat# 300-07
Recombinant human TGF- β1	R&D systems	Cat# 240-B-010
Recombinant human TPO	Peprotech	Cat# 300-18
Recombinant human FLT3L	Peprotech	Cat# 300-19
Recombinant human IL-4	Peprotech	Cat# 200-04
Recombinant human M-CSF	Peprotech	Cat# 300-25
Recombinant murine GM-CSF	Peprotech	Cat# 315-03
GlutaMAX	ThermoFisher	Cat# 35050038
Pennicilin/Streptomycin	PAA	Cat# P11-010
Retronectin	Takara	Cat# T100B
Lymphoprep	Axis Shield	Cat# 1114547
RNase-Free DNase Set	Qiagen	Cat#79254
Red Blood Cell Lysing Buffer Hybri-Max	Sigma-Aldrich	Cat# R7757
DNAse I	Stem Cell	Cat# 07900
Liberase	Roche	Cat# 5401119001
Hoechst 33342	ThermoFisher	Cat# H3570
Imiquimod 5% creme	Perrigo	N/A
Zombie NIR Fixable Viability Kit	Biolegend	Cat# 423105
EasySep human CD34 positive selection kit	StemCells	Cat# 18056
CD14 MicroBeads, human	Miltenyi	Cat# 130-050-201
TaqMan MicroRNA Assay hsa-miR-424-5p	ThermoFisher	Cat# 4427975 (Assay ID: 000604)
TaqMan MicroRNA Assay hsa-miR-424-3p	ThermoFisher	Cat# 4427975 (Assay ID: 002309)
TaqMan MicroRNA Assay hsa-miR-503-5p	ThermoFisher	Cat# 4427975 (Assay ID: 001048)

TaqMan MicroRNA Assay U6 snRNA	ThermoFisher	Cat# 4427975 (Assay ID: 001973)
miRNeasy Mini kit	Qiagen	Cat# 217004
TaqMan microRNA reverse transcription kit	ThermoFisher	Cat# 4366596

Table published in (1).

3.2 Primary cell isolation

3.2.1 $CD34^+$ hematopoietic stem cell (HSC) isolation

Ethical approval approval (EK 26- 520 ex 13/14) was obtained from the Medical University of Graz Institutional Review Board. Informed consent was provided to patients in accordance with the Declaration of Helsinki. Cord blood obtained from healthy donors during full-term deliveries was heparinized and diluted 1:2 with 1xPBS. Then, mononuclear cells (MNCs) were separated be the gradient separation with Lymphoprep™ (Axis Shield, Norway). Briefly, 20 ml of Lymphoprep was pipetted on top in 50 ml tube and centrifuged without break for 30 min at 1400 rpm. The interphase was collected and cells were further resuspended in 1xPBS and centrifuged low break at 1600 rpm. Red blood cells were lysed in 5ml of ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM Na2EDTA, pH = 7.2 – 7.4) for 10 minutes on ice and washed with 1xPBS. Then, cells were resuspended in the MACS buffer and isolated by positive selection using EasySep™ human CD34 selection kit (Stem Cell Technologies, Vancouver, Canada) according to manufacturer's protocol. Samples with cell purity more than 95% after isolation were used in subsequent experiments. Before freezing $CD34^+$ HSCs were expanded for 3 days in serum-free X-Vivo media supplemented with Glutamax, penicillin/streptomycin, 50 ng/ml SCF, 50 ng/ml FLT3L, and 50 ng/ml TPO.

3.2.2 $CD14^+$ monocytes, neutrophils

Buffy coats from healthy donors were obtained from the Transfusion Medicine Department of Medical University of Graz. Cells were washed with 1xPBS and resuspended in MACS buffer. $CD14^+$ cells were isolated by magnetic sorting using Milteny Biotec positive selection kit according to manufacturer's protocol. After isolation cell purity was assessed by flow cytometry. Neutrophils were isolated from peripheral blood by density-gradient centrifugation using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) according to the manufacturer's protocol.

3.3 Human *in vitro* differentiation cultures

3.3.1 $CD34^+$ stem cell - derived LCs and moDCs

For moDCs and LCs differentiation previously described two-step culture model was applied with slight modifications (212,226). Briefly, $CD34^+$ cells were plated (5 x 10^4 to 1x 10^5 / ml) in 24-well tissue culture plate in in CellGro DC (CellGenix, Freiburg, Germany) medium supplemented with Glutamax (2.5 mmol/L), penicillin/streptomycin (125 U/ml), 10% FCS, 100 ng/mL GM-CSF, 20 ng/mL stem cell factor (SCF), 50 ng/mL FMS-like tyrosine kinase 3 ligand (FLT3L), and 2.5 ng/mL TNF-α. After 3-5 days cells were washed, counted and replated into RPMI (Sigma, St Louis, Mo; +10% FCS) either with 100 ng/mL GM-CSF, 35 ng/mL IL-4 for moDCs or 100 ng/mL GM-CSF, 2.5 ng/mL TNF-α, and 1 ng/mL TGF-β1 for LCs. Differentiated cells were harvested after 5-6 days and analyzed by flow cytometry. For direct LC differentiation from $CD34^+$ cord blood cells were plated 5 x 10^4 ml per well directly in CellGro DC medium and supplemented with Glutamax (2.5 mmol/L), penicillin/streptomycin (125 U/ml), 2.5 ng/mL TNF-α, 100 ng/mL GM-CSF, 50 ng/mL FLT3, 20 ng/mL SCF and 1 ng/mL TGF-β1.

3.3.2 $CD14^+$ monocyte-derived moDCs

For moDC differentiation directly from $CD14^+$ monocytes, cells were cultured for 6 days in RPMI medium supplemented with 10% FCS, Glutamax (2.5 mmol/L), penicillin/streptomycin (125 U/ml), 100 ng/mL GM-CSF, 35 ng/mL IL-4 or 100 ng/mL GM-CSF alone.

3.3.3 moMacrophages

moMacrophages were generated for 6-7 days from $CD14^+$ monocytes in RPMI medium with addition of 100 ng/mL GM-CSF, 2 ng/mL M-CSF, Glutamax (2.5 mmol/L), penicillin/streptomycin (125 U/ml) and 10% FCS.

3.3.4 Granulocytes

Granulocytes were generated from $CD34^+$ cord-blood cells by plating 300.000 cells in serum free RPMI medium with 100 μg/mL G-CSF, 20 μg/mL SCF, Glutamax (2.5 mmol/L), penicillin/streptomycin (125 U/ml). On day 5 and 8 of differentiation cells were replated in a fresh medium and finally collected on day 10.

3.4 Lentiviral transfection, transduction of $CD34^+$ HSCs

miR-424 silencing in HSCs was performed using lentiviral vectors including antisense hsa-miR-424 (MZIP424-PA-1) and corresponding scramble control lentiviral vectors (MZIP000-

PA-1. Lentiviral constructs were purchased from SBI (System Biosciences). For miR-424 overexpression hsa-miR-424 human lentiviral construct (PMIRH424PA-1-GVO-SBI) and scramble control hairpin vector (PMIRH000PA-1-GVO-SBI) were used (purchased from SBI). Transfection of 293T packaging cell line was performed using calcium-phosphate protocol. Transduction was performed as previously described (227). Briefly, lentiviral supernatant was added to $CD34^+$ cells in the precoated with RetroNectin (Takara) 24-well suspension plate according to manufacturer protocol. Lentiviral supernatant was added to cells on 3 consequent days. Transduction efficiency was assessed by flow cytometry and usually was in the range of 50-85%. After 5 days cells were replated into previously described moDC or LCs lineage-specific cytokine mix and finally differentiated. Next, cells were harvested, stained and FACS sorted for GFP^+ gate. Knockdown efficiency was assessed in FACS sorted for GFP^+ cells by TaqMan RT-PCR using hsa-miR-424 (Assay ID 002309) probe and normalized to U6 snRNA endogenous control (Assay ID 001973). On day 5 after replating into lineage-specific cytokine cocktail, cells were activated with either 100ng/mL LPS (for moDCs) or 5μg/mL PGN (for LCs) and analyzed by flow cytometry on day 8.

3.5 Flow cytometry

Flow cytometry analysis was carried out using LSRII and LSR Fortessa flow cytometers (BD Biosciences, USA). The data was analyzed using Flowjo software (Tree Star, Inc.USA). FACS sorting was done on the FACS Aria instrument (BD Biosciences). For FACS staining cells were harvested, resuspended in 40 μm of staining buffer and incubated for 15 minutes with Fc block. Then, cells were incubated with flow cytometry antibodies on ice ice for at least 1 hour. The list of flow cytometry antibodies used in the experiments can be found in Table 3.

Table 3. Flow cytometry antibodies.

Antibodies (Clone)	SOURCE	Cat #
Anti-human CD1a (HI149)	BD Biosciences	Cat# 563938
Anti-human CD83 (HB15e)	BD Biosciences	Cat# 551073
Anti-human CD80 (L307.4)	BD Biosciences	Cat# 56113
Anti-human CD86 (FUN-1)	BD Biosciences	Cat# 561128
Anti-human CD11b (ICRF44)	Biolegend	Cat# 301306

Anti-human CD207 (DCGM4)	Beckman Coulter	Cat# IM3577
Anti-human CD209 (eB-h209)	eBioscience	Cat# 12-2099-42
Anti-human/mouse CD207(929F3.01)	Acris Antibodies	Cat# DDX0362
Anti-mouse MHC II/ I-A/I-E (M5/114.15.2)	Biolegend	Cat# 107607
Anti-mouse CD103 (2E7)	eBioscience	Cat# 50-150-43
Anti-human/mouse CD207 (4c7)	Biolegend	Cat# 144205
Anti-human/mouse CD11b (M1/70)	Biolegend	Cat# 101207
Anti-mouse MHC II/ I-A/I-E (M5/114.15.2)	Biolegend	Cat# 107619
Anti-mouse Ly-6C (HK 1.4)	Biolegend	Cat# 128017
Anti-mouse CD86 (GL-1)	Biolegend	Cat# 105013
Anti-mouse CD11c (418)	Biolegend	Cat# 117325
Anti-mouse CD45 (30-F11)	Biolegend	Cat# 103108
Anti-mouse EpCAM (G8.8)	Biolegend	Cat# 118227
Anti-mouse CD64 (290322)	R&D	Cat# FAB20741c
Anti-mouse MerTK (108928)	R&D	Cat# FAB5912N
Anti-mouse CD117/c-kit (2B8)	Biolegend	Cat# 105815
Anti-mouse CD135 (A2F10)	Biolegend	Cat# 135309
Anti-mouse CD115/CSF-1R (AFS98)	Biolegend	Cat# 135531
Anti-mouse CD172a/SIRPα (P84)	Biolegend	Cat# 144011
Anti-mouse CD16/32 (93)	Biolegend	Cat# 101301

Table published in (1).

3.6 RNA isolation and RT-PCR

RNA was extracted using miRNAeasy Mini Kit (Qiagen) with DNase I treatment according to the manufacturer's protocol. RNA concentration was checked on the Nanodrop ND-1000. RT-PCR was performed using TaqManTM MicroRNA Assay or specific primers (Table 4). Reverse transcription was performed using High Capacity cDNA (Applied Biosystems by ThermoFisher) or TaqMan microRNA reverse transcription kit (Applied Biosystems) on a C1000TM Thermal Cycler (Biorad, Minneapolis, MN, USA). Probes for TaqManTM MicroRNA Assay (hsa-miR-424-3p, hsa-miR-424-5p, has-miR-503-5p, U6 snRNA control) were purchased from ThermoFisher. The relative expression of target genes was normalized to

U6 snRNA or GABDH housekeeping gene expression. Primers, used for RT-PCR are listed in Table 4.

Table 4. List of RT-PCR primers.

Gene	Orientation	Sequence 5'→3'
HOXA2	Fw	CCCCTGTCGCTGATACATTTC
	Rev	TGGTCTGCTCAAAAGGAGGAG
GRK5	Fw	GGGGTGGAGGAAAGCGCAAA
	Rev	GAATGTAGCACTCCAGCCCAG
IL7	Fw	TGGCCTAGTCTCCCCGATCA
	Rev	TCACATTCAGACTCGTTTTTGGCT
Tinagl1	Fw	ACAAACGCAAGCAGTGGTTC
	Rev	GTTTCCCAACGCCCATACTG
Kpna4	Fw	TTCTGCTCCCTAAGAAGAGGGTT
	Rev	CCCTGCGCCAACGACTCC
RUNX3	Fw	AGGCAATGACGAGAACTACTCC
	Rev	CGAAGGTCGTTGAACCTGG
TCF4	Fw	CAAGCACTGCCGACTACAATA
	Rev	CCAGGCTGATTCATCCCACTG
ETV2	Fw	GAAGGAGCCAAATTAGGCTTCT
	Rev	GAGCTTGTACCTTTCCAGCAT
BMPR1a	Fw	GGACACTGCCCAGATGATGC
	Rev	CGTAGCTGGGCTTTCGGTGA
GABDH	Fw	AACTTTGGCATTGTGGAAGG
	Rev	CACATTGGGGGTAGGAACAC

Fw – forward; Rev – reverse

3.7 Mouse experiments

3.7.1 MiR-424(322)/503 knockout mouse model

Animal maintenance and experiments were done according to the Institutional Animal Care and Use Committee (IACUC) guidelines. IACUC protocol number is IACUC-2018-0008. Experiments were performed with 8-12 weeks old male miR-424(322)/503$^{-/-}$ and WT mice with homogeneous FvB/NJ background. miR-424(322)/503 $^{-/-}$ model is a full knockout model of miR-424 and miR-503 previously reported Llobet-Navas et al. (228). Expression of miR-424(322) in BMDCs isolated from miR-424(322)/503 $^{-/-}$ mice was checked by qPCR and was virtually undetectable. Imiquimod-induced inflammation in miR-424(322)/503 $^{-/-}$ mice was more pronounced in male than female mice. To exclude the impact of the estrogen levels in our model, we included only male subjects to our studies.

