Dissertation

The role of C/EBPα in early dendritic cell development

C/EBPalpha in der frühen Entwicklung von dendritischen Zellen

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

Anirudh Subramanian Muralikrishnan
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Disclosures

This thesis has been published in the following original paper:

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TNFα Rescues Dendritic Cell Development in Hematopoietic Stem and Progenitor Cells Lacking C/EBPα

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"Both the man of science and the man of action

live always at the edge of mystery,

surrounded by it"

J. Robert Oppenheimer

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Abbreviations and Definitions (in order of appearance in text)

APC	-	Antigen presenting cells
DC	-	Dendritic cells
NK cells	-	Natural Killer cells
cDC	-	Conventional/Classical dendritic cells
pDC	-	Plasmocytoid dendritic cells
IFN-γ	-	Type 1 Interferon gamma
TNFα	-	Tumor Necrosis Factor alpha
iNOS	-	inducible nitric oxide synthase
LC	-	Langerhans Cells
FLT3	-	FMS like tyrosine kinase 3
FLT3L	-	FMS like tyrosine kinase 3 ligand
LN	-	Lymph Node
MHC II	-	Major histocompatibility complex II
moDCs	-	Monocyte derived dendritic cells
HSC	-	Hematopoietic stem cells
HSPC	-	Hematopoietic stem and progenitor cells
BM	-	Bone marrow
MPP	-	Multi-potent Progenitors
CMP	-	Common Myeloid Progenitor
GMP	-	Granulocyte and Monocyte Progenitor
MP	-	Myeloid Progenitor
CLP	-	Common Lymphoid Progenitor
MDP	-	Monocyte and Dendritic cell progenitor

CDP	-	Common dendritic cell progenitor
MEP	-	Megakaryocyte and erythroid progenitor
Sfpi1 / PU.1	-	Spi-1 Proto-Oncogene ETS domain binding transcription factor
IRFs	-	Interferon regulatory factors
BATF3	-	Basic leucine zipper transcription factor ATF-like 3
ID2	-	Inhibitor of DNA binding protein 2
C/EBPα	-	CCAAT/enhancer binding protein alpha
BR-LZ	-	Basic region leucine zipper
AML	-	Acute Myeloid leukaemia
GM-CSF	-	Granulocyte macrophage colony stimulating factor (CSF1)
M-CSF	-	Macrophage colony stimulating factor (CSF 2)
TNF R1 & R2	-	Tumor necrosis factor receptor 1 & 2
IL1β	-	Interleukin 1 beta
MIP1 & 2	-	Macrophage inflammatory protein 1 & 2
pIpC	-	Polyinosinic:polycytidylic acid
WT	-	Wild type
KO	-	Knock out
PCR	-	Polymerase chain reaction
qRT-PCR	-	quantitative real time PCR
EYFP	-	Enhanced yellow fluorescent protein
HBSS	-	Hanks balanced salt solution
RPMI1640	-	Roswell park memorial institute media 1640
DNA	-	Deoxyribonucleic acid
RNA	-	Ribonucleic Acid

cDNA	-	Complementary DNA
RIN	-	RNA integrity number
MLR	-	Mixed lymphocyte reaction
EPD	-	Eukaryotic promoter database
SD	-	Standard Deviation
FDR	-	False discovery rate

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Zusammenfassung

Dendritische Zellen (DCs) sind zentrale Effektorzellen des adaptiven Immunsystems, die aus hämatopoetischen Stammzellen und bestimmten Vorläuferzellen des Knochenmarks gebildet werden. Das Zytokin FLT3L (FMS-related tyrosine kinase 3 ligand) ist zentral an der Differenzierung von multipotenten Vorläuferzellen (MPPs) über gemeinsame myeloische Vorläufer (CMPs) zu monocytischen dendritischen Vorläuferzellen (MDPs) und anschließend zu gemeinsamen dendritischen Vorläuferzellen (CDPs) beteiligt. Schließlich differenzieren CDPs entweder in konventionelle DCs oder in plasmozytoide DCs. C/EBPα (CCAAT/Enhancer-Bindungs-protein Alpha) ist ein zentraler Transkriptionsfaktor in der Blutbildung und unverzichtbar für die Bildung reifer Granulozyten und Monozyten. Mäuse, denen das *Cebpa*-Gens in Knochenmarkszellen fehlt, weisen eine normale Anzahl von CMPs auf, es fehlen jedoch die nachgeschalteten Granulozyten/Monozyten-Vorläufer (GMPs). C/EBPα ist daher für die frühe Myelopoese essentiell, indem es die Differenzierung von CMPs zu GMPs ermöglicht. Obwohl C/EBPα in der Myelopoese gut untersucht ist, ist wenig über seine Rolle in der Entwicklung von DCs bekannt.

Unter Verwendung eines *Cebpa*^{Cre}-EYFP-Reporter-Mausmodells konnten wir in dieser Studie zeigen, dass die Mehrheit der konventionellen DCs in der Milz von *Cebpa*-exprimierenden hämatopoetischen Stamm- und Vorläuferzellen (HSPCs) abstammen. Eine detaillierte Analyse der DC-Vorläuferzellen ergab einen Anstieg der *Cebpa*/EYFP^{+} -Zellen von frühen Vorläuferzellen wie MPPs über CMPs auf ihr Maximum im MDP-Stadium. Da in späteren Differenzierungsstadien kein weiterer Anstieg der Cebpa/EYFP^{+} -Zellen beobachtet werden konnte, zeigten diese Ergebnisse zusammen mit Daten aus öffentlich zugänglichen Genexpressionsstudien, dass *Cebpa* vor allem während der frühen DC-Entwicklung exprimiert wird. Um eine funktionelle Rolle von C/EBPα in der frühen DC-Entwicklung zu untersuchen, verwendeten wir als nächstes ein induzierbares knochenmarkspezifisches *Cebpa*-Knockout (KO)-Mausmodell (*Mx*1^{Cre}*Cebpa*$^{F/F}$-Mäuse), isolierten HSPCs dieser Mäuse und untersuchten ihre Fähigkeit, reife DCs nach *in vitro* Kultur mit FLT3L zu bilden. Während *Cebpa*-Wildtyp (WT) -HSPCs eine hohe Anzahl reifer DCs produzierten, zeigten HSPCs von *Cebpa*-KO-Mäusen eine deutliche Verminderung an reifen DCs. In einer detailierten Analyse konnten wir eine verringerte Bildung von MDPs mit einem fehlenden Übergang zu CDPs in KO-HSPCs beobachten, während WT-HSPCs eine vollständige Differenzierung über MDP- und CDP-Stadien zu reifen DCs durchliefen. In einer umfangreichen Genexpressionsanalyse von

FLT3L-stimulierten HSPCs aus *Cebpa* WT- und KO-Mäusen fanden sich signifikante Veränderungen von Transkriptionsfaktoren, wie IRF8 und PU.1, die in der Entwicklung von DCs eine wichtige Rolle spielen, von entzündlichen Zytokinen, wie TNFα und IL1β, und mehrerer Gene, die, wie zB. *Cx3cr1,* mit der DC-Entwicklung oder mit dem TNFα- oder NFκB-Signalweg assoziiert sind. Zusätzlich war auch die Sekretion des TNFα-Proteins von mit FLT3L-stimulierten *Cebpa* KO-HSPCs reduziert. Interessanterweise konnten wir durch Zugabe von TNFα zu HSPCs, die *in vitro* mit FLT3L kultiviert wurden, die DC-Bildung aus *Cebpa*-KO-HSPCs wiederherstellen. Dieses Phänomen war mit einer erhöhten Expression von *Sfpi1* (dieses Gen kodiert für den Transkriptionsfaktor PU.1) und *Cx3cr1* assoziiert und führte zu funktionstüchtigen DCs.

Zusammenfassend ist es uns mit dieser Studie gelungen, eine zentrale Rolle von C/EBPα in der frühen DC-Entwicklung zu identifizieren. Die Expression von *Cebpa* war für die Bildung von MDPs und deren Übergang zu CDPs während der FLT3L-induzierten DC-Entwicklung unverzichtbar. Eine Zugabe von TNFα konnte diesen Reifungsblock durch Induktion der *Sfpi1*- und *Cx3cr1*-Expression überwinden. Es sind jedoch weitere Studien erforderlich, um die detaillierten molekularen Mechanismen, durch die C/EBPα die frühe DC-Bildung beeinflusst, aufzuklären.

Abstract

Steady-state dendritic cells (DCs) are crucial effector cells of the immune system, which are derived from hematopoietic stem cells and distinct progenitors. In the presence of FMS-related tyrosine kinase 3 ligand (FLT3L), multipotent progenitors (MPPs) and common myeloid progenitors (CMPs) differentiate to monocytic dendritic progenitors (MDPs) and subsequently to common dendritic precursors (CDPs). Finally, CDPs differentiate either into conventional DCs or plasmocytoid DCs. C/EBPα (CCAAT/enhancer binding protein alpha) is a basic region-leucine zipper transcription factor and indispensable for formation of mature neutrophils and eosinophils. Mice with a homozygous deletion of the *Cebpa* gene have normal to elevated numbers of CMPs, but completely lack downstream granulocyte/monocyte precursors (GMPs) and all subsequent granulocytic stages indicating that C/EBPα is essential for early myelopoiesis by enabling transition of CMPs to GMPs. Though the gene is well studied in myelopoiesis, little is known about its role in early steps of development of DCs.

Using a *Cebpa*^{Cre}-EYFP reporter mouse model, we identified the majority of splenic conventional DCs to be derived from *Cebpa*-expressing hematopoietic stem and progenitor cells (HSPCs). Detailed analysis of DC progenitor stages revealed an increase of *Cebpa*/EYFP^{+} cells in these mice from early progenitors, such as MPPs via CMPs to its maximum at the MDP stage. Since in later stages no further increase of *Cebpa*/EYFP^{+} cells was observed, these results together with data from published gene expression studies clearly indicated *Cebpa* expression during early DC development. To assess a functional role of C/EBPα in early DC development, we next used an inducible bone marrow-specific *Cebpa* knockout (KO) mouse model (Mx1^{Cre}*Cebpa*$^{F/F}$ mice), isolated HSPCs of these mice and studied their capacity to form mature DCs after *in vitro* culture with FLT3L. While *Cebpa* wildtype (WT) HSPCs produced high numbers of mature DCs after eight days of culture, HSPCs from *Cebpa* KO mice exhibited a profound reduction in DC numbers. In a stepwise analysis, we observed decreased formation of MDPs and a block in their transition to CDPs in KO HSPCs, whereas WT HSPCs underwent successful transition through the MDP and CDP stages towards mature DCs. Gene expression analysis of FLT3L-stimulated HSPCs from *Cebpa* WT and KO mice revealed a significant change in transcription factors associated with DC development, like IRF8 and PU.1, inflammatory cytokines like TNFα and IL1β and several genes related to DC development, such as *Cx3cr1*, as well as related to the TNFα- and NFκB-pathways. Interestingly, TNFα was also reduced in supernatants of *Cebpa* KO HSPCs stimulated with FLT3L. Accordingly,

addition of TNFα to HSPCs cultured *in vitro* with FLT3L partially restored DC formation in *Cebpa* KO cells by upregulating *Sfpi1* (the gene for PU.1) and *Cx3cr1* expression. Mixed lymphocyte reactions showed that DCs generated from *Cebpa* KO HSPCs by TNFα addition were functionally fully active.

In conclusion, we identified a critical role of C/EBPα in early DC development in this study. Expression of *Cebpa* was indispensable for the formation of MDPs and their transition to CDPs during FLT3L-induced DC development. The inflammatory cytokine TNFα was able to partially overcome this maturation block probably by interfering with *Sfpi1* and *Cx3cr1* expression. However, further studies are needed to reveal the detailed molecular mechanisms, through which C/EBPα affects early DC formation.

1. Introduction

1.1 Dendritic cells

Both innate and adaptive immunity are essential for the functioning of a healthy immune system. Dendritic cells (DCs) are a special class of antigen presenting cells (APCs) which form a bridge between innate and adaptive immunity, facilitating immune reactions towards pathogenic antigens. DCs are well distributed in the body and function via the uptake and recognition of pathogens, activation of natural killer cells (NK cells), processing and presentation of antigens and subsequent induction of T and B cell based adaptive immune responses (Steinman and Idoyaga, 2010). DCs carry out their primary function by residing in the peripheral tissues in an immature state and scanning their environment for pathogenic signals using pattern recognition receptors like Toll-like receptors. The identification and subsequent phagocytosis of the pathogenic antigen causes DCs to attain a mature phenotype, which enables them to act as antigen presenting cells and activate T- and B-cells to illicit the necessary adaptive immune response (Mogensen, 2009). In a secondary but equally important role, DCs also control onset and prevention of autoimmunity by inducing and maintaining tolerance towards self-antigens in the steady, non-diseased state. DCs present self-antigens to autoreactive T-cells causing their anergy or deletion and induction of regulatory T- cells. This function of the DCs is specific dependent on multiple factors like the activation status and the method of antigen uptake (Ganguly *et al.*, 2013).

Since the coining of the term dendritic cells in 1973 by Steinman and Cohn (Steinman and Cohn, 1973), the understanding of their development and function has expanded substantially. Especially in the last decade, DCs have been identified to be a heterogeneous group of cells with a varied number of functions based on the type. Over the years multiple methods for classifying DCs based on activation status (mature or immature DCs), the physiological state they show up in (steady-state or inflammatory DCs), and the anatomical location (tissue resident, migratory or circulating DCs) (Heath and Carbone, 2009); (Coquerelle and Moser, 2010); (Guilliams *et al.*, 2010) have been introduced.

1.2 Classification of DCs

Though multiple classification systems have been used to classify DCs in the past, the most comprehensive and simplified model of classification is based on the functional grouping of DCs (Guilliams *et al.*, 2010). According to this model, there are three major classes of DCs namely steady-state DCs, inflammatory DCs and Langerhans cells.

1.2.1 Steady-state DC subgroups

In a steady-state, defined by the absence of infection or inflammatory stimuli, DCs are present as sentinels of the immune system. In steady-state, DCs can further be classified as classical DCs (cDCs) and plasmocytoid DCs (pDCs) (Shortman and Naik, 2007).

1.2.1.1 Classical DCs

Classical DCs form the majority of the steady-state DCs and function via antigen processing and presentation. In the immature state they possess high phagocytic activity and upon maturation function by active cytokine release. These are short lived cells that are replenished constantly from bone marrow precursors. Though defined under the steady-state category, cDCs function by regulating T-cells both in steady-state and in inflammatory conditions. cDCs can be found in both lymphoid as well as non-lymphoid tissue and also as migratory DCs (Merad *et al.*, 2013).

1.2.1.2 Plasmacytoid DCs

Plasmocytoid DCs, as opposed to cDCs, are phenotypically non-dendritic round cells, which are long lived and function via type 1 Interferon (IFN-γ) production (Liu, 2005). These cells exhibit lymphoid-like molecular features which differentiates them from cDCs though both are produced from the same progenitors. pDCs functionally specialise in antiviral defence by the upregulation of IFN production and subsequent activation of NK cells. In a steady-state, pDCs are non-dendritic but upon stimulus gain more dendritic cells like cytoplasmic protrusions.

1.2.2 Inflammatory DCs

Even though DCs are present in steady-state, there are some DCs that transiently appear after an infection or inflammation. Monocytes circulate in the blood and are present in the spleen but do not proliferate in steady-state. But, upon infection, monocytes can give rise to macrophages or inflammatory DCs based on the pathogen recognition patterns (Merad *et al.*, 2013). Tip DCs are another example of inflammatory DCs identified in *listeria monocytogenes* infected animals as a subset that produce Tumor necrosis factor alpha (TNFa) and inducible nitric oxide synthase (iNOS) (Serbina *et al.*, 2003). Inflammatory DCs disappear with the alleviation of the inflammatory stimuli.

1.2.3 Langerhans cells

Langerhans cells were the first class of DCs identified in 1868 by Paul Langerhans though they were called nerves cells of the skin and not DCs. Langerhans cells were identified to be a part of the DC family almost after 100 years of their discovery. They are found in the epidermis, the genital mucosa and the oral epithelium. Langerhans cells differ from the all other DCs due to their ontogeny and homeostatic properties (Ginhoux and Merad, 2010). Primarily DCs are derived from bone marrow precursors during adult life, but LCs are derived prenatally from LC precursors that infiltrate the epidermis and are restored by local self-renewal. LCs are locally self-renewed from two specific embryonic myeloid progenitors, yolk sac-derived macrophages and fetal liver monocytes. LCs are characterised by the expression of high levels of langerin (Schuler, Romani and Steinman, 1985) and function like the cDCs in steady-state but are replaced by monocytes during inflammatory conditions.

1.3 Identification of murine dendritic cell subsets

DCs in the mouse can in general be identified based on their surface expression of CD11c and MHC II. Therefore, CD11c and MHC II can be considered as pan markers for DC identification albeit there can be subset specific differences in the levels of expression of these markers (Merad *et al.*, 2013). Additionally, most DCs are formed through expression of FMS-like tyrosine kinase 3 (Flt3) and the stimulus provided by its ligand Flt3 ligand (Flt3l). The expression of Flt3 can thus be used to discern cDCs from macrophages (McKenna *et al.*, 2000). The various subsets of murine DCs are then classified based on the additional surface markers expressed as outlined in the next paragraphs.

1.3.1 Murine steady-state DC subsets

Steady-state DCs represent the majority of DCs in both lymphoid and non-lymphoid tissues. As generally described above, in the mouse steady-state DCs can also further be classified into two classes – classical or conventional DCs and plasmocytoid DCs.

1.3.1.1 Classical/ Conventional DCs

In general, classical DCs can be further divided into two main types, based on their surface marker namely $CD8^+$ DCs and $CD11b^+$ DCs.

In the lymphoid tissue, the $CD8^+$ subset forms about 20-40 % of cDCs in the spleen and lymph nodes and most of the thymic cDCs (Shortman and Heath, 2010). $CD8^+$ cDCs show low to no expression of CD11b and other macrophage markers making them easily discernible. They express high levels of Flt3 and proliferate in response to Flt3l (Waskow *et al.*, 2008). In contrast to the migratory DCs that reach the LNs in a mature form, the $CD8^+$ subset is present as phenotypically immature cells in steady-state. The $CD8^+$ DCs mature upon antigenic stimulation enabling them to execute effector functions. They distinctly express lectins like CD205 and Clec9a, which are not found on the $CD11b^+$ subset (Shortman and Heath, 2010). In the non-lymphoid organs, like connective tissues, the $CD103^+$ subset replaces the $CD8^+$ cDCs but shares its ontogenic roots with the $CD8^+$ cDCs found in lymphoid organs (Ginhoux *et al.*, 2009). The expression of CD103 is tissue specific and also controlled by production of Csf-2 cytokine (Melanie Greter *et al.*, 2009). As with $CD8^+$ cDCs, the non-lymphoid tissue-resident $CD103^+$subset lacks the expression of macrophage markers, like Cx3Cr1, CD11b and CD115, but they express high levels of Flt3 on their surface and respond to stimulation by Flt3l.

The $CD11b^+$ DC subset lacks expression of CD8 and represents the predominant fraction in most lymphoid organs other than the thymus. It can also be found in non-lymphoid organs. The $CD11b^+$ DCs also proliferate in response to Flt3l stimulation and are reduced accordingly in Flt3 negative mice, but are affected to a lesser extent than the $CD8^+$ subset. In the spleen the $CD11b^+$ are considered to contain two further subsets, $ESAMhiCD11b^+$ which arise from the cDC precursors and the $EASMloCD11b^+$ which arise from circulating monocytes (Lewis *et al.*, 2011). This subset is hence a mixture of cDCs and macrophages, but the two ontogenically distinct subsets can be differentiated in a tissue specific manner by using for example FcγRI expression in the muscles (Langlet *et al.*, 2012) and based on CD103 expression in the *lamina propria* of the intestine (Schulz *et al.*, 2009).

Classical non-lymphoid DCs are also present in peripheral lymph nodes and are called the tissue migratory DCs (Randolph, Angeli and Swartz, 2005). The nature of the tissue migratory DCs is dependent on the site of LN drainage. The migration of the cDCs to draining LNs is CCR7 dependent and can be characterised based on their high expression of MHCII and low intermediate expression of CD11c in the steady-state.

1.3.1.2 Plasmocytoid DCs

Plasmocytoid DCs are the second class of steady-state DCs and are present in small numbers throughout peripheral tissues (Reizis, 2019). These cells are characterized by their expression of B220, Ly6C, and CD317. They also express Siglec H and Ly6D, which is why they have been considered to be closely related to the lymphoid lineage. In addition to their crucial role in IFN production during viral infections, pDCs also have the potential to act as APCs, as they express MHC II and co-stimulatory molecules and continue to synthesize peptide-MHC II complexes after activation (Villadangos and Young, 2008).

1.3.2 Inflammatory DCs

Inflammatory DCs can be generated from steady-state monocytes in the presence of inflammation. These inflammatory DCs are characterised by their expression of Ly6c, CD11b, MHC II and low level of CD11c and are called monocyte derived DCs (moDCs) (Domínguez and Ardavín, 2010). moDCs specifically express the transcription factor Zbtb46, which can be used to differentiate them from the other subset of inflammatory DCs, the so-called Tip DCs (Satpathy *et al.*, 2012).

1.3.3 Epidermal LCs

LCs are DCs that are present in the epidermal layer of the skin. They are distinct from all other types of DCs majorly due to their ontogeny. They are characterised by a lower expression of MHCII and intermediate levels of CD11c expression. The express very high level of CD207 (langerin), CD11b and F4/80 while lacking CX3CR1 expression (Merad, Ginhoux and Collin, 2008). Due to their origin they develop independent of the effects of Flt3 and Flt3l.

