“The role of Id2 (inhibitor of differentiation/ DNA binding) in dendritic cell development in steady state and inflammation”

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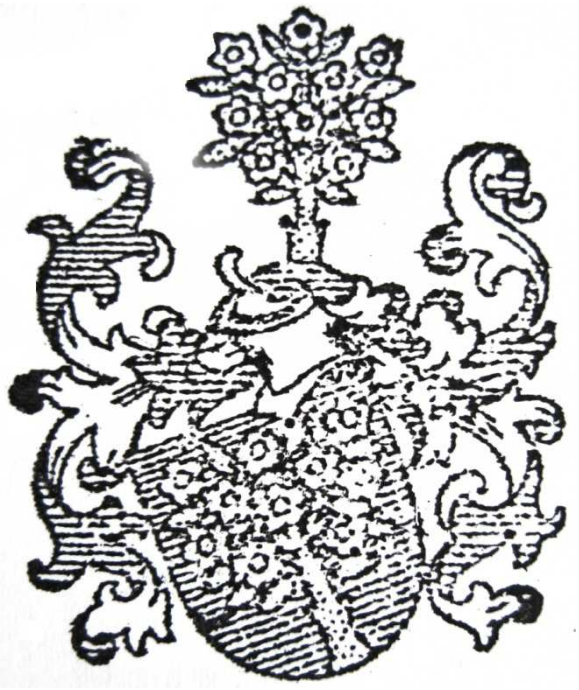
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Content

Abbreviations ..... **IV**

Zusammenfassung ..... **VI**

Abstract ..... **VII**

1 Introduction **1**

1.1 Basic principles of immunity 1

1.2 Dendritic cells 3

1.2.1 Migratory dendritic cells *4*

1.2.2 Conventional dendritic cells *6*

1.2.3 Circulating dendritic cells *7*

1.3 Development and activation of dendritic cells 8

1.3.1 Overview of dendritic cell development *8*

1.3.2 Role of cytokines in dendritic cell development *10*

1.3.3 Role of TLRs in dendritic cell activation *12*

1.3.4 Role of transcription factors in dendritic cell development and activation *14*

1.4 Aim of the study 19

2 Material and Methods **20**

2.1 Material 20

2.1.1 Chemicals *20*

2.1.2 Size standards *20*

2.1.3 Media *20*

Basic culture medium *20*

Flt3 precursor amplification medium *20*

FL amplification medium *20*

GM-CSF medium *20*

Tissue culture additives: *21*

2.2 Methods 24

2.2.1 Mice *24*

Injections *24*

Irradiation *24*

Adoptive transfer *24*

UV irradiation *24*

2.2.2 Flow Cytometry *25*

Compensation *25*

Cell preparation *25*

Intracellular staining *25*

2.2.3 Isolation of primary cells *26*

Splenocytes/ thymocytes *26*

Bone Marrow cells *26*

Blood monocytes *26*
In vivo labelling of blood monocytes *26*
Skin dendritic cells *26*
2.2.4 Dendritic cell cultures *27*
DC generation from Flt3$^+$CD11b$^+$ progenitor cells (two-step cultures) *27*
DC generation from GM-CSF bone marrow cultures *28*
DC generation from Flt3L bone marrow cultures *28*
2.2.5 Miscellaneous protocols *28*
Polymerase chain reaction (PCR) *28*
TLR stimulation of DCs and Flt3$^+$ progenitor cells *31*
Cytometric bead assay (CBA) *31*
Fluorescence microscopy *31*
Confocal microscopy and live cell imaging *32*
Tissue preparation for transmission electron microscopy *32*
Statistics *33*
Microarray preparation *33*

3 Results 34

3.1 Characterization of an in vitro amplified Flt3$^+$ precursor 34
3.1.1 Surface marker profile of the Flt3$^+$ precursor *35*
3.1.2 TLR repertoire of the Flt3$^+$ precursor cells *35*
Comparative gene expression analysis for TLR pathway components *36*
Expression and up-regulation of TLR mRNA in Flt3$^+$ precursors and differentiated DCs *38*
Protein expression of TLRs in Flt3$^+$ precursors and differentiated DCs *38*
3.1.3 Functional analysis of TLRs on Flt3$^+$ precursor and differentiated DCs *40*
3.1.4 Analysis of the in vivo developmental potential of the Flt3$^+$ precursor *42*
3.2 Development of two distinct types of Langerhans cells in steady state and inflammation 46
3.2.1 Id2 deficiency results in lack of LCs and langerin$^+$ dDCs *47*
3.2.2 Flt3$^+$ bone marrow progenitor cells rescue LC deficiency in Id2$^{-/-}$ mice *48*
3.2.3 Id2$^{-/-}$ Gr-1^{hi} monocytes infiltrate inflamed skin *53*
3.2.4 LC reconstitution after UV irradiation is Id2-independent *55*
3.2.5 LC maintenance after UV irradiation is Id2-dependent *57*
3.2.6 Reconstituted Id2$^{-/-}$ LCs differ from Id2$^{+/+}$ LCs *58*
3.2.7 Id2 is dispensable for in vitro LC development *61*
3.3 The influence of Id2 in lymphoid organ DC development 66
3.3.1 Id2 deficiency results in decreased cDC numbers in spleen *67*
3.3.2 Id2 mice lack CD8α$^+$ and CD8α$^+$langerin$^+$ DCs in spleen and thymus *68*
3.3.3 Rescue of Id2$^{-/-}$ phenotype by adoptive transfer *69*
3.3.4 Id2 deficiency leads to increased pDCs *70*
3.3.5 Id2 is essential for CD8 T cell maintenance in spleen and thymus *72*
3.4 Id2 antagonizes the function of activating bHLH proteins 74
3.4.1 E47 deletion overcomes the need for Id2 in the development of langerin$^+$ skin DCs *75*
3.4.2 The double-deficiency of Id2 and E47 does not restore the Id2$^{-/-}$ phenotype in spleen DCs *77*

3.4.3 Loss of E47 fails to restore CD8α$^+$langerin$^+$ cDCs spleen or thymus of Id2$^{-/-}$ mice 79
3.4.4 Only E47$^{-/-}$Id2$^{-/-}$ double deficiency leads to increased pDCs 80
3.4.5 Id2 remains essential for CD8$^+$ T cell maintenance in spleen and thymus 82

4 Discussion 85
4.1 Characterization of an in vitro amplified Flt3$^+$precursor 85
4.2 Development of two distinct types of Langerhans cells in steady state and inflammation 87
4.3 The influence of Id2 in lymphoid organ DC development 91
4.4 Id2 antagonizes the function of activating bHLH proteins 92

5 References 96

Appendix 104

Acknowledgements 108

Abbreviations

APC	Antigen presenting cell
BCR	B cell receptor
bHLH	Basic helix-loop-helix
CBA	Cytometric bead assay
CCL	Chemokine ligand
CCR	Chemokine receptor
CD	Cluster of differentiation
cDC	Conventional DC
CDP	Common DC progenitor
CLP	Common lymphoid progenitor (identified as lin^- $Sca\text{-}1^-$ $c\text{-}kit^+$ $IL\text{-}7R^+$)
CMP	Common myeloid progenitor (identified as lin^- $CD34^+$ $Fc\gamma R^{lo}$)
CpG	Cytosine (unmethylated)-phosphate-guanosine
DC	Dendritic cell
dDC	Dermal dendritic cell
dhet	Double heterozygote; $E47^{+/-}Id2^{+/-}$
dko	Double knockout; $E47^{-/-}Id2^{-/-}$
$E47^{ko}$	E47 knockout; $E47^{-/-}Id2^{+/-}$
EDTA	Ethylenediamine tetra acetic acid
FACS	Fluorescence activated cell sorter (flow cytometry)
FCS	Fetal calf serum
FITC	Fluoresceinisothiocyanat
Flt3	c-fms-like tyrosine kinase 3
Flt3L	c-fms-like tyrosine kinase 3 ligand
FSC	Forward scatter
GALT	Gut- associated lymphoid tissue
GFP	Green fluorescent protein
GM-CSF	Granulocyte macrophage-colony stimulating factor
hi	high
HLH	Helix-loop-helix
HSC	Haematopoietic stem cell
Hyper IL-6	IL-6/IL-6R fusion protein
i.v.	Intravenous
Id	Inhibitor of differentiation/ DNA binding
$Id2^{-/-}$	Id2 deficient; $Id2^{-/-}$
$Id2^{ko}$	Id2 knockout; $E47^{+/-}Id2^{-/-}$
IFN	Interferon
IGF-1	Insulin-like growth factor 1

IL	Interleukin
iNOS	Inducible nitric-oxide synthase
IRF	Interferon regulatory factor
ko	Knock out
LC	Langerhans cell
LN	Lymph node
lo	low
LPS	Lipopolysaccharide
LTi cells	Lymphoid tissue inducer cells (defined as $CD3^{-}CD4^{+}IL\text{-}7R\alpha^{+}$)
M-CSF	Macrophage colony-stimulating factor
MDP	Macrophage and DC progenitor
MHC	Major histocompatibility complex
MHC-II	Major histocompatibility complex class II
min	Minutes
Mo-LCs	Monocyte derived LCs
NALT	Nasal-associated lymphoid tissue
NF-κB	Nuclear factor kappa-B
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid DC
PP	Peyer's patch
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcriptase polymerase chain reaction
SCF	Stem cell factor
SD	Standard deviation
SSC	Side scatter
STAT	Signal transducers and activators of transcription
TCR	T cell receptor
TGFβ	Transforming growth factor β
Th1	T helper cell response type 1
Th2	T helper cell response type 2
TLR	Toll-like receptor
TNFα	Tumour necrosis factor alpha
TRAF6	TNF receptor associated factor 6
UV	Ultraviolet light
WT	Wild type

Zusammenfassung

Dendritische Zellen gehören zu den professionellen antigenpräsentierenden Zellen, die Antigene aufnehmen und in das Lymphgewebe wandern, wo sie antigenspezifische Immunreaktionen initiieren. Verschiedene Subtypen von Dendritischen Zellen wurden bereits charakterisiert, abhängig von ihrer Funktion und Lokalisation. Jeder dieser Subtypen scheint eine besondere Rolle, sowohl in Immunität als auch in Toleranz zu haben. Hingegen ist es weniger klar, wie sich diese verschiedenen Subtypen von Dendritischen Zellen aus ihren hämatopoetischen Vorläufern entwickeln und welche molekularen Mechanismen an der Regulation der verschiedenen Entwicklungsstufen beteiligt sind.