3.7.2 Topical treatment with Imiquimod (IMQ)

Mice were topicaly applied of 62 mg of 5% Aldara IMQ cream on both ears, which was previously determined as the most optimal dose to induce ear inflammation (229). IMQ treatment was repeated every 24 hours during 7 consequent days. Ear thickness was measured daily before IMQ application using electronic measurement device C1X018 (Kroeplin, GmbH). Values were normalized to the day 0 measurement.

3.7.3 BMDC cultures

BMDCs were generated *ex vivo* using fresh-isolated mouse BM precursors from femur and tibiae. Both sides of the bones were cut out and cells were flushed out with ice-cold PBS, washed and incubated for 1 minute in 1 ml Red Blood Cell Lysing Buffer (Hybri-Max, Sigma-Aldrich). Then, cells were counted and plated in 24-well tissue culture plate (0.5 x 106 /ml per well) in DMEM supplemented with 1% penicillin/streptomycin (Lonza #CC-4136), 10% FCS and 1% MEM non-essential amino acids solution (ThermoFisher) and 20 ng/ml GM-CSF. Medium was exchanged on day 3 of differentiation and on day 7 BMDCs were analyzed by flow cytometry.

3.7.4 Immunofluorescence on mouse epidermal sheets

Epidermal sheets of mouse ears were separated and floated on ammonium thiocyanate for 15 minutes at 37°C. The epidermis was peeled off, fixed in 4% PFA for 30 minutes and incubated for 2 hours at room temperate in blocking solution containing 1% BSA, 0,05% horse serum and 20%Triton in PBS. Staining was performed using 1:1500 diluted Hoechst 33342, 1:500 diluted MHCII/I-A/I-E PE and 1:200 diluted CD207 AF488 fluorescent antibodies. Epidermal sheets were washed twice with PBS and analysed on the Zeiss Axio Obzerver Z1 microscope. Images were processed using ImageJ software.

3.7.5 Epidermal thickness measurement

Mouse ears were collected and fixed in formalin for 24-48 hours. Paraffin embedded 4 μm sections were stained hematoxylin and eosin (Sigma, USA). Images were obtained on Leica DM4000 B microscope (Leica Cambridge Ltd) equipped with Leica DFC320 Video camera (Leica Cambridge Ltd). Epidermal thickness (10X magnification) was measured in 10 random fields on 3 pictures per sample. Analysis was performed using ImageJ software.

3.7.6 AGO2 immunoprecipitation and PAR-CLIP

BMDCs were harvested in approximately 100 x 106 cells per condition. PAR-CLIP was performed as described previously (230-232) with several minor modifications. Briefly, cells were treated with 50 μM 4-thiouridine (T4509; Sigma) overnight and cross-linked on ice at 150 mJ/cm2 365 nm UV. Then cells were lysed with lysis buffer 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.

3.8 RNA sequencing and data analysis

BM cells for total RNA sequencing were extracted from mouse BM. Cells were collected fresh day 0 and on day 2 of GM-CSF differentiation. Samples were 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 library preparation. Stranded RNA libraries were prepared with 100ng total RNA using the KAPA RNA HyperPrep Kit with RiboErase (HMR) (Roche). RNA sequencing was performed on Illumina NovaSeq 6000 system (Illumina). Gene expression was quantified in counts using Salmon. Each data point has 3 biological replicates. In both day 0 and day 2, differential expression between miR-KO and wild type was estimated using DESeq2. Differential expressed genes were selected based on the adjusted P-value with cut off equal 0.05. The RNA-seq data was deposited in the NCBI Gene Expression Omnibus (GEO) with accession number GSE169717.

3.9 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6 software 6 (GraphPad Software Inc.). Two-tailed Student's t-test was used for comparing differences between two groups. Multiple groups were subjected to one-way or two-way analysis of variance (ANOVA) analysis. P values ≤ 0.05 were considered statistically significant.

4. RESULTS

4.1 miR-424/503 is inversely regulated during moDC vs. LC differentiation from monocytic progenitor cells

Previous studies have shown that miRNAs are able to mediate DC differentiation, maturation and function (225). Our group has previously identified the list of differentially expressed miRNAs in LCs and moDCs generated *in vitro* from $CD34^+$ cord blood hematopoietic precursor (212). Microarray analysis revealed forty-six miRNAs to be at least 2-fold differentially expressed in moDCs vs. LCs (212). miR-424 was of particular interest for further studies because it was 6 times higher expressed in moDCs than in LCs (212). Another miRNA-503, which forms polycistronic cluster with miR-424, was 3 times upregulated in moDCs showing similar expression pattern to miR-424 (212). Together miR-424 and miR-503 are members of miR-16 family and share many features, including seed sequence and putative target profile (204). It has been previously shown that miR-503 has a much lower expression level (~100 times) than miR-424 in breast cancer cell lines (228). Thus, we first evaluated the expression of miR-503 in DC subsets (Figure 4A). Indeed, miR-503 had the same pattern of expression as miR-424 and its expression was higher in moDC than in LCs.

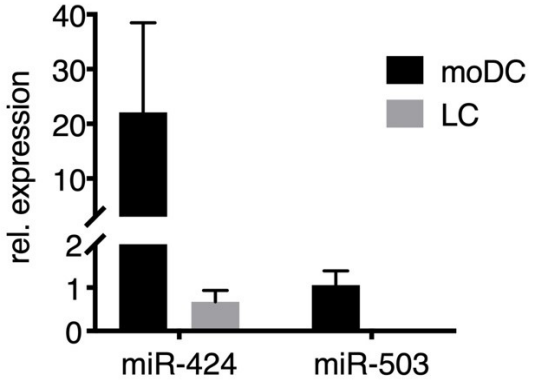

Figure 4. Relative expression of miR-424 and miR-503 in moDC vs. LCs.

moDCs and LCs were generated from $CD34^+$ HSC via the step of intermediate monocytic progenitors as previously described by Caux et al. (148) . Relative expression of miR-424 and miR-503 was measured by Taqman RT-PCR on day 4 of differentiation in lineage-specific cytokine cocktail (n=3, ± SEM). Figure published in (1).

However, in comparison to miR-424 its expression in moDCs was very low, in LCs it was almost undetectable. Given the fact that miR-424 plays a non-redundant functional role in human monocyte/macrophage differentiation (233,234), we assumed that miR-424 expression is representative for miR-424/503 gene cluster expression in all subsequent experiments.

Upon processing, miR-424/503 cluster generates Pre-miR-424 primary transcript, which is subsequently processed into miR-424 -5p and -3p strands (Figure 5).

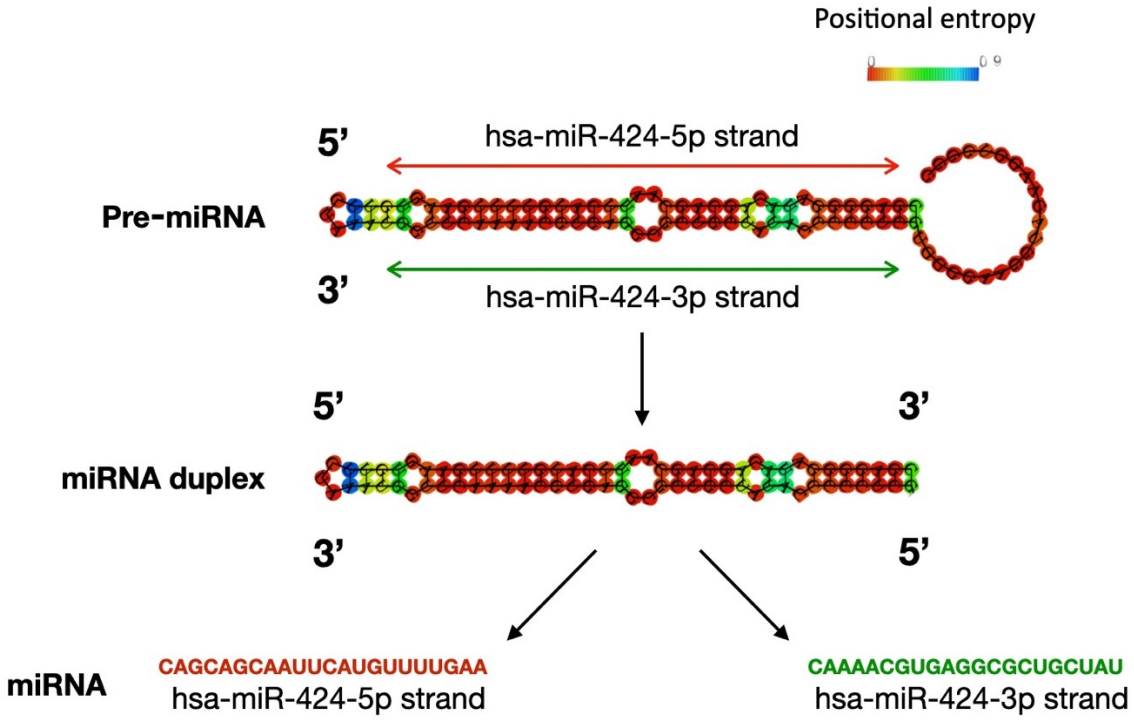

Figure 5. Processing of miR-424-5p and -3p strands from primary miRNA transcript.
Pre-miR-424 similar to other miRNAs is fold into precursor hairpin structure, which is cleaved by RNases to produce miRNA-424 duplex. RISC complex unwinds the miRNA duplex with generation of 5'-strand and 3'-strand of mature miR-424. Most often 5'-strand is dominant and conserved across the species and 3'-strand undergoes degradation (235,236). However, sometimes both strands avoid strand selection and contribute as functional miRNA. This process is inconsistent and may vary depending on many factors, including location, cell type and differentiation stage (237). miR-424 sequence is taken from miRbase (238). Secondary RNA structure was predicted using RNAfold web server software (239).

To address the role of miR-424/503 cluster in DC subsets we assessed expression of miR-424-5p and -3p on different stages of monocyte to LC vs. moDC differentiation *in vitro*. As schematically represented on Figure 6A, moDCs and LCs were generated using either IL-4 or TGF- β1-cytokine mix from intermediate monocytic precursors (MoPs). Figure 6B shows that miR-424 has a low expression in both $CD34^+$ hematopoietic progenitor cells and $CD14^+$ monocytes. MoPs exhibited intermediate levels of miR-424, which was inversely expressed by fully differentiated LCs and moDCs independently of their origin (Figure 6B). The findings were in line with the previously published microarray data (212). The broad comparison of miR-424 expression in different myeloid cell types showed that moDCs had the highest levels of miR-424 expression in comparison to GM-CSF-induced monocyte-derived macrophages (moMac), fresh isolated neutrophils and $CD34^+$HSC-derived granulocytes (G precursors). miR-424-5p and miR-424-3p exhibited similar expression pattern, although miR-424-5p was higher expressed in myeloid cells than miR-424-3p strand. Therefore, we focused on miR-424-5p strand expression in all subsequent experiments.

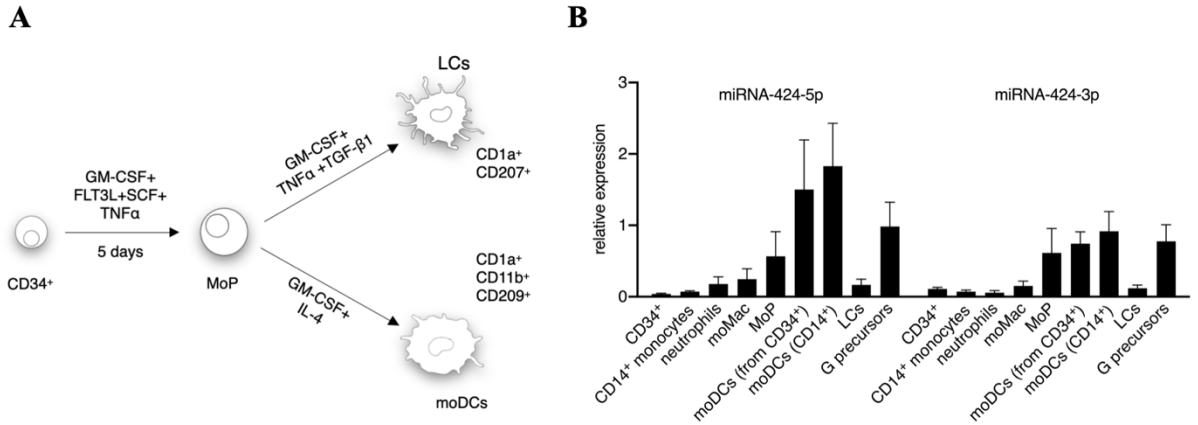

Figure 6. miR-424 profiling in myeloid cells.

(A) Schematic representation of moDCs and LC differentiation model from $CD34^+$ cells *in vitro*. (B) Relative expression of miR-424-5p and miR-424-3p in different human myeloid cell types measured by Taqman RT-PCR (n=3-5, ± SEM). Figure published in (1).

4.2 Involvement of TGF-β1 and IL-4 in DC subset differentiation correlates with miR-424 expression

TGF-β1 is a key cytokine instructing LC differentiation in human and mice (161-163). Omission of TGF-β1 in the LC generation cultures leads to the abrogation of LC differentiation, in favor of $CD11b^+$ cells that exhibit similarities with interstitial/dermal DCs (227). On the other hand, addition of IL-4 cytokine to TGF-β1-dependent LC generation cultures represses LC characteristics (148,240).

To further study if exogeneous TGF-β1 can modulate miR-424 expression, we omitted TGF-β1 in 7 day LC generation cultures (i.e. containing GM-CSF and TNFα). Comparison of miR-424 expression levels in the differentiated cells has shown that miR-424 was elevated in the absence of TGF-β1 (Figure 7A). Similarly, addition of IL-4 to $CD14^+$ monocyte-derived moDC cultures (+ GM-CSF) promoted miR-424 expression in comparison to GM-CSF alone condition (Figure 7B). Interestingly, miR-424 expression levels gradually increased upon IL-4 stimulation within 48 hours of MoPs to moDC differentiation (Figure 7C), indicating that IL-4 promoted miR-424 induction during moDC differentiation.