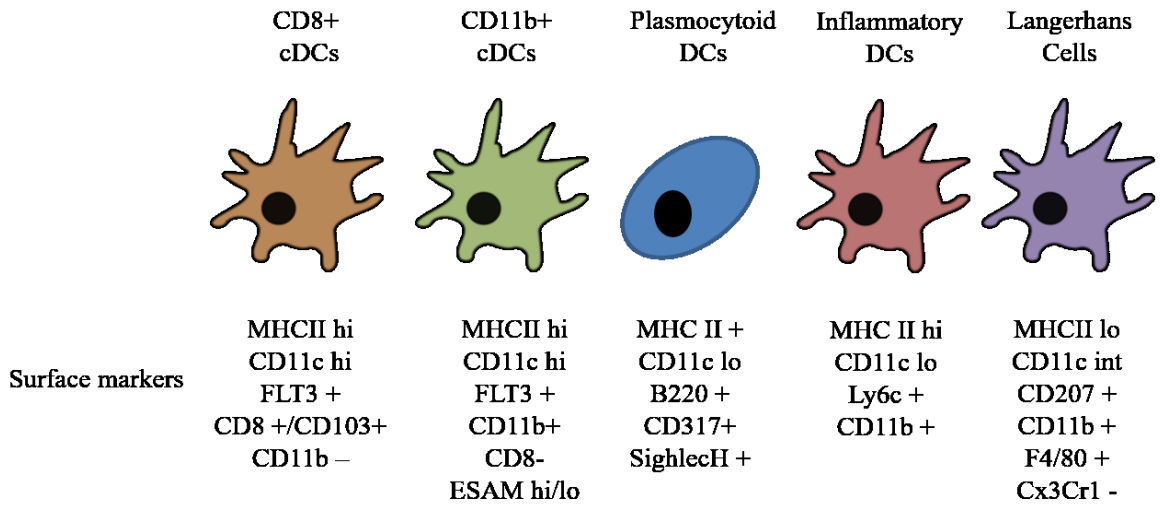

Figure 1.1 – Phenotype of murine DC subsets. A comprehensive view of the different defined subsets in mice along with the surface markers used to discern each subset. DC denotes dendritic cell; cDCs conventional DCs; + positive; - negative; hi high; lo low.

1.4 Ontogeny of steady-state DCs

DC are dependent on self-renewing hematopoietic stem cells (HSCs) for their establishment and replenishment like all hematopoietic cells (Merad *et al.*, 2013). Development of mature steady-state DCs is a process that occurs via the sequential formation of lineage restricted progenitors. Described below are the multiple stages progenitors pass through before producing mature DCs.

1.4.1 Early Progenitors during DC development

The first progenitors are the multi-potent progenitors (MPPs) which lack the ability to self-renew, but can give rise to all the lineages of the hematopoietic system. After losing the capacity to give rise to lymphoid cells, the MPPs give rise to the common myeloid progenitors (CMPs) (Manz *et al.*, 2001). It has alternatively also been suggested that lineage marker negative (lin^-), $Sca1^-$, CD117hi (c-Kit) myeloid progenitors (MP) that phenotypically overlap with CMPs contain the first immature DC precursors (Liu and Nussenzweig, 2010). These first immature DC precursors within CMPs also express CD135 (FLT3), the receptor for FLT3L (D'Amico and Wu, 2003). The identification of these progenitors has been through studies based on adoptive transfer experiments. Adoptive transfer of CMPs is successfully able to produce both cDCs and pDCs (Traver *et al.*, 2000). Transplantation of isolated CLPs and CMPs into congenic mice resulted in the formation of high numbers of DCs in the spleen, thymus and

lymph nodes. The ratios of DCs produced by CLPs and CMPs are quite similar but given that the number of CMPs present in the bone marrow is normally higher than CLPs most DCs are assumed to derive from myeloid lineage. Data has also shown that CD8 expression is not indicative of lymphoid origins, but most DC are just derived from a common progenitor for all myeloid lineage cells at least as far as most extra-thymic DCs are concerned (Traver *et al.*, 2000) .

1.4.2 Intermediate Progenitors

Through the activity of FMS-related tyrosine kinase-3 ligand (FLT3L), which binds to the FLT3 receptor (CD135), the CMPs/MPs differentiate into the committed progenitor stage called the monocytic dendritic cell progenitors (MDPs). The MDPs reflect an intermediate progenitor stage and are defined by surface expression of CD135, CD115 and CX3CR1. They have lost all granulocytic potential and are thus fully committed to produce mononuclear phagocytes including steady-state DCs and monocytes (Fogg D *et al.*, 2006). Downstream of MDPs, common dendritic cell progenitors (CDPs) are defined as the first fully restricted progenitors for DCs. Characterised by CD135 and CD115, they are phenotypically similar to MDPs except for their intermediate levels of CD117 expression (Onai *et al.*, 2007). Through adoptive transfer it has been proven that CDPs have the potential to give rise to all steady-state DCs like $CD8^+$, $CD11b^+$, $CD103^+$ and pDC subsets (Liu K *et al.*, 2009).

1.4.3 Late Progenitors

The CDPs can give rise to pDCs and immature intermediates of cDCs, the pre-DCs, which then give rise to $CD8^+$ and $CD11b^+$ mature DCs. Pre-DCs are an intermediate late progenitor that directly arise from CDPs and can be found in the bone marrow, blood and peripheral organs. They are characterised by their expression of high levels of CD11c and lack of expression of MHCII (Liu K *et al.*, 2009). The pre-cDCs leave the bone marrow niche continuously and travel to the lymphoid organs and non-lymphoid organs to remain as immature cDCs awaiting activation through antigenic stimuli. Pre-DCs have high clonal efficiency and are able to produce all the types of cDCs ($CD8^+$, $CD1b^+$ and $CD103^+$) upon adoptive transfer, but fail to form pDCs (Diao *et al.*, 2004). Monocytes arising either from GMPs or MDPs were initially also thought to be the precursors of cDCs, but were later proven to be so only in inflammatory conditions (Naik *et al.*, 2007). Even though they are majorly precursors of inflammatory DCs, they have recently been shown to play a minor role in steady-state development of very specific $CD11b^+$ DCs (Lewis *et al.*, 2011), discussed in section 1.3.1.1)

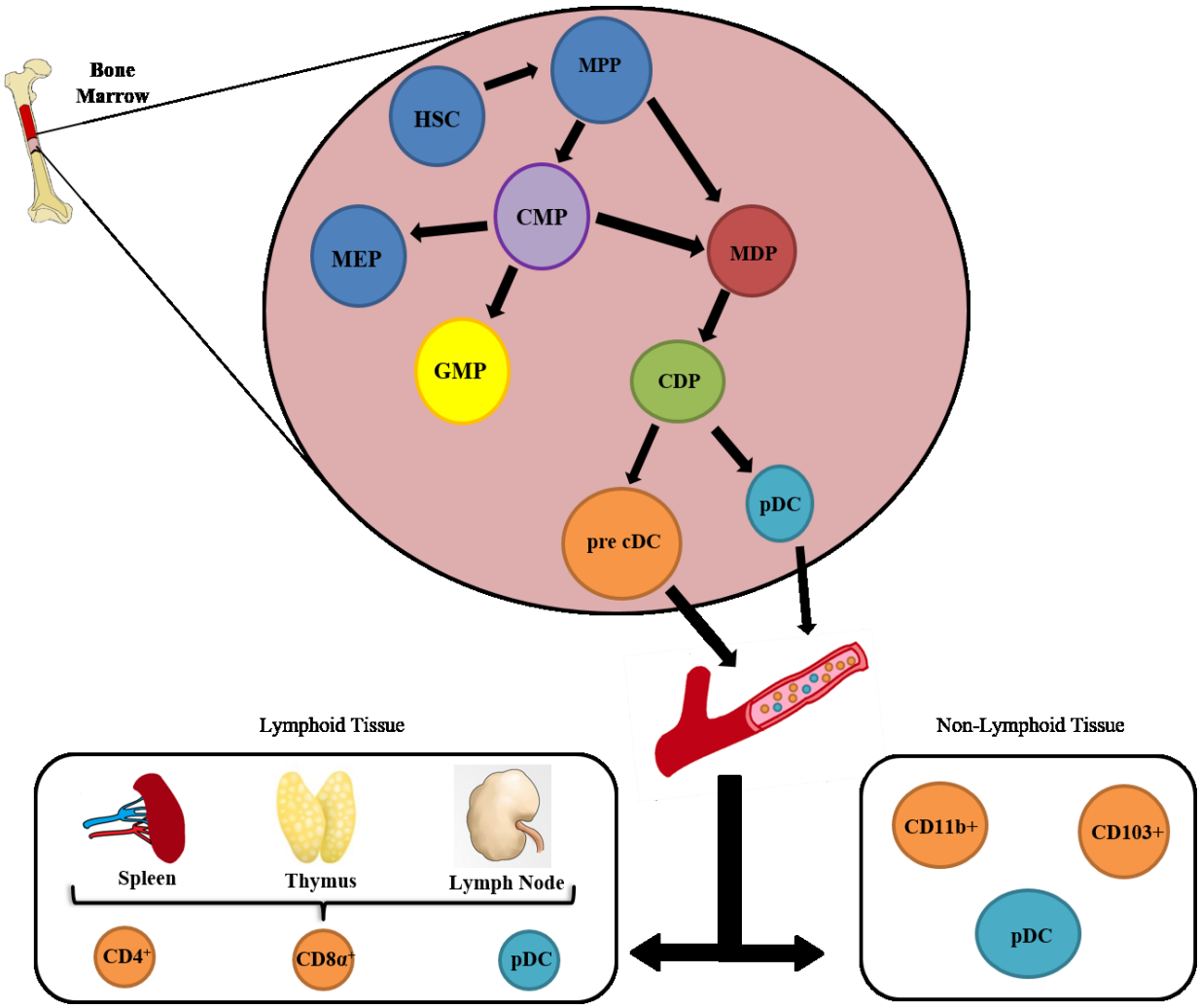

Figure 1.2 – Steady-state DC ontogeny. A schematic representing the most accepted model of DC development with the distinct steps of the development from the earliest progenitors in the bone marrow to the mature DCs in the peripheral organs. The earliest committed progenitors for the DC lineage are found within MPPs arising from the HSCs. MPPs in turn give rise to MDPs or CMPs. MDPs are the major precursors of all DCs and after going through the intermediate CDP step give rise to pre-cDCs or pDCs which migrate to the tertiary sites and await signals for differentiation into their final formation and subsequent activation to perform their respective functions. HSC denotes hematopoietic stem cell; MPP multipotent progenitor; CMP common myeloid progenitor; MEP megakaryocyte erythroid progenitor; GMP granulocyte monocyteprogenitor; MDP monocyte dendritic cell progenitor; CDP common dendritic cell progenitor; pre cDC pre conventional dendritic cell; pDC plasmacytoid dendritic cell.

1.5 Transcriptional control of steady-state DC development

As outlined in the previous chapter, all steady-state DCs are derived from hematopoietic stem and progenitor cells (HSPCs), which undergo successive differentiation steps passing through multiple restricted progenitor stages and eventually forming the mature cells. The decisions of cell fate are controlled by multiple transcription factors that play important roles at different steps for the successful differentiation and formation of specific DCs. In the next paragraphs, the most crucial transcription factors involved in DC development are discussed.

1.5.1 PU.1

PU.1 is a master transcription factor belonging to the ETS family of transcription factors encoded in mice by the *Sfpi1* gene. PU.1 plays multiple roles in fate decisions in haematopoiesis. In the DC lineage, PU.1 acts as a pioneering transcription factor and is expressed in all cDCs and pDCs. PU.1 deficient mice exhibit a marked reduction in the $CD11b^+$ and $CD8^+$ cDCs along with multiple other hematopoietic deficiencies (Guerriero *et al.*, 2000). HSPCs from PU.1 deficient mice fail to differentiate into DCs upon treatment with FLT3L and GM-CSF *in vitro* (Anderson *et al.*, 2000). Re-introduction of PU.1 restores DC development and also increases expression of FLT3. It has also been shown that induction of FLT3 in normal MEPs increases PU.1 expression exhibiting a positive feedback loop. Conversely, induction of FLT3 expression in PU.1 negative HSPCs does not restore DC development and PU.1 expression, indicating that PU.1-dependent cofactors other than FLT3 are required for proper DC development to occur (Carotta *et al.*, 2010). Deficiency of PU.1 also affects the proper formation of the DC progenitor stages like MDPs and CDPs.

1.5.2 Interferon regulatory factors (IRFs)

Multiple IRFs have been identified to play a role in DC development (see Table 1). The most crucial one, IRF8, has been shown to be important for myeloid cell differentiation by influencing a fate choice promoting monocyte/DC development and inhibiting granulopoiesis. Accordingly, *Irf8* null mice are deficient of monocytes, splenic $CD8^+$ DCs and $CD103^+$ DCs, while having an increased number of granulocytes (Tamura, Kurotaki and Koizumi, 2015). *Irf4* deficient mice on the other hand show a reduction only in the CD8- cDCs and no defect in the $CD8^+$ compartment (Suzuki *et al.*, 2004). In addition to its role at progenitor stages, Irf8 has also been shown to be important for DCs function. It affects maturation of DCs, production of

IL12 and controls the migration of DCs to the draining lymphnodes. Furthermore, Irf8 has a role in inducing tolerogenic functions of DCs (Schiavoni *et al.*, 2002).

1.5.3 BATF3

The basic leucine zipper transcription factor ATF-like 3 (BATF3) has a very specific role in DC development. Mice deficient for BATF3 show a reduction in $CD8^+$ DCs and no effect on the rest of the DC subsets (Hildner *et al.*, 2008). This suggests a more downstream action of BATF3 as compared to PU.1 and IRFs. Lack of BATF3, however, can be compensated by cytokines, like IL12 and IFN-γ. This compensatory mechanism is elicited through BATF and BATF2 molecules interacting with other transcription factors like IRF8 with similar efficiency as BATF3 due to their conserved nature (Grajales-Reyes *et al.*, 2015).

1.5.4 ID2

Inhibitor of DNA binding protein 2 **(**ID2) is a protein that acts by inhibiting the binding of HLH protein E2-2 to the DNA. E2-2 and ID2 act antagonistically. Since E2-2 expression is required for pDC development and negatively affects cDC development (Ghosh *et al.*, 2010), ID2 deficient mice show a marked reduction in the $CD8^+$ and $CD103^+$ DCs but an increase in pDCs (Hacker *et al.*, 2003). Conversely, ectopic expression of ID2 in early HSPCs causes an increase in the formation of cDCs and inhibits the formation of pDCs.

Transcription factor KO	CD8+ cDCs	CD11b+ cDCs	pDCs	Langerhans Cells
PU.1	- - -	- - -		
IRF2	=	- -		
IRF4	=	- -	-	
IRF8	- - -	=	- -	-
ID2	- - -	=	+	- - -
BATF3	- - -	=	=	=

Table 1.1 – Crucial transcription factors in DC development. Here the impact of their respective deletion in HSPCs on the change of distinct DC subpopulations is summarized. "+" stands for increase, "–" stands for decrease and " =" stands for no change. HSPC denotes hematopoietic stem and progenitor cell; cDc conventional dendritic cell; pDC plasmacytoid dendritic cell.

1.6 C/EBPα

CCAAT/enhancer binding protein alpha (C/EBPα) belongs to the family of basic region-leucine zipper transcription factors and is encoded by the intron-less gene *Cebpa*. C/EBPα functions by homo-dimerizing and binding to consensus sequences on certain promoters and enhancer regions, but can also hetero-dimerize with other C/EBP family members as well as with other transcription factors, such as c-JUN. While its C-terminal basic region leucine zipper (BR-LZ) domain mediates DNA-binding and protein–protein interactions with other transcription factors, the two transactivation domains are responsible for E2F repression and represent binding sites for proteins such as SWI/SNF and CDK2/CDK4 (Ramji and Foka, 2002). C/EBPα was identified as a key regulator of adipogenesis and lipid accumulation in fat cells along with affecting glucose and lipid metabolism in the liver (Fuchs *et al.*, 2010).

C/EBPα also plays a well-defined role in steady-state haematopoiesis specifically by eliciting a lineage-instructive function in MPPs towards the myeloid lineage (Wölfler *et al.*, 2010) and also affects malignant haematopoiesis (Zhang *et al.*, 2016). C/EBPα expression is observed at low levels in HSCs, it increases towards CMPs with its highest levels found in GMPs. In contrast, its expression decreases as CMPs differentiate to MEPs (Hasemann *et al.*, 2014). *Cebpa* deficient mice have normal numbers of CMPs but are deficient in GMPs and the successive granulocytic stages, indicating that C/EBPα is indispensable for CMP to GMP transition (Zhang *et al.*, 2004). Accordingly, C/EBPα is absolutely needed for the formation of granulocytes and monocytes (Zhang *et al.*, 1997). A heterozygous deletion of *Cebpa* does not have any effect on the development of myeloid lineage, showing that mono-allelic expression of C/EBPα suffices for normal function (Wouters *et al.*, 2009).

Concerning DC development, C/EBPα has been implicated to play a role in early stages of DC differentiation but is known to be dispensable for later stages and their maturation and functioning (Welner *et al.*, 2013)**.** However, little is known about the mechanisms through which C/EBPα might influence DC development.

1.7 Cytokine control of steady-state DC development

Along with the intrinsic control of DC development offered by transcription factors, the stepwise differentiation of DC is extrinsically controlled by multiple cytokines. The most crucial cytokines and their role in DC development are discussed in the next paragraphs (Metcalf, 2009).

1.7.1 FLT3 Ligand

FMS like tyrosine kinase 3 ligand (FLT3 ligand/FLT3L) is the most pivotal cytokine in the development of DCs. FLT3 (CD135), the receptor for FLT3L, is expressed on HSCs, MPPs CLPs and a subset of CMPs. All steady-state DCs arise from these FLT3 expressing cells. FLT3 expression is also maintained on the DC progenitor stages like MDPs, CDPs and pre-cDCs. In contrast, FLT3 expression is lost as progenitors become committed to non-DC lineages (Merad *et al.*, 2013). FLT3 is also expressed in bone marrow derived DCs cultured *in vitro* with FLT3L, but is lost in the moDCs. FLT3L is ubiquitously expressed by endothelial cells, T-cells and the stroma of multiple tissues.

FLT3- or FLT3L-deficient mice are deficient in MDPs, CDPs and all subsequent DCs, underpinning the crucial role of this axis in DC development (McKenna *et al.*, 2000). Reintroduction of FLT3 causes a spurt in the number of DCs formed in the blood and the tissues. Inhibition of FLT3 and deficient formation of cDCs causes an increase in the levels of FLT3L in the serum, suggesting the presence of a negative feedback loop that causes the high levels of FLT3L to induce DC development (Onai *et al.*, 2006).

1.7.2 GM-CSF

CSF2/GM-CSF is a cytokine that is necessary for myelopoiesis. It was shown to have an effect on *in vitro* generation of DCs from progenitors (Inba *et al.*, 1992). Though *in vivo*, mice deficient for GM-CSF or its receptor have normal number of lymphoid-resident $CD8^+$ cDCs but has a minor effect causing reduction of the non-lymphoid $CD103^+$ and $CD11b^+$ subsets (Vremec *et al.*, 1992). It has been implicated to have a more important role in the final maturation of cDCs by inducing antigen cross presentation abilities. In contrast to its rather neglectable role in steady-state DC formation, GM-CSF is upregulated under inflammatory conditions thereby inducing moDC development. Thus, the current understanding is that GM-CSF plays an important role in emergency DC development but is dispensable in steady-state (Banchereau and Palucka, 2005).

1.7.3 M-SCF

CSF1/M-CSF regulates the survival, proliferation and differentiation of monocytes and macrophages. Its receptor CSF1R/CD115 is expressed in the myeloid lineage specifically at the CMP and GMP stage (Schmid *et al.*, 2010). It is also expressed at the MDP stage but at lower levels and decreases even more as progenitor cells are committed towards the DC lineage through low to almost no expression at the CDP stage (Pixley and Stanley, 2004). M-CSF deficient mice show no changes in steady-state DC subsets, but display a significant reduction in numbers of epidermal LCs. Thus, M-CSF is assumed to be dispensable for steady-state DC development but has a role in LC and moDC formation.

1.8 Tumor necrosis factor alpha (TNFα)

TNFα is a highly pleotropic cytokine, which has important effects on multiple physiological processes like inflammation, anti-tumour responses and homeostasis. TNFα works through its two cognate receptors TNF-R1 and TNF-R2 (Aggarwal, 2003)). TNF-R1 is constitutively expressed on most cell types, whereas TNF-R2 expression is induced by inflammatory stimuli. Hence it is assumed that TNFα elicits most of its functions through its interaction with TNF-R1 (Grell *et al.*, 1995). Concerning haematopoiesis, TNFα has been shown to exert various, and sometimes even opposite effects. Some studies revealed that TNFα inhibits the proliferation of colony forming cells from $Lin^{-}Sca^{+}$ HSPCs indicating detrimental effects on HSPC functions (Zhang *et al.*, 1995). However, other studies suggest the opposite, showing that TNFα induces the secretion of IL3 and GMCSF in human $CD34^{+}$ cells *in vitro* (Caux *et al.*, 1992). Concerning DC development, TNFα was shown to induce CSF2R upregulation in DC precursors and to induce IL3 production leading to generation of DCs from human cord blood (Ouaaz *et al.*, 2002). Defective engraftment of Lin^{-} BM isolated from TNFα deficient mice in WT recipient mice and subsequent loss of hematopoietic reconstitution along with the other observations suggest that TNFα might play the role of a hematopoietic inducer in the bone marrow niche (Pearl-Yafe *et al.*, 2010). More recently, consistent with past studies on the pleotropic nature of TNFα in the hematopoietic process, it has been shown that TNFα induces apoptosis of myeloid progenitors while promoting HSC survival and hematopoietic regeneration through an NFκB based axis. It has also been identified that TNFα causes further myeloid differentiation of HSCs by driving PU.1 upregulation (Yamashita and Passegué, 2019).