Die vorliegende Arbeit hatte das Ziel einen bereits beschriebenen Vorläufer für Dendritische Zellen, den sogenannten „Flt3^{+} progenitor“, weiterführend zu charakterisieren und sein Entwicklungspotenzial *in vivo* zu untersuchen. Dabei zeigte sich, dass der „Flt3^{+} progenitor“ nicht nur Dendritische Zellen bildet, sondern sich auch in alle anderen Zellen des hämatopoetischen Systems entwickeln kann.
Des Weiteren bearbeitet diese Studie den Einfluss des HLH Transkriptionsfaktors Id2 auf die Entwicklung von verschiedenen Subtypen Dendritischer Zellen, insbesondere Langerhans Zellen. Da bereits gezeigt wurde, dass Monozyten Vorläufer der Langerhans Zellen sein können, wurden Gr-1^{hi} Monozyten sowie der „Flt3^{+} progenitor“ auf ihre Fähigkeit Langerhans Zellen zu bilden untersucht. Dafür wurde ein Mausmodell verwendet, in dem Id2 genetisch inaktiviert war. Hierbei wurde gefunden, dass Id2 unter normalen Bedingungen essentiell für die Bildung von Langerhans Zellen ist, während deren Bildung aus Gr-1^{hi} Monozyten in Antwort auf eine Entzündung unabhängig von Id2 verläuft.

Es ist bekannt, dass Id2 die Aktivität von E-Proteinen, wie zum Beispiel E47, verändert, indem es ihre Bindung an DNS verhindert. Daher wurde der Einfluss von Id2 und E47 auf die Entwicklung von Dendritischen Zellen analysiert und diese Arbeit zeigt, dass der molekulare Mechanismus, durch den Id2 die Entwicklung von verschiedenen Subtypen Dendritischer Zellen kontrolliert, auf einer Beeinflussung von E-Proteinen basiert.
Zusammen genommen identifiziert diese Arbeit einige der wesentlichen Schritte in der Entwicklung von Dendritischen Zellen aus frühen hämatopoetischen Vorläuferzellen. Weiterhin betrachtet sie den Beitrag verschiedener Vorläufer zur Entwicklung von Langerhans Zellen, sowohl im Normalzustand, als auch während der Entzündung. Abschließend zeigt sie die Bedeutung des Zusammenspiels von Id2 und E-Proteinen, wie zum Beispiel E47, in der Entwicklung und Differenzierung von Dendritischen Zellen.

Abstract

Dendritic cells are professional antigen presenting cells that capture antigens and migrate to lymphoid tissues where they elicit specific immune responses. Several DC subtypes have previously been characterised, depending on their function and location. Each subtype appears to have a specialised role in both immunity and tolerance. However, less clear are the processes by which these different DC subsets develop from haematopoietic precursors, and the molecular mechanisms involved in the regulation of the different stages during DC development.

The research described herein had the aim to continuatively characterize a previously described DC precursor, the $Flt3^+$ progenitor, downstream of the haematopoietic stem cells, and to assess its developmental potential *in vivo*. Hereby, it was found that the $Flt3^+$ progenitor does not only develop into dendritic cells, but can also generate all other cells of the haematopoietic compartment.

Furthermore, this study examined the role of the HLH transcriptional regulator Id2 on the development of different DC subtypes, especially Langerhans cells. As monocytes had been assumed to be LC precursors, Gr-1^{hi} monocytes as well as the $Flt3^+$ progenitor were tested for their LC precursor potential. Using an Id2 knockout mouse model, it was found that Id2 is essential for the formation of Langerhans cells in steady state. Conversely, their development from Gr-1^{hi} monocytes in response to inflammation is independent of Id2.

Id2 is known to modulate the activity of E-proteins, such as E47, by antagonising their DNA binding. Therefore, the impact of Id2 and E47 on DC development was studied and this work provides new insights into the molecular mechanisms controlling the development of distinct DC subsets. It shows that the molecular mechanism by which Id2 controls the development of different DC subtypes is based on a modulation of E-proteins.

Taken together, this research identified some of the key steps in the development of DCs downstream of the early haematopoietic precursors. It also dissected the contribution of different precursors for the Langerhans cell development in both the steady-state and during inflammation. Finally it provides evidence for the importance of Id2 and E proteins, such as E47 working in concert to regulate DC development and differentiation.

1 Introduction

1.1 Basic principles of immunity

Invading pathogens can swiftly replicate and spread within an organism, frequently causing severe damage to the host. Due to the constant exposure to potentially harmful pathogens, an intricate combination of cellular and humoral factors is required to prevent invasion or confine dissemination of pathogens and finally eliminate an infection - the immune system. It consists of two major parts; the innate immune system, and the acquired or adaptive immune system. Pathogens first encounter innate immunity, comprising, among other components, natural barriers such as the skin and mucosae. Should these be overcome by a pathogen, it faces a group of soluble factors, the complement system, which can bind to and lyse infectious agents. In addition, the cells of the innate immune system constantly circulate through the entire body in search of conserved molecular patterns that signal the presence of pathogens. Once detected, these are eliminated through phagocytosis by neutrophil granulocytes and macrophages [1]. The majority of infections is dealt with by the innate part of the immune system without the need for involving adaptive immunity. However, many pathogens have developed successful strategies to evade innate immunity, necessitating a more specific form of an immune response.

Whereas the innate immune system mediates a fast, but unspecific reaction, the adaptive immune system gives rise to a delayed, but highly specific form of an immune response. It is based on two types of effector cells, the B and T lymphocytes. These lymphocytes carry specific antigen-recognition receptors, the B cell receptor (BCR) and the T cell receptor (TCR), respectively. Upon recognition of an antigen by its receptor, a cell enters a phase of rapid proliferation, a process called clonal selection. This leads to a population of cells with receptors specific for this particular antigen. After the activation, lymphocytes of the adaptive immune system contribute to host defense either by producing antibodies (B cells), facilitating the functions of other immune cells such as B cells or macrophages (T helper cells), or eliminating infected host cells (cytotoxic T cells). A particularly important part of adaptive immunity is the existence of a so-called memory cell repertoire. Upon subsequent contact with a previously encountered antigen, memory cells will be reactivated, leading to a faster, but still highly specific and efficient response of the adaptive immune system [2].

To mount an adaptive immune response a naïve T cell needs to be activated by recognition of a foreign antigen via its TCR [3, 4]. But as already said, the TCR of a T cell is antigen specific, each T cell carries only one kind of TCR, and the number of T cells specific for a certain antigen is very limited. Therefore the chance for a T cell to encounter its "matching" antigen is rather low, which makes T cell activation a highly unlikely event [5]. To overcome this problem, the body spatially confines naïve lymphocytes to a limited set of tissues. The main organs to fulfil this function are spleen and lymph nodes, but also different types of mucosa-associated lymphoid tissues, such as Peyer's patches [6, 7]. Naïve lymphocytes are continuously circulating between these tissues in vasculature and lymph vessels.

Moreover, these tissues are specialized to recruit phagocytic cells that carry ingested antigen and promote their interaction with naïve lymphocytes, thereby significantly increasing the likelihood for an interaction between an antigen and its cognate receptor. This is necessary because pathogens often invade peripheral tissues, but not the lymphoid system to which naïve T cells are confined. Therefore the professional Antigen-presenting cells (APCs) overcome the separation between the site of antigen capture, and the site of its presentation, by monitoring tissues and organs in the periphery for infection [2, 4].

APCs represent a heterogeneous family of cells, including macrophages, monocytes, and dendritic cells (DCs). They patrol peripheral tissues, capturing both self and foreign antigen. APCs process the antigens into short peptides, which are then loaded onto major histocompatibility complex (MHC) molecules, where they are presented to antigen-specific T cells [8, 9]. Two types of antigen presentation can be distinguished, depending on the origin of the antigen. Peptides that are endogenously derived, including viral peptides, cellular and tumour antigens are presented to cytotoxic $CD8^+$ T cells via MHC class I (MHC-I). On the other hand, peptides that are derived from exogenous antigens, which are acquired by endocytosis, are presented to $CD4^+$ T helper cells via MHC-II molecules [10-12]. However, there is a certain exception, which is called cross-presentation. During this, immature DCs are able to present exogenous antigens on MHC-I molecules, allowing a virus-specific activation of cytotoxic T cells, even if a certain virus does not infect APCs [13]. MHC-I is expressed by all nucleated cells of the body, but APCs are the only cells that express MHC-II molecules [14]. However, antigen presentation by

MHC alone is not sufficient for proper activation of naïve T cells. This requires an additional signal, mediated by co-stimulatory molecules such as CD40, CD80, and CD86 (the latter also being known as B7.1 and B7.2, respectively). These co-stimulatory molecules are only expressed on APCs, making them the only group of cells capable of activating naïve T cells [2, 15, 16].