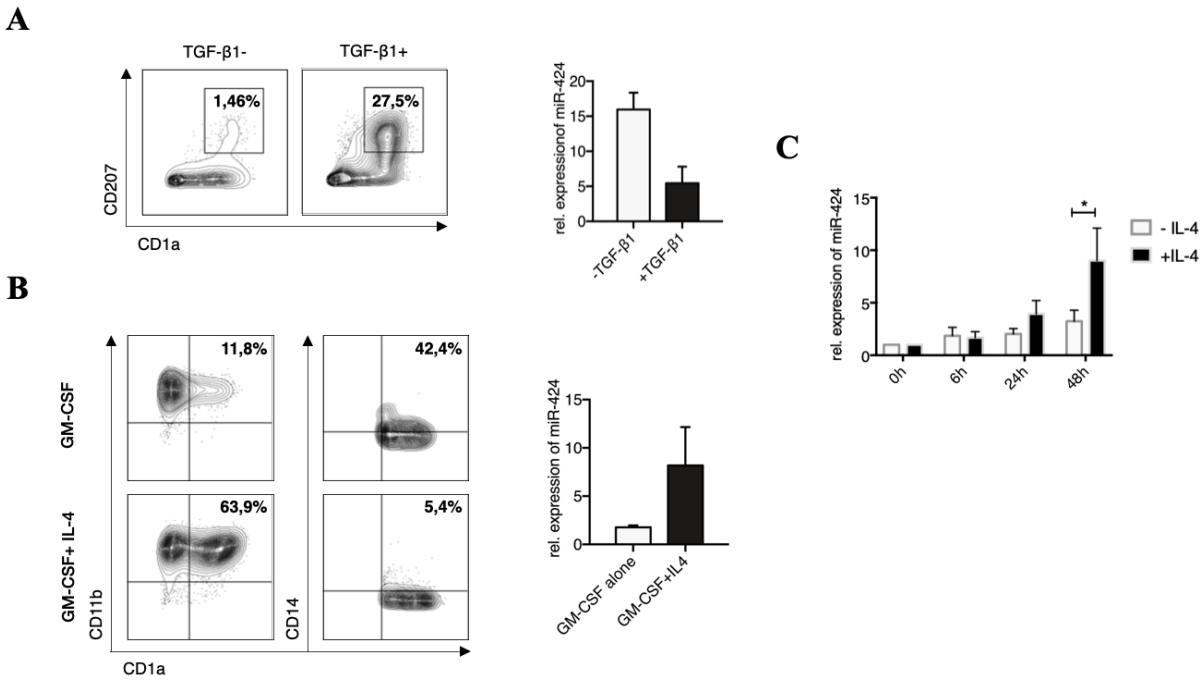

Figure 7. Involvement of TGF-β1 and IL-4 in DC subset differentiation correlates with miR-424 expression.

(A) Representative flow cytometry analysis of $CD34^+$ cells differentiated for 7 days into LCs in cytokine cocktail with and without TGF-β1. Graph shows relative expression of miR-424 in +/- TGF-β1 LCs on day 7 after differentiation from $CD34^+$ progenitors (n=3, ± SEM). (B) Representative flow cytometry

analysis of monocytes, cultured 7 days either with GM-SCF or GM-CSF+IL-4. Graph shows relative miR-424 expression in +/- IL-4 moDCs generated from $CD14^+$ monocytes (n=3, ± SEM). (C) Graph shows relative miR-424 expression in +/- IL-4 moDCs generated from MoPs (n=6, ± SEM). Data were analyzed using paired 2-tailed Student's t-test (*P<0.05). Figure published in (1).

4.3 miR-424/503 is required for human moDC but not for LC differentiation

Given the observation that miR-424/503 ectopic expression is higher in moDCs than in LCs, we next studied whether miR-424/503 is required for moDC vs. LC differentiation in our *in vitro* model. Therefore, we knocked-down miR-424 in $CD34^+$ progenitor cells using lentiviral vector and induced them to differentiate to moDC and LCs under lineage-specific conditions. The expression of miR-424 and miR-503 in GFP^+ sorted cells was on average 50-70% decreased in comparison to control transduced cells (Figure 8A). Flow cytometry analysis for lineage-markers for moDCs ($CD11b^+CD209^+CD1a^+$) and LCs ($CD1a^+CD207^+$) on the 4^{th} day of differentiation from MoPs has shown that the percentages of moDCs were decreased relative to control cultures (Figure 8B, upper panel). However, the frequency of LCs remained unimpaired and even had a tendency to increase in miR-424 knockdown cells (Figure 8B, lower panel).

We next examined whether the kinetics of differentiation is simply delayed in the moDCs condition and analyzed the moDC/LC phenotype at the later time point. Consistent with the diminished frequencies of moDCs on day 4, the phenotype of moDCs and LCs remained unchanged on the day 7 of differentiation (Figure 8C). Thus, miR-424/503 promoted $CD11b^+CD209^+CD1a^+$ moDCs differentiation but failed to impair $CD1a^+CD207^+$LC differentiation.

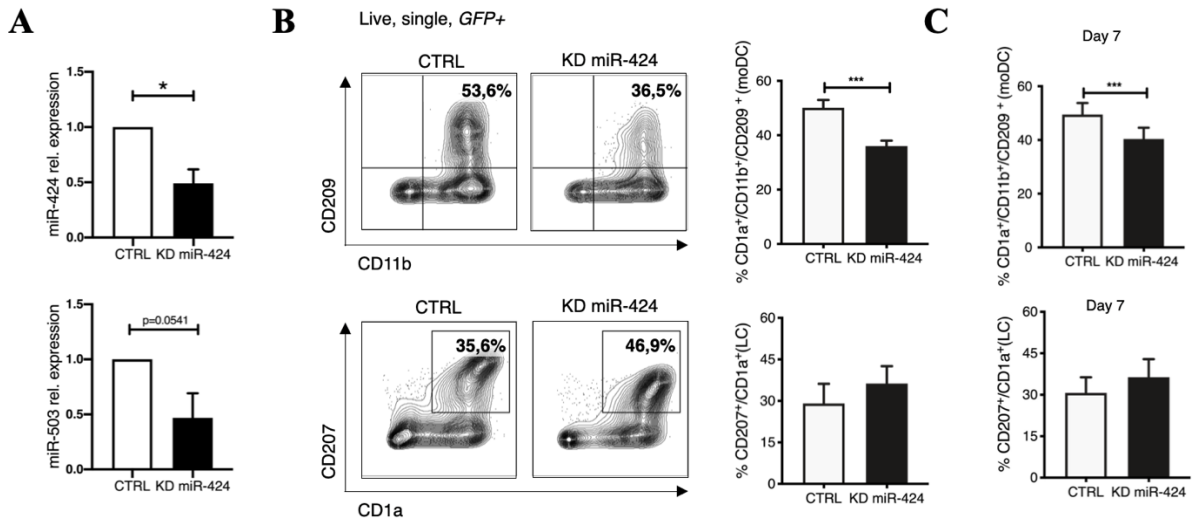

Figure 8. miR-424/503 is required for human moDC but not for LC differentiation.

(A) miR-424 and miR-503 expression in sorted GFP$^+$ cells on day 4 of differentiation under lineage-specific conditions (n=3, ± SEM, 2-tailed Student's t test, *P<0.05) (B) Lineage marker profile of gated GFP$^+$CD1a$^+$ miR-424 knockdown cells on day 4 of differentiation into moDCs or LCs under lineage specific conditions. Data represent the mean ± SEM of 3-6 donors, 2-tailed Student's t test, *** P<0.001. (C) Graph shows the percentages of moDCs and LCs after miR-424 knockdown on day 7 of differentiation. Data represent the mean ± SEM of 3 donors, 2-tailed Student's t test, *** P<0.001. Figure published in (1).

4.4 Ectopic expression of miR-424/503 does not influence DC subset activation

Since miR-424/503 expression was shown to be required for moDC vs. LCs differentiation *in vitro*, we next analyzed whether ectopic miR-424/503 expression might influence DC subset activation. moDC and LC were cultured for 72 hours with TLR-agonists LPS and PGN. We then assessed the activation markers expression CD80, CD83 and CD86 (Figure 9A and B). GFP$^+$ cells from miR-424 knockdown and control cultures showed similar percentages of CD80, CD83 and CD86 cells, demonstrating that miR-424/503 expression does not interfere with DC subset activation.

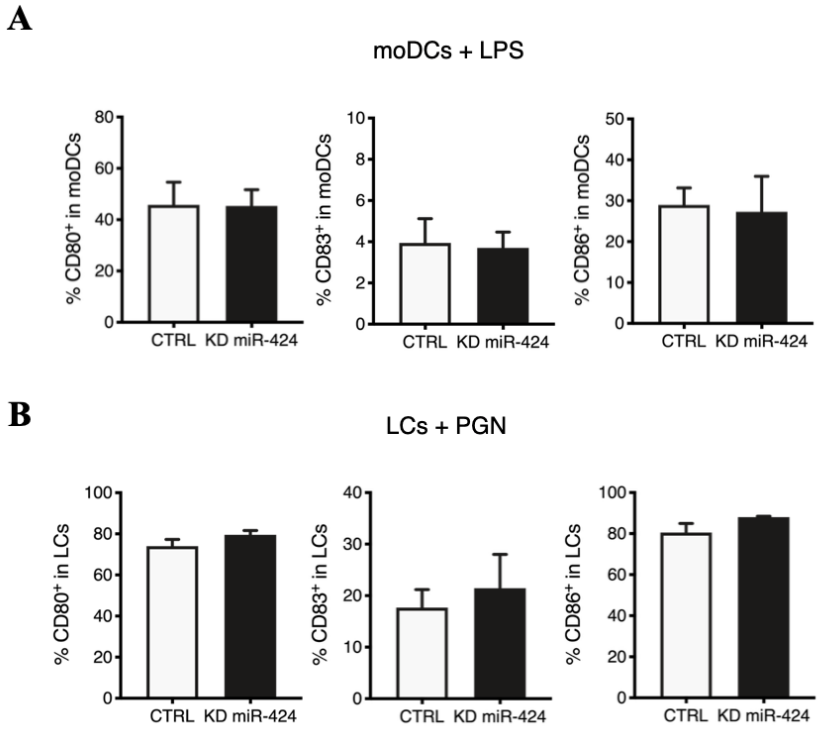

Figure 9. Ectopic expression of miR-424 does not influence DC subset activation.
(A) Expression of CD80, CD83 and CD86 activation markers in LPS-stimulated moDCs in miR-424 KD vs. transduced control cells (n=3, ± SEM, 2-tailed Student's t test). (B) Expression of CD80, CD83 and CD86 activation markers in PGN-stimulated LCs in miR-424 KD vs. transduced control cells (n=3, ± SEM, 2-tailed Student's t test). Figure published in (1).

4.5 miR-424(322)/503 knockout mice displayed diminished psoriatic-like skin inflammation

To validate our findings on the role of miR-424/503 in moDC differentiation *in vivo*, we turned our studies to miR-424(322)/503 knockout model (from now will be referred to as miR-KO model for simplification). MiR-322 is known as the murine orthologue of human miR-424. This mouse model has a full body miR-424(322)/503 knockout, including moDCs and LCs, and was previously characterized by Llobet et al. (228,232). We did not observe any phenotype differences in the miR-KO and WT (wild-type) mice in the steady-state. To induce psoriatic-like skin inflammation, mouse ears were topically treated with TLR7 agonist Imiquimod (IMQ) during 6 consecutive days (Figure 10A). During this time period, the ear swelling and body weight was assessed. IMQ treated miR-KO mice exhibited significantly decreased ear thickness in comparison to WT mice (Figure 10B), which was additionally confirmed by

microscopical measurements of the epidermis on day 7 (Figure 10C). However, there was no difference in body weight loss between the groups during treatment (Figure 10D).

Taken together, these results indicate that miR-KO mice displayed decreased ear swelling upon IMQ treatment, which is associated with diminished psoriatic-like skin inflammation.

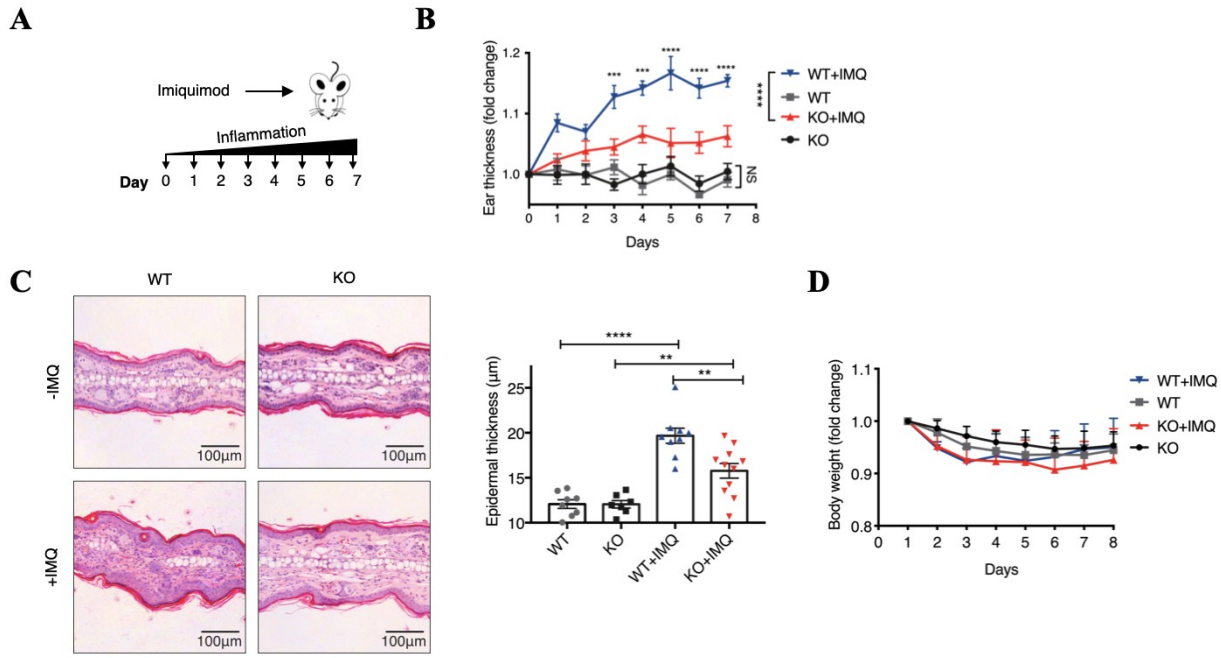

Figure 10. miR-424(322)/503 knockout mice displayed diminished psoriatic-like skin inflammation.