2. Hypothesis and aim of the thesis

DCs are essential immune cells and develop from HSCs via several specification steps and intermediate progenitor stages. Each of these differentiation steps involves cell fate decisions that successively restrict developmental potential. Distinct cytokines and several transcription factors are known to play important roles in these processes. The transcription factor C/EBPα has a well-documented function in myelopoiesis, but its role in DC development has not yet been fully established. Given the fact that the vast majority of steady-state DCs is derived from myeloid progenitors, we hypothesise that C/EBPα does play a crucial role in early DC development. Byusing transgenic mouse models enabling lineage tracing of *Cebpa*-expressing hematopoietic cells as well as inducible bone marrow-specific knock-out of the *Cebpa* gene, we specifically aim for identifying the distinct developmental step during DC formation at which C/EBPα exerts its critical role. Furthermore, we employ gene expression studies as well as *in vitro* primary cell culture studies to elucidate probable molecular mechanisms through which DC development is controlled by C/EBPα. Given the reported interaction of C/EBPα with other transcription factors as well as distinct cytokines known to be important for DC development, we hypothesise that C/EBPα exerts its role in early DC development via interaction and modulation of these transcription factors and cytokines, like PU.1, Irf8 as well as TNFα, respectively.

3. Materials and Methods

3.1 Mouse models

All mice were on a C57Bl/6 background and housed in specific pathogen-reduced conditions at the central animal facility of the Medical University of Graz. Mouse experiments were approved by the Austrian Federal Ministry for Science, Research, and Economy (GZ: BMWF-66.010/0017-II/3b/2014, GZ: BMWFW-66.010/0027-WF/V/3b/2017).

3.1.1 Bone marrow specific conditional *Cebpa* knockout mice (*Mx1^{Cre}/Cebpa$^{F/F}$ mice)*

Mx1^{Cre}/Cebpa$^{F/F}$ mice were obtained from Prof. Daniel G Tenen, Department of Hematology, Harvard University, Boston, MA, USA. As previously described (Zhang *et al.*, 2004), the bone marrow specific conditional knockout of the *Cebpa* gene is achieved by Mx1 promoter induced Cre based excision of a 6.2 kb region which includes the single exon of *Cebpa* while the rest includes 3.3 kb upstream of the transcription start site on 5‘ end and 1.6 kb downstream on the 3‘ end (Represented in Figure 3.1).

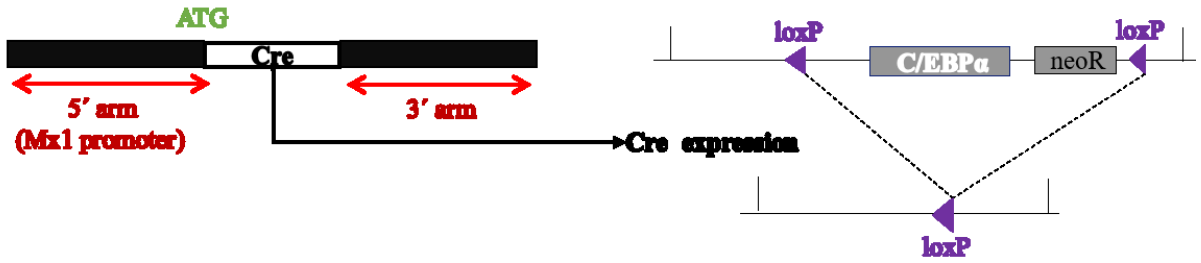

Figure 3.1 – Schematic representation of *Mx1^{Cre}/Cebpa$^{F/F}$* mice. Cre denotes Cre recombinase.

3.1.1.1 Breeding strategy

Cebpa$^{F/F}$ mice were maintained by breeding them with mice with the same genotype. The breeding strategy to obtain *Mx1^{Cre}/Cebpa$^{F/F}$* experimental as well as *Cebpa$^{F/F}$* control (wildtype, WT) mice is depicted in figure 3.2.

The *Cebpa$^{F/F}$* mice were also considered as the control (WT) for *in vitro* experiments and the *Mx1^{Cre}/Cebpa$^{F/F}$* were considered the experimental mice (KO). The rest of the breeding strategy is shown in detail in the figure 3.2.

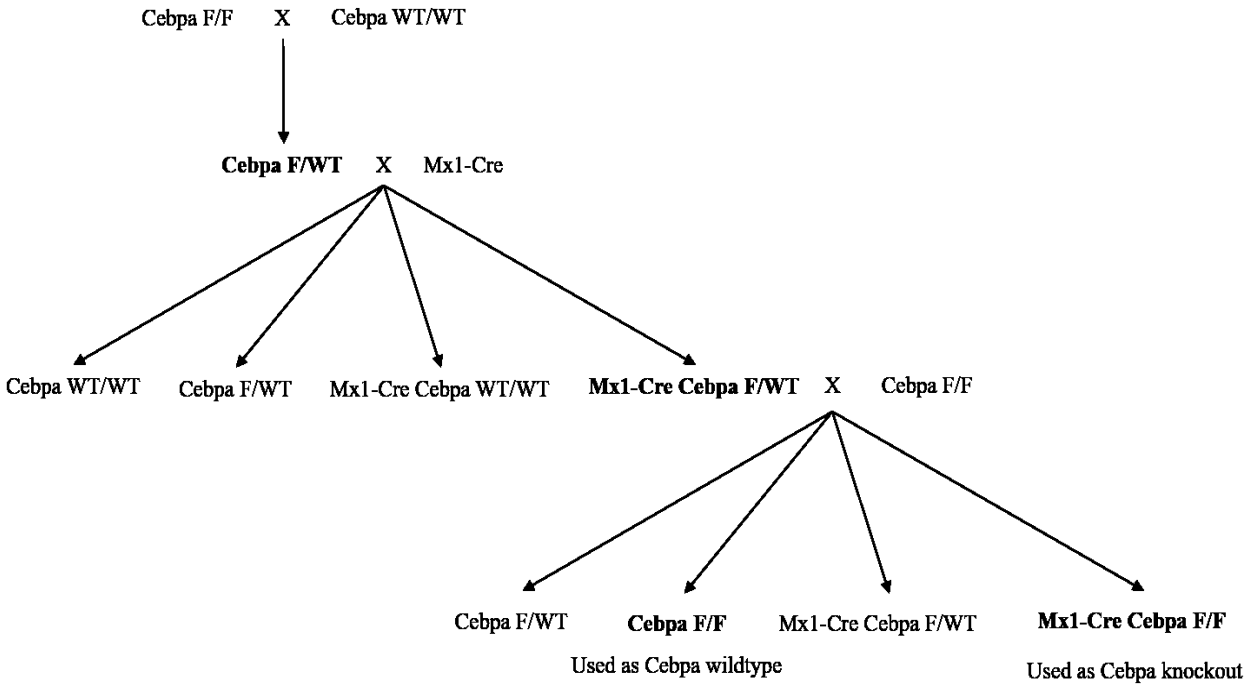

Figure 3.2 – Breeding strategy used to obtain *Mx1^{Cre}/Cebpa$^{F/F}$* mice. Cre denotes Cre recombinase; F floxed; WT wild type.

3.1.1.2 Inducing BM-specific deletion of *Cebpa* by pIpC injection

To induce the knockout of the *Cebpa* gene, mice aged 8 – 14 weeks were given intraperitoneal injections of polyinosinic: polycytidylic acid (pIpC, Sigma) four times every other day. The mice were then sacrificed 18-21 days after the last pIpC injection for further processing.

3.1.1.3 Genotyping of *Mx1^{Cre}/Cebpa$^{F/F}$* mice

DNA extraction was performed according to manufacturer´s protocols using the KAPA express Extraction kit (KAPA Biosystems) from tail tips or ear punches. To analyze the *Cebpa$^{F/F}$* locus, DNA was amplified with HotStarTaq DNA polymerase kit (Qiagen, Germany), while for targeting the *Mx1^{Cre}* locus the KAPA2G fast Genotyping mix (KAPA biosystems) was used. Specific primers for both loci and PCR programs used are listed in Table 3.1.

The PCR products were then assayed electrophoretically at constant voltage of 100V for 40 – 60 minutes on a 2-4 % agarose (BioZym ME agarose) gel containing 8µL/100mL of GelRed Nucleic acid Stain (Biotium), along-with a 100bp GeneRuler DNA ladder (Thermofisher). The imaging was done using the ChemiDoc imaging system (Bio-Rad technologies). An example for genotyping of *Mx1^{Cre}/Cebpa$^{F/F}$* mice is given in figure 3.3.

Primer	Sequence
Cebpa F/F 1	TGG CCT GGA GAC GCA ATG A
Cebpa F/F 2	CGC AGA GAT TGT GCG TCT TT
Mx1-Cre 1	TCC CCG CAG AAC CTG AAG ATG TTC G
Mx1-Cre 2	GCC AGA TTA CGT ATA TCC TGG CAG C

PCR-Program - Cebpa Floxed			
Initial activation	94°C	15min	
Denaturation	94°C	30 sec	35 cycles
Annealing	60°C	30sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	
Storage	4°C	Hold	

PCR-Program - Mx1-Cre			
Initial activation	95°C	3min	
Denaturation	95°C	40 sec	30 cycles
Annealing	62°C	40sec	
Extension	72°C	40 sec	
Final extension	72°C	10 min	
Storage	4°C	Hold	

Table 3.1 – Sequence of primers used in PCR and PCR cycler program used to genotype *Mx1^{Cre}/Cebpa$^{F/F}$* mice. Cre denotes Cre recombinase; F floxed; WT wild type.

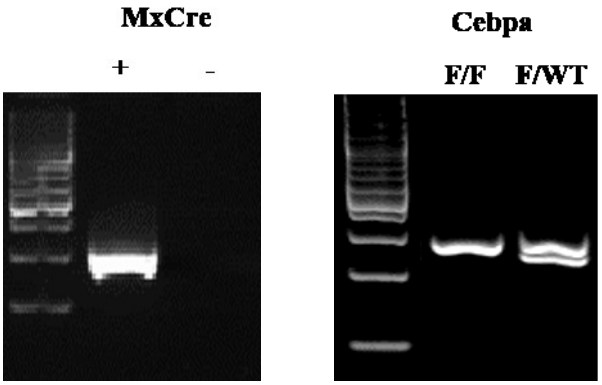

Figure 3.3 – Representative gel images of the PCR products from genotyping PCR of *Mx1^{Cre}/Cebpa$^{F/F}$* mice. Cre denotes Cre recombinase; F floxed; WT wild type.

3.1.2 *Cebpa^{Cre} ROSA26 EYFP* reporter mice

In the *Cebpa^{Cre}* mouse model the gene *Cre recombinase* is expressed under the endogenous *Cebpa* promoter. Crossing these mice with homozygous ROSA26 EYFP (enhanced yellow fluorescent protein) reporter strains, which have a floxed STOP cassette upstream of an introduced EYFP gene into the continuously expressed ROSA26 locus (Represented in figure 3.4), was used to trace *Cebpa*-expressing cells and their progeny in various hematopoietic cell compartments as described (Wölfler *et al.*, 2010).

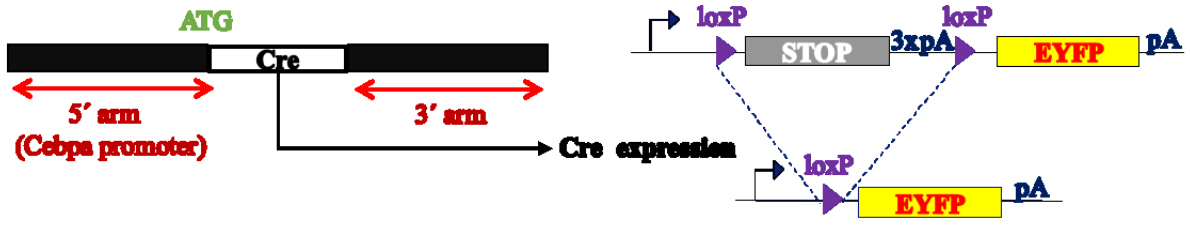

Figure 3.4 – Schematic representation of the reporter mice. EYFP denotes enhanced yellow fluorescence protein.

3.1.2.1 Breeding strategy

To obtain *Cebpa*^{Cre} *ROSA26 EYFP* reporter mice, the following breeding strategy was used (figure 3.5).

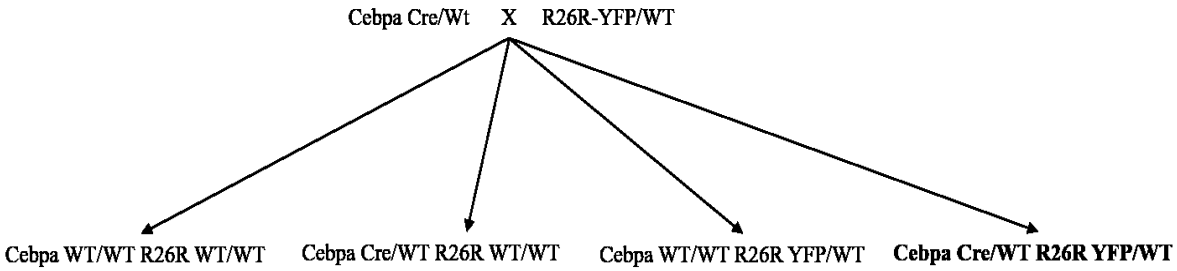

Figure 3.5 – Breeding strategy used to obtain *Cebpa*^{Cre} *ROSA26 EYFP* reporter mice. EYFP denotes enhanced yellow fluorescence protein; Cre Cre recombinase; WT wild type.

3.1.2.2 Genotyping

DNA extraction for genotyping of these mice was done as described above (3.1.1.3). For specific DNA amplification, the HotStarTaq DNA polymerase kit (Qiagen, Germany) was used with primers and PCR conditions as shown in Table 3.2. An example for genotyping of *Cebpa*^{Cre} *ROSA26 EYFP* mice is given in figure 3.6.

Primer	Sequence
CreSeqF1	CGC TAA GGA TGA CTC TGT T
CebpaR1	GTC TCA AGG AGA AAC CAC CAC
CebpaF3	GCT CTA AGA CCC AGC AGC C
CebpaExR1	CGG CTC CAC CTC GTA GAA GTC

PCR-Program - Cebpa Floxed			
Initial activation	95°C	5min	
Denaturation	94°C	30 sec	35 cycles
Annealing	50°C	30sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	
Storage	4°C	Hold	

Primer	Sequence
Primer Rosa2	GCG AAG AGT TTG TCC TCA ACC
Primer Rosa3	GGA GCG GGA GAA ATG GAT ATG
Primer LacZ-F-981	ACC TTT CTG GGA GTT CTC TGC TG

PCR-Program - Cebpa Floxed			
Initial activation	95°C	3min	
Denaturation	95°C	30 sec	35 cycles
Annealing	65°C	30sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	
Storage	4°C	Hold	

Table 3.2 – Primer sequence and PCR conditions of Cebpa Cre and Rosa26 EYFP

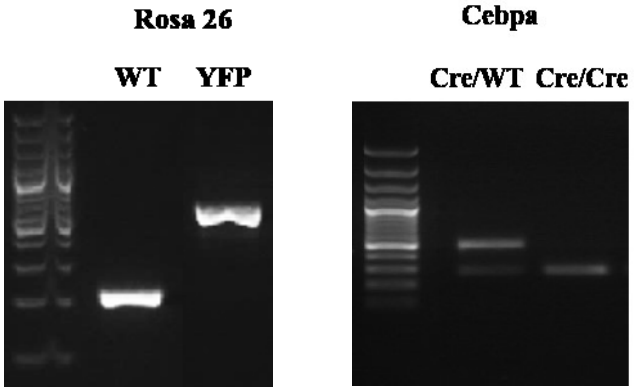

Figure 3.6 – Representative gel images of the PCR products from genotyping PCR of *Cebpa*^{Cre} *ROSA26 EYFP* mice; EYFP denotes enhanced yellow fluorescence protein; Cre Cre recombinase; WT wild type.

3.1.3 *Mx1*^{Cre}*/Cebpa*$^{F/F}$ R26 EYFP mice

For experiments where we co-cultured *Cebpa* KO and *Cebpa* WT BM progenitor cells, we introduced a ROSA26 EYFP reporter allele for identification of *Cebpa* KO cells.

3.1.3.1 Breeding strategy

The breeding strategy to obtain these mice is depicted in figure 3.7.

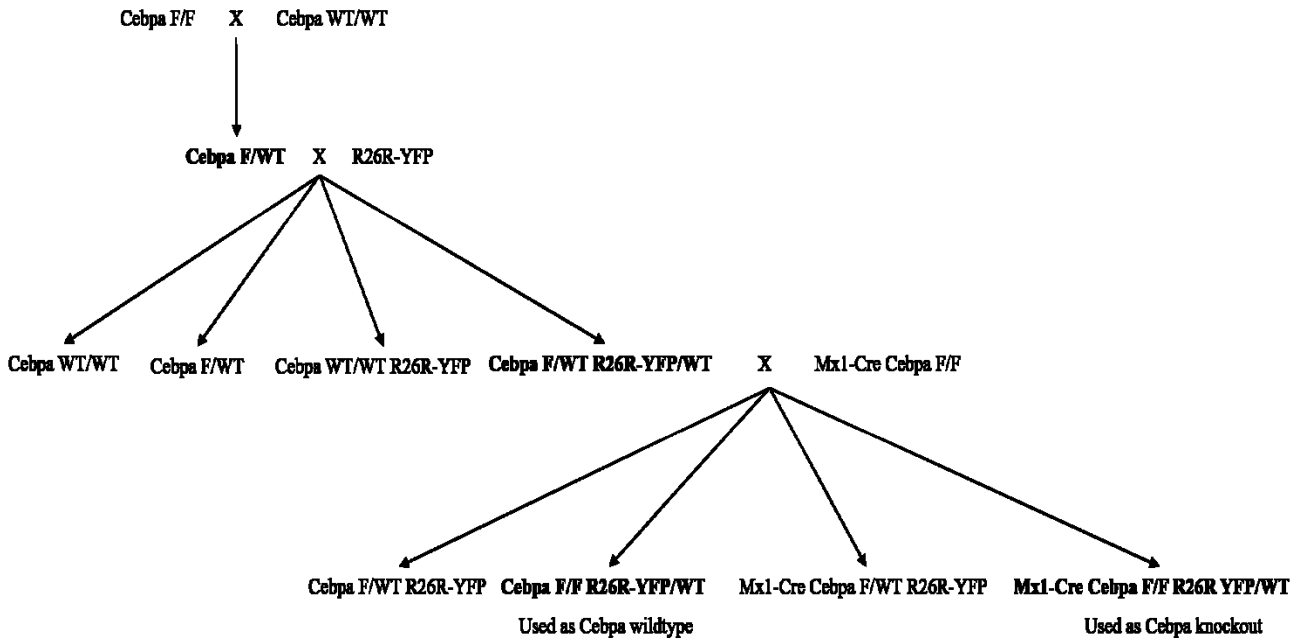

Figure 3.7 – Breeding strategy used for generation of *Mx1^{Cre}/Cebpa$^{F/F}$ ROSA26 EYFP* mice; YFP denotes enhanced yellow fluorescence protein; Cre Cre recombinase; WT wild type.

3.1.3.2 Genotyping

Genotyping of these mice was done as explained previously (3.1.1.3). Mx1Cre and Cepba Floxed are assayed as decribed indetail in section 3.1.1.3. R26R-EYFP was analysed using the KAPA2G fast Genotyping mix (KAPA biosystems) with specific primers and PCR conditions as described in section 3.1.2.2.

3.2 Mice sample preparation

The samples were obtained from mice that were sacrificed via ethical sacrifice methods involving anesthetization of mice using 4% Isoflurane mixed with O2 (1.5L/min) and subsequent cervical dislocation.

3.2.1 Bone marrow preparation

This was done for all bone marrow (BM) cell isolations. The sacrificed mice were dissected to obtain the hind limbs, sternum, pelvic girdle and spine. The bones were macerated in a mortar and pestle using Hanks balanced salt solution (here after referred to as HBSS buffer) (Thermofisher scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) (Thermofisher scientific, Waltham, MA, USA) and 1% penicillin-streptomycin (Thermofisher scientific, Waltham, MA, USA). The macerated tissue was then passed through a 70-µm nylon filter (Thermofisher scientific, Waltham, MA, USA) for removal of fibrous material. The cells were washed with HBSS buffer and centrifuged at 1500 rpm for 5 mins. The supernatant was discarded and the cell pellet was used for further processing. For erythrocyte lysis, cells were resuspended in 10% PharmLyse solution (BD Biosciences, San Jose, CA, USA) for 2-3 minutes depending on requirement. The cells were then resuspended in 40 mL of HBSS buffer and centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 1 mL of HBSS buffer and used for further experiments based on requirement.

3.2.2 Spleen preparation

The spleen was sliced into small pieces for ease of maceration. The spleen was then macerated and passed through a 70-µm nylon filter for removal of fibrous material. The cells were washed with 40 mL of HBSS buffer and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the cell pellet resuspended in 1 mL of 10% PharmLyse solution for 2-3 minutes depending on requirement. The cells were then resuspended in 40 mL of HBSS buffer and centrifuged at 1500rpm for 5 minutes. The supernatant was discarded and the cells were resuspended in 1 mL of HBSS buffer and used for further experiments based on requirement.