1.2 Dendritic cells

Among APCs, there is one group which is of special importance and most efficient in activating T cells, these are the DCs. DCs were first discovered in 1868; by the observation of Langerhans cells (LCs) of human epidermis; although they were originally thought to be nerve cells [17]. However, it took another century to demonstrate that these cells are in fact APCs and belong to the immune system. The seminal work of Steinman and Cohn in 1973 led to the detection of DCs in lymphoid tissue [18-20]. Following their identification and purification, it was realized that DCs have an extraordinary capacity as APCs, being efficient inducers of both primary and memory immune responses as well as immunological tolerance [21].

Today, it is known that DCs cover virtually all tissues of the body (Figure 1-1). Immature DCs mature in response to stimulation different pathogens and migrate to secondary lymphoid organs were they present processed antigen [9]. During their migration, DCs acquire full maturity, which is accompanied by an upregulation of MHC molecules and co-stimulatory molecules, as well as a shut-down of antigen uptake. This process permits DCs to be the most efficient activators of T cell stimulation [22].

All DCs have the ability to take up antigens, process them into antigenic peptides, and present these to naïve T cells, resulting in their activation. However, DCs are not a homogenous cell population, rather, they are a group of different subtypes, each locating to particular tissues of the body, where they fulfil distinct functions [18, 23].
As the body has to cope with a high number of diverse pathogens, DCs need to adapt to different routes of infection, like skin, tissue, mucosa, or blood. And they must be able to trigger the appropriate immune response tailored to the specific pathogen [21, 23, 24].

Therefore several functionally distinct DC subsets have developed, of which the most important will be briefly summarised here according to their various roles.

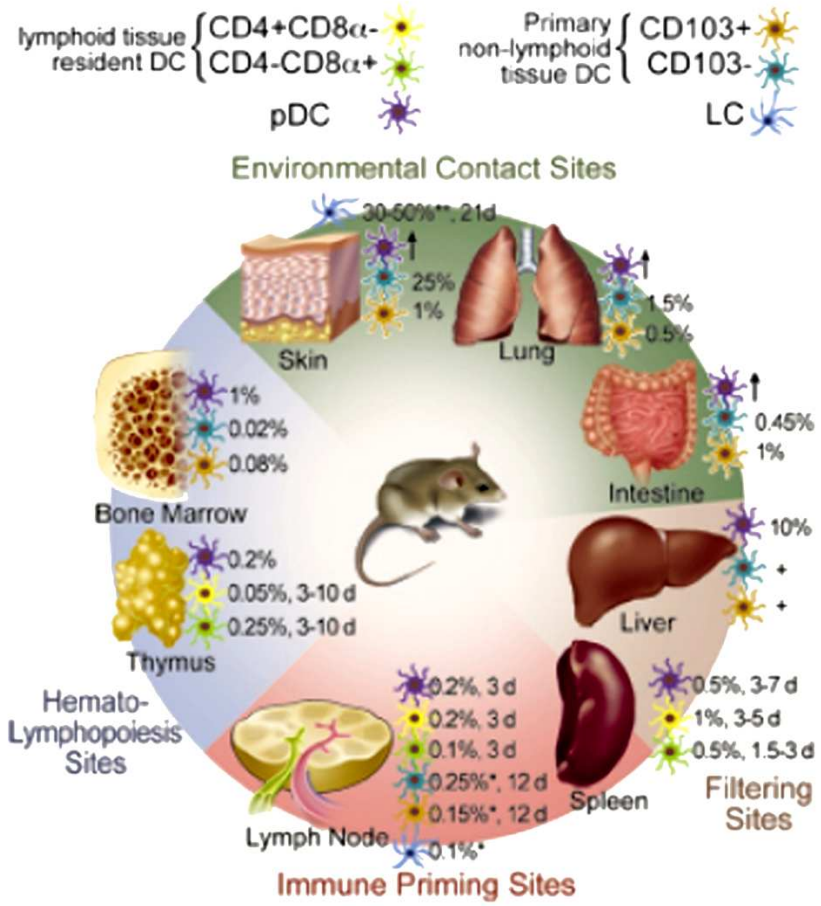


Figure 1-1: Mouse DC populations, location, and turn-over in steady-state.
Dendritic cells are distributed throughout the body. The major DC sub-populations at haematopoietic sites, environmental contact sites, filtering sites, and immune priming sites are depicted. Frequencies are given as percent (%) of total nucleated haematopoietic cells. Approximate time to about 50% renewal in steady-state is given in days (d). * Skin draining lymph node. ** Epidermis. (+) Present, but exact numbers are not known. (↑) Present in inflammation. Picture adapted from [25].

1.2.1 Migratory dendritic cells

Migratory DCs, including for example epidermal LCs and interstitial or tissue DCs, are DCs that collect antigens in the periphery and subsequently travel to the draining lymph nodes, where they present these antigens. LCs are the only DC type of the epidermis and account for 3–5% of all nucleated cells. They form a continuous cellular network through their extended dendrites and thereby providing the first immunological barrier to the external environment [26]. Even though LCs were integrated into the DC family, they

remain a special subgroup among DCs that show certain singularities [26, 27]. One of these singularities, which serves as a hallmark of LCs, is the presence of tennis racket-shaped intracellular structures called Birbeck granules, whose function still remains poorly understood [26]. Another characteristic feature is the expression of langerin (also known as CD207). Initially, langerin was thought to be solely expressed in cells containing Birbeck granules. In the meantime, however, it has also been detected in other cell types not containing these structures [26, 28]. The LCs of the epidermis are the classical model for migratory DCs [29, 30]. They reside as antigen-sampling sentinels in the skin. From here, a small fraction of these cells constantly migrates to the draining lymph nodes [29, 30]. During inflammation, the number of migrating cells is significantly upregulated, ensuring that in case of an infection an increased amount of antigens from the affected area is presented in the lymphoid tissues [8, 31-34]. LCs, as well as other migratory DCs remain in an immature state in the periphery. Here, they express only intermediate levels of MHC-II and no co-stimulatory molecules. Upon migration in the lymphatics, cell maturation leads to a shutdown of antigen uptake and a high expression of MHC-II and co-stimulatory molecules, whereby DCs reach the maximal capacity to stimulate naive T cells once they have reached the lymph nodes [33, 35]. Nevertheless, it still remains to be shown if LCs really present their antigens directly to T cells, or if antigens are transferred to lymph node- resident $CD8\alpha^+$ cDCs for antigen presentation [36-38].

Tissue or interstitial DCs are the main DC type found in peripheral tissues. They constitute several diverse populations which reside in the periphery, including the DCs of the dermis or the lamina propria of mucosae [39, 40]. As LCs, these DCs also migrate to draining lymph nodes, both in steady state and during inflammatory processes. However, interstitial DCs differ from LCs not only in their location, but in their function. One example is that interstitial DCs localize close to the B cell follicles in the outer paracortex of the lymph node were they are able to induce naive B cells to differentiate into IgM-secreting B-1 cells. LCs, on the contrary, settle in the inner paracortex were they mix with lymph node-resident $langerin^+$ cells [41]. Another example is their assumed role in peripheral tolerance. There is evidence that interstitial DCs are able to act on mature T cells in the periphery that escaped negative selection in the thymus, deleting auto-reactive T cell clones; a mechanism also referred to as “recessive tolerance” [42].

1.2.2 Conventional dendritic cells

Conventional DCs (cDCs) are also called resident DCs, which highlights a major difference to the migratory DCs discussed above: cDCs do not migrate into lymphoid organs from the lymphatics. Instead, they reside in lymphoid organs like lymph nodes, where they take up and present antigens coming either from the lymph or from migratory DCs. It has been estimated that approximately half of the DCs in lymph nodes are resident DCs, whereas the remainder has migrated there from the periphery [33, 43]. Not only are resident DCs present in significant numbers in all lymphoid organs, they even constitute the majority of thymic and splenic DCs [43-45]. In spleen these cells can be further subdivided, based on their expression of $CD8\alpha^+$ [45, 46].

The subset of $CD8\alpha^+$ cDCs accounts for up to 15% of splenic DCs and is the dominant DC subtype in the thymus [7, 47]. CD8α DCs show some unique functions, which separate them from other resident cDCs. Although they are a rather minor population in spleen, $CD8\alpha^+$ DCs are the major producers of interleukin (IL)-12 upon activation. During infection with intracellular pathogens they are the major subset that efficiently cross-presents pathogenic antigens on MHC-I complexes, thereby promoting $CD8^+$ T-cell responses to the invading pathogens [47]. In steady state, $CD8\alpha^+$ DCs locate primarily to the T cell areas of spleen and lymph nodes [48]. Here they are believed to be involved in immune regulatory properties and maintaining self-tolerance by inactivation of potentially auto-reactive T cells [7, 47, 49].

The $CD8\alpha^-$ cDCs constitute the major subset of DCs in the spleen. In steady state, $CD8\alpha^-$ DCs are predominantly found in the marginal zones of the white pulp, but in response to contact with agents that stimulate pattern recognition receptors they migrate into the T cell zones [23]. According to their expression of CD4, $CD8\alpha^-$ DCs can be sub-divided into $CD4^+CD8\alpha^-$ and $CD4^-CD8\alpha^-$ cDCs. However, due to their high degree of functional similarity, they are frequently considered one single subset, irrespective of the presence or absence of CD4 [45, 50]. To date, there is still no information on the particular role of $CD4^-CD8\alpha^-$ cDCs during an infection or in the induction of tolerance, and their physiological function remains to be identified.