(A) Representative scheme illustrating the time span of the IMQ treatment. (B) Ear swelling of both mouse ears in WT and miR-KO mice measured on the indicated days (n=6-8 mice per group, ± SEM), two-way Anova, *** P<0.001, **** P<0.0001. (C) Epidermal thickness of each mouse measured on H&E staining on experimental day 7 (n=4 mice per group, ± SEM, one-way ANOVA, **P<0.01, **** P<0.0001). Each dot represents a mean of 4-5 measurements. The left panel shows representative H&E staining picture of the mouse ear. (D) Body weight in WT and miR-KO mice on the indicated days (n= 6-8 mice per group, ± SEM). Figure published in (1).

4.6 miR-424(322)/503 is required for moDC differentiation *in vivo*

Previous study has demonstrated that moDCs are principal contributors to psoriatic-like skin inflammation *in vivo* (92). Next, we extended our study to investigate whether the development of moDCs was disturbed in miR-KO mice in comparison to WT mice. Therefore, we analyzed the frequencies of Ly6C^{int}MHCII^{high} (T3) and Ly6C^{low}MHCII^{high}(T4) moDCs in the skin of IMQ-treated mice (Figure 11A). Similar to our *in vitro* findings, moDCs subsets were diminished in the dermis of miR-KO relative to WT mice upon IMQ treatment, whereas only low numbers of moDCs even in both conditions were identified in the steady-state (Figure 11B). In addition to moDCs, the reduced frequencies of T1 macrophages were exhibited in the inflamed dermal tissue of miR-KO mice (Figure 11 C). However other immune cell subsets, including CD103^{+}cDCs, CD11b^{+}cDCs and Ly6C^{high} monocytes(T2), were not significantly different in miR-KO mice vs. WT controls (Figure 11C).

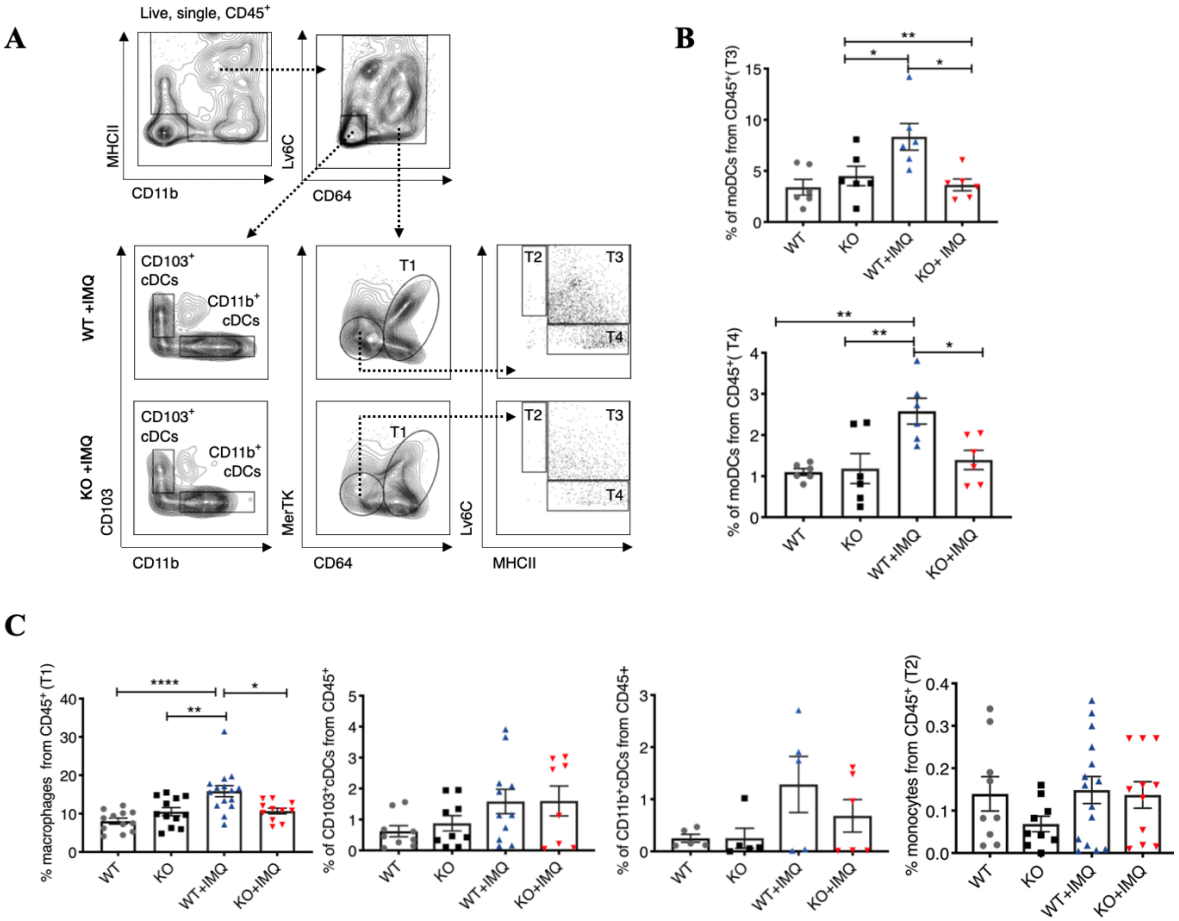

Figure 11. miR-424(322)/503 is required for moDC differentiation *in vivo.*
(A) Phenotypical identification of macrophages (T1), monocytes (T2), Ly6C^{++} moDCs (T3), Ly6C^{+} moDCs (T4) in the dermis of mouse ears on day 7 of experiment in miR-KO and WT mice. (B) Graph represents percentage of phenotypically defined moDCs subsets T3 and T4 in mouse dermis quantified by flow cytometry on day 7 of experiment (n= 6, ± SEM, one-way ANOVA, *P<0.05, **P<0.01). (C) Graph represents percentage of phenotypically defined macrophages (T1), CD103^{+} cDCs, CD11b^{+} cDCs and monocytes (T2) in mouse dermis quantified by flow cytometry on day 7 of experiment (n=6-10, ± SEM, one-way ANOVA, *P<0.05, **P<0.01**** P<0.0001). Figure published in (1).

Taken together, these data suggest that miR-KO mice displayed decreased frequencies of moDC subsets T3 and T4, suggesting that miR-424(322)/503 is required for moDC differentiation *in vivo.* Moreover, the abrogation of moDC differentiation was associated with diminished psoriatic-like skin inflammation in miR-KO mice.

4.7 miR-424(322)/503-deficient mice exhibit on average slightly elevated numbers of non-activated LCs

Based on the previous finding that miR-424 knockdown in CD34^{+} progenitor cells impaired moDCs but not LCs differentiation *in vitro*, we next studied whether miR-424(322)/503 deficiency influenced LC differentiation *in vivo* in steady-state and inflammation. Comparison of the histologically stained epidermal sheets of untreated miR-KO and WT mice revealed an undisturbed network of LCs, with on average slightly increased frequencies of CD207^{+} LCs in miR-KO relative to WT mice (Figure 12A). Further flow cytometry analysis of mouse epidermis has shown that LCs from miR-KO mice exhibited less activated phenotype. Particularly, the percentages CD86^{+} cells were reduced in miR-KO compared to WT mice upon IMQ treatment (Figure 12B). Activated LCs emigrate from the epidermis to the skin-draining lymph nodes. Frequencies of LCs among CD45^{+} cells in draining lymph nodes also has shown a tendency to decrease in IMQ treated miR-KO mice compared to WT mice, however the difference was not significant (Figure 12C).

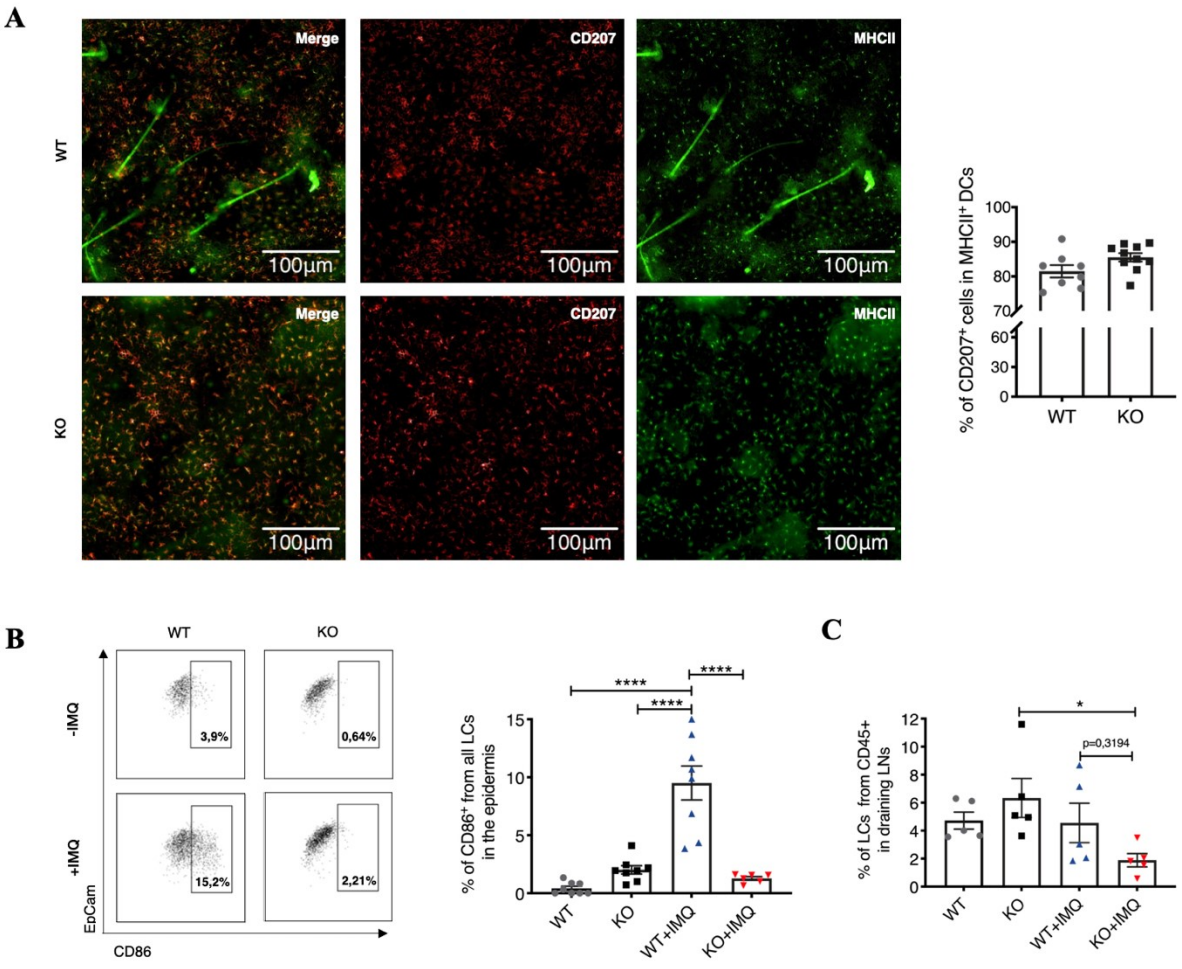

Figure 12. miR-424(322)/503-deficient mice exhibit on average slightly elevated numbers of non-activated LCs.

(A) Mouse epidermal sheets staining for $CD207^+$ and $MHCII^+$ DCs in steady-state. Frequency of CD207 LCs in MHCII DCs in immunofluorescent staining of mouse epidermal sheets on day 7 of IMQ experiment (n=4-5 mice per group, ± SEM). (B) Activation of LCs in the epidermis of the ear on day 7 of IMQ experiment. Graph shows frequency of $CD86^+$ LCs in the epidermis of mouse ear (n=8 mice per group, ± SEM, one-way ANOVA **** P<0.0001). (C) Graph shows frequency of LCs in draining lymph nodes on day 7 of IMQ experiment (n= 5 mice per group, one-way ANOVA, *P<0.05). Figure published in (1).

4.8 moDC precursors in the bone marrow are present at equivalent frequencies in WT and miR-424(322)/503-deficient mice

To further study the role of miR-424(322)/503 in moDCs differentiation, we tested whether the diminished frequencies of moDCs observed in IMQ-treated miR-KO mice are associated with the defects in their BM-DC precursors. It was previously described that murine Ly6C$^+$ monocytes in the BM comprise a heterogeneous population and give rise to iNOS$^+$ macrophages (R1), inflammatory moDCs upon GM-CSF exposure (R2) or FLT3 dependent DCs (R3) (120). Therefore, we phenotypically analyzed the precursors of moDCs (lin$^-$ CD115$^+$Ly6C$^+$FLT3$^+$CD11c$^-$; i.e. R2 cells) in the freshly isolated BM cells of WT vs. miR-KO mice (Figure 13, upper panel). All three precursor populations R1, R2 and R3 displayed equivalent frequencies in the WT vs. miR-KO mice (Figure 13, lower panel), suggesting that monocytic precursors were not impaired in miR-KO vs. WT mice.