3.2.3 Enrichment of hematopoietic stem and progenitor cell (HSPCs) from bone marrow

After isolation of total BM cells as indicated above (see 3.2.1), cells were counted using a Neubauer chamber and diluted to a concentration of $20x10^6$/mL. Enrichment of lineage negative (lin^-) HSPCs was done using the IMag Hematopoietic Progenitor Enrichment Kit (BD Biosciences) according to manufacturer's protocol. In brief, the diluted BM cells were mixed with a cocktail of primary antibodies against the lineage markers TER119, CD11b, Gr-1, CD11c, CD3e, and B220 at a concentration of 5µl of cocktail per 1 $x10^6$ cells. The cells were resuspended well and incubated for 15 minutes on ice. Afterwards cells were resuspended in 10% IMag buffer (BD Biosciences) and centrifuged at 300g for 7 mins. The supernatant was discarded and the pellet was resuspended in secondary antibodies tagged to magnetics particle with the volume corresponding to the volume of primary antibody. After incubation at 4° C for 30 minutes, the mixture was resuspended in 10% IMag buffer and adjusted to a total volume of 3.5 – 4 mL. The tubes were then moved to a magnetic separator for 15 minutes for negative selection of the Lin^- fraction in the solution. After careful collection of the remaining suspension it was centrifuged at 1500rpm for 7 mins. For higher purity the magnetic particles are resuspended in IMag buffer and subjected to the magnetic separator a second time. The cells obtained after two rounds of magnetic separation are resuspended in HBSS/RPMI based of requirement of experiment as described in the specific experiments and used for all further experiments.

3.2.4 Hematopoietic stem and progenitor cell (HSPC) culture set up for *in vitro* dendritic cell generation

Lin^- cells were used after enrichment for culture set up to emulate the development of DCs *in vitro*. For these experiments, lin^- cells were counted using a Neubauer chamber and plated into 24 well plates at 1 $x10^6$ cells per mL of RMPI1640 containing 10%FBS, 1% penicillin – streptomycin, 1% glutamine and 0.1% β-mercaptoethanol (complete culture medium) as well as the indicated cytokines. For co-culture experiments regular plating was complemented with an additional well containing 5x 10^5 lin^- cells from *Cebpa* WT mixed with 5x 10^5 cells from *Cebpa* KO mice.

All cytokines and chemokines were purchased from Peprotech (Rocky Hill, NJ, USA). FLT3L was used at 200ng/mL, whereas TNFα, IL-1β, MIP1a, MIP2 were used at 20ng/mL. The total culture period was 8 days, with re-supplementing of cytokines once on day 3/4, and media twice on days 3/4 and 5/6.

3.3 Flow Cytometry Analysis and Cell Sorting

Flow cytometry analysis was done using a LSRII flow cytometer (BD Biosciences), and cell sorting was performed on a FACS Aria II (BD Biosciences). Samples were analyzed as single cell suspensions with different antibody panels as per the requirement of the experiment and number of events acquired also depended on the individual experiment. For analysis of mature DCs in both the spleen and BM either before or after culture, antibody cocktail containing antibodies against MHCII and CD11c were used. Specifically for the step wise analysis a cocktail with antibodies against CD117, CD115, FLT3, and the lineage panel and CD11c were used. For exclusion of dead cells 7AAD was used in all cocktails. Flow cytometry data analysis was done using Kaluza software (Beckman Coulter, Krefeld, Germany). The list of all antibodies used is given in table 3.3. The bone marrow progenitor populations were defined by the following markers: MPPs: lin^{-} $FLT3^{+}$ $CD117^{+}$ $CD115^{-}$, MEPs: $lin^{-}CD117^{+}$ $Sca\text{-}1^{-}$ $CD16\text{-}32^{-}CD34^{-}$, CMPs: lin^{-} $CD117^{+}$ $Sca\text{-}1^{-}CD16\text{-}32^{-}CD34^{+}$, GMPs: lin^{-} $CD117^{+}$ $Sca\text{-}1^{-}CD16\text{-}32^{+}CD34^{+}$, MDPs: lin^{-} $FLT3^{+}$ $CD117^{+}$ $CD115^{+}$ and CDPs: lin^{-} $FLT3^{+}$ $CD117^{int}$ $CD115^{+}$. Mature DCs in the spleen or bone marrow were defined as $CD11c^{+}MHCII^{+}$ cells.

Table 3.3 – Antibodies used for Flow cytometry and flow assisted cell sorting.

Anitbodies	**Supplier**	**Identifier**
Biotin Mouse Lineage Panel	BD bioscience	# 559971
PE-Cy7 conjugated Ly6 - A/E (sca1) (Clone D7)	eBiosceince	# 25-5981-82
APC conjugated CD117(Ckit) (Clone 2B8)	BD pharmigen	# 553356
7-AAD	BD pharmigen	# 559925
APC conjugated CD11c (Clone HL3)	BD pharmigen	# 550261
APC-Cy7 conjugated MHC II (Clone M5/114.15.2)	Biolegend	# 107627
PE-Cy7 conjugated CD117(Ckit) (Clone 2B8)	eBioscience	# 25-1171-82
APC conjugated FLT3 (Clone A2F10)	Biolegend	# 135310
PE-Conjugated CD115 (Clone AFS98)	eBioscience	# 12-1152-82

3.4 mRNA microarray analysis

Lin^- HSPCs were isolated from bone marrow of 4 *Cebpa* WT and KO mice as described before (see 3.2.1 and 3.2.3). $Lin^-CD117^+FLT3^+$ HSPCs were double sorted and used either for immediate gene expression analysis (untreated cells, UT) or treated (T) with FLT3L for 4 h. RNA was isolated from the WT/KO cells with or without FLT3L treatment (referred to hereafter as WT(T)/WT(UT) and KO(T)/KO(UT)) using the RNeasy Micro Kit (Qiagen, Hilden, Germany). The quality of RNA was checked by using the Bioanalyzer BA2100 (Agilent, Foster City, CA, USA) and samples with a RNA integrity number (RIN) value greater than 8 were used for further processing. cDNA amplification was done using Ovation Pico WTA System (NuGEN technologies inc. Redwood city, CA, USA). The hybridization and expression data were analyzed on Gene-expression Console v.1.1, while the Mouse Gene 1.0 ST array was used for whole transcriptome analysis both as per manufacturer instructions (Affymetrix, Santa Clara, CA, USA). The data was normalized and analyzed using Partek genomic suite software (Partek inc. St. Louis, MO, USA). The gene expression data were further analyzed in detail using BioVenn (Hulsen, de Vlieg and Alkema, 2008) and detailed pathway analysis was done on Network Analyst 3.0 web-based freeware (Xia, Gill and Hancock, 2015; Zhou *et al.*, 2019). All microarray expression data are available at GEO: GSE146288.

3.5 RNA isolation and Quantitative RT-PCR

All experiments using RNA were performed in an RNase free environment. $5x10^5$ cells were resuspended in 300 µl of resuspension buffer (provided by manufacturer) and passed through a syringe needle for homogenization. The mixture was used for RNA isolation using RNeasy Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer´s protocol. The RNA quality and quantity were checked with the NanoDrop™ 2000/2000c (Thermofisher scientific, Waltham, MA, USA). cDNA synthesis was done with 200 ng of RNA using the High Capacity cDNA kit (Applied Biosystems, Foster City, CA, USA) and the PCR protocol used was as per manufacturers protocol (see Table 3.4). The cDNAs were then assayed with TaqMan™ Universal PCR Master Mix and TaqMan™ probes (Applied Biosystems) for *Cebpa, Irf8, Sfpi1, Cx3Cr1, β-Actin*, and *Gapdh* using the PCR protocol recommended by the manufacturer (see Table 3.5) in the LightCycler 480 96 well plate PCR machine (Roche, Penzberg, Germany). The expression was calculated using the ΔΔCt method and represented as x-fold change.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Table 3.4 – **cDNA synthesis** - reverse transcription PCR conditions.

	Polymerase activation	Denature	Anneal/Extend	Hold
Temperature (°C)	95	95	60	4
Time	10 min	15 secs	1 min	∞

Table 3.5 – **RT-qPCR**- Thermocycler conditions for relative gene expression analysis.

Gene Name	Assay ID
Cebpa	Mm00514283_s1
Gapdh	Mm99999915_g1
β-Actin	Mm02619580_g1
Cx3Cr1	Mm02620111_s1
Irf8	Mm00492567_m1
Sfpi.1	Mm00488140_m1

Table 3.6 – Gene specific p**robes used to identify and normalize expression of indicated genes.**

3.6 Cytokine Profiling Assay

Lin$^-$ HSPCs were isolated from bone marrow of 6 *Cebpa* WT and KO mice each using the procedure described before (3.2.1 and 3.2.3). The HSPCs were then set in culture for 6 h in complete culture medium with 200 ng/mL of FLT3L to emulate the start of DC lineage development *in vitro*. After 6 h, the supernatants from the cultured HSPCs were collected for multiplex cytokine analysis. 100 µL of supernatant was used to detect differences in the cytokine profile between *Cebpa* WT and KO HSPCs cultured with FLT3L. Cytokine profiling was done in a 96-well plate format using the Bio-Plex™ system (Biorad Laboratories Inc., Hercules, CA, USA) with a custom cytokine panel (Table 3.4) according to the manufacturer's protocol. In brief, 100 µL beads coupled with specific antibodies is added to each well. The wells are washed twice with 100 µL of wash buffer post coating with beads. Then pipette 100

µL of standards, samples and controls. The plate is then incubated in a shaker at 850 rpm for 15 minutes. The wells are washed thrice with 100 µL of wash buffer, then 100 µL of streptavidin-PE was added and plate was incubated at 850 rpm for 10 minutes. The wells were washed again thrice with 100 µL of wash buffer. 125 µL of assay buffer was added to the wells and resuspended by shaking for 30s. The plate was the inserted into the Bio-Plex system to read the output.

CCL2/JE/MCP-1	G-CSF
CCL3/MIP-1 alpha	GM-CSF
CCL4/MIP-1 beta	IFN-gamma
CCL5/RANTES	IL-1 alpha
CCL20/MIP-3 alpha	IL-1 beta
CXCL1/KC	IL-4
CXCL2/MIP-2	M-CSF
CXCL10/IP-10/CRG-2	TNF-alpha
CXCL12/SDF-1 alpha	

Table 3.7 – List of cytokines assayed for in the custom Bio-Plex™ assay.

3.7 Allogenic Mixed Lymphocyte Reaction (MLR) Assay

This assay was performed to determine the T-cell activation potential of *in vitro* generated DCs. DCs were generated *in vitro* from both *Cebpa* WT and KO HSPCs as described above (see 3.2.1 and 3.2.3) and used on day 8. The *in vitro* generated DCs are irradiated at with 30grays for 1 cycle and used as stimulator cells. BALB/c mice were sacrificed ethically and dissected to obtain the spleen. The spleen was processed as described above (3.2.2). The splenic cell pellet was enriched for T-cells with the magnetic particle based T-cell enrichment kit (BD Biosciences) as per manufacturer's protocol. The procedure for T-cell enrichment is similar to the lineage depletion protocol described in section (3.2.3) with antibodies cocktail specific to T-cells. 10^5 splenic T-cells were then seeded with increasing numbers of *in vitro* generated DCs (10^3, 3×10^3, 10^4, and 3×10^4) in triplicates. After 5 days of co-culture, radioactive thymidine was added to the culture and uptake was calculated as a measure of T-cell proliferation after 16 h.

3.8 Preparation of DC cytospins and Pappenheim staining

To determine the morphology of *in vitro* generated DCs, 100 µL of cells after culture period were collected separately. The cells were pipetted into the funnel of the cytospin slide setup (Figure 3.8) and the whole merged unit is spun at 400rpm for 10 minutes. The slides were then further used for Pappenheim staining. Briefly, the slides were incubated for 4 minutes in a May Grunwald solution. The slides were then rinsed with distilled water, followed by 20 minutes of incubation in Giemsa solution. After washing thoroughly in distilled water to remove all traces of excess stain, the slides were air dried, and coverslips were mounted with mounting fluid and allowed to set. The images were captured on a bright-field inverted microscope at 40X and 100X magnification.

3.9 In-silico promoter and transcription factor binding site prediction

To predict the presence of C/EBPα binding sites on the promoter region of possible target genes, we extracted the distal promoter regions for the genes from Ensembl genome browser (Yates *et al.*, 2020). The extracted sequence was confirmed using the Eukaryotic promoter database (EPD) (Dreos *et al.*, 2015) and Promo DB catalogued promoter sequences by using n-Blast to align all sequences. The confirmed promoter sequence was then loaded into the PROMO 3.0 (Messeguer *et al.*, 2002; Farré *et al.*, 2003) online platform with the C/EBPα transcription factor as the query. The prediction was conducted with a dissimilarity cut off of 15 to remain stringent. The predicted promoter binding sites were confirmed using a second online platform named CONSITE. The genes that showed a minimum of 3 predicted binding sites for C/EBPα were used for further analysis.

3.10 Statistical Analysis

Prism 6 software (GraphPad, La Jolla, CA, USA) was used to perform statistical analysis. Unless otherwise stated, a two-sided t-test with Welch's correction (two-tailed) was used for comparisons of two groups (mostly *Cebpa* WT vs. *Cebpa* KO). All results in this thesis are presented as the mean ± SD, and a p-value of < 0.05 was considered statistically significant.

4. Results

The following section reports in detail the results obtained in this project. A large part of the data presented here has been published in Anirudh MS et al. (Anirudh *et al.*, 2020))

4.1 Tracing *Cebpa* expression during DC lineage development

C/EBPα has a well-documented role in myelopoiesis. Previous studies have traced its expression in the myeloid and lymphoid lineages and across multiple progenitor stages (Wölfler *et al.*, 2010), but little is known about its expression pattern in the DC lineage. Hence, we aimed to trace the expression of *Cebpa* in the DC lineage using the *Cebpa*^{Cre}-EYFP reporter mouse model, which enables tracking of cells that either express C/EBPα or are the progeny of C/EBPα-expressing (*Cebpa*/EYFP^{+}) cells in steady-state haematopoiesis (Wölfler *et al.*, 2010).

With this approach, we identified that about 60% of mature CD11c^{+}MHCII^{+} cDC in the spleen were *Cebpa*/EYFP positive (Figure 4.1). Since *Cebpa* is known to be hardly expressed at the mature DC stage (Welner *et al.*, 2013), these data indicate that the origin of the majority of splenic DCs is from *Cebpa*/EYFP expressing progenitors. Indeed, detailed analysis of DC progenitors stages revealed that there is an increase in the percentage of *Cebpa*/EYFP^{+} cells from MPPs, which have ~6% *Cebpa*/EYFP^{+} cells, via CMPs with ~34% *Cebpa*/EYFP^{+} cells, increasing to its maximum at the MDP stage with ~64% *Cebpa*/EYFP^{+} cells (Figure 4.1). In accordance to previous findings, the percentage of *Cebpa*/EYFP^{+} cells was highest among GMPs (Wölfler *et al.*, 2010). The gating strategies of all progenitor stages as well as a respective example of EYFP expression for each progenitor population are shown in Figures 4.2 - 4.4. The fact of no further increase in the percentage of *Cebpa*/EYFP^{+} cells was found beyond the MDP stage indicates that *Cebpa* is not expressed or at least at lower levels in late progenitors as well as mature DCs. This finding is well in line with findings that C/EBPα is dispensable in later stages of DC development (Welner *et al.*, 2013).

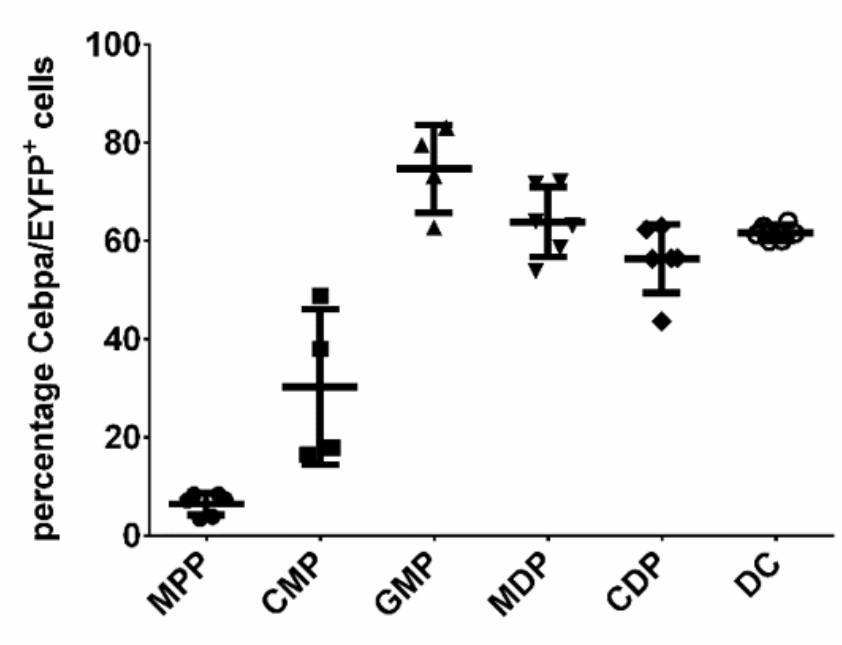

Figure 4.1 – Percent of *Cebpa*/EYFP positive cells in distinct progenitor cell compartments and mature splenic CD11c^{+}MHCII^{+} DCs. An increase in the percentage of *Cebpa*/EYFP positive cells is observed which corresponds to the more differentiated state of the cells and the percentage reaches its highest level at the GMP and MDP stages. Data represent the mean (+ SD) percentage of *Cebpa*/EYFP positive cells of the given subpopulation obtained from 4–6 mice. [Figure reproduced from (Anirudh *et al.*, 2020)]. EYFP denotes enhanced yellow fluorescence protein; DC dendritic cells; SD standard deviation.

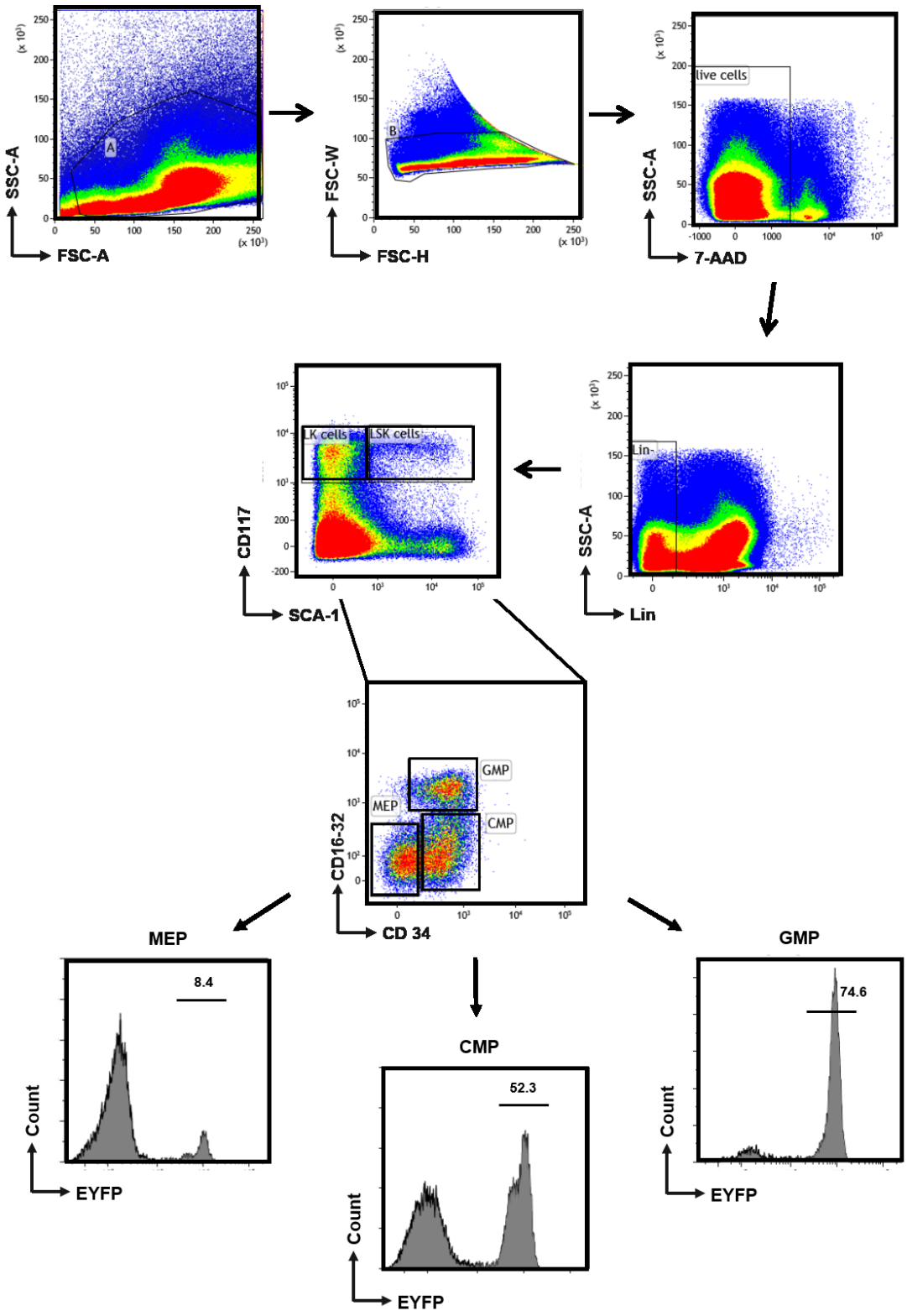

Figure 4.2 – Gating strategy used to identify MEP, CMP and GMP populations. Representative FACS plots and histograms for EYFP expression are shown. MEPs are defined as $Lin^{-}CD117^{+}$ $Sca\text{-}1^{-}$ $CD16\text{-}32^{-}CD34^{-}$ cells, CMPs as Lin^{-} $CD117^{+}$ $Sca\text{-}1^{-}CD16\text{-}32^{-}CD34^{+}$ cells and GMPs as Lin^{-} $CD117^{+}$ $Sca\text{-}1^{-}CD16\text{-}32^{+}CD34^{+}$ cells. MEP denotes MEP megakaryocyte erythroid progenitor; CMP common myeloid progenitor; GMP granulocyte monocyte progenitor.