1.2.3 Circulating dendritic cells

The circulating DC was long thought to be a cDC that migrates through the blood and subsequently seeds lymphoid organs. However, cDCs with their characteristic expression of $CD11c^{hi}MHC\text{-}II^{+}$ are not found in blood, which indicates that these DCs are not generally circulating [51]. Instead, circulating DCs can rather be seen as DC precursors (pre-DCs), including the type-1 Interferon (IFN) producing plasmacytoid pre-DCs (pDCs) [52]. Plasmacytoid DCs are thought to develop in the bone marrow. From here, they travel through the blood into the lymphoid organs. Comparable to B cells, pDCs seem to be developed and already functional cells [53]. Nevertheless, as indicated by their classification as pre-DCs, their maturation seems to be less pronounced than that of cDCs [51, 54, 55]. Activation of pDCs occurs after contact with various kinds of pathogens, such as viruses and bacteria, but also with pathogen-associated molecular patterns (PAMPs), including single stranded RNA and CpG-containing DNA, which are ligands for the Toll-like receptors (TLR)-7 - or -9, respectively, which are discussed below (section 1.3.3) [56-59]. As a consequence, pDCS react with the production of inflammatory chemokines and cytokines. Among others, there is a characteristic burst of IFNα release that is crucial for activating neighbouring DCs and limiting viral replication [60].

Another relevant cell type in the circulation are monocytes. In blood, these cells normally express only low levels of Gr-1 ($Gr\text{-}1^{lo}$ monocytes), whereas reserves of the less mature $Gr\text{-}1^{hi}$ subset are retained in the bone marrow. It is still unclear if these monocytes are constantly present in the circulation, or if they have the potential to extravasate from lymphoid organs in the steady-state, as well [61]. It is, however, known that monocytes constitute a precursor with the potential to give rise to fully mature DCs. However, this only occurs upon infection or inflammation [62, 63]. Therefore, blood monocytes are precursors from which inflammatory DCs can develop. Multiple studies have investigated the role of monocytes in the generation of inflammatory dendritic cells, yielding the conclusion that at least a proportion of the inflammatory DCs are the progeny of monocytes. These have been further characterized as a subset of monocytes that not only express CD115, which is typical for the entire monocytic lineage, but also Gr-1, CCR2, and L-Selectin [61]. In experiments in which these $Gr\text{-}1^{+}CCR2^{+}L\text{-}Selectin^{+}$ monocytes were transferred into uninfected, non-irradiated mice, no sign of DC progeny of these cells was

found in lymphoid organs, indicating that monocytes do not serve as DC precursors in steady-state [64-66]. In contrast, injection of Gr-1^{+}CCR2^{+}L-Selectin^{+} monocytes during inflammation gave rise to DC populations in peritoneum and spleen [64, 66].

1.3 Development and activation of dendritic cells

1.3.1 Overview of dendritic cell development

In general, the remaining lifespan of the majority of DCs is rather short. This necessitates a constant replenishment from a pool of precursor cells [67, 68]. The precursors of DCs and the developmental pathways that lead to the different DC subtypes are therefore not only important for the initial establishment of the many different DC populations throughout the body, it is also crucial to preserve effective antigen surveillance by DCs. Although the knowledge of DC function is continuously growing, the developmental origin of DCs remains rather controversial. One reason for this lies in the great variety of DCs, their different locations and function, and their different maturation states.

All cells of the immune system are derived from bone marrow-resident precursors, the self-renewing haematopoietic stem cells (HSC), which can develop into a progression of cells with an increasing degree of specialization into the different lineages. One of the initial steps involves the development of precursors for lymphoid and myeloid cells, the common lymphoid progenitor (CLP) or the common myeloid progenitor (CMP), respectively (Figure 1-2) [69, 70].

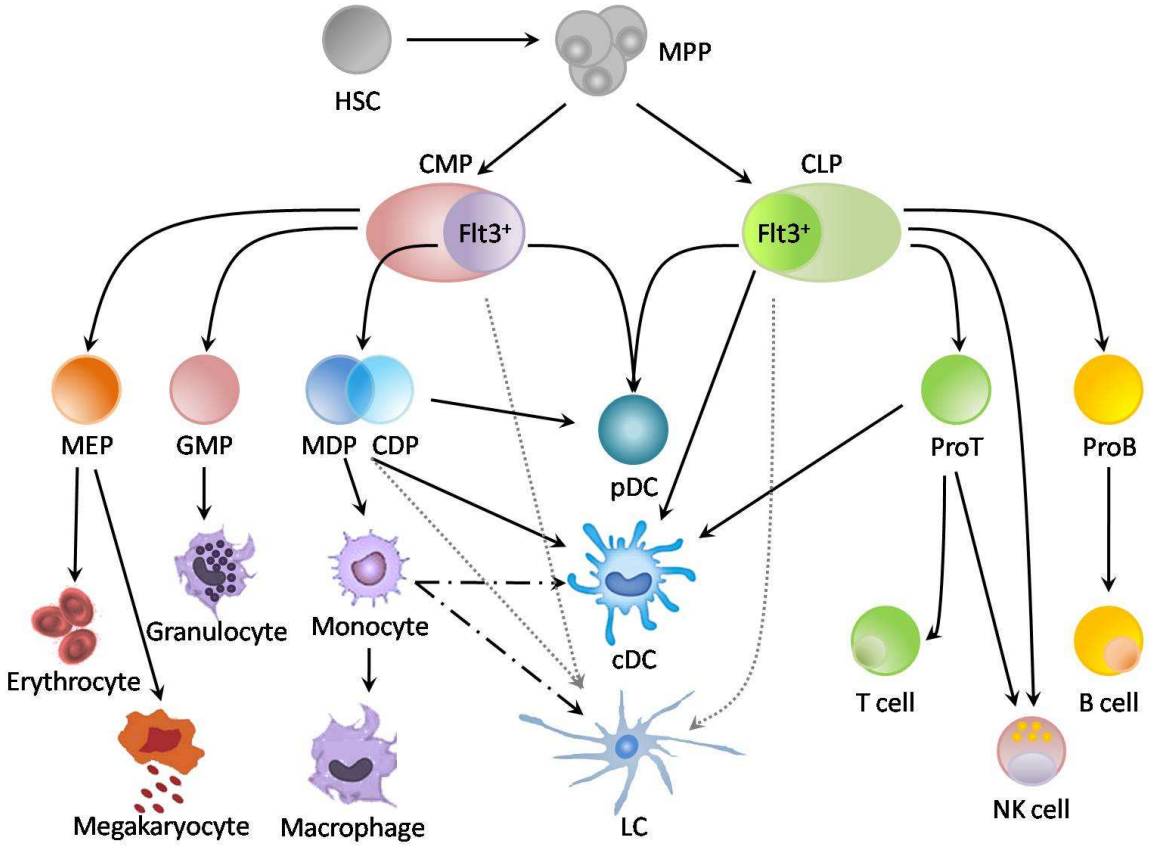


Figure 1-2: Scheme of mouse DC development from bone marrow HSCs and lineage-committed progenitors.
Both cDCs and pDCs can be generated from the Flt3 expressing early myeloid or lymphoid progenitors. Flt3 ligand is essential for the development of steady-state DC populations. Under inflammatory conditions, i.e., in the presence of GM-CSF, monocytes can differentiate into cDCs and LCs (black dash-dotted line). LCs are suggested to be maintained locally and independent of circulating precursors in the steady-state. However, they most probably also develop from Flt3$^+$ CLPs and CMPs via the MDP/CDP (grey dotted line). Abbreviations: CDP, common DC progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; GMP, granulocyte and macrophage progenitor; HSC, haematopoietic stem cell; MDP, macrophage and DC progenitor; MEP, megakaryocyte and erythrocyte progenitor; MPP, multipotent progenitors. Picture adapted and modified from [24].

Based upon a number of similarities with macrophages, DCs were thought to derive from a myeloid origin. This was supported by the observation that not only granulocytes, but also macrophages and DCs could be grown from a myeloid precursor cell treated with granulocyte-macrophage colony-stimulating factor (GM-CSF) [71]. Moreover, splenic and thymic cDC and pDC populations were replenished by transfer of CMPs into irradiated mice [72-74].

However, the view of DCs developing from a myeloid precursor and continuously migrating via the bloodstream into their respective organs where they give rise to progeny was too simple. As stated above, cDCs in thymus, spleen and lymph nodes carry CD8α and CD4, as well as some other markers that are normally found on lymphoid cells

[46]. This indicates that at least a fraction of DCs may be derived from the CLP. Later it was found that a lymphoid precursor cell from murine thymus, which should be a precursor for T lymphocytes, did not only yield T cells, but in addition developed into $CD8\alpha^+$ thymic cDCs and both $CD8\alpha^+$ and $CD8\alpha^-$ DCs in the spleen [73, 75, 76]. A conclusion of these experiments was that the CMP might give rise to $CD8\alpha^-$ cDCs, whereas $CD8\alpha^+$ cDCs are derived from the lymphoid lineage. This idea was overthrown by a detailed analysis of the ability of isolated CLPs and CMPs to form different DC subtypes [73, 77, 78]. Hereby, it was found that all known DC subtypes could be generated from either precursor *in vivo* (Figure 1-2) [72, 73, 77, 79]. This demonstrates a high degree of flexibility in the development of DCs, which has to be determined downstream of the separation of lymphoid and myeloid lineage. Consequently, the expression of the receptor c-fms-like tyrosine kinase 3 (Flt3) was detected on approximately one-third of all CMPs and two-thirds of CLPs. Only these $Flt3^+$ progenitor cells are capable of developing into DCs (Figure 1-2) [80-82].

However, one DC subpopulation, the LCs, does fit into this concept. In contrast to an origin on the bone marrow, LC development could involve a local precursor rather than a supply of cells from the circulation [83]. For this, two options exist: First, LCs could have the potential for self-renewal, even as mature cells. Second, they could develop from a local precursor in the skin rather than bone marrow. One region where such a LC precursor could reside is the bulge region of the hair follicle. They contain keratinocytes, melanocytes and mast-cell progenitors, and experiments indicate that these follicles are sufficient to replace LCs lost as a consequence of damage to the epidermis [84]. Notably, this seems to be true only for the steady state. Under inflammatory conditions, LCs are replaced from a pool of $Gr\text{-}1^+$ monocytes in blood. This process requires the macrophage colony-stimulating factor receptor (M-CSFR), pointing towards an important role of cytokines in DC development, which is discussed in more detail in the next section [63].