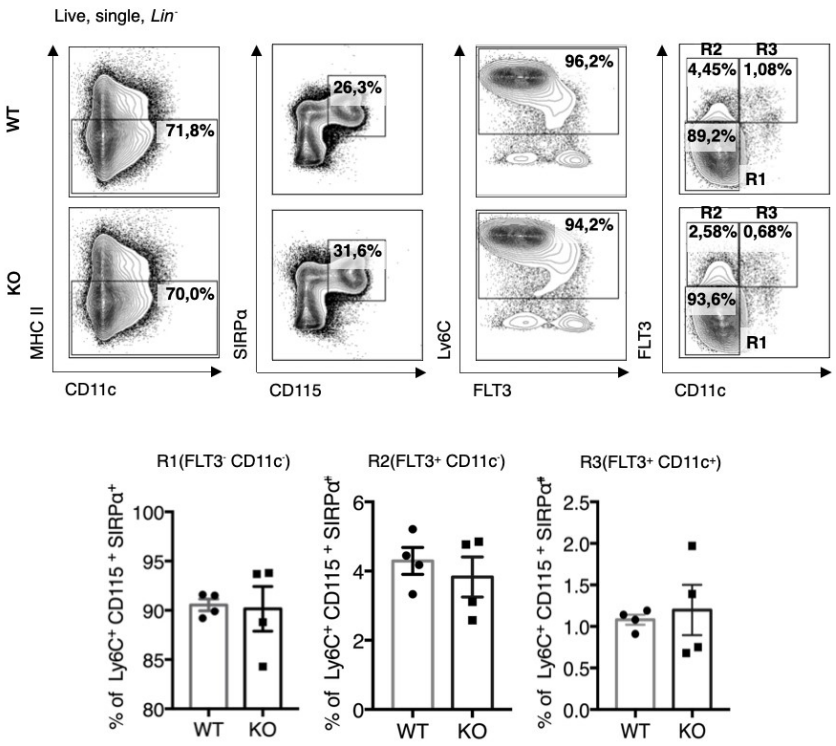

Figure 13. moDC precursors in the bone marrow are present at equivalent frequencies in WT and miR-424(322)/503-deficient mice.

Upper panel shows flow cytometry analysis of murine BM for DC precursors. Bar graph at the lower panel shows the frequencies of R1 (precursors for iNOS macrophages), R2 (moDCs precursors), R3 (pre-DC precursors) from the Ly6C$^+$CD115$^+$SIRPα$^+$ population. Figure published in (1).

4.9 miR-424(322)/503 promotes *ex vivo* BMDC differentiation

BM precursor cells cultured with GM-CSF have been extensively used as *ex vivo* model for generation $CD11c^{+}MHCII^{+}$ BM-derived DCs (BMDCs) (146). Indeed, BMDCs cultures comprise two (sub)lineages that correspond to monocyte-derived macrophages and CDP-derived DCs (107). In order to see whether miR-424(322) is required for BMDC differentiation, we isolated BM cells from miR-KO vs. WT mice and cultured them with GM-CSF. Flow cytometry analysis on day 7 of differentiation revealed the diminished generation of $MHCII^{+}Ly6C^{+}CD11c^{+}CD11b^{+}$ BMDCs in miR-KO mice in comparison to parallel WT BMDC cultures (Figure 14A). To better understand the kinetics of miR-424(322) regulation during BMDC differentiation we next monitored the miR-424(322) levels at four different time points. Interestingly, the miR-424(322) levels revealed a transient upregulation at the early time point (48 hours) after GM-CSF stimulation (Figure 14B). These data suggest that miR-424(322) promotes BMDC generation at the early stages of cell differentiation.

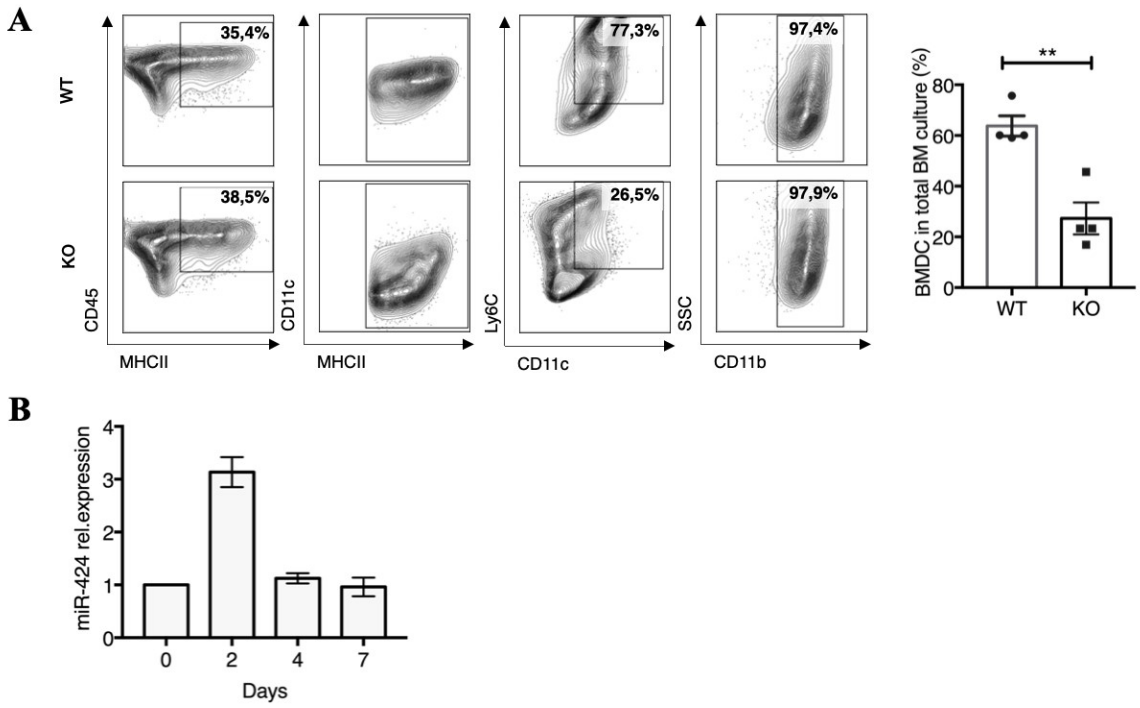

Figure 14. miR-424(322)/503 promotes *ex vivo* BMDC differentiation.

(A) Flow cytometry analysis of CD11c, Ly6C and CD11b expression on CD45+MHCII+ cells. Graph shows percentage of BMDCs in GM-CSF BM cultures (n= 4 mice per group, ± SEM, 2-tailed Student's t test, ** P<0.01). (B) MiR-424(322) endogenous levels in BMDCs cultures over time. Figure published in (1).

4.10 Altered gene expression profile in GM-CSF stimulated miR424(322)/503$^{-/-}$ vs. WT BM cells

Given the observed induction of miR-424(322) after 48 hours in GM-CSF-stimulated BMDC cultures, we then focused our studies on the changes in the transcriptional profile that occurred on the early stages of BMDC differentiation. In order to fully characterize the genes up-or downregulated in response to miR-424(322) deficiency in macrophage/DC precursors, we performed RNA sequencing of the samples collected at day 0 and day 2 of differentiation. As expected from the analysis of BM-derived DC precursors, only minor differences in gene expression were observed on day 0 in unstimulated BM cells from WT vs. miR-KO mice (Figure 15A). Next, we compared the gene expression profiles at day 0 and after 48 hours of GM-CSF stimulation in WT mice. We identified 418 genes to be significantly upregulated or downregulated in the WT condition in response to GM-CSF treatment (Figure 15B).

Then we determined those genes among GM-CSF response genes that are differentially expressed by WT vs. miR-KO cells at 48 hours (Figure 15C). A large number of genes was substantially upregulated in response to GM-CSF stimulation in both conditions, with portions of these being stronger or weaker expressed in miR-424(322) deficient cultures relative to WT control. Notably, 19 genes were downregulated, with some being stronger downregulated in WT (e.g. *Tinagl-1*, *Sh3fc2*); others were stronger downregulated miR-KO cells (e.g. *Dntt*). What could be observed however, is that miR-424(322)/503 deficiency in 48 hours GM-CSF stimulated BM cells resulted in altered expression of several GM-CSF response genes (Figure 15C). Overall, 1395 genes were differentially expressed on day 2 between GM-CSF stimulated WT vs. miR-KO BM cells (Figure 15D). Furthermore, among them we identified 47 differentially expressed transcription factors (TFs) (Figure 15E), including previously described in the context of myelopoiesis RUNX3, ID3 and GATA2 TFs.

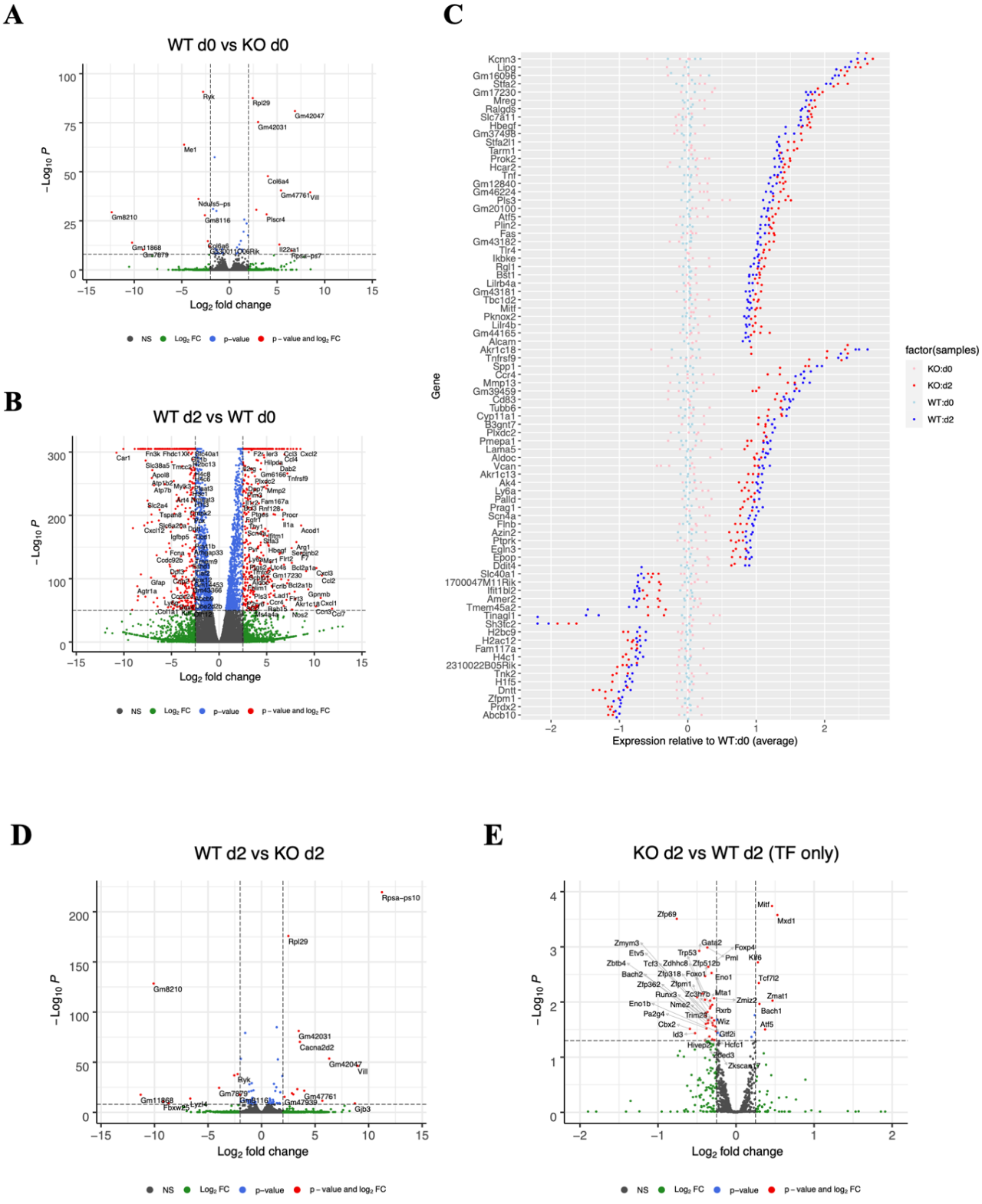
A
WT d0 vs KO d0
B
WT d2 vs WT d0
C
Gene
Expression relative to WT:d0 (average)
factor(samples)
KO:d0
KO:d2
WT:d0
WT:d2
D
WT d2 vs KO d2
E
KO d2 vs WT d2 (TF only)
−Log10 P
Log2 fold change
NS
Log2 FC
p−value
p − value and log2 FC

Figure 15. Altered gene expression profile in GM-CSF stimulated miR424(322)/503$^{-/-}$ vs. WT BM cells.

(A)Volcano plot representation of differential expression analysis of the genes in unstimulated BM cells from WT vs. miR-KO mice (day 0). (B) Volcano plot representation of differential expression analysis of genes in BM cells from WT mice on day 0 vs. day 2 of GM-CSF stimulation. (C) The graph shows differential gene expression between WT vs. miR-424(322)/503 KO cells at day 0 and day 2. (D) Volcano plot representation of differential expression analysis of genes in BM cells from WT vs. miR-KO mice on day 2. (E) Volcano plot representation of differential expression of transcription factors in BM cells from WT vs. miR-KO mice on day 2. Figure published in (1).

4.11 Identification of miR-424(322)/503 putative targets in BMDCs

Since miR-424(322)/503 deficiency in 48 hours GM-CSF stimulated BM cells results in altered expression of several GM-CSF response genes, we next studied whether some of those genes can be direct targets of miR-424(322)/503. Thus, we crossed the miR-424(322)/503 predicted targets from TargetScan with the list of day 2 miR-424(322) KO upregulated genes in our BM RNA seq data (Figure 16A). Computational analysis revealed 19 genes that could be potentially targeted by miR-424(322)/503 in BMDC (padj<0.05) (Table 4). We assessed expression of several genes that might be interesting for further studies by qPCR (Figure 16B). Finally, we analyzed which pathways might be involved in the miR-424(322)/503 downstream regulation through targeting those 19 genes. The pathway enrichment analysis performed on miR-424(322)/503 putative targets has shown that TGF- β1 signaling pathway was significantly enriched (Figure 16C).