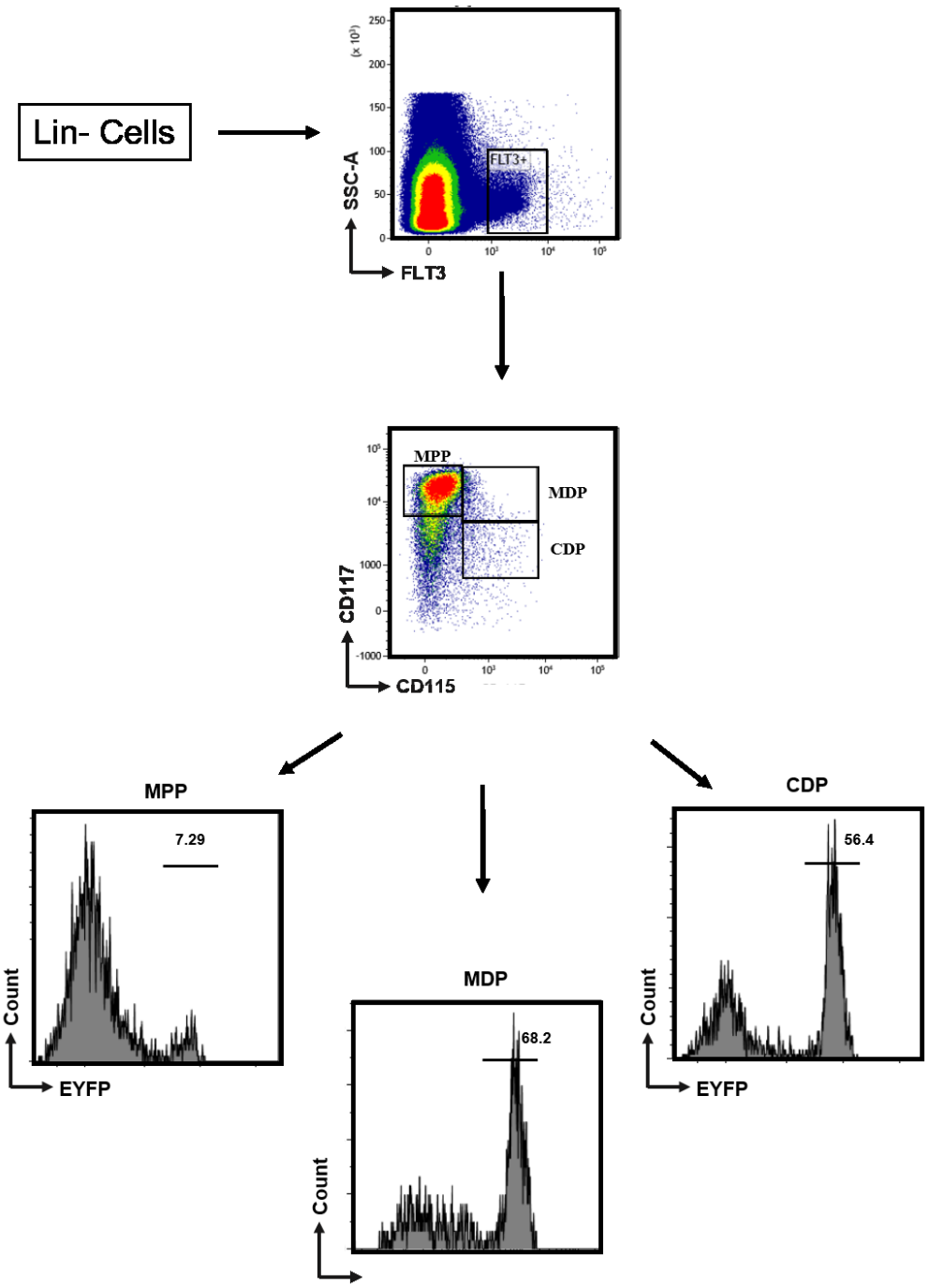

Figure 4.3 – Gating strategy used to identify DC progenitor stages (MPPs, MDPs and CDPs). Representative FACS plots and histograms for EYFP expression are shown. MPPs are defined as Lin^{-} $FLT3^{+}$ $CD117^{+}$ $CD115^{-}$ cells, MDPs as Lin^{-} $FLT3^{+}$ $CD117^{+}$ $CD115^{+}$ cells and CDPs as Lin^{-} $FLT3^{+}$ $CD117^{int}$ $CD115^{+}$ cells. MPP denotes multipotent progenitor; MDP monocyte dendritic cell progenitor; CDP common dendritic cell progenitor

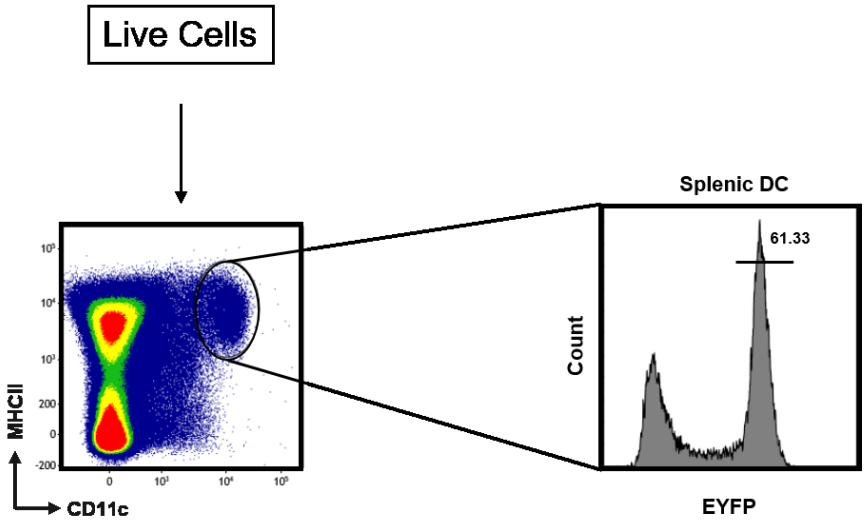

Figure 4.4 – Gating strategy used to identify mature splenic DCs. Representative FACS plots and histograms for EYFP expression are shown. DC population as a whole (encompassing pDCs and cDC) are identified as $CD11c^{+}$ MHC $II^{+/int}$ cells. DC denotes dendritic cells; pDC plasmacytoid dendritic cell; cDC conventional dendritic cell.

Since the *Cebpa*^{Cre}-EYFP reporter mouse model does not measure actual *Cebpa* expression levels, we next analysed mRNA expression of *Cebpa* in the respective progenitor populations. For this purpose, we used publicly available data sets from gene expression studies in murine myeloid progenitors. The most comprehensive data set was published by Miller and colleagues in 2012 (Miller *et al.*, 2012). In accordance with our results in *Cebpa*^{Cre}-EYFP reporter mice, we found an increase in *Cebpa* mRNA expression from CMPs towards MDPs, but no further increase in the more differentiated CDPs (Figure 4.5). As expected, the highest levels of *Cebpa* mRNA expression were found in GMPs, as reported by our group previously (Wölfler et al., 2010)

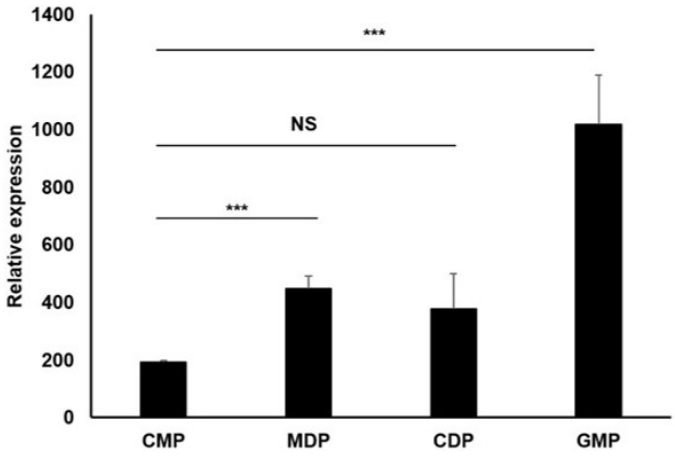

Figure 4.5 – Database based analysis of *Cebpa* mRNA expression - (Miller *et al.*, 2012)(GEO: GSE15907) in DC progenitors (*** $p < 0.001$; NS denotes not significant). *Cebpa* mRNA expression increases from CMP to MDP and GMP stages but remains stable after MDP stage and shows no significant change at the CDP stage. CMP denotes common myeloid progenitor; MDP monocyte dendritic cell progenitor; GMP granulocyte monocyte progenitor; CDP common dendritic cell progenitor. [Figure reproduced from (Anirudh *et al.*, 2020)]

4.2 Role of C/EBPα in *in vitro* DC development

The study of DC development and biology has been challenged by the lack of cells lines that can recapitulate accurately all the intermediate developmental stages. Hence, to study physiological DC development, we used primary BM HSPCs isolated from $Mx1^{Cre}Cebpa^{F/F}$ mice, in which pIpC treatment results in deletion of the *Cebpa* gene in BM cells including HSPCs (Zhang *et al.*, 2004). DC development was then assayed by using a FLT3L based *in vitro* HSPC culture system mimicking *in vivo* DC formation (Naik *et al.*, 2007).

4.2.1 *Cebpa* deletion significantly reduces DC formation from HSPCs *in vitro*

To assess the effect of *Cebpa* deletion on *in vitro* DC formation, we isolated HSPCs from both WT and KO mice and analysed numbers of mature $CD11c^{+}MHCII^{+}$ DCs after stimulation with FLT3L for 8 days. The WT HSPCs were able to give rise to mature DCs by the end of the culture period. As compared to the WT HSPCs, in the KO HSPCs DC formation was hindered and the number of mature DCs formed was reduced to about 20% (Figure 4.6). This result clearly indicates a role for *Cebpa* in FLT3L-induced formation of DCs *in vitro*.

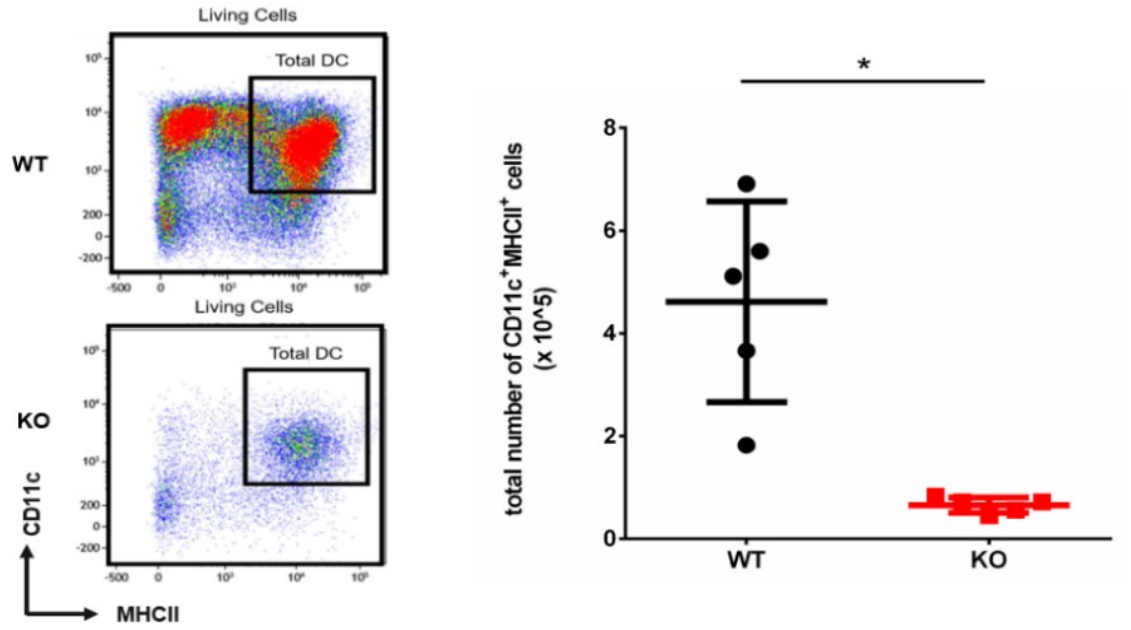

Figure 4.6. FLT3L-induced *in vitro* formation of mature CD11c$^+$MHCII$^+$ DC is significantly reduced in *Cebpa* KO, as compared to WT HSPCs ($p < 0.01$, $n = 5$ mice per group). Results of one experiment representative from three independent experiments are shown. A representative FACS plot for the analysis of mature CD11c$^+$MHCII$^+$ DC is shown on the left side (upper panel: WT, lower panel: KO). DC denotes dendritic cells. [Figure reproduced from (Anirudh *et al.*, 2020)].

4.2.2 *Cebpa* deletion in HSPCs reduces the formation of MDPs and block their transition to CDPs

To understand the timing and at which specific progenitor stage *Cebpa* deletion plays a role in DC development a stepwise analysis of early DC progenitor formation after FLT3L-stimulation was performed. In accordance to literature reporting that a lack of *Cebpa* results in increased formation of FLT3$^+$ MPPs (Ye *et al.*, 2013), higher numbers of CD117$^+$FLT3$^+$ progenitors were observed in *Cebpa* KO mice (Figure 4.7 and 4.8). Although CD117$^+$FLT3$^+$ cells were higher in number, the percentage as well as total numbers of cells maturing and forming CD117^{hi}CD115$^+$ MDPs was reduced and almost none of the MDPs transitioned to CD117$^{lo/int}$CD115$^+$ CDPs on days 1 and 3 in cultures with *Cebpa* KO HSPCs (Figure 4.7, 4.8 and 4.9). These results clearly indicate that decreased formation of mature CD11c$^+$MHCII$^+$ DCs from *Cebpa* KO HSPCs is due to reduced numbers of MDPs and a stop in transition to CDPs after FLT3L-stimulation.

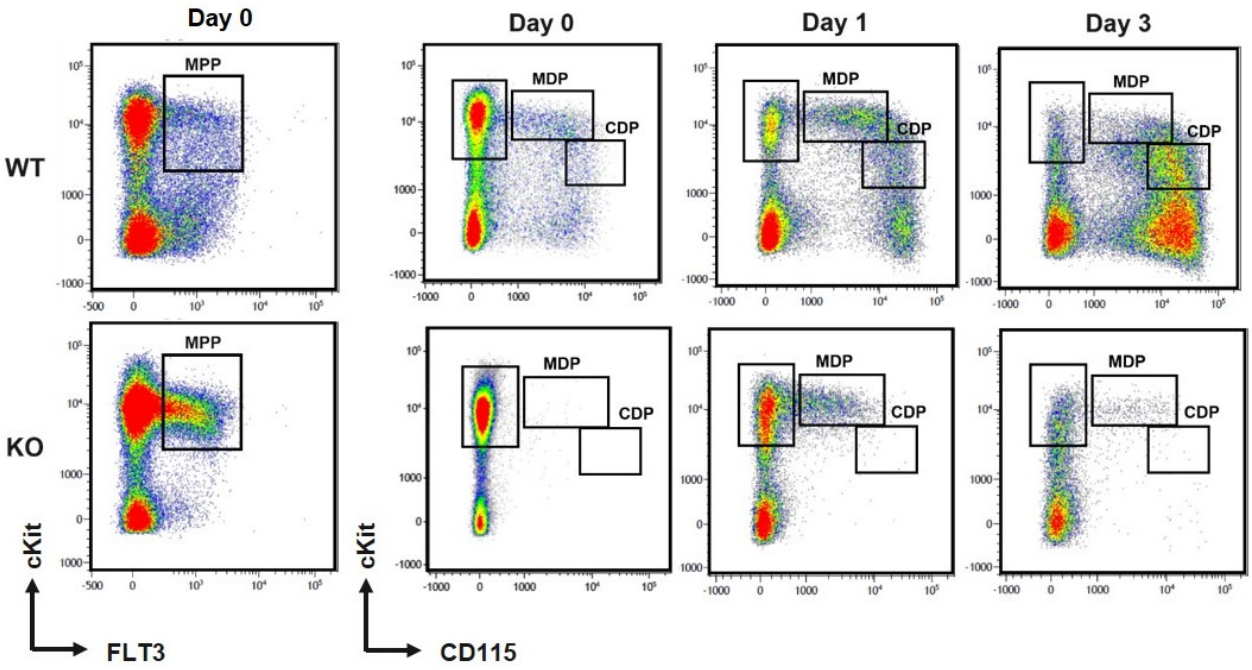

Figure 4.7 – *Cebpa* deletion causes MDP reduction and blocks transition to CDPs - Cells analyzed by flow cytometry on days 0, 1, and 3 of culture (upper panels: WT, lower panels: KO). While KO HSPCs do not show progression through the different progenitor stages during *in vitro* culture, WT cells progress through the stages towards a mature phenotype with time. HSPCs denotes hematopoietic stem and progenitor cells; MPP multipotent progenitor; MDP monocyte dendritic cell progenitor; CDP common dendritic cell progenitor. [Figure reproduced from (Anirudh *et al.*, 2020)].

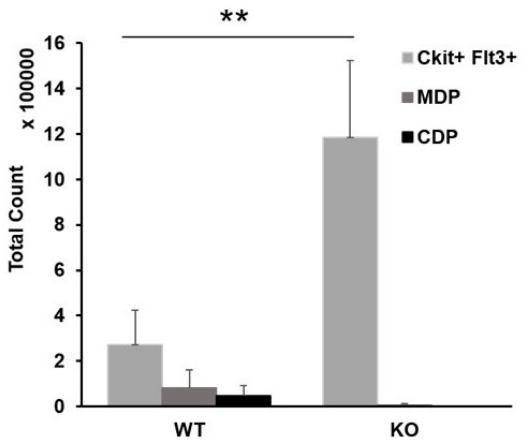

Figure 4.8 – Day 0 comparison of progenitor populations - On day 0 higher numbers of $FLT3^{+}CD117^{+}$ progenitors are present in the bone marrow of Cebpa KO mice, whereas there is almost no detectable presence of MDPs and CDPs as compared to WT mice. All data represent mean + SD of 5 mice (** $p < 0.001$). MDP denotes monocyte dendritic cell progenitor; CDP common dendritic cell progenitor. [Figure reproduced from (Anirudh *et al.*, 2020)].

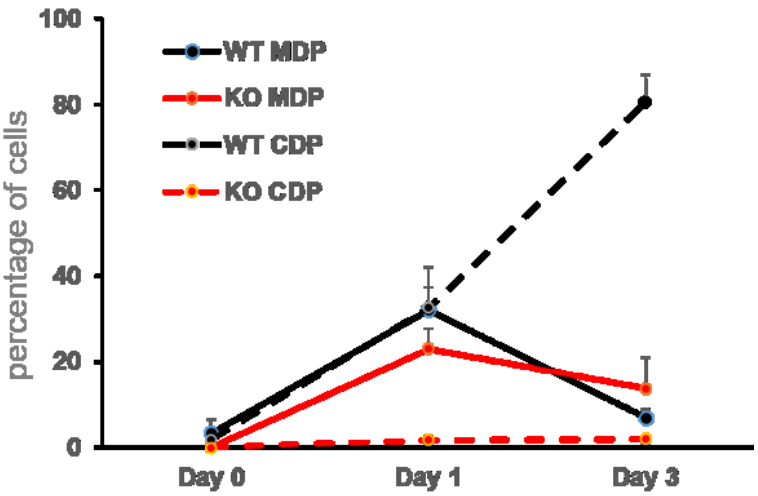

Figure 4.9 – Formation of successive progenitor stages with time - Analysis of early differentiation after FLT3L treatment *In vitro* shows a reduced formation of MDPs and a block of transition towards CDPs in *Cebpa* KO HSPCs. The graph shows the percentage of MDPs and CDPs of total CD117^{+} HSPCs on days 0, 1 and 3 to trace change in the progenitor progression with time. All data represent mean + SD of 5 mice. HSPCs denotes hematopoietic stem and progenitor cells; MDP monocyte dendritic cell progenitor; CDP common dendritic cell progenitor. [Figure reproduced from (Anirudh *et al.*, 2020)]

4.3 Lack of C/EBPα has a strong effect on the transcriptional landscape of early DC progenitors and on their cytokine secretory profile

Having deciphered that DC development is indeed affected by the absence of C/EBPα at stages as early as MDPs and their transition to CDPs, we next aimed to investigate mechanisms by which C/EBPα exerts its role in early DC development. To this end, we investigated mRNA expression in FLT3^{+} HSPCs of Cebpa WT and KO mice to reveal involved mechanisms.