1.3.2 Role of cytokines in dendritic cell development

Among the many cytokines involved in the regulation of haematopoiesis, four stand out as being of particular importance for DC development: M-CSF, GM-CSF, the ligand of the

Flt3 receptor (Flt3L), and transforming growth factor β (TGFβ). There influence on DC development is shortly summarized in Table 1-1.

Table 1-1: The influence of cytokines in DC development.

Cytokine level alteration	DC subsets				
	pDCs	**CD8α$^+$ cDC**	**CD8α$^-$ cDC**	**LCs**	**Inflammatory DCs**
GM-CSF null	↔	↔	↔	ND	ND
GM-CSFR null	↔	↔	↔	↓	ND
GM-CSF transgenic	ND	↔	↔	ND	ND
GM-CSF administration	ND	↔	↑↑(?)	↑	↑↑(?)
GM-CSF over-expression	ND	↑	↑↑(?)	ND	↑↑(?)
Flt3L null	↓↓	↓↓	↓↓	↔	ND
Flt3L administration	↑↑	↑↑	↑	↓	ND
M-CSFR null	↔	↔	↔	↓↓	ND
TGFβ null	↔	↔	↔	↓↓	ND

Abbreviations: ↑, increased; ↓, decreased; ↔, unaffected; ND, not determined.
Table adapted from [53].

As indicated above, mice lacking the receptor for M-CSF cannot repopulate LCs in the epidermis depleted by inflammation. In fact, the animals are devoid of monocytes and LCs [63, 85]. Whereas M-CSF seems to be required for the formation of LCs, and possibly also other types of migratory DCs from Gr-1$^+$CCR2$^+$L-Selectin$^+$ monocytes under inflammatory conditions, the influence of M-CSF during steady-state is most likely only of minor importance, if any.

Application of GM-CSF to myeloid precursor cells leads to their differentiation into granulocytes, monocytes and DCs [71], and administration of GM-CSF increases the development of both monocytes and haematopoietic progenitors into myeloid DCs, in particular the development of myeloid CD8α$^-$ cDCs in the spleen [86]. The effect of GM-CSF has been utilized as an *in vitro* culture model, allowing to obtain large numbers of DCs from isolated murine bone marrow by a relatively simple procedure [87, 88]. As a consequence, the so-called bone marrow derived DCs have become a frequently used model system for the investigation of DCs *in vitro* [88].

In contrast to the strong effects *in vitro*, neither the GM-CSF receptor nor the cytokine itself are necessary for DC development; mice deficient in either protein were normal with respect to cDC and pDC development [89, 90]. This may be based on the fact that

GM-CSF is predominantly expressed during inflammation, and its *in vivo* role may be, similar to M-CSF, in the development of inflammatory DCs from myeloid precursors, rather than maintenance of steady state DC levels [91, 92].

Flt3L is the ligand for Flt3, which has been mentioned in section 1.3.1 as a characteristic marker for the fractions of CLPs and CMPs that have the ability to differentiate into DCs. A functional relevance for Flt3 in these events is indicated by the fact that Flt3L is required for pDC and cDC development in the steady state [56, 86]. Mice that lack Flt3L or have compromised Flt3-dependent signalling pathways show a marked reduction of pDC and cDC levels. However, Flt3 is not only involved in the development of DCs; e.g., NK cell levels are also affected by a lack of Flt3L. Comparable to GM-CSF, administration of Flt3L to mice also leads to elevated levels of DCs [93-96]. Thus, Flt3L has also been used as an agent to induce the development of DCs from bone marrow cultures *in vitro* [93, 97-100].

TGFβ does not have an effect on general DC development. Mice deficient for TGFβ have normal levels of pDCs and cDCs of myeloid and lymphoid background. However, TGFβ seems to be crucial for LC development, since these mice lack LCs in their epidermis completely [101]. As for M-CSF, GM-CSF, and Flt3L, the effects of TGFβ have also been investigated using *in vitro* cultures. There it was found that TGFβ strongly augmented the generation of LCs from $CD34^+$ haematopoietic progenitor cells [102]. Another study aimed to pinpoint the event where TGFβ is required for LC development. When lethally irradiated wild type mice were injected with bone marrow cells from $TGF\beta^{-/-}$ mice, the transplanted cells were still able to form LCs. This indicates that the absence of LCs in $TGF\beta^{-/-}$ mice is not caused by an absence of LC precursors in the bone marrow, but most likely based on a requirement of LCs for paracrine stimulation with TGFβ [103].

1.3.3 Role of Toll like receptors in dendritic cell activation

During an infection, optimal host defence by the adaptive immune system depends on the presentation of a sufficient amount of antigen in the lymphoid tissues. Hence, an activating signal is required to increase the number of migratory DCs carrying antigens from the infected tissue to the site of antigen presentation. Whereas cytokines mediate the development DCs, another group of substances is important for their activation.

These are pathogen associated molecular patterns (PAMPs), molecular structures that are only found in pathogens, but not the host organism, and can therefore serve as a reliable indicator for the presence of an infection [1, 104]. These are detected by so-called pattern recognition receptors, most importantly the family of Toll-like receptors (TLRs). Thirteen TLRs are known in mammals, an overview can be found in table 1-2.

Table 1-2: Toll like receptors

TLR	Expression	Ligand(s)	References
1	surface	triacyl lipopeptides (as TLR1/2 dimer)	[105]
2	surface	various components of Gram-positive and -negative bacteria, mycoplasms and yeast, such as peptidoglycan, lipopeptides, zymosan	[106-108]
3	cytoplasm	viral double-stranded RNA	[109]
4	surface	lipopolysaccharide from Gram-negative bacteria	[110-113]
5	surface	bacterial flagellin	[114]
6	surface	diacyl lipopeptides (as TLR2/6 dimer)	[115]
7/8	cytoplasm	TLR8 in humans and TLR7 in mice sense viral single-stranded RNA and similar synthetic molecules such as imidazoquinolines. The respective other TLR is inactive in that species.	[58, 116-120]
9	cytoplasm	unmethylated CpG-containing sequences in bacterial DNA	[121-124]
10	unknown	ligand unknown (TLR10 is functional in humans but not in mice)	[125]
11	unknown	profilin-like protein from Toxoplasma gondii, unknown component(s) of uropathogenic E. coli (TLR11 is not found in humans)	[126-128]
12	unknown	TLRs 12 and 13 have only been identified as cDNA in mice, no ligands are known	[129]
13	unknown		

Activation of DCs via their TLRs induces maturation, which has three main features:

a. DCs react to contact with PAMPs with the secretion of various soluble mediators, such as inflammatory cytokines, type I IFN, and chemokines.
b. Activation of DCs is accompanied by increased surface expression of co-stimulatory molecules such as CD40, CD80, and CD86.
c. Triggering of TLRs increases MHC-II presentation on the cell surface.

The last point bears a potential pitfall. DCs constantly sample antigens from their environment for MHC-II presentation to T cells, regardless if these antigens are derived

from pathogens or the host. In immature DCs, most MHC-II remains in the late endosomes. Upon activation, however, DCs must be able to distinguish between endosomes containing self and foreign antigens in order to avoid accidental presentation of self-antigens and inducing autoimmunity. Here, TLRs on the surface of the endosomes seem to monitor their content, selecting the antigens that the DCs present [130].

TLRs do not only sense the presence of pathogens in general. TLRs are specific for PAMPs associated with a particular group of infecting agents. TLRs 3 and 7 detect double- or single-stranded RNAs, respectively, which are of viral origin [109, 116]. In contrast, several other TLRs, including TLR 2, 4 and 5, sense bacterial PAMPS. TLR4, e.g., is activated by lipopolysaccharide, which is a component of Gram negative bacteria [110-113]. Additional specificity results from heterodimerisation. A case in point is TLR2. In addition to homodimerisation, it also associates with TLR1 and TLR6. TLR2 homodimers recognise peptidoglycans, whereas TLR1/2 hetero-dimers respond to triacyl lipopeptides, such as Pam_3CSK_4, and TLR2/6 detects diacyl lipopeptides, such as MALP-2 [105-108, 115]. It has been shown that some aspects of DC activation, especially cytokine production, depend on the type of TLR by which they were activated. This has an impact on T helper cell polarization, stimulating the formation of T cells that mediate an immune response which is adapted to the type of pathogen [4].

1.3.4 Role of transcription factors in dendritic cell development and activation

The developmental and activating signals that DCs receive from cytokines and PAMPs via their respective receptors result in the downstream activation of several transcription factors. Differential activation of several transcription factors is involved in orchestrating the development of DCs into the different subsets. Table 1-3 summarizes the effects excerted by deletion of single transcription factors on the expression of DC subsets in mice.