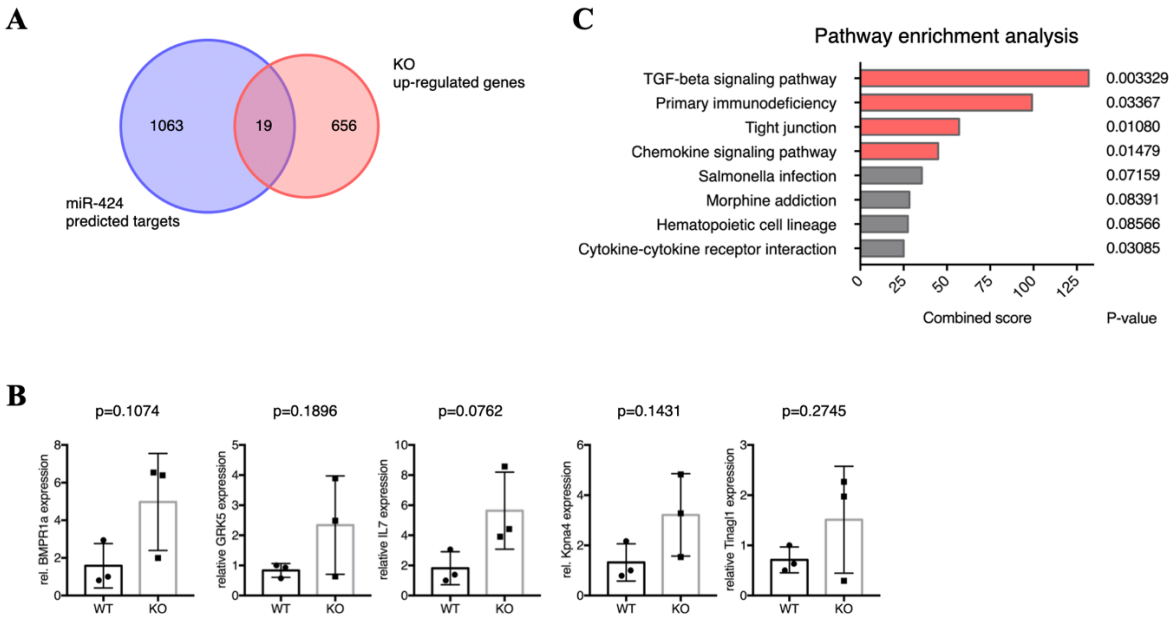

Figure 16. Identification of miR424(322)/503 putative targets in BMDCs.

(A) The Venn diagram shows the number of genes unregulated in miR-KO mice on day 2 overlapped with bioinformatically predicted miR-424(322)/503 targets from TargetScan. (B) RT-PCR validation of selected genes from mRNA screening. Expression is normalized to GABDH, n=3. H. (C) KEGG pathway enrichment analysis showed pathway enrichment in miR-424(322)/503 related genes. An expressed gene pool (19 genes) was used as the background for pathway enrichment analysis. Figure 16A published in (1).

Table 4. The list of predicted targets of miR424(322)/503 in BMDCs.

	Gene	d0:log2FC	d0:pvalue	d0:padj	d2:log2FC	d2:pvalue	d2:padj
1	*Sh3bgrl2*	-0,56	1,58E-03	1,04E-01	0,77	2,06E-12	1,22E-09
2	*Kpna4*	-0,22	9,77E-02	8,06E-01	0,21	6,76E-04	2,59E-02
3	*Rybp*	-0,20	1,46E-01	9,02E-01	0,26	1,76E-04	1,04E-02
4	*Grk5*	-0,11	3,00E-01	9,94E-01	0,34	1,31E-04	8,41E-03
5	*Nbeal1*	-0,10	3,86E-01	9,94E-01	0,27	9,50E-05	6,78E-03
6	*Rap2c*	-0,09	5,99E-01	9,94E-01	0,32	1,94E-04	1,11E-02
7	*Rock1*	-0,06	5,52E-01	9,94E-01	0,23	8,70E-04	3,12E-02

8	*1810013L24Rik*	-0,03	7,93E-01	9,94E-01	0,25	3,44E-04	1,66E-02
9	*Tmcc1*	-0,02	8,68E-01	9,94E-01	0,40	5,33E-07	1,17E-04
10	*Cask*	0,00	9,99E-01	1,00E+00	0,32	4,17E-04	1,92E-02
11	*N4bp1*	0,00	9,62E-01	9,98E-01	0,25	4,74E-04	2,09E-02
12	*Msl1*	0,11	3,43E-01	9,94E-01	0,23	1,57E-03	4,53E-02
13	*G0s2*	0,11	5,77E-01	9,94E-01	0,48	2,22E-04	1,22E-02
14	*Dach1*	0,13	4,11E-01	9,94E-01	0,33	2,49E-06	4,24E-04
15	*Phip*	0,14	1,28E-01	8,74E-01	0,27	1,73E-04	1,03E-02
16	*Tmem154*	0,21	1,03E-01	8,17E-01	0,27	5,30E-04	2,23E-02
17	*Il7r*	0,21	7,00E-02	7,25E-01	0,33	6,88E-04	2,63E-02
18	*Bmpr1a*	0,23	1,58E-01	9,22E-01	0,56	6,04E-05	4,95E-03
19	*Ago3*	0,27	6,06E-03	2,23E-01	0,31	3,44E-05	3,36E-03

Table published in (1).

4.12 Loss of miR-424/503 facilitates TGF-β1-dependent LC differentiation at the expense of moDC differentiation

As our pathway enrichment analysis suggested that TGF- β1 pathway is enriched in potential target gene dataset, we next investigated whether the TGF- β1 signature was increased in our miR-424(322)/503 BMDC cultures. Therefore, we performed gene set enrichment analysis (GSEA) to see whether previously described TGF-β1 response genes were enriched in the upregulated genes in miR-KO BMDC cultures. As a reference dataset we have used the microarray data from the previous study that identified the genes induced (at 6 and 24 h) by TGF-β1 during LC commitment from purified human $CD34^+$ progenitor cells (241). Indeed, GSEA has shown that TGF-β1 signature was increased in the pool of miR-KO upregulated genes, suggesting the loss of miR-424(322)/503 might induce TGF-β1 signaling in BM cultures (Figure 17A). Consequently, we identified 33 genes among those TGF-β1-LC response genes to be significantly induced in miR-KO vs. WT cells (Figure 17B). Among those genes (Table 5) there were transcriptional regulators that have been previously shown to be essential for LC commitment, such as RUNX3 (242) and ID2 (243); TGF-β1 (244) as well as surface molecule CDH1/E-cadherin, functionally implicated in LC differentiation (245,246). In line with this,

loss of miR-424(322)/503 in GM-CSF stimulated BM cells led to the induction of genes previously shown to be essential for LC commitment and differentiation.

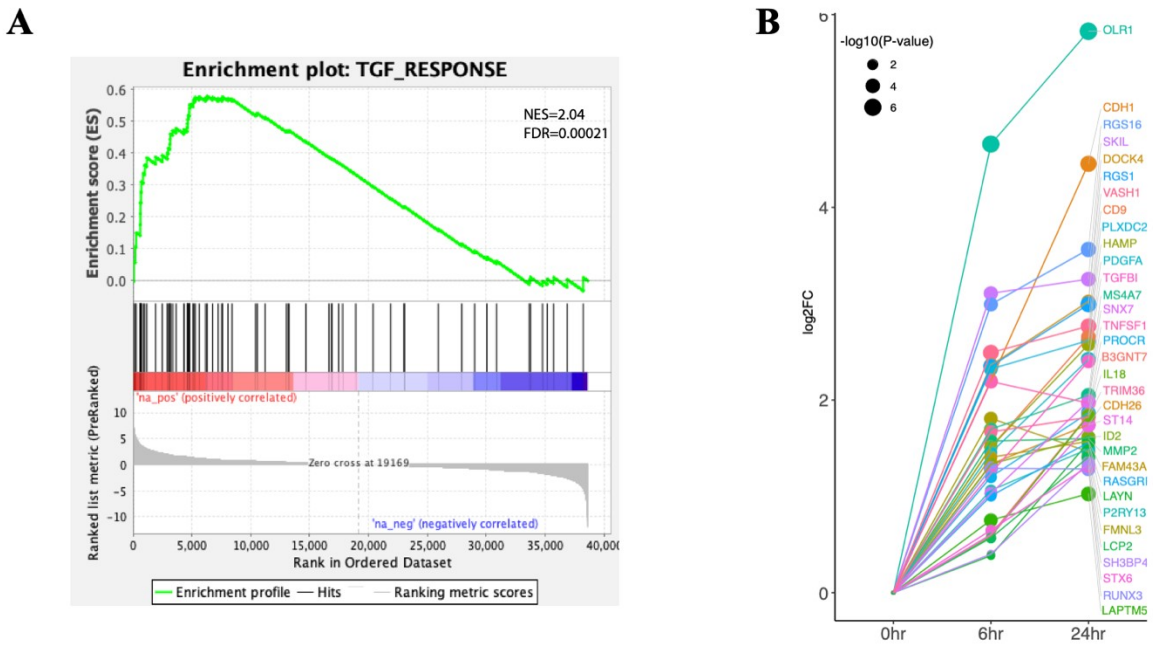

Figure 17. Gene set enrichment analysis (GSEA) of TGF-β response genes.

(A) Gene set enrichment analysis (GSEA) performed on comparisons of TGF-β1 and miR-424(322)/503 KO signature genes. (B) Gene expression data of TGF-β1 signature genes on different time points of LC differentiation overlapped with genes upregulated in miR-424(322)/503 KO mice. Figure published in (1).

Table 5. The list of TGF-β1 response genes upregulated in miR-424(322)/503 knockout BMDCs.

	Gene	6h.logFC	6h.P.Value	24h.logFC	24h.P.Value
1	OLR1	4,66	8,16E-07	5,83	5,16E-07
2	SKIL	3,11	2,27E-04	3,26	1,19E-04
3	RGS16	2,99	3,06E-04	3,56	4,21E-05
4	VASH1	2,49	1,57E-05	2,77	2,22E-05
5	DOCK4	2,36	1,51E-04	3,02	5,14E-04
6	RGS1	2,35	5,54E-05	2,99	1,33E-05
7	PLXDC2	2,32	4,23E-04	2,62	5,11E-05
8	CDH1	2,20	7,81E-04	4,45	8,31E-06
9	TNFSF14	2,19	3,59E-04	1,97	1,50E-04
10	FMNL3	1,81	5,38E-04	1,46	5,42E-04
11	MS4A7	1,70	5,39E-04	2,05	1,04E-04
12	TRIM36	1,67	9,38E-04	1,82	1,77E-04
13	MMP2	1,57	4,72E-04	1,60	6,92E-04
14	CD9	1,51	8,91E-04	2,65	8,37E-05
15	HAMP	1,51	1,25E-03	2,58	6,23E-04
16	PDGFA	1,45	2,01E-03	2,42	4,83E-05
17	FAM43A	1,41	1,63E-03	1,57	9,84E-04
18	CDH26	1,34	8,31E-04	1,74	2,60E-04
19	ID2	1,33	6,90E-04	1,62	3,47E-04
20	RUNX3	1,29	1,99E-04	1,28	3,98E-04
21	TGFBI	1,24	1,70E-02	2,40	7,24E-04
22	PROCR	1,20	5,63E-03	1,87	1,25E-04
23	P2RY13	1,06	6,57E-03	1,49	6,05E-04
24	SNX7	1,03	1,90E-03	1,99	3,32E-04
25	RASGRP3	1,00	1,40E-02	1,56	7,88E-04
26	LAPTM5	0,75	4,84E-04	1,03	8,98E-05
27	STX6	0,65	2,48E-02	1,31	9,56E-04
28	B3GNT7	0,60	2,59E-01	1,85	7,85E-04
29	ST14	0,60	7,71E-02	1,74	2,54E-04
30	IL18	0,58	2,80E-01	1,84	8,27E-04
31	LCP2	0,57	3,11E-02	1,41	4,05E-04
32	SH3BP4	0,40	1,86E-01	1,34	9,14E-04
33	LAYN	0,38	1,61E-01	1,56	7,34E-04

Table published in (1).

In order to fully comprehend how miR-424(322) regulates the lineage fate decisions LC and moDCs, we next studied whether miR-424 knockdown in human $CD34^+$ progenitor cells indeed results in augmented TGF-β1-dependent LC differentiation. Based on our previous finding that miR-424(322)/503 was induced after 48 hours of GM-CSF stimulation in BM cells, we investigated if the miR-424 KD cells pretreated for 48 hours with GM-CSF/IL-4 could potentially switch lineage decision in favor of LC differentiation.

It was previously described that IL-4 addition to GM-CSF/TNFα containing cultures of $CD34^+$ hematopoietic progenitor cells repressed TGF-β1-induced LC differentiation in favor of DCs and macrophages (148). In line with this, the addition of IL-4 repressed LC differentiation in favor of moDC differentiation in our *in vitro* cultures (Figure 18A). Next, we pre-treated monocytic progenitors with GM-CSF plus IL-4 for 48 h to induce miR-424/503 and additionally stimulated them with TGF-β1-containing LC cytokine mix for another 48 hours. As a control we used parallel cultures stimulated with GM-CSF plus IL-4 for 4 days. Strikingly, miR-424/503 knockdown cultures contained higher percentages of LCs and lower percentages of moDCs relative to control transduced cultures on the 4^{th} day of differentiation (Figure 18B). This suggests that miR-424/503 induced concomitant with moDC differentiation functionally interferes with TGF-β1-dependent LC differentiation. Consistent with the induction TGF-β1 LC-response genes in miR-KO vs. WT BM cells, miR-424/503 knockdown facilitated *in vitro* TGF-β1-dependent LC differentiation from 48 h GM-CSF/IL-4 pre-stimulated monocytic cells. Overall, these findings support that miR-424/503 is involved in alternative lineage fate decisions of monocytes towards LC vs. moDCs.