4.3.1 Gene expression analysis reveals robust changes in the transcriptional profile of KO FLT3^{+} HSPCs

For gene expression analysis, double sorted FLT3^{+} HSPCs were isolated from both WT and KO mice and either treated with FLT3L for 4 hours or left untreated. Genes were considered to be differentially regulated when their level of expression displayed a >1.5 fold change between groups and they also showed a p value < 0.05 after 5% FDR correction. The number of differentially expressed genes after FLT3L stimulation was 2149 in WT FLT3^{+} HSPCs and 2128 in FLT3^{+} KO HSPCs. Interestingly, only 1104 genes were commonly deregulated in both groups (Figure 4.10)

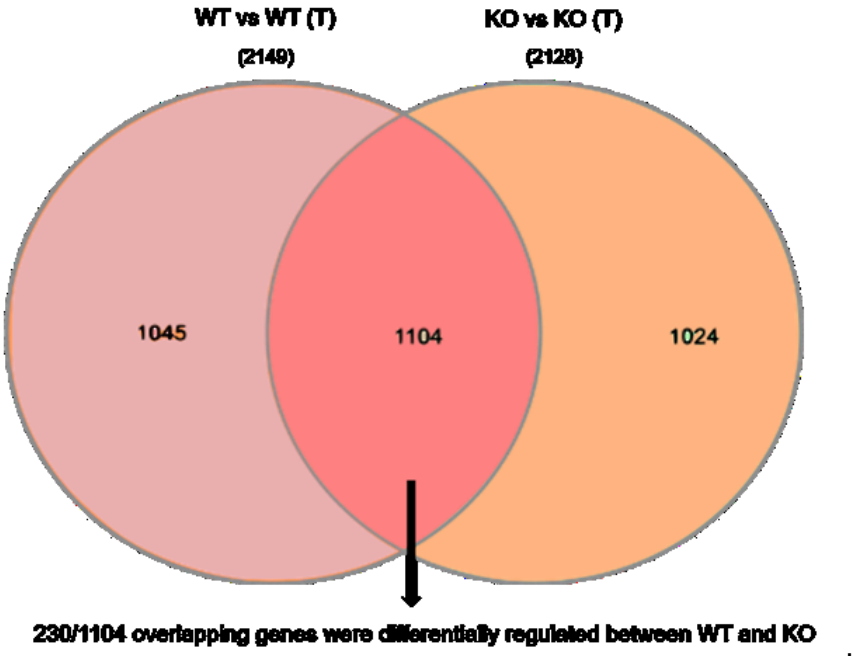

Figure 4.10 - Venn diagram showing the overlap between differentially regulated genes in FLT3$^+$ HSPCs after FLT3L stimulation in the presence and absence of C/EBPα. All genes included for analysis show >1.5 fold difference with a FDR 5% corrected significance of < 0.05. HSPCs denotes hematopoietic stem and progenitor cells; FDR false discovery rate. [Figure reproduced from (Anirudh *et al.*, 2020)]

The obtained gene lists were further analysed for pathway network identification. We first analysed the exclusive WT vs. WT (treated, T) (1045 genes) and the exclusive KO vs. KO (T) (1024 genes) lists. This analysis revealed that genes linked to the TNFα signalling cascade and to NFκB signalling were among the most prominently affected pathways found in WT FLT3$^+$ HSPCs after FLT3L stimulation. In complete contrast, these pathways were not affected in KO FLT3$^+$ HSPCs (Figure 4.11) indicating that the changes in TNFα- and NFκB-signalling pathways are strongly related to the presence of C/EBPα.

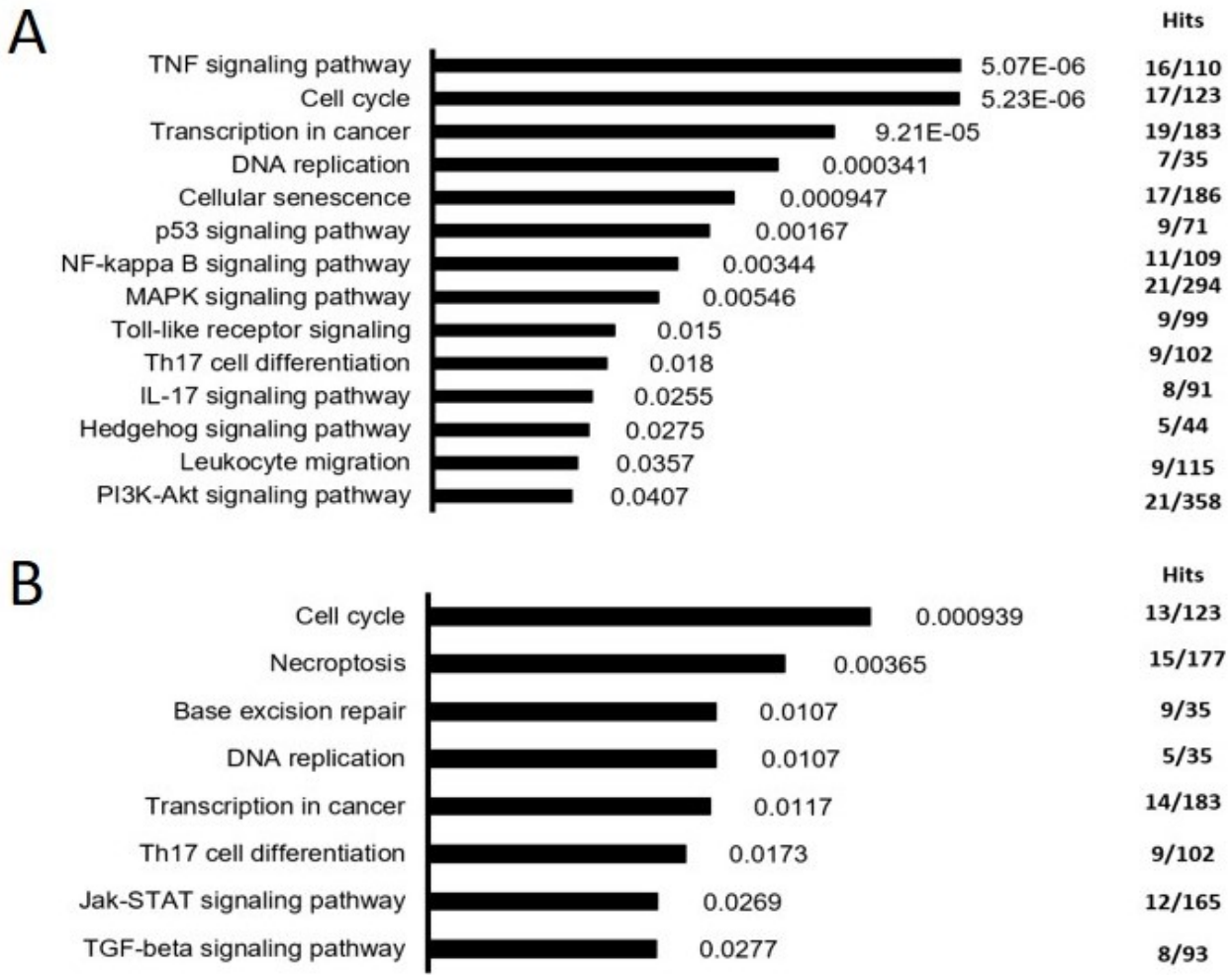

Figure 4.11 - Pathway analysis of gene lists differentially regulated exclusively in (A) WT vs WT(T) and (B) KO vs KO (T)FLT3$^+$ HSPCs. Bars represent the significance of hits. On the right, the number of differentially expressed genes (hits) as well as total number of genes per pathway. HSPCs denotes hematopoietic stem and progenitor cells; WT wild type; KO knock-out, T treated. [Figure reproduced from (Anirudh *et al.*, 2020)]

To get an even more comprehensive picture of C/EBPα-dependent mechanisms for future analysis, we used a combined list of genes containing the 1045 genes exclusively changed in *Cebpa* WT FLT3$^+$ HSPCs along with 230 genes out of the 1104 commonly changed genes, which were significantly differentially regulated between *Cebpa* WT and KO FLT3$^+$ HSPCs (see also Figure 4.10). Again, TNFα- and NFκB-signalling were among the most significant pathways along with a few other prominent pathways like cytokine-cytokine receptor interactions and haematopoietic cell lineage that were identified to be affected (see Figure 4.12). Interestingly, we also identified the genes for TNFα and IL-1β (*Tnf* and *Il1b*) to be the

most prominently differentially regulated cytokines on mRNA levels. Cytokine genes like *Csf1* and *Csf2* on the other hand were not changed at all between WT and KO FLT3^{+} HSPCs (Figure 4.13).

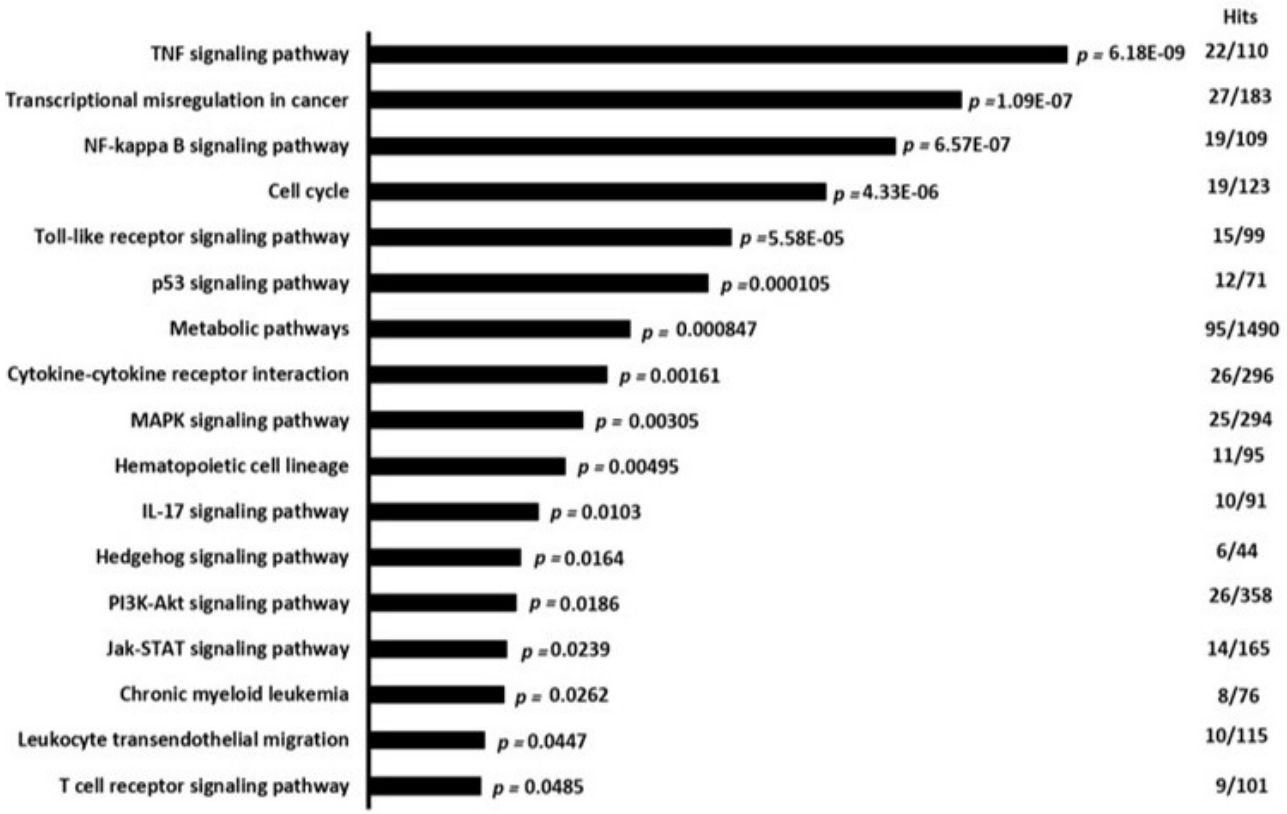

Figure 4.12 - Pathway analysis including all differentially modulated genes (n=1275) between WT and KO HSPCs after FLT3L treatment. On the right (hits), the number of differentially expressed genes are given as numerator, while the total number of genes per pathway are given as denominator. HSPCs denotes hematopoietic stem and progenitor cells; WT wild type; KO knock-out, T treated. [Figure reproduced from (Anirudh *et al.*, 2020)]

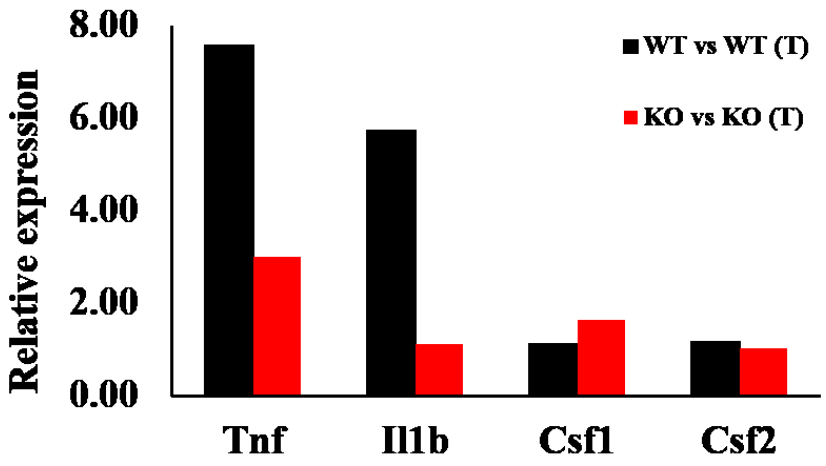

Figure 4.13 – Upregulation of relative expression of cytokine genes involved in DC development after FLT3L-stimulation of *Cebpa* WT vs. KO FLT3$^+$ HSPCs. Specific genes that encode for cytokines are seen to be affected in a C/EBPα - dependent manner, especially Tnf and Il1b, both of which are known to play roles in steady state DC development, whereas Csf1 and Csf2 are not affected. HSPCs denotes hematopoietic stem and progenitor cells; WT wild type; KO knock-out, T treated.

4.3.2 Bio-plex analysis reveals distinct cytokine secretory profile of WT and KO HSPCs

Having observed an effect of C/EBPα on genes involved in TNFα-signalling pathways as well as C/EBPα-dependent changes at mRNA levels for multiple cytokines including TNFα itself, we were next interested in investigating whether a change could also be seen in the secretory profiles of these cytokines in WT versus KO HSPCs after FLT3 stimulation. The supernatants of FLT3L-treated *Cebpa* WT and KO HSPCs were therefore assayed for the presence of a comprehensive panel of chemokines and cytokines. A stark reduction was observed in the levels of proinflammatory chemokines like CCL3 (MIP-1α), CCL4 (MIP-1β), and CCL5 (RANTES) in *Cebpa* KO HSPCs (Figure 4.14). Although not as pronounced as with the mentioned chemokines, inflammatory cytokines, like TNFα and IL-1β, were also significantly reduced in KO as compared to WT cell supernatants. Interestingly, cytokines like *Csf2* (GM-CSF) and *Csf1* (M-CSF) showed no C/EBPα-dependent changes consistent with the finding from the microarray data (Figure 4.14).

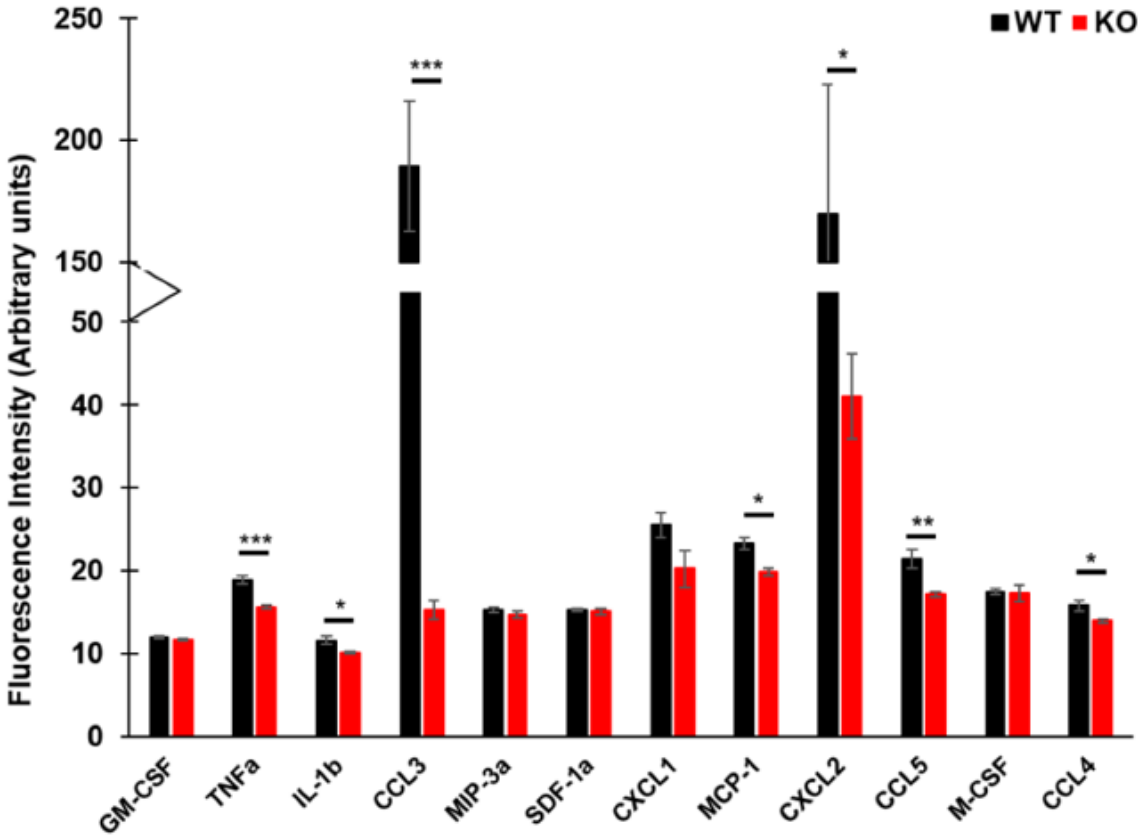

Figure 4.14 - Bioplex analysis of various cytokines and chemokines from supernatants isolated from FLT3L-treated WT and KO HSPCs ($n = 6$ mice, * $p < 0.01$, ** $p < 0.001$, and *** $p < 0.0001$). Cytokines and chemokines like CCL3, CXCL2, CCL5 and TNFa are significantly upregulated in WT HSPCs after FLT3L treatment as compared to KO HSPCs. HSPCs denotes hematopoietic stem and progenitor cells; WT wild type; KO knock-out. [Figure reproduced from (Anirudh *et al.*, 2020)].

4.4 Evaluation of a role of cytokines and/or chemokines in C/EBPα-dependent DC development

As outlined above, inflammatory cytokines and chemokines specifically CCL3, CXCL2, CCL5, TNFα and IL-1β were significantly upregulated in *Cebpa* WT as compared to KO HSPCs after FLT3L stimulation. Thus, we next tested whether the supplementation of these cytokines would have an effect on DC development in the absence of C/EBPα and/or whether such a C/EBPα-dependent paracrine effect of cytokines/chemokines was required for proper DC development.

4.4.1 WT HSPCs cannot rescue *Cebpa* KO HSPCs via a paracrine effect.

To answer whether direct physiological paracrine signalling of distinct cytokines and/or chemokines secreted from *Cebpa* WT HSPCs could restore formation of DCs from *Cebpa* KO HSPCs, we carried out co-culture experiments of HSPCs isolated from $Mx1^{Cre}/Cebpa^{F/F}$ *ROSA26 EYFP* mice. In this setting, *Cebpa* KO HSPCs express *EYFP* upon deletion of *Cebpa* making KO and WT HSPCs discriminable. Interestingly, although starting with a 50:50 ratio of WT vs. KO cells, by the end of the culture period the KO HSPCs were almost completely depleted and only WT HSPCs were able to produce mature DCs (Figure 4.15). These results clearly show that paracrine effects of secreted cytokines and/or chemokines are not sufficient to overcome the need for C/EBPα in early DC development. Instead, the lack of *Cebpa* probably reflects a cell intrinsic effect hampering early steps of DC development.

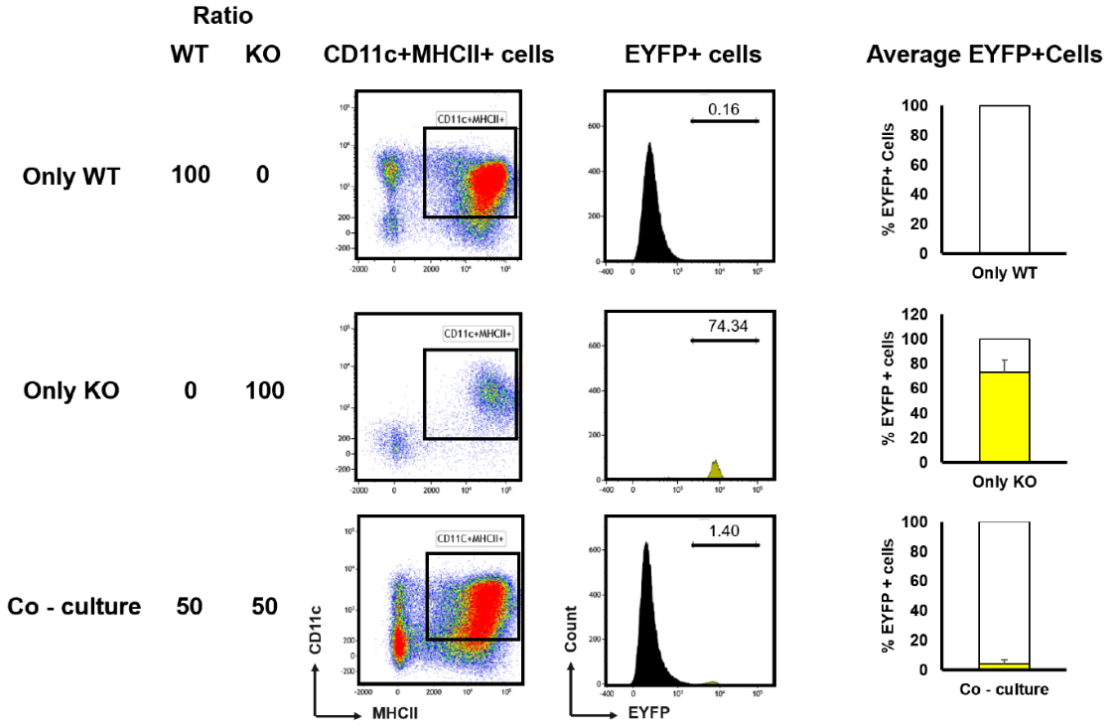

Figure 4.15. Paracrine effects of *Cebpa* WT HSPCs cannot restore DC formation in *Cebpa* KO HSPCs stimulated with FLT3L. In the *Cebpa* WT only setting HSPCs differentiate into mature DCs (upper panel), while in the *Cebpa* KO only setting DC differentiation is impaired and a few $EYFP^+$ DCs were formed (middle panel). In the co-culture setting using *Cebpa* WT and KO HSPCs in a 50:50 ratio at start of the culture period, $EYFP^+$ KO cells were almost completely depleted after 8 days and only WT HSPCs were able to produce mature DCs (lower panel). Data represent mean + SD (n = 4 mice). HSPCs denotes hematopoietic stem and progenitor cells; WT wild type; KO knock-out, EYFP enhanced yellow fluorescence protein.