Table 1-3: Differential requirements for transcription factors in DC development

Transcription factor	DC subsets				
	$CD4^-CD8\alpha^+$	**$CD4^+CD8\alpha^-$**	**$CD4^-CD8\alpha^-$**	**pDC**	**LC**
$RelB^{-/-}$	+++	-	+++	ND	+++
$IRF\text{-}2^{-/-}$	+++	+	ND	+++	++
$IRF\text{-}4^{-/-}$	+++	-	+++	+	ND
$IRF\text{-}8^{-/-}$	-	+++	+++	-	++
Ikaros DN	-	-	-	ND	+++
$Ikaros^{-/-}$	+	-	-	ND	ND
$PU.1^{-/-}$	++	-	-	ND	ND
$STAT3^{-/-}$	-	-	-	++?	ND
$Gfi1^{-/-}$	+	+	+	+	++++
$Id2^{-/-}$	-(+)	++++	+	++++	-
$Runx3^{-/-}$	++++		++	ND	-

A panel of transcription factor knockout mice and their DC phenotypes. The impact of the targeted gene is depicted as increased (++++), normal (+++), reduced (++) or low (+) presence or complete absence (-) of the respective DC populations. ND = not determined. Table adapted from [24, 53, 131]

Transcription factors from the nuclear factor (NF)-κB/rel and interferon regulatory factors (IRF)-families are involved in both development and activation of DCs. NF-κB is a major mediator for TLR-signaling, accompanied by other transcription factors such as AP-1, and three members of the IRF-family, IRF-3, -5, and -7 [132]. Especially signals involving the adaptor protein myeloid differentiation primary response gene (MyD)88-pathway, which leads to the expression of inflammatory cytokines, depend on NF-κB. In contrast, expression of type I interferons and co-stimulatory molecules are controlled, amongst others, by IRF-3 [132].

In addition to their role in TLR-signaling, NF-κB/rel family transcription factors are also involved in DC development and subset formation [133-135]. NF-κB/rel factors control the development and function of DCs in a complex and subunit-specific fashion. Hereby, the RelB subunit seems to have the most profound impact [131]. Deletion of RelB has no effects on $CD4^-$ DCs and LCs, but abrogates the development of splenic $CD4^+$ DCs. A predominant role for RelB in the development of $CD4^+$ DCs, but not $CD4^-$ DCs, corresponds well to the comparatively higher expression levels of this transcription factor that were reported in splenic $CD4^+$ cDCs [133, 134, 136, 137]. Additional indirect evidence

for a requirement for RelB in these events comes from defects in $CD4^+$ cDC development in mice deficient of TNF receptor associated factor (TRAF) 6, which is located upstream in the signalling cascade leading to RelB activation [137].

Whereas IRF-3, -5, and -7 are involved in DC activation by TLRs, three additional members of the IRF family participate in the development of DC subsets. Absence of functional IRF2 has no impact on pDCs and $CD8\alpha^+$ cDCs, but leads to diminished levels of $CD4^+CD8\alpha^-$ cDCs and LCs [138]. A similar defect was also observed in IRF4-deficient, which have a selective defect in splenic $CD11b^{high}CD8\alpha^-$. Moreover, *in vitro* cultures of bone marrow from these mice also showed impaired development of $CD4^+CD8\alpha^-$ DCs [139]. IRF8, which is also known as ICSBP, has a different effect than IRF-2 and -4. Lowered numbers of splenic $CD8\alpha^+$ cDCs were found in the spleens of IRF8 knockout mice. Furthermore, other DC population, namely pDCs and LCs, were also diminished [140-143]. Finally, IRF-4 and -8 double knockout mice showed severe impairment in the development of all pDCs and cDCs, indicating that at least one of these transcription factors seems to be involved in the development of all DC subsets [144].

A number of additional transcription factors have been identified, which have no role in DC activation or TLR-signalling, but are nevertheless involved in DC development. Some of them belong to the family of Ets proteins. Their ability to regulate the expression of a wide variety of genes in many tissues and cell types is based on interacting with other, structurally unrelated transcription factors [145]. One Ets protein is PU.1, and its interaction with IRF-4 and -8 already indicates that it should have an influence on DC development. High expression levels of Pu.1 are found in haematopoietic cells that are known to be DC precursors, such as HSCs, CLPs, and $Flt3^+$ CMPs [146]. Bone marrow cells obtained from Pu.1-deficient mice were not able to develop into DCs during *in vitro* culture with GM-CSF and IL-4 [131]. DCs express high levels of PU.1, and PU.1 is required for the development of both cDCs and pDCs [147, 148]. Another transcription factor that interacts with PU.1 is Spi-B, which also belongs to the Ets family. In contrast to PU.1, it is not expressed in cDCs or myeloid cells, but has been detected in pDCs, B cells, and $CD34^+$ haematopoietic progenitors [149, 150]. When cultures of the latter were subjected to Spi-B or PU.1 knockdown by RNA-interference, they lost their ability to develop into pDCs. In the case of Spi-B this instead led to differentiation into B cells and myeloid cells, whereas PU.1 knockdown reduced the development of all three cell types [149].

Ikaros belongs to the Krüppel family of zinc finger transcription factors. Observations in mice expressing a dominant-negative form of Ikaros indicated that this transcription factor has a general role in haematopoietic development. Not only were these mice lacking all splenic DCs, but also all lymphocytes, namely T, B, and NK cells. Notwithstanding, Ikaros knockout mice, which should show a comparable phenotype, were only deficient in $CD8\alpha^-$ cDCs and pDCs and had impaired development of residual $CD8\alpha^+$ cDC, indicating that the role of this transcription factor in DC development requires further investigation [151-153].

Signal transduction by many cytokines involves one of the seven members of the signal transducer and activator of transcription (STAT) family. This is also true for two regulators of DC development, Flt3 and GM-CSF. STAT3 is involved in signalling downstream from Flt3. Treatment of wild type mice with Flt3L led to a strong increase in splenic DC numbers, which was completely abrogated in $STAT3^{-/-}$ mice, demonstrating that STAT3 is absolutely necessary for Flt3L-dependent DC development [154]. The transcriptional repressor Gfi1 is involved in STAT3 signalling during DC development. Gfi1-deficient progenitor cells have decreased STAT3 activity. In accordance with the essential function of STAT3 in DC development, the absence of Gfi *in vivo* resulted in a reduction of myeloid and lymphoid DC numbers and function in all lymphoid organs, but elevated numbers of LCs in the epidermis. *In vitro*, hematopoietic progenitor cells from Gfi-1-deficient mice were not able to differentiate into DCs in the presence of GM-CSF and IL4 [155]. Flt3L-mediated pDC development is reduced by addition of GM-CSF in favour of elevated formation of cDCs both *in vivo* and *in vitro*. It has been shown that STAT5 activation by GM-CSF is required for shifting the balance from pDC to cDC generation in response to this cytokine [24].

In section 1.3.2, TGFβ was introduced as a cytokine critical for LC development. In DCs, TGFβ induces expression of the transcription factor Inhibitor of differentiation 2 (Id2). Notably, Id2 is also upregulated during development of DCs *in vitro* [156]. Moreover, Id2 as well as TGFβ-deficient mice both lack LCs. Hence, it has been suggested that TGFβ acts on LC development through regulating the expression of Id2 [131]. Experiments in which Id2 was overexpressed in an *in vitro* culture system of haematopoietic stem cells showed that Id2 has no impact on cDC development, whereas it inhibits the development of pDCs [157]. The helix-loop-helix (HLH) protein Id2 does not regulate gene expression by binding to promoter regions, but instead by binding other transcription factors that also belong to

the HLH family of transcriptional regulators. Overexpression of two of these factors, HEB and E2A in haematopoietic progenitor cultures promotes the formation of pDC [149]. This observation suggests that Id2 might antagonize pDC development by binding, and thereby inactivating, HEB or E2A. Similar to Id2, Runx3 is another transcription factor which is markedly expressed in mature DCs, is under the control of TGFβ, and affects DC development. It is unclear if this is based on an interaction with Id2, or if both proteins act independently from each other. Regardless, knockout of Runx3 or Id2 in mice results in similar alterations in DC subsets. On the one hand, development of LCs in response to TGFβ is also impaired in Runx3$^{-/-}$ mice, on the other hand the inhibitory effect of TGFβ on other DCs is abrogated in the absence of Runx3 [158].

1.4 Aim of the study

One of the current aims of developmental biology is to understand the mechanisms that direct multipotent cells to commit to a single fate. The development of mature lymphoid and myeloid cells occurs through the progressive restriction of the developmental options of HSCs. Before committing to a particular lineage, HSCs differentiate into multipotent progenitors that lack the extensive self-renewal capacity. This thesis continuatively examines the previously described $Flt3^+$ DC precursor from mouse bone marrow, especially its developmental potential *in vivo*.

Furthermore, the mechanisms that promote the development of lineage restricted cell types, such as DCs from early progenitors, are beginning to be understood at the molecular level and include the activity of numerous transcriptional regulatory proteins, many of which are member of the HLH protein family. HLH protein expression and activity can be regulated by multiple signalling pathways that are active during lineage determination. However, the relationship between these pathways and HLH activity during the commitment process is currently not known. This research aims to determine the importance of Id2 for the development of LCs and other skin DCs as well as for DC development in lymphoid organs. Additionally, it analyses whether the development of other lymphocytes, especially T cells, is also regulated by Id2. Finally, it determines the impact of the antagonism of Id2 and E2A for the development of different DC subsets and addresses the question whether lowering E protein activity in $Id2^{-/-}$ mice by deletion of the E2A gene product E47 results in the restoration of previously absent DC subtypes.

2 Material and Methods

2.1 Material

2.1.1 Chemicals

Unless stated otherwise, all chemicals were obtained from Merck, Fluka or Sigma in analytical grade quality.

2.1.2 Size standards

DNA size marker: Gene Ruler 1kb ladder
Gene Ruler 100bp ladder plus
Gene Ruler 50bp ladder
Protein size marker: Page Ruler Prestained Protein Ladder
All from Fermentas.

2.1.3 Media

Basic culture medium: RPMI1640 containing 2mM L-glutamine, 100U/ml penicillin, 100µg/ml streptomycin, 50µM β-mercaptoethanol, and 10% FCS (heat inactivated).

Flt3 precursor amplification medium: Basic culture medium complemented with 30U/ml muSCF, 5ng/ml hyperIL-6, 25ng/ml Flt3L, 40ng/ml huIGF-1 long range, 25U/ml muGM-CSF, and 1pM Dexamethasone.