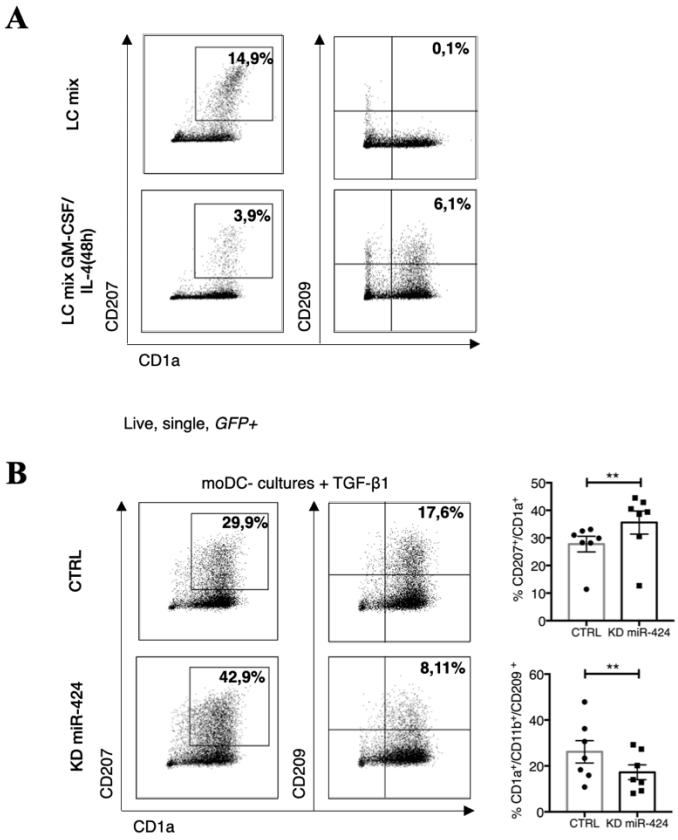

Figure 18. Loss of miR-424 facilitates LC at the expense of moDC differentiation.

(A) Lineage marker profile of human *in vitro* generated LCs and LCs cultured with IL-4/GM-CSF cytokines. (B) Phenotype of human gated GFP^+ miR-424 knockdown vs. CTRL cells. *In vitro* generated MoPs were first stimulated with GM-CSF plus IL-4, followed by LC cytokine mix to generate LCs. Data represent the mean ± SEM of 7 donors, 2-tailed Student's t test, **P<0.01. Figure published in (1).

5. DISCUSSION

Monocytes arise from monocyte-committed progenitor cells and can differentiate into proinflammatory monocyte-derived DCs or anti-inflammatory Langerhans cells. Previous findings have shown that DC differentiation critically depends on microRNAs, which regulate key biological processes in the immune cells. Given that moDCs vs. LCs rely on distinct molecular and cellular processes for differentiation, we searched for microRNAs that are differentially expressed by human moDCs vs. LCs generated from human $CD34^+$ hematopoietic progenitor cells *in vitro*. In our screen, miR-424/503 cluster stood out as the most strongly differentially expressed by these human DC subsets; miR-424/503 was strongly upregulated in moDCs vs. LCs. Moreover, we identified miR-424/503 to be strongly upregulated in moDCs in comparison to other myeloid cells and their precursors. Using lentiviral approach, we showed that miR-424/503 is required for *in vitro* moDC differentiation and its knockdown led to decrease of moDC subset in parallel transduced cultures. In contrast, the knockdown of miR-424 in LCs in the same experimental settings did not impair LC differentiation. Our results also revealed that miR-424 expression is gradually increased in GM-CSF/ IL-4 stimulated MoPs and expressed at low levels in TGF β1- induced LCs suggesting that miR-424/503 might play a prominent role in the signaling pathways regulating moDC vs. LC differentiation *in vitro*.

To gain more *in vivo* relevance, we induced psoriasis-like inflammation in miR-424(322)/503 knockout mice and assessed the distribution of immune cell subsets in their skin. Upon treatment with IMQ in miR-424(322)/503 knockout mice displayed diminished ear swelling relative to WT mice that was associated with reduced psoriatic-like skin inflammation. Extending on these analyses, we here showed that moDCs subsets were significantly reduced in the dermis of miR-424(322)/503 knockout mice under inflammatory conditions in comparison to WT mice. Interestingly, other DC subsets in the skin of miR-424(322)/503 knockout mice including LCs were not impaired. Similarly, we demonstrated that BM - derived DCs differentiation was promoted by miR-424(322)/503 *ex vivo*. The percentages of DC-precursors in the fresh isolated BM from miR-424(322)/503 knockout mice were equal to WT mice, suggesting that miR-424/503 expression acts on the early stages of DC subset differentiation but does not interfere with BM precursors. Consequently, our murine data

corroborate our findings in human moDC vs. LC differentiation that moDCs were selectively dependent on miR-424(322)/503 expression.

To gain more insights into the molecular basis of DC differentiation by miR-424(322)/503, we performed RNA-sequencing of the BMDC cultures generated using BM-precursors from miR-424(322)/503 knockout vs. WT mice. Our data revealed the altered gene expression profile after 48 hours of cell stimulation with GM-CSF in the miR-424(322)/503 knockout condition but not in the untreated precursors. Moreover, by performing gene set enrichment analysis (GSEA) we found that miR-424(322)/503 knockout BMDCs were enriched in TGF- β1 signature genes, suggesting that TGF- β1 signaling pathway was preferentially activated in miR-424(322)/503 knockout BMDCs. Furthermore, we demonstrated that miR-424/503 knockdown facilitates *in vitro* TGF-β1-dependent LC differentiation from 48 h GM-CSF/IL-4 pre-stimulated monocytic cells. Therefore, miR-424/503 expression facilitates LC differentiation at the expenses of moDC differentiation by modulating TGF- β1 signaling genes.

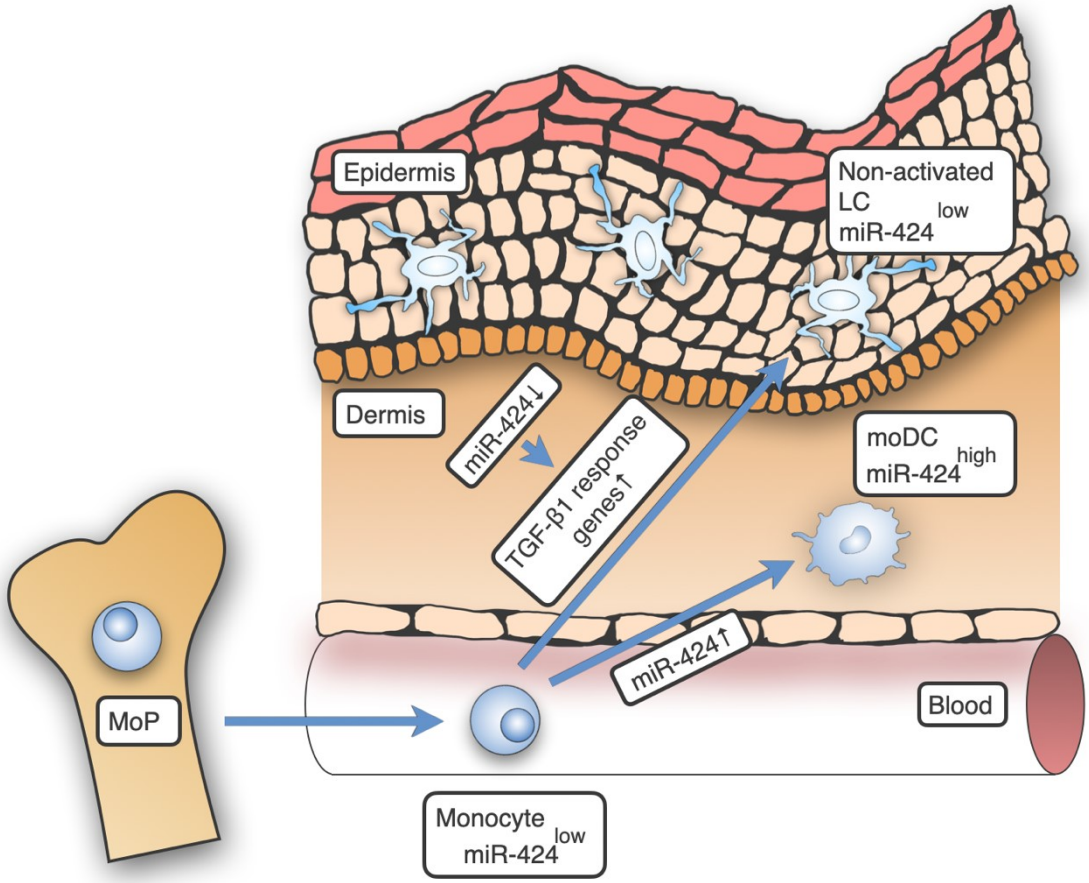

Figure 19. Model of miR-424(322)/503 involvement in LC vs. moDC differentiation from monocytes during skin inflammation.

Proposed model suggests that miR-424(322)/503 acts as a molecular switch for LCs vs. moDCs cell fate lineage decision via modulating TGF-β signaling. miR-424(322)/503 is induced during moDC differentiation from monocytes and its upregulation is required for inflammation-linked moDC differentiation. In contrast, low miR-424(322)/503 expression facilitates the differentiation and accumulation of non-activated LCs, associated with augmented expression of genes of the TGF-β family. Figure published in (1).

There is a considerable amount of literature on the role of microRNA-424/503 in cancer cell proliferation (247) and epithelial- mesenchymal transition (248). Over the last few years there has been growing interest in the tumor suppressive function of miR-424/503 in different types of cancer (249-251). Rodrigues-Baarrueco et al. has shown that loss of miR-424(322)/503 promotes chemoresistance by the upregulation of BCL-2 and insulin-like growth factor-1 in breast cancer (252). Tumor microenvironment contains monocyte-derived tumor associated DCs subpopulations at different maturation stages (253). Interestingly, moDC transcriptional

signature was revealed in breast cancer tissue and correlated with prognosis of breast cancer patients (254). Given the role of tumor-derived TGF-β1 in DC maturation (255), it would be important to further study if the tumor infiltrates of miR-424(322) deficient mice have altered moDC population.

Notably, for our study we utilized the miR-424(322)/503-KO mice that were previously described as a breast cancer tumor model. These mice have inbred FVB background and present enhanced sensitivity to tumorigenesis, which is favored for cancer-related studies. As it was previously described in the literature (228,232), 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 and H&E staining. As it was described by Rodrigues-Baarrueco et al. (252), 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. However, heterozygous miR-424(322)/503$^{+/-}$ mice showed the intermediate phenotype. Additionally, Ki-67 immunohistochemistry revealed that these lesions presented a higher proliferative index than the mammary epithelia of WT 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 the 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. While the majority of the invasive lesions was microscopic, a small fraction of the animals (two out of 40) also developed palpable tumors. Overall, these studies 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 our experiments were 8- 16 weeks old and at this age the didn't show any signs of tumorigenesis. It allowed us to successfully incorporate FVB miR-424(322)/503 KO mice into our experimental settings.

As mentioned above, miR-424(322)/503 was mostly studied in the context of cancer biology. The role of miR-424(322)/503 in immune cell biology have not been fully elucidated. Recent study has identified that miR-424(322) promoted T-cell response by regulation of PD-L1 expression in ovarian cancer. Similar findings on T-cell related immune response in breast cancer were published by Dastmalchi et al. (256). Another study demonstrated that miR-424 levels in lymphocytes and neutrophils correlated with the severity of acute stroke (257). *In vivo* miR-322 (a murine analog of miR-424) was identified as a negative regulator of inflammatory response and promotor of LPS-induced murine macrophage proliferation (258). Rosa et al. described that miR-424 regulates monocyte/macrophage differentiation by targeting the transcription factor NFI-A in human (234). However, the role of miR-424(322)/503 in dendritic cells have been poorly understood. The key finding of our study is that miR-424(322)/503 promoted moDCs but not LC differentiation acting as a molecular switch between those DC subsets. However, due to the fact that miR-424(322)/503 is broadly expressed by T cells and epithelial cells, we cannot exclude that changes in the inflammatory response in miR-424(322)/503 KO mice upon IMQ treatment are secondarily mediated by other immune cells in the skin. Therefore, further studies including BM transfer experiments would be necessary to correctly address the role of miR-424(322)/503 in the skin inflammation.

It has been previously shown that dermal moDCs contribute to inflammation through production of the inflammatory cytokines (92). As expected, miR-424(322)/503 KO mice revealed the decrease in the inflammatory response resulted in less ear swelling upon IMQ treatment. IMQ is known to induce murine psoriatic-like skin inflammation via the IL-23/IL-17 axis (229). Notably, miR-424(322)/503 KO mice exhibited less activated LCs in the epidermis upon inflammation in comparison to WT mice, which could be explained by the fact that miR-424(322)/503 KO mice generally had reduced inflammation and consequently less microenvironmental stimuli to activate LCs in the skin. Our study confirmed our *in vitro* observations that the number of phenotypically defined LCs in the epidermis of miR-424(322)/503 KO mice was not impaired. Then, we checked if miR-424(322)/503 expression can be involved in the ability of LCs migrate to the draining lymph nodes upon inflammation. Frequencies of steady-state and activated LCs in the draining lymph nodes were also not significantly different between the groups. One of the limitations of our study is that we did not functionally assessed LCs from miR-424(322)/503 deficient mice vs. WT mice, which would be important for supporting our *in vivo* data. On the other hand, we performed *in vitro*

studies by activation of miR-424/503 KD LCs vs. transduced control cells by PGN that showed undisturbed upregulation of T cell co-stimulatory molecules in respond to miR-424/503 KD. Therefore, our experiments argue that miR-424(322)/503 is important for DC differentiation but not activation. This is in line with the previous study that have reported miR-424 and miR-503 to be induced by phorbol myristate acetate (PMA) activation in THP-1 cells, suggesting that miR-424/503 at least partially promoted monocytic differentiation and directly targeted multiple cell-cycle regulators (204).