4.4.2 TNFα has a rescuing effect on KO HSPCs and increase formation of mature DCs

As the gene expression results strongly pointed to deregulated TNFα- as well as NFκB-signalling in the absence of C/EBPα, we next wondered whether inducing these pathways by addition of pharmacological amounts of TNFα to FLT3L might be able to rescue DC formation in *Cebpa* KO HSPCs. Indeed, *Cebpa* KO HSPC cultured *in vitro* with FLT3L and TNFα formed increased numbers of mature $CD11c^+$ $MHCII^+$ DC almost comparable to numbers of cells derived from WT HSPCs (Figure 4.16 and 4.17). These results indicate that addition of TNFα at least partially can overcome the cell intrinsic block of DC development in *Cebpa* KO HSPCs.

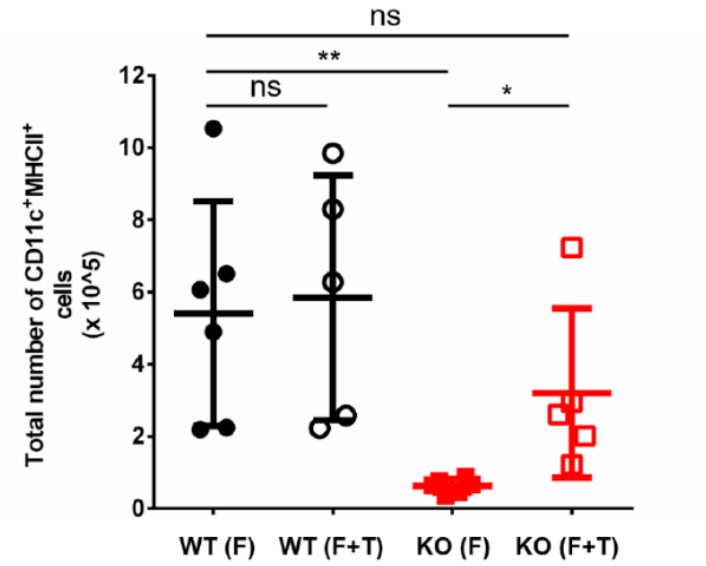

Figure 4.16. Addition of TNF increases the number of mature DCs formed from *Cebpa* KO HSPCs. *In vitro* formation of $CD11c^+MHCII^+$ DCs is increased, when KO HSPCs are cultured with a combination of FLT3L (F) and TNFα (T), as compared to FLT3L only. Data represent mean + SD (n = 5–6 mice, * $p < 0.01$, ** $p < 0.001$). NS denotes nonsignificant; HSPCs hematopoietic stem and progenitor cells; WT wild type; KO knock-out; F FLT3L and T TNFα. [Figure reproduced from (Anirudh *et al.*, 2020)]

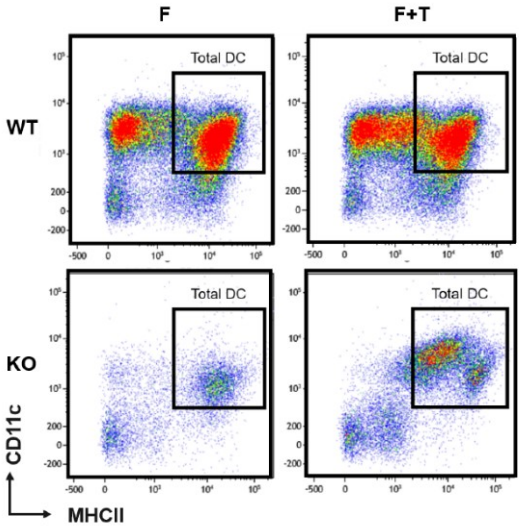

Figure 4.17. Addition of TNF rescues formation of mature DCs in *Cebpa* KO HSPCs. Representative FACS plots show an increased *in vitro* formation of $CD11c^{+}MHCII^{+}$ DCs in KO HSPCs cultured with a combination of FLT3L (F) and TNFα (T), as compared to FLT3L only. HSPCs denotes hematopoietic stem and progenitor cells; WT wild type; KO knock-out; F FLT3L and T TNFα. [Figure reproduced from (Anirudh *et al.*, 2020)]

In contrast to TNFα, treatment with a combination of FLT3L and MIP-1α (CCL3) or MIP-2 (CXCL2) had no effect on the potential of *Cebpa* KO HSPCs to form mature DCs (Figure 4.18).

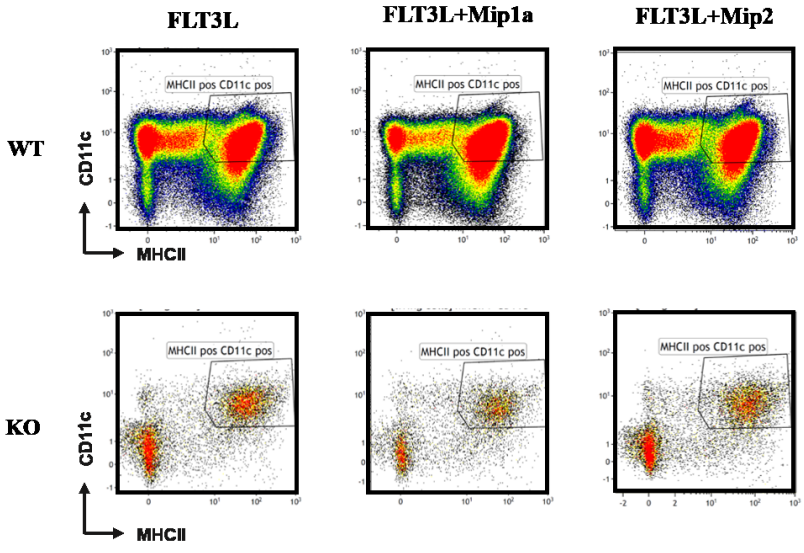

Figure 4.18 –MIP-1α and MIP-2 cannot restore DC development in *Cebpa* KO HSPCs. Representative FACS plots do not show increased *in vitro* formation of $CD11c^{+}MHCII^{+}$ DCs in KO HSPCs cultured with a combination of FLT3L and MIP-1α or MIP-2 as compared to FLT3L alone. HSPCs hematopoietic stem and progenitor cells; WT wild type; KO knock-out. [Figure reproduced from (Anirudh *et al.*, 2020)]

4.4.3 DCs generated from *Cebpa* KO HSPCs by TNFα and FLT3L are functionally active and morphologically identical to WT cells

To prove that the DCs generated with the combination treatment of FLT3L and TNFα from *Cebpa* KO HSPCs were fully functional, mature DCs, we checked for cellular morphology and found them to be comparable to DCs generated from WT HSPCs (Figure 4.19). Furthermore, an allogenic mixed lymphocyte reaction revealed that DCs generated from *Cebpa* KO HSPCs treated with FLT3L and TNFα could indeed activate naïve T-cells in a comparable manner to WT cells obtained with FLT3L (Figure 4.20), while sufficient numbers of mature DC were not obtained from KO HSPCs treated with FLT3L alone to perform the assay. This suggests that additional TNFα stimulation might indeed bypass the early differentiation block in MDPs caused by the lack of C/EBPα.

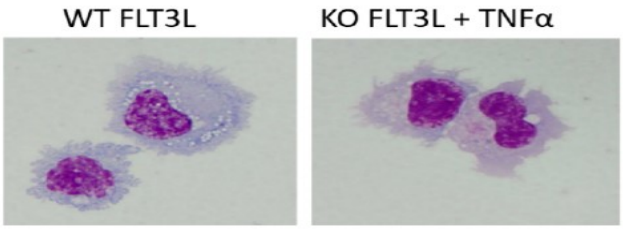

Figure 4.19. Morphology of DCs formed from KO HSPCs by TNFα and FLT3L are similar to WT DCs. Morphological comparison of DCs from WT HSPCs treated with FLT3L and DCs of KO HSPCs treated with TNFα and FLT3L for 8 days. HSPCs hematopoietic stem and progenitor cells; WT wild type; KO knock-out. [Figure reproduced from (Anirudh *et al.*, 2020)]

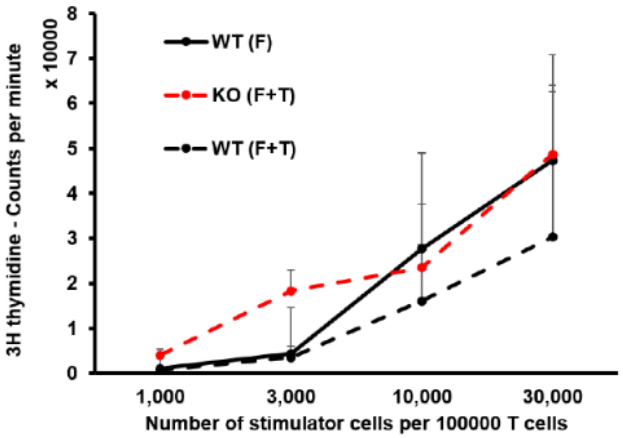

Figure 4.20. Allogenic MLR after 5 days of stimulation shows comparable T-cell activation potential of DCs from WT HSPCs treated with FLT3L and DCs of KO HSPCs treated with TNFα and FLT3L. Data represent mean + SD (n = 4–6 mice). HSPCs hematopoietic stem and progenitor cells; WT wild type; KO knock-out; F FLT3L and T TNFα. [Figure reproduced from (Anirudh *et al.*, 2020)]

4.5 TNFα treatment partially restores C/EBPα-dependent transcriptional programs in early DC development

Interestingly, when we compared FLT3L-stimulated KO versus WT FLT3$^+$ HSPCs for their gene expression profile, we observed that distinct transcription factors, like *Irf8, Sfpi1, Irf4* and *Klf4,* as well as other crucial receptors, such as *Cx3cr1*, known to be involved in DC development, were regulated in a *Cebpa* dependent manner (Figure 4.21).

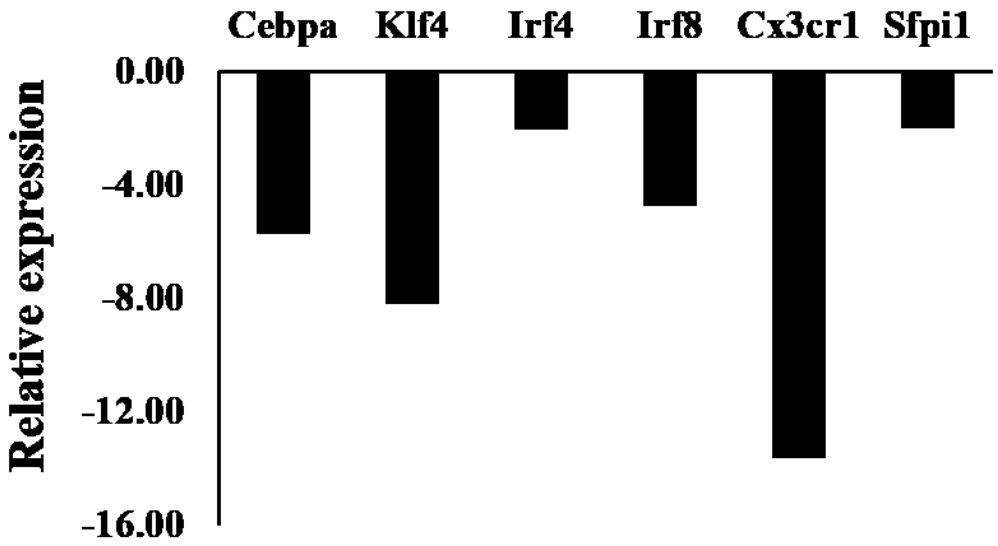

Figure 4.21– Transcription factors and Cxc3r1 are downregulated in FLT3$^+$ HSPCs treated with FLT3L in a *Cebpa* dependent manner. Bar graphs represent expression changes of selected genes known to be important for DC development, in KO as compared to WT HSPCs treated with FLT3L. HSPCs hematopoietic stem and progenitor cells; WT wild type; KO knock-out;

In accordance, in silico analysis of the promoter regions of *Sfpi1*, *Irf8 and Cxc3r1* showed the presence of three putative binding sites for C/EBPα in the promotor region of *Sfpi1* and four binding sites in the *Irf8* and *Cxc3r1* promoter regions, respectively (Figure 4.22).

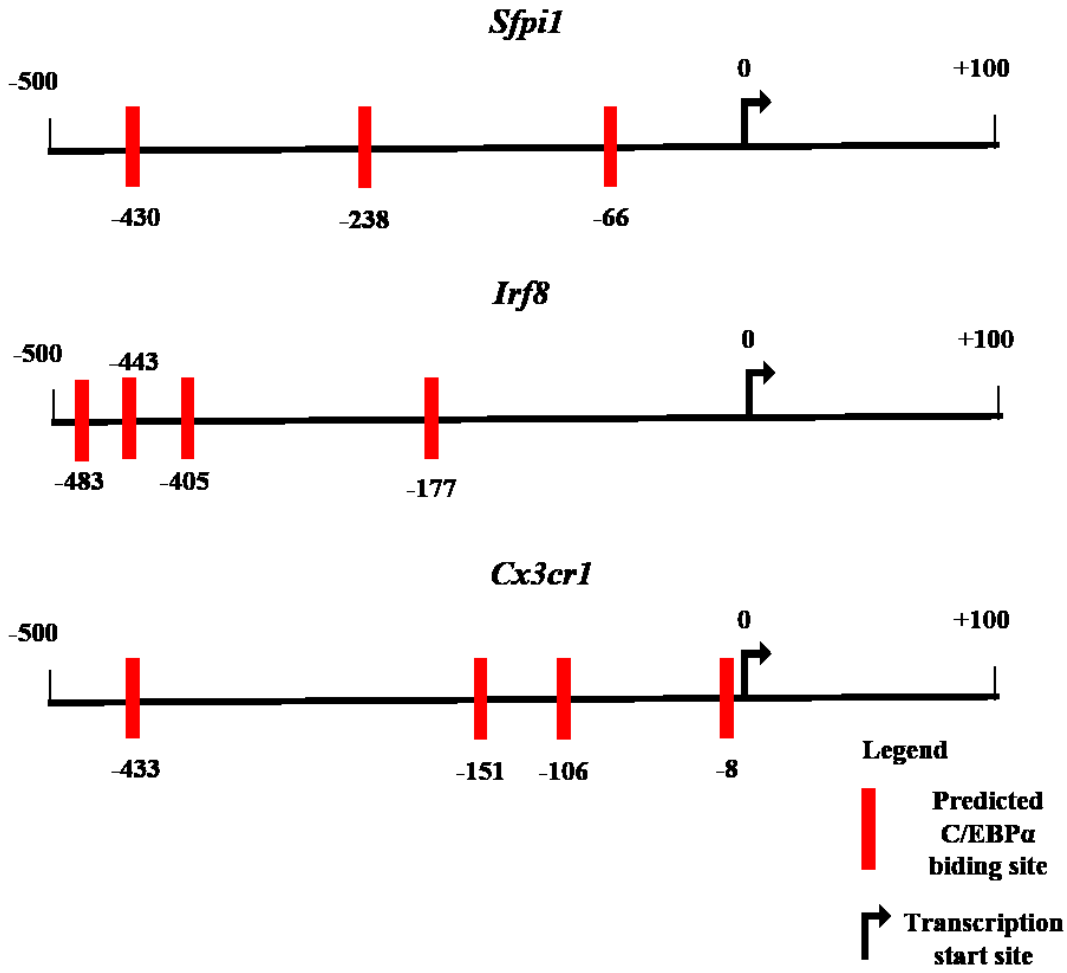

Figure 4.22 – C/EBPα binding sites in the promoter regions of *Sfpi1, Irf8* and *Cx3cr1*, predicting a possible direct interaction between the genes and C/EBPα protein.

Due to the known roles for *Sfpi1* and *Irf8* in early DC development and the fact that expression of *Cx3cr1* is among the first characteristics attributable to MDPs, we next wondered, whether the three genes could be probable candidates for C/EBPα-dependent mechanisms in DC development, which could be overcome by TNFα in the absence of *Cebpa*. First, we investigated the expression pattern of *Sfpi1, Irf8* and *Cx3cr1* through analysis of already published microarray data (Miller *et al.*, 2012) and observed, as expected, that in all cases a significant increase is seen in expression levels with progressively restricted stages being lowest at the CMP stage increasing at the MDP stage and reaching the peak of expression at the CDP stage (Figure 4.23). Interestingly, their pattern of expression was similar to the pattern of *Cebpa* in CMPs and MDPs. However, in contrast to these three genes no further increase is seen for *Cebpa* from MDPs to CDPs. Furthermore, while *Cebpa* expression is highest in GMPs (Figure 4.5), *Sfpi1, Irf8* and *Cx3cr1* reach their highest levels at the CDP stage.

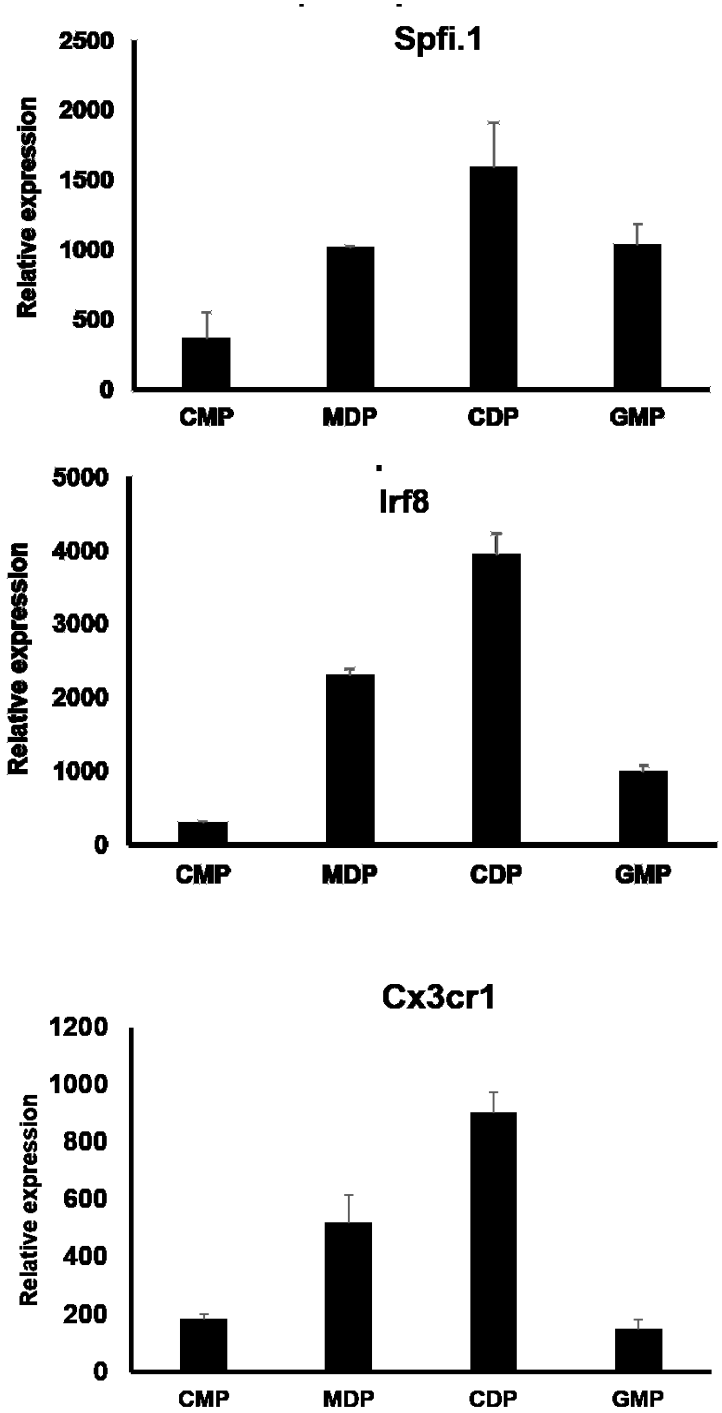

Figure 4.23 – Relative expression of genes *Sfpi1, Irf8* and *Cx3cr1* in different progenitors stages analysed from (Miller *et al.*, 2012)(GEO: GSE15907) All three genes show an increase in mRNA expression levels as the differentiation during DC development progresses reaching their highest values at the CDP stage. CMP denotes common myeloid progenitor; MDP monocyte dendritic cell progenitor; CDP common dendritic cell progenitor; GMP granulocyte monocyte progenitor; DC dendritic cell.

Having observed these similarities in the published expression patterns, we next checked mRNA levels of these genes by quantitative RT-PCR in HSPCs isolated from FLT3L-

stimulated WT and KO mice. Expression of *Irf8*, *Sfpi1* and *Cxc3r1* was significantly reduced in the KO HSPCs (Figure 4.24). Importantly, combinatorial treatment with FLT3L and TNFα increased the expression of *Sfpi1* and *Cxc3r1* in the KO HSPCs, but did not affect *Irf8* expression levels. These data indicate that TNFα may overcome lack of *Cebpa* by inducing *Sfpi1* and *Cx3cr1* expression.