FL amplification medium: Modified Flt3 precursor amplification medium. Basic culture medium complemented with 30U/ml muSCF, 5ng/ml hyperIL-6, 25ng/ml Flt3L, 40ng/ml huIGF-1 long range.

GM-CSF medium: Basic culture medium complemented with 200U/ml muGM-CSF

Tissue culture additives:

- All media, L-glutamine, and penicillin/streptomycin were purchased from Gibco-BRL
- β-mercaptoethanol was obtained from Sigma-Aldrich
- Fetal Calf Serum (FCS) was supplied by PAA and was heat-inactivated for 30min at 56°C prior to medium preparation
- Recombinant murine SCF was produced in SF900 insect cells using baculovirus generated with the pETH2a HIS-mSCF plasmid (S.M. Kurz, PhD thesis 2000)
- Recombinant IL-6/IL-6R fusion protein (Fischer, et al., 1997) was kindly provided by S. Rose-John, Christian Albrecht University, Kiel
- Flt-3L was purchased from PeproTech EC
- recombinant long-range IGF-1 was obtained from Sigma-Aldrich
- Murine GM-CSF was produced in *E. coli* transformed with the plasmid pETH2aHIS-mGM-CSF (S.M. Kurz, PhD thesis, 2000)
- Dexamethasone was from Sigma-Aldrich

Table 2-1: TLR ligands

Name	Ligand of	Description	Concentration	Working concentration
Pam_3CSK_4	TLR1/2	Synthetic Lipoprotein, mimics the acylated amino terminus of bacterial lipoprotein	1mg/ml	1µg/ml
HKLM	TLR2	Heat killed *Listeria monocytogenes*	10^{10} cells/ml	10^{8} cells/ml
Poly(I:C)	TLR3	Polyinosine-polycytidylic acid, a synthetic analog of double stranded RNA	10mg/ml	10µg/ml
LPS-EK	TLR4	Lipopolysaccharide from *E.coli* K12	1mg/ml	1µg/ml
FLA	TLR5	Bacterial flagellin; major component of the flagella filament	1mg/ml	1µg/ml
FSL-1	TLR6/2	Synthetic lipoprotein, represents the N-terminal part of lipoprotein LP44 of *Mycoplasma salivarium*	1mg/ml	1µg/ml
ssRNA	TLR7	single stranded RNA, complexed with LyoVec, a cationic lipid; substitues viral ssRNA	2.5mg/ml	2.5µg/ml
Gardiquimod	TLR7	Synthetic ligand for TLR7	1mg/ml	1µg/ml
CpG 1826	TLR9	CpG ODN are synthetic oligonucleotides containing unmethylated CpG motifs that are found in a high frequency in bacterial DNA	5mM	5µM

All TLR ligands were from Invivogen

Table 2-2: Kits

Kit	Manufacturer
Cytometric Bead Assay	BD Bioscience
Mouse IL-6 Flex Set (bead B4)	
Mouse IL-10 Flex Set (bead C4)	
Mouse IL-12p70 Flex Set (bead D7)	
Mouse MCP-1 Flex Set (bead B7)	
Mouse MIG Flex Set (bead D9)	
Mouse TNFα Flex Set (bead C8)	
High Capacity cDNA Reverse Transcription Kit	Applied Biosystems
NucleoSpin RNA II	Macherey & Nagel
NucleoSpin Tissue	Macherey & Nagel
RNeasy Mini Kit	Qiagen
RNeasy Midi Kit	Qiagen
T7 Mega Script Kit	Ambion

Table 2-3: Buffer and solutions

Buffer	Reagents
10x TBE	0.89M Tris/HCl, 0.89M boric acid, 20mM EDTA
17% sucrose buffer	88ml 0.1M B2 buffer, 12ml ddH_2O, 17g sucrose, pH 7.4
20mM EDTA	20mM EDTA in PBS
B2 buffer	13mM NaH_2PO_4 x H_2O, 87mM Na_2HPO_4 x $2H_2O$, pH 7.4
Collagenase solution	1mg/ml Collagenase V in RPMI1640
EDTA/PBS	2mM EDTA in PBS
Hank's buffered saline solution (HBSS)	Purchased from Gibco-BRL
HBSS/PBS	50% HBSS in PBS
Monocyte wash buffer	0.5% BSA, 2mM EDTA in DMEM
PFA/PBS	2% PFA in PBS
Phosphate Buffered Saline (PBS)	Purchased from PAA
Red blood cells (RBC) lysis buffer	150mM NH_4Cl, 100mM $NaHCO_3$, 10mM EDTA in ddH_2O, pH adjusted to 7.4
Saponin buffer	3% FCS, 2mM EDTA, 1% saponin, 0.02% Thimerosal in HBSS/PBS
Staining buffer	3% FCS, 2mM EDTA, 0.02% Thimerosal in HBSS/PBS
Trypsin/EDTA	10x trypsin/EDTA purchased from Gibo-BRL

Table 2-4: Antibodies for flow cytometry

Name	Cluster of differentiation	mAB clone	Source
	CD3ε	145-2C11	eBioscience
Armenian hamster IgG			BD Bioscience
B220	CD45R	RA36B2	eBioscience
B4	CD19	MB19-1	eBioscience
BD CompBead Set anti rat and hamster			BD Bioscience
CCR7 ligand-IgG fusion			Ulrich Kunzendorf
CSF-1 receptor	CD115	AFS98	eBioscience
E-cadherin		114420	R&D systems
F4/80, Ly-71		CI:A3-1	AbD Serotec
Gr-1, Ly6C/6G		RB6-8C5	BD Bioscience
Heat Stable Ag	CD24	M1/69	BD Bioscience
Integrin α6 chain	CD49f	GoH3	BD Bioscience
Integrin-αX	CD11c	N418	eBioscience
Langerin	CD207	929F3	Alexis
Ly-2	CD8a	YTS169.4	eBioscience
Ly-4	CD4	GK1.5	eBioscience
Ly-5	CD45	30-F11	BD Bioscience
Mac-1	CD11b	M1/70	Caltag
MHC-II		M5/114	eBioscience
PDCA-1	CD317	eBio927	eBioscience
Prominin	CD133	AC133	eBioscience
Rat IgG2a			eBioscience
Rat IgG2b			eBioscience
Siglec H		440c	eBioscience
Ter119		TER-119	BD Bioscience
TLR1	CD281	TR23	eBioscience
TLR2	CD282	6C2	eBioscience
TLR3	CD283	Polyclonal	Alexis
TLR4	CD284	HTA125	eBioscience
TLR5		Polyclonal	Imgenex
TLR6		Polyclonal	Imgenex
TLR7		Polyclonal	Imgenex
TLR9		Polyclonal	Imgenex

Table 2-5: Antibodies for immunohistochemistry

Alternative Name	Cluster of differentiation	mAB clone	Source
langerin	CD207	929F3	Alexis
I-A/I-E		2G9	BD Bioscience
DAPI			Vector Laboratories

2.2 Methods

2.2.1 Mice

All experimental mice used were between 8-16 weeks of age. All mice were bred and maintained under specific pathogen-free conditions at the University Hospital Aachen animal breeding facility, according to institutional guidelines. Mice were killed using cervical dislocation.

Table 2-6: Mice used for experiments in this thesis

Mouse	Genotype	Background
BL/6	BL/6	BL/6
SV129	SV129	129/SvJ
Id2	Id2 deficient	129/SvJ
Id2/E47	Id2 and E47 deficient	129/SvJ x FVB/NJ
eGFP	eGFP knock-in	BL/6

Injections - All intravenous (*i.v.*) injections were performed using 30-gauge needles. For *i.v.* injections, mice were left under a heat lamp for 15min prior to injection to dilate the tail vein. The volume injected was between 50 and 200µl.

Irradiation - For mice that required lethal irradiation prior to cell transfer experiments, γ-irradiation was performed using ^{60}Co from a Telecobalt source. Mice were given 5.5 Gy twice, 4 hours apart, and cell transfer was performed within 2 hours.

Adoptive transfer of bone marrow and Flt3$^+$ progenitor cells - *In vitro* amplified Flt3$^+$ progenitor cells were generated from bone marrow and transplanted into lethally irradiated recipient mice. Different concentrations of Flt3$^+$ progenitor cells or crude bone marrow were injected intravenously. In case of Flt3$^+$ progenitor cells transfer, mice were always reconstituted with $2x10^4$ unfractionated bone marrow cells to ensure mouse survival.

UV irradiation - For LC analysis in normal and inflamed skin ears of sex- and age-matched Id2$^{-/-}$ and Id2$^{+/+}$ mice were incubated for 10min in depilation crème (veet) to remove hair. One day later, mice were exposed to UV-C light (wavelength = 254 nm) for 30min at

a distance of 30cm to induce ear skin inflammation as described [83] or left untreated. Mice were sacrificed at indicated timepoints and ears were taken for further analysis.

2.2.2 Flow Cytometry

Analytical flow cytometry was carried out using a FACS Calibur or FACS Canto II (Beckton Dickinson). Cell sorting was carried out employing a FACS Aria (Beckton Dickinson). Analysis of data was performed using FlowJo software (Treestar).

Compensation - Prior to cell analysis anti-rat/ anti-hamster compensation beads (Beckton Dickinson) were used to eliminate spillover of fluorescence from one conjugate into the detection channels for other conjugates. Compensation was performed using either the instrument software or acquired manually.

Cell preparation - Cells from various preparations (see below) to be analysed or sorted by flow cytometry were usually incubated in FcR blocking reagent and 3% mouse serum before staining of surface markers. Cells were resuspended at $5x10^5$ cells in 100µl of staining buffer containing 0.1µg of the respective antibodies (Table 2-4), stained for 30min at 4°C, and washed with about 1ml of EDTA/PBS. Prior to analysis, propidium iodide (Calbiochem) was added to the sample at a final concentration of 0.5µg/ml to label dead cells for subsequent dead cell exclusion.