The role of miR-424/503 in DC differentiation was also supported by the observation that miR-424(322) was upregulated after 48 hours of BMDC differentiation and then decreased to the baseline levels. Thus, miR-424(322)/503 impaired differentiation capacity of BMDCs on the early stages of differentiation. 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 (259), we wondered if the lack of moDCs in miR-424(322)/503 KO mice might be a consequence of the lack of monocytic precursors but not the direct effect of impaired moDCs differentiation. Therefore, we checked whether miR-424(322)/503 mediates cell differentiation pathways already on the stage of PU.1^{hi}Flt3^{+}MHCII^{+} precursors by phenotyping of BM-DC precursors. Our data showed no significant changes in the precursors of iNOS^{+} macrophages, moDC precursors or pre-DCs in miR-424(322)/503 KO vs. WT mice. Therefore, miR-424(322)/503 KO rather impacts the DC differentiation early during cell differentiation from precursors and does not interfere with the number of monocytic bone marrow progenitors.

It has been previously shown that PU.1 is an upstream regulator of miR-424 in human monocyte/macrophage differentiation (205). We demonstrated here that TGF-β1 co-signaling represses miR-424 during LC differentiation, whereas IL-4 co-stimulation induces miR-424 during moDC differentiation. It was previously observed that IL-4 inhibits TGF-β1-dependent LC differentiation (148,240), whereas TGF-β1 addition to GM-CSF/IL-4 moDC cultures promotes certain LC characteristics (148). This suggests that miR-424/503 might be a downstream factor mediating TGF-β1 vs. IL-4 effects on LC vs. moDC differentiation. To gain more insight into the transcription regulation driving miR-424/503 expression in DCs, we carried out bioinformatic analysis of the miR-424/503 promotor. Some myeloid/DC-associated transcription factors (i.e. TCF4, TCF3, ETV2 and HOXA2, data not shown) were predicted to bind to the miR-424 promoter. In this context, it is interesting that TCF4 is a downstream

mediator of Notch/WNT signaling (260), which is involved in LC differentiation (241). Additionally, the RNA seq data of GM-CSF stimulated BM precursors revealed a short-list of differentially expressed transcription factors that might be upregulated in miR-424(322)/503 KO mice as a compensatory mechanism for the loss of miR-424(322)/503. Among those we identified the transcription factor RUNX3, that according to previous studies, is controlled by Notch-dependent repression of KLF4 in moDCs (241). Moreover, the miR-424 promoter has a predicted binding site for RUNX3, indicating that RUNX3 might repress miR-424 downstream of TGF-β1. It is interesting to speculate whether miR-424 induction in moDCs might inhibit RUNX3 in a negative feedback loop, potentially mediating impairment in LC differentiation in GM-CSF/IL-4 supplemented moDC differentiation cultures.

Given the fact that GM-CSF derived BMDCs are a relevant model of *in vitro* moDCs that normally appear during infection or inflammation (261), we characterized BMDC transcriptome of miR-424(322)/503 KO vs. WT mice by performing RNA-sequencing analysis. GM-CSF-dependent DC differentiation from bone marrow cells is regulated by transcription factors and signaling proteins early during differentiation (262). Our results suggest that the difference of the gene expression profile between miR-424(322)/503 KO vs. WT BMDCs could be identified already after 48h of GM-CSF stimulation, although unstimulated precursors had only minor differences on transcriptional level. Interestingly, among all the genes there were some of the differentially expressed genes that were previously known in the context of myeloid cells. For instance, TLR4 and CCR4 are associated with antigen-primed T cell binding to dendritic cells (263,264). Consistently, Fas ligand (CD95) and TNF induce the phenotypical and functional maturation of DCs (265,266). Moreover, lymphoid associated transcript *Dntt*, which is known to contribute to hematopoietic stem cell differentiation (267), and *Tinagl1* gene were strongly downregulated in miR-424(322)/503 KO mice. These results show that several genes responsive for myeloid cell development had reduced expression in miR-424(322)/503 KO mice.

To gain more comprehensive understanding on miR-424(322)/503- mediated moDC vs. LC differentiation, we identified putative downstream targets of miR-424(322)/503 in BMDCs. 19 genes upregulated in miR-424(322)/503-KO BMDCs on day 2 of differentiation overlapped with the list of predicted miR-424(322)/503 targets in TargetScan database. Among the nineteen picked genes that were identified as potential miR-424 targets, we noticed BMPR1a gene, which has previously been detected as a key molecule in TGF-β1/BMP-7 LC

differentiation (268). Since TGF- β1 family ligands utilize the BMPR1a-RUNX3 signaling cascade to induce LC commitment and differentiation, we reasoned that miR-424(322)/503 repression might induce upregulation BMPR1a, enabling TGF-β1 ligands to induce BMPR1a-mediated LC differentiation. This hypothesis was also supported by the pathway enrichment analysis that highlighted TGF- β pathway enrichment in the putative target genes of miR-424(322)/503. Further mechanistical studies, such as PAR-CLIP analysis, are required to prove that BMPR1a is enriched in the RISC complex of miR-424(322). We believe that apart from looking for single genes targeted by miR-424(322)/503, future research should consider that miR-424(322)/503 may target multiple TGF-β1 pathway components simultaneously in direct or indirect manner. This agreed with the previous observation that miR-424(322)/503 fine-tunes a network of genes in order to coordinate critical biological processes such as cell differentiation, proliferation and metabolism (269).

TGF-β1 was demonstrated as a critical factor for LCs development (165). TGF-β1 is not only required for proper LC differentiation, but also maintains LC and memory T cell residence in the epidermis (163). Moreover, TGF-β1 can be also secreted by keratinocytes in autocrine/paracrine manner and the autocrine TGF-β1 is known to be crucial for epidermal residence of LCs (162,166). One of the most important findings of the study was that miR-424(322)/503 knockout BMDCs exhibited upregulation of TGF-β1 signature genes, suggesting that miR-424(322)/503 modulates TGF-β1 signaling pathway. miR-424(322)/503 has previously been functionally linked to TGF-β signaling in epithelial cells: mechanistically, TGF-β1 exposure increased levels of miR-424(322)/503 in the mammary epithelium cells (228), showing opposite regulation than here observed in TGF-β1-induced LC differentiation. Interestingly, E-cadherin shows opposite regulation by TGF-β1 in epithelial cells vs. during LC differentiation (245,270). Follow up studies revealed that upregulation of miR-424(322)/503 primary transcript by TGF- β1 results in the cascade of molecular processes, including targeting of cell cycle regulator CDC25A and mediation of insulin-like growth factor 1 (IGF1) signaling (232). Others have described that miR-424(322) can inhibit Smad7 that subsequently activated the TGF-β1/Smad3 signaling pathway (271). Wei et al. described that miR-424-5p in gastric cancer could inhibit TGF-β1 signaling pathway by targeting Smad3 (272). Li et al previously found that miR-424/503 suppresses expression of two key inhibitory factors of TGF-β signaling Smad7 and Smurf2 (273). Hence, miR-424(322)/503 shows an

opposite effect on TGF-β signaling in different cell types, which is not surprising because miRNA regulation and function is often cell-context dependent (189).

We here demonstrated a positive regulatory effect of miR-424/503 in moDC differentiation, however, there is an evidence that miR-424/503 is also indispensable for regulation of LC development. Based on our results, we propose a model whereby TGF-β pathway repress miR-424/503 in monocytic LC precursors within the epidermis, which in turn augments TGF-β genes required for TGF-β1/BMP7-mediated LC differentiation. TGF-β1 signaling is expressed supra-basally within the epidermis, whereas BMP7 is strongly induced in all the keratinocyte layers in the lesional psoriatic epidermis (168). Moreover, previous studies have shown that TGF- β1 signaling synergizes with BMP7 -signaling to induce LC differentiation and preserve LC in a quiescent state (268). Further studies of BMP7 and TGF-β1 interaction might be important to address the role of miR-424/503 in the interplay between LC signaling pathways. BMP7 can fully replace TGF- β1 in LC differentiation through the selective BMPR1a receptor activation (169), which has been identified among the putative targets of miR-424/503 in our mRNA screen. The possibility that miR-424/503 might be directly or indirectly implicated in BMP7-driven LC differentiation warrants further investigation.

At this stage of understanding we believe that low expression of miR-424/503 may participate in a feed forward signaling loop by promoting LC commitment and differentiation within the epidermal microenvironment. Evidence for the existence of such a mechanism is provided by our observations (1) that TGF-β addition to GM-CSF and TNFα (LC cytokine mix) led to the low expression of miR-424/503 concomitant with LC differentiation from monocytic cells; (2) that miR-424/503 deficiency during BMDC differentiation *ex vivo* led to enrichment of large pool of TGF-β1 signature genes. Contrary to this hypothesis we did not see the significant increase in LC percentages after overexpression of miR-424/503 *in vitro*, although generally miR-424/503 KD cultures had a tendency to the increased numbers of LCs. Regardless, further research could continue to explore whether the repression of miR-424/503 may impact the expression of previously described TGF-β1-inducible anti-inflammatory effector genes in LCs, such as *Axl* (274).

In the study we provided several novel insights into the mechanisms underlying differentiation of two functionally different dendritic cell subsets moDCs and LCs from the common monocytic precursor. Firstly, we described for the first time that miR-424(322)/503

expression promotes moDC differentiation both in human and mouse. Secondly, we found that low expression of miR-424(322)/503 impairs TGF-β1 signaling pathway in order to skew differentiation rather towards LCs than moDCs lineage. Using IMQ-induced psoriatic-like inflammation model, we characterized the role of miR-424(322)/503 in DC subset specification in the inflamed skin microenvironment. Previously identified as a regulator of monocyte/macrophage differentiation, miR-424(322)/503 was shown to be functionally involved in the inflammatory response in the skin by regulating moDCs generation. Further studies that will consider that miR-424(322)/503- mediated effects on non-myeloid cells, such as epithelial cells and T cells, would complement our initial findings. Therefore, BM transfer experiments are required to specifically address effects of miR-424(322)/503 deficiency in moDCs.

To our knowledge, our investigation is the first to describe that miR-424(322)/503- KO cells exhibit altered transcriptional profile in BMDCs with a strong induction of TGF-β1 signature genes. Using the computational analysis, we pointed out that several transcription regulators as well as potential targets of miR-424(322)/503 were upregulated in the miR-424(322)/503- KO mice, suggesting that miR-424(322)/503 might be implicated in the whole network of upstream and downstream molecular events. Future research should further validate these gene candidates and address their role in the lineage specification from the monocytic progenitors.

In conclusion, our data revealed that miR-424(322)/503 is critically required for the differentiation of inflammation-associated moDCs from monocytic precursors that in turn contribute to the progression of the inflammatory response in the skin. Low levels of miR-424(322)/503 in monocytic LC precursors augment TGF-β signature genes and thereby contribute to TGF-β-mediated LC differentiation. Taken together, our study identified that miR-424(322)/503 represent a cell intrinsic regulator of myeloid/DC fates both in human and mice.

6. APPENDIX

To investigate downstream targets of miR-424(322) 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.

We developed conditions to specifically immuoprecipitate endogenous murine Ago2 after cross-linking of the RISC complex in BMDCs. 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 (228,230,231) to BMDC cultures.

Protocol:

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 1ml PBS per plate, transfer to 50 ml tube and centrifuge for 5min ,500g, 4 degree. 100 x 10^6 cells ideally should come up as 1ml of wet pellet (For BMDCs harvest BMDC by incubation in PBS on ice for 10 min, wash twice with PBS).

2.Add as minimum as possible volume of 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) to keep protein concentrated, make sure that whole pellet is resuspended completely. Incubate in ice 10 min and take out the clear lysate after centrifugation 13000g ,15 min , 4ºC and proceed further with it, discard the pellet.

3.Perform mild (5U/uL) RNase-T1 (Fermentas #EN0541) digestion at 22ºC for 15 minutes in water bath (temperature and duration are critical), cool down 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 the beads 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+ 40 uL beads and incubate overnight at 4ºC.

Troubleshooting: if the enrichment isn't good enough, double the amount of beads and antibody.

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.

9.Finally extract was lysed and RNA extracted using the Qiagen miRNA isolation Kit according to the instructions provided, follow the protocol for small RNA extraction with increased percentage of Ethanol instead of total RNA extraction protocol. Target-mRNA can be quantified by real-time PCR using specific primers.

As shown on the Figure 20, 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.

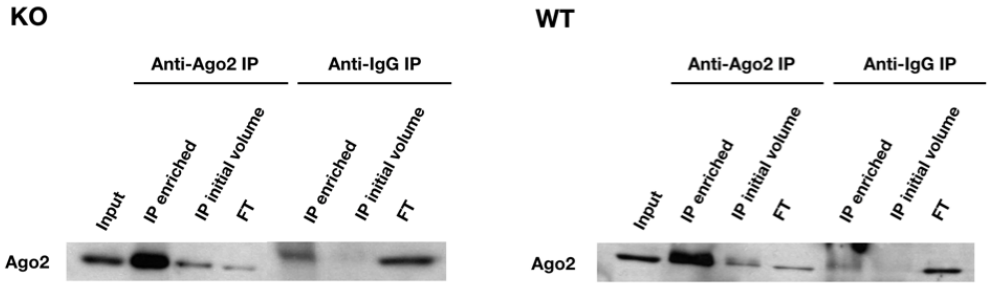

Figure 20. CLIP analysis for identification of miR-424 targets.

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.

Reagents:

Lysis buffer:

2.5 mM HEPES pH7

50 mM NaCl

10% glycerol

1% Tryton X-100;

4-thiouridine (T4509; Sigma)

proteinase inhibitor Roche 04693159001 (1 tablet in 10ml buffer)

0.2 mM dithiothreitol DTT

1 U/µL RNAseOUT 10777-019 Invitrogen (250 uL/ 10 ml buffer)

5 U/µL RNase-T1 EN0541; Fermentas

IP: A/G magnetic beads (88802, Pierce, Thermofisher Scientific)

Anti Ago2 antibody H00027161-MO1; Abnova

Washing buffer 1:

50 mM Tris pH 7.5

150 mM NaCl

0.1% NP-40

1mM EDTA

Washing buffer 2:

50 mM Tris pH 7.5

500mM Nacl

0.1% NP-40

Proteinase K P8102; New England Biolabs

Qiagen miRNA isolation Kit

Primers

microRNA reverse transcription kit (4366596) with Pul-RT primers

Fast Sybr green master mix

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