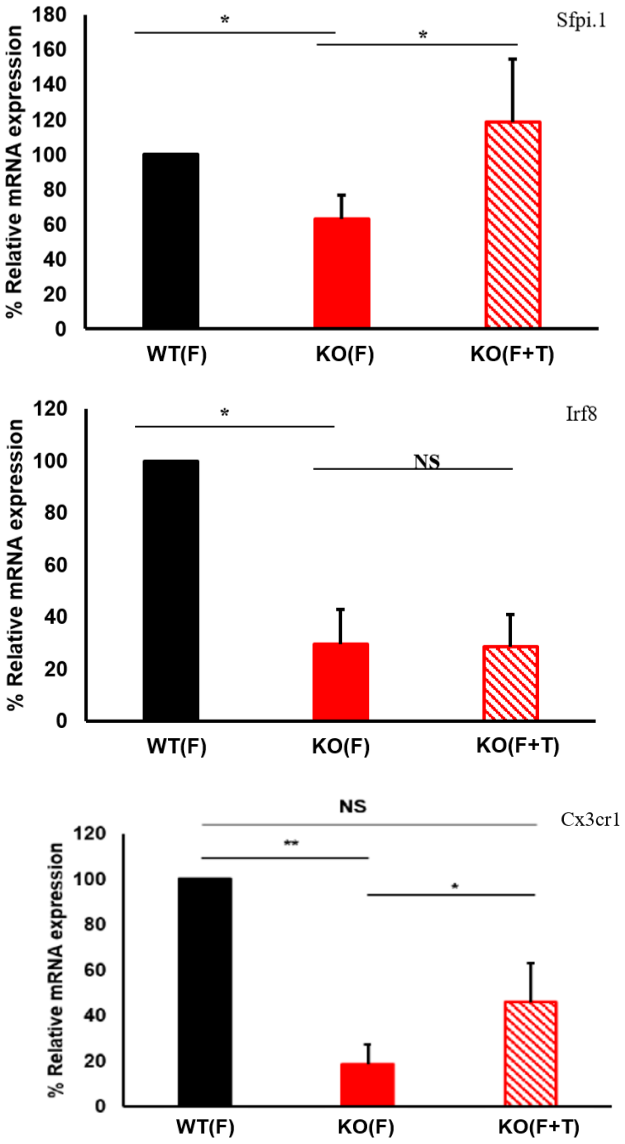

Figure 4.24 – Relative expression of genes *Sfpi1, Cx3cr1* and *Irf8* in *in vitro* cultured HSPCs from WT and KO mice after treatment with FLT3L or FLT3L + TNF α. (n = 3 per group, * p<0.01, **p <0.001) Expression of *Sfpi.1* and *Cx3cr1* is reduced in KO HSPCs after treatment with FLT3L alone but is increased after combinatorial treatment with or FLT3L + TNFα. In contrast, Irf8 is reduced in KO HSPCs also after combinatorial treatment. NS denotes not significant; HSPCs denotes hematopoietic stem and progenitor cells; WT wild type; KO knock-out; F FLT3L and T TNFα

5. Discussion

Development of mature steady-state DC through stepwise differentiation of BM-resident HSPCs via DC progenitors is a process tightly controlled by distinct transcription factors and cytokines (Shortman and Naik, 2007) (Schmid *et al.*, 2010). The gene *Cebpa* has been known for more than two decades to encode for an important transcription factor in haematopoiesis. Specifically, C/EBPα exhibits a lineage defining role in early stages of myeloid cell development (Wölfler *et al.*, 2010) and was proven to be essential for formation of granulocytes and monocytes in steady-state conditions (Zhang *et al.*, 1997) (Zhang *et al.*, 2004). However, little is known about its role in the development of DCs. Thus, in this project we used primary HSPCs from an inducible knockout mouse model targeting *Cebpa* and studied the development of DCs using a well-established *in vitro* assay based on FLT3L-stimulation to elucidate a role of C/EBPα in this process. Through data from mRNA microarray analysis and additional cellular experiments, we elucidated probable molecular mechanisms that underlie the role of C/EBPα in early steady-state DC development.

5.1 C/EBPα is important in early DC development

To know if C/EBPα plays a role in DC development it was first necessary to investigate whether or not *Cebpa* is expressed in the DC lineage. To this end we used a Cebpa-Cre induced EYFP expressing mouse model, which enables lineage tracing of *Cebpa* expressing cells *in vivo*. We observed that the percentage of *Cebpa*/EYFP-positive cells gradually increased in myeloid progenitor compartments with the highest percentage, not surprisingly, in the GMP population, whereas in the erythroid lineage only a small population was Cebpa/EYFP positive comparably to what has been reported in the literature (Wölfler *et al.*, 2010). Concerning DC progenitors, we noted an increase in the percentage of *Cebpa*/EYFP-positive cells at the MDP stage as compared to CMPs and even more pronounced to MPPs, which both are thought to be the main upstream progenitors of MDPs (Traver *et al.*, 2000) (Manz *et al.*, 2001) indicating increasing *Cebpa* expression during transition of MPPs and CMPs towards MDPs. Interestingly, in more downstream progenitors, such as CDPs, as well as mature splenic DCs the percentage of *Cebpa*/EYFP-positive cells remained approximately at the same level (about 60%), indicating that *Cebpa* is rather expressed at early stages during DC development. Although we found increasing levels of *Cebpa* mRNA expression levels in MDPs and CDPs as compared to CMPs and MPPs when analysing published data (Miller *et al.*, 2012), the fact that only about 60% of MDPs, CDPs and mature splenic DCs were EYFP-positive raises the

question whether *Cebpa* is indeed expressed in all DC progenitors at a distinct stage and therefore indispensable for DC development. Concerning this question, it has to be noted that Cre-based reporter mouse models are known to sometimes display an incomplete process of Cre recombination explaining why only a majority but not all late progenitors and splenic DCs are positive for *Cebpa*/EYFP (Wölfler *et al.*, 2010) (Schlenner *et al.*, 2010)

To test whether C/EBPα is indeed needed for DC development we used lineage depleted, BM-HSPCs for *in vitro* culture with FLT3L stimulation mimicking DC development (Naik *et al.*, 2007). After 8 days of culture, the number and percentage of CD11c and MHCII positive cells were analysed. These two markers allow identification of all mature steady-state DCs formed *in vitro*. HSPCs derived from *Cebpa* knockout (KO) mice showed a marked reduction in their potential to form $CD11c^{+}MHCII^{+}$ DCs as compared to the normal HSPCs, proving that DC development does indeed depend on C/EBPα to a significant extent. This also coincides with previous findings that C/EBPα is necessary as an early mediator for DC development (Welner *et al.*, 2013). Through the use of bone marrow specific knockouts Welner et al. showed that lack of C/EBPα reduced DC formation. However, data from a CD11c-Cre based knockout suggested that C/EBPα was only required in the early steps of DC development before CDP formation. Though these findings suggested an early role, the specific step at which C/EBPα plays its vital role was not investigated. Hence, we were interested at what exact stage C/EBPα is needed during DC development. For this, we performed a stepwise analysis of *Cebpa* KO and WT HSPCs set in culture with FLT3L and revealed that HSPCs lacking *Cebpa* expression contained higher numbers of more immature $lin^{-}FLT3^{+}cKit^{+}$ progenitors as compared to HSPCs expressing normal levels of *Cebpa*. This finding is consistent with data from the literature reporting an expansion of the HSC and $FLT3^{+}$ MPP compartment in the absence of C/EBPα (Zhang *et al.*, 2004) (Ye *et al.*, 2013). Importantly, even though HSPCs lacking *Cebpa* expression contained higher numbers of $FLT3^{+}cKit^{+}$ progenitors they failed to go through the stepwise progression of DC development displaying a reduced number of MDPs and a block in the transition of existing MDPs to CDPs. This data along with the data from *Cebpa*/EYFP expression unequivocally revealed that Cebpa is indeed required for early development of DCs and is specifically involved in the formation of MDPs and their subsequent transition to CDPs. Interestingly, similar to what we observed for C/EBPα, studies have shown that the expression levels of two other important transcription factors in early DC development, namely IRF8 and PU.1, vary at different progenitor stages and affect the formation of different progenitor stages upon deletion. Specifically, studies with *Irf8-EGFP* reporter mice revealed a gradual increase

in the levels of *Irf8* expression as the HSPCs progress through the stepwise formation of GMPs via the MPP and CMP stages. The expression of *Irf8-EGFP* was also homogenously high in MDPs, CDPs and pDCs whereas it was found high only in a few of mature splenic steady-state DCs (Wang *et al.*, 2014). Ablation of *Irf8* on the other hand had no effect on the $lin^{-}Sca1^{+}$ $cKit^{+}$ encompassing MPPs or the GMP compartment, whereas it caused a marked reduction in CMPs and CDPs. Interestingly, deletion of *Irf8* resulted in a high number of MDPs to be formed, but these MDPs were not able to differentiate into the downstream cell types (Schönheit *et al.*, 2013). Comparably to what we found in *Cebpa* KO HSPCs, this suggested a possible role for Irf8 at the MDP to CDP transition. Similar studies have been carried out to investigate the role of PU.1 in myeloid cell development. Though, using Mx1-Cre $PU.1^{F/F}$ mice, it was shown that there is a significant reduction in the numbers of HSCs, CMPs and GMPs upon deletion of PU.1 (Iwasaki *et al.*, 2005). Although no direct data has been presented about numbers of the DC progenitor stages like MDPs and CDPs, it was shown that ablation of PU.1 in MPP or CMP stages all caused a marked reduction in the formation of mature DCs suggesting that PU.1 plays an important role at multiple steps during formation of DCs (Carotta *et al.*, 2010). Lineage tracing using *in vitro* culture of HSPCs from PU.1-GFP knockin mice have been used to only determine the number of cDCs and pDCs but not for the earlier progenitor stages (Carotta *et al.*, 2010). Since a role of PU.1 has never investigated at the MDP stage, it would be interesting to see whether the expression levels of PU.1 at the MDP stage go in parallel with *Cebpa* expression and whether lack of PU.1 in MDPs can affect their transition to CDPs (Merad, 2010).

5.2 *Cebpa* deletion has a significant effect on the transcriptional and cytokine secretory profile of HSPCs after FLT3L stimulation

Steady-state DC development is a process controlled by a vast network of transcription factors and cytokine/chemokine interactions. Steady-state DCs are formed from progenitors which are positive for the FLT3 receptor. In order to discover pathways and possible downstream effectors and targets, we performed gene expression analysis using mRNA microarrays of lineage depleted BM-derived $FLT3^{+}$ HSPCs from both KO and WT mice sorted twice for FLT3 positivity to obtain a pure DC generating HSPC population. To identify FLT3L-induced genes, which are dependent on C/EBPα, isolated cells were either left untreated or treated with FLT3L for 4 hours. Data obtained from the microarray analysis indicated a significant dysregulation in the expression pattern of a large number of genes. Grouping of the genes based on their

functional roles revealed a pattern for C/EBPα-dependent deregulation in multiple pathways. These included the TNFα signalling pathway, NFkB signalling pathway, Chemokine-Chemokine receptor interaction pathway as well as multiple transcription factors like *Sfpi1* (Merad and Manz, 2009) and *Irf8* (Tamura, Kurotaki and Koizumi, 2015) which were included in the hematopoietic cell lineage pathway and are well known for their role in DC development. However, the most prominently deregulated pathway was the TNFα-signalling pathway. Interestingly, expression of the gene TNF itself was also significantly decreased in *Cebp*a KO HSPCs as were other inflammatory cytokines, such as IL1β. A deregulation of pathways involving inflammatory cytokines was also further corroborated by the finding that the most affected signalling pathway by inflammatory cytokines, namely the NFκB pathway was also deregulated in *Cebp*a KO HSPCs.

Having observed such significant changes in the cytokine levels in the gene expression analysis, we questioned whether a difference also existed in the secretory profiles of KO as compared to WT HSPCs. To this end, we performed a BioPlex analysis to check the secretory levels of a list of cytokines/chemokines. Indeed, we observed specific differences in the levels of multiple cytokines like MIP1, MIP2, TNFα and IL1β between *Cebp*a KO and WT HSPCs. Interestingly there was no difference in the levels of cytokines like CSF2, which is known to be required more specifically in the development of inflammatory DCs (Van De Laar, Coffer and Woltman, 2012).

5.3 Cytokine and/or chemokine play a role in *Cebpa* dependent DC development

Data from our gene expression combined with the cytokine secretion analysis pointed towards a possible role of cytokines and chemokines in C/EBPα-dependent DC development. Though cytokines like GM-CSF have been accepted as dispensable for development of most steady-state DC subsets (Greter *et al.*, 2012), others like FLT3L are absolutely essential for normal steady-state DC development to occur (Merad and Manz, 2009). By having observed a significant difference in the secretory levels of multiple cytokines and chemokines, we hypothesised that the paracrine presence of low amounts of these cytokines is necessary for DC development and might overcome the lack of *Cebpa*. However, our data from the co-culture experiments clearly demonstrated that the presence of WT HSPCs cannot restore DC formation in KO HSPCs indicating a cell-intrinsic effect of *Cebpa* loss. So we next questioned whether direct supplementation of higher amounts of cytokines/chemokines would be able to

overcome this cell-intrinsic defect in DC development. But addition of the most prominently affected chemokines, MIP-1 and MIP-2, to the culture along with FLT3L, failed to have any effect on the DC development potential of HSPCs lacking *Cebpa.* This is not surprising, since MIP-1 is known more for its role in maturation and activation rather than in early development and has functions specifically in DC trafficking of moDC (Caux *et al.*, 2000). MIP-2 on the other hand is known for its role in mobilisation of HSPCs, but nothing has been reported so far about a role in HSPC development (Wang *et al.*, 1997).

In contrast to MIP-1 and MIP-2, TNFα was shown to affect DC development via NFκB signalling (Ouaaz *et al.*, 2002) and also has been used for *in vitro* generation of DCs from human and murine HSPCs in combination with other cytokines (Shortman and Naik, 2007). Given the prominent deregulation of TNFα signalling in our gene expression analyses, we therefore tested addition of TNFα on FLT3L-DC development. We observed that treating of KO HPSCs with a combination of TNFα and FLT3L almost completely restored DC formation capacity as compared to WT HSPCs. Of note, KO DCs generated by this combinatorial treatment were fully active in their function as antigen-presenting cells as viewed by morphology and assayed by allogenic MLR. The role of TNFα in haematopoiesis and early myelopoiesis has been extensively studied, but remains controversial. A recent study reported inflammation-induced elimination of differentiated myeloid progenitors by TNFα in the bone marrow (Etzrodt *et al.*, 2019). However, TNFα treatment of mice prevented necroptosis in HSC and initiated emergency myelopoiesis through NF-kB-dependent mechanisms, hence promoting HSPC regeneration (Yamashita and Passegué, 2019). TNFα itself can directly and rapidly upregulate PU.1 protein in HSCs by recruiting NFκB to bind to the enhancer element of PU.1 (Etzrodt *et al.*, 2019). From all these data, it is tempting to speculate that TNFα might overcome lack of *Cebpa* in HSPCs by directly inducing PU.1 thus restoring early DC development. In line with this argumentation, treatment with inflammatory cytokines was also shown to be able to restore formation of GMPs and mature granulocytes from *Cebpa* KO fetal liver cells in culture and *in vivo* (Hirai *et al.*, 2006). In mouse models of acute myeloid leukemia (AML) using MLL-AF9 and MOZ-TIF2 fusion genes, C/EBPα-induced formation of GMPs was critical for leukemia development, since deletion of *Cebpa* prevented initiation of AML. Interestingly, treatment of *Cebpa* KO HSPCs of these mice with inflammatory cytokines re-established AML transformation capacity (Ye *et al.*, 2015).

5.4 Cebpa-dependent transcriptional programs may play an important role in control of early DC development

Most processes induced by cytokines are mainly controlled by an intricate network of transcription factors (Bosteels and Scott, 2020). As an example, as early as the MPP stage, it was shown that deletion of the master transcription factor *Ikaros* leads to the formation of reduced number of DCs (Wu *et al.*, 1997). The central cytokine regulating DC development, FLT3L, induces the expression of transcription factor PU.1 and effects the differentiation of HSPCs into all types of steady-state DCs, through its receptor FLT3. A lack of either *Flt3* or *Sfpi1* (the gene encoding PU.1) causes defective DC differentiation both *in vitro* and upon *in vivo* transfer (Carotta *et al.*, 2010). Although activation of PU.1 causes FLT3 expression to increase, suggesting a positive feedback loop between FLT3 and PU.1, restoring FLT3 expression in PU.1-deficient HSPCs does not restore their potential to give rise to DCs (Carotta *et al.*, 2010), indicating that in addition to FLT3, other targets of PU.1 are vital for DC differentiation. Given this role of PU.1 as a master regulator of early DC development, it is tempting to argue that at least some of the effects of C/EBPα we have observed rely on its interaction with PU.1. This argumentation is also corroborated by the fact that both transcription factors were shown to exhibit a well-defined coordinating effect on early HSPC development as early as the MPP and CMP stage (Yeamans *et al.*, 2007).

The role of *Cebpa* in myelopoiesis has been defined extensively as an early master regulator of myeloid fate choices. (Wölfler *et al.*, 2010). It was also shown that loss of C/EBPα causes a differentiation block preceding the GMP stage which is a distinct precursor population immediately downstream of the CMPs (Iwasaki and Akashi, 2007). This also correlates with our data, which shows a similar block at the MDP stage downstream of CMPs. The observations make it plausible to predict that C/EBPα plays an important role as a pioneering factor on selected enhancers during early DC development, possibly via its switch/sucrose non-fermentable (SWI/SNF) domain, which was shown to be important for enhancer binding in adipocyte differentiation, where C/EBPα is also known to be crucial (Madsen *et al.*, 2014). As already mentioned, C/EBPα has been described to coordinate these fate choices as well as early myeloid differentiation steps mostly by interacting with PU.1. It has been shown, via integrated analysis of enhancer kinetics, transcription factor dynamics and proximal gene expression in *Cebpa* knockout HSPCs, that there is a profound reduction in PU.1 binding at many of enhancers that are active during early myelopoiesis identifying a surprising C/EBPα

dependency for binding of PU.1 (Yeamans *et al.*, 2007). When *Cebpa* KO HSPCs treated with FLT3L alone were assayed via qRT-PCR for expression levels of *Sfpi1* we observed a decrease, when compared to WT HSPCs, which could be restored by addition of TNFα. Analysis of mRNA microarray data from GEO databases also confirms that the expression pattern of *Sfpi1* is similar to the expression pattern of *Cebpa* in that it increases from the CMP stage to the MDP stage. In silico promoter analysis has shown the presence of C/EBP binding sites on the *Sfpi1* distal promoter. These results combined with the described effect of TNFα on PU.1 levels (Etzrodt *et al.*, 2019) make it tempting to speculate on a probable mechanism involving TNFα working via NFkB to increase PU.1, in turn partially restoring the formation of DC in absence of C/EBPα. However, simple overexpression of PU.1 was not able to restore DC formation in *Cebpa* KO HSPC (Welner *et al.*, 2013), indicating that additional mechanisms other than PU.1 induction are indispensably regulated via C/EBPα during early DC development.

Another transcription factor known to be necessary for multiple differentiation steps in the DC lineage is IRF8. IRF8 is known specifically for its role in the development of the $CD8^+$ DC subset (Yáñez *et al.*, 2015) and works in tandem with its own family member IRF4 to regulate formation of either $CD8^+$ or the CD8- subsets (Kurotaki *et al.*, 2018). We observed a significant reduction in the expression level of *Irf8* in the *Cebpa* KO HSPCs as compared to the WT HSPCs. The expression data from the GEO analysis shows that *Irf8* mRNA also increases from the CMP stage to the MDP stage and continues to increase further to the downstream stages. In silico promoter analysis also predicts binding sites for C/EBPα in the promoter of the *Irf8* gene. Furthermore, PU.1 has been described to induce specific remodelling of the higher-order chromatin structure at the *Irf8* gene to initiate DC fate choice (Schönheit *et al.*, 2013). Therefore, it would be very interesting to investigate an interaction of these three transcription factors during early DC development in more detail in future studies.

Since we observed a downregulation of *Cx3cr1* in *Cebpa* KO HSPCs, and addition of TNFα partially alleviated the expression levels resulting in formation of fully functional, mature DCs, one could speculate that *Cx3cr1* is also a critical gene induced by C/EBPα. *Cx3cr1* expression has been used as an early marker for identification of MDPs in murine bone marrow (Fogg D, *et al.*, 2006) and in silico analysis revealed C/EBP binding sites in its promoter region. Furthermore, it has also been shown that expression of CX3CR1 protein promotes the generation of DCs under steady-state conditions (Łyszkiewicz *et al.*, 2011). Consistently, lack of CX3CR1 affects DC differentiation from bone marrow myeloid cells induced by CSF2 (GM-CSF) and interleukin-4 (IL-4) *in vitro* (Sutti *et al.*, 2019)

6. Conclusion

Since the discovery of DCs in the late nineteenth century by Paul Langerhans and their subsequent naming by Ralph Steinman and Zanvil A Cohn in 1973, researchers have extensively tried to investigate the molecular processes involved in the stepwise differentiation and formation of these specialized antigen presenting cells. Multiple mechanisms have been brought to light over the last decade or so, including transcription factors and cytokine signalling pathways. In this study, we investigated one such gene, namely *Cebpa*, which has been implicated as a master regulator of myelopoiesis. We have established that *Cebpa* does indeed play an important role in DC development especially in the formation of MDPs and their transition to CDPs. We have also elucidated that a certain cytokine, TNFα, could help to overcome the absence of C/EBPα. Through this study we thus add important information to the existing knowledge of the role C/EBPα and open up avenues for investigation which can lead to a better understanding of the exact mechanisms C/EBPα is involved in during DC development.

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