Intracellular staining - For intracellular staining cells were fixed by resuspending them in 200µl PFA/PBS and incubated for 10min at room temperature. Cells were washed in ETA/PBS and permeabilized in 200µl saponin buffer containing FcR blocking reagent and 3% mouse serum and incubated for 10min at 4°C. Subsequently cells were washed, resuspended in 100µl saponin buffer containing the respective antibodies and incubated for 30min at 4°C before further analysis.

2.2.3 Isolation of primary cells

Splenocytes/ thymocytes

Splenocytes and thymocytes were prepared as follows. Spleens and thymii were chopped with scissors and then forced through a cell strainer. Splenocytes were subjected to RBC lysis for 5min at room temperature and washed twice in EDTA/PBS. Cells were resuspended in staining buffer and stained for FACS analysis.

Bone Marrow cells

Bone marrow cells were extracted from the tibia and femur with EDTA/PBS using a 1ml syringe and 23-gauge needle. The single cell suspension was centrifuged and the pellet resuspended in about 1ml RBC lysis buffer for 5min. Cells were washed 2-3 times and passed through a cell strainer to remove bone fragments and dead cell clumps. The pellets were resuspended in staining buffer and stained for FACS analysis.

Blood monocytes

To isolate blood monocytes, blood was collected by retro-orbital puncture into heparinised tubes. The blood was subsequently subjected to RBC lysis for 5min at room temperature and washed twice in Monocyte wash buffer to remove platelets. Cells were centrifuged at 4°C at 1400g for 4min, resuspended in staining buffer and stained for FACS analysis. All steps were performed at room temperature.

In vivo labelling of blood monocytes

For labelling of Gr-1^{hi} monocytes, 250µl of liposomes containing clodronate were injected intravenously into the tail, followed by i.v. injection of 250µl FITC-conjugated latex microspheres 0.5µm in diameter (LX-beads; Polysciences) 18h later [62]. Clodronate (a gift from Roche) was incorporated into liposomes as described [159].

Skin dendritic cells

Ears were cut off at the base and incubated for 5min in veet depilation crème sensitive (veet). Ears were washed twice in PBS and separated into dorsal and ventral halves by tearing them apart. Each half was floated on trypsin/EDTA (0.5% for ventral, 0.3% for dorsal halves) containing ~20U DNase per ml and incubated for 1h at 37°C. The reaction

was stopped by adding 1ml FCS to each well, and the epidermis was carefully detached from dermis with forceps. Epidermis and dermis were washed twice in PBS and cut into small pieces using fine scissors. Further digestion was performed in 1ml collagenase solution for 30- 60min at 37°C on a shaker. Single cell suspension was obtained by pipetting the sample up and down several times through a 23-gauge syringe and was passed through a cell strainer. Cells were resuspended in staining buffer for further analysis.

2.2.4 Dendritic cell cultures

DC generation from $Flt3^+CD11b^+$ progenitor cells (two-step cultures)

Two-step DCs were generated in culture as described (Figure 2-1) [160]. Bone marrow cells were extracted from the tibia and femur of mice and adjusted to $2x10^6$ cells/ml in amplification medium. Three days after culture, cells were applied to density gradient centrifugation using Ficoll-Hypaque. To recover factor dependent progenitor cells, cells from the interphase were collected, washed in RPMI containing 10% FCS, and adjusted to $2x10^6$ cells/ml in amplification medium. Medium was renewed every second day. At day 7 of culture differentiation of DC from the proliferating progenitor cells was initiated. Cells were washed, transferred to a new dish and adjusted to $2x10^6$ cells/ml in GM-CSF medium. Medium was renewed every second day for another 8 days until a population of differentiated DCs developed. In some experiments further maturation of DCs was achieved by adding TNFα for another 24 hours.

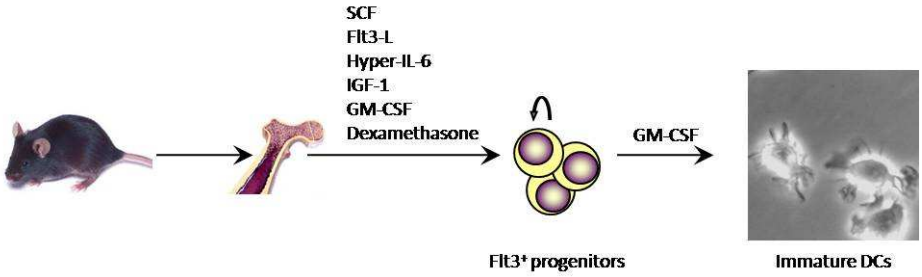


Figure 2-1: Generation of $Flt3^+$ progenitor cells and two-step DCs.
$Flt3^+$ progenitor cells were amplified from mouse bone marrow using the indicated cytokines. After 7 days of amplification, two-step DCs were differentiated from $Flt3^+$ progenitors by GM-CSF.

DC generation from GM-CSF bone marrow cultures

GM-CSF generated DCs from mouse bone marrow were prepared as previously described [161, 162]. Bone marrow cells were isolated by flushing femur and tibia. Cells were resuspended in GM-CSF medium and adjusted to 1 $x10^6$ cells/ml. The medium was renewed by half every 3 days and fresh GM-CSF was added. Cells were cultured for 8 days until a population of differentiated DCs developed.

DC generation from Flt3L bone marrow cultures

Flt3L DCs were generated in culture as described, but slightly modified [160]. Bone marrow cells were extracted from the tibia and femur of mice and adjusted to $2x10^6$ cells/ml in FL-amplification medium. After three days of culture cells were applied to density gradient centrifugation using Ficoll-Hypaque to recover factor dependent progenitor cells. Cells were washed and adjusted to $2x10^6$ cells/ml amplification medium. Medium was renewed every second day. At day 7 of culture differentiation of DCs from the proliferating progenitor cells was initiated. Cells were washed, transferred to a new dish and adjusted to $1x10^6$ cells/ml in FL medium. Medium was renewed every third day for another 10 days until a population of differentiated DCs developed.

2.2.5 Miscellaneous protocols

Polymerase chain reaction (PCR)

RNA prepared from tissue culture cells was purified using the NucleoSpin RNA II Kit (Macherey & Nagel) according to manufacturer's instructions. RNA concentration was measured by spectrophotometry using a NanoDrop (Thermo Scientific). Up to 2µg RNA were reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit following manufacturer's protocol. Semi-quantitative PCR was performed to determine the expression of indicated genes using the Eppendorf Master Cycler (Eppendorf, Germany); 50ng cDNA was used per PCR reaction. The standard PCR protocol is given in Table 2-7

Table 2-7: Standard PCR protocol

Step	Temperature	Cycles
Initial activation	5min at 95°C	1
Denaturation	45sec at 95°C	28-35
Annealing	45sec at 55-60°C	
Extension	1min at 72°C	
Final extension	5min at 72°C	1

Primer for PCR analysis are listed in Table2-8. Hprt1 (encoding hypoxanthine guanine phosphoribosyl transferase) or β-actin was used as 'housekeeping' gene control. PCR products were resolved on 1% agarose gels.

Table 2-8: Primer used for RT-PCR

Gene	Annealing temperature	Cycle No.	Primer	Sequence
Beta actin	60°C	32	beta actin-for	cgt gcg tga cat caa aga gaa
			beta actin-rev	cca tac cca aga agg aag gct
CCR2	55°C	30	CCR2-for	aca tgt gct aag aat tga ac
			CCR2-rev	cct atc aat tgt cag gag ta
CCR4	60°C	35	CCR4-for	att gct tca tag act gtc ct
			CCR4-rev	gat ctt gca cag acc tag t
CCR7	55°C	30	CCR7-for	aga gag aca aga acc aaa ag
			CCR7-rev	act tat aga tgc caa aga tg
CCR9	55°C	35	CCR9-for	cat gtt cct ttt gaa ttt ag
			CCR9-rev	agg tat gaa tga tga tgg ta
CD11c	55°C	35	CD11c-for	acc tgg ata gcc ttt ctt ctg c
			CD11c-rev	tca aaa tct gtg gaa ctg atg c
HPRT	60°C	32	HPRT-for	gct tgc tgg tga aaa gga cct c
			HPRT-rev	tca aaa gtc tgg gga cgc ag
E-cadherin	58°C	30	E-cadherin-for	gct gga ccg aga gag tta
			E-cadherin-rev	tcg ttc tcc act ctc aca t
Id1	60°C	32	Id1-for	cct gct cta cga cat gaa cg
			Id1-rev	tcc tca gaa atc cga gaa gc
Id2	55°C	28	mId2-2 for	gca tcc ccc aga aca aga ag
			mId2-2445 rev	cgt gtt ctc ctg gtg aaa tgg
Id3	60°C	37	Id3-for	gca ctg ttt gct gct tta gg
			Id3-rev	aga tcg aag ctc atc cat gc
Id4	57°C	33	Id4-for	aac aag aaa gtc agc aaa gtg g
			Id4-rev	agc ggt cat aaa aga aga aac g
Langerin	57°C	32	Langerin-for	aag agt gat gcc cag atg ttg aaa gg
			Langerin-rev	ttg ggg tgc gtg aaa agt aat aga ag
M-CSFR	57°C	28	M-CSFR-for	atg agt ccc tct tca ctc cg
			M-CSFR-rev	acc ttc agc act gca tct tc
TLR1	60°C	32	TLR1-for	gtt ggt gaa gaa ctc agg cg
			TLR1-rev	tca gct tgg aca atg aga gg
TLR2	58°C	30	TLR2-for	atg gca gaa gat gtg tcc g
			TLR2-rev	gtc acc atg gcc aat gta gg
TLR3	58°C	30	TLR3-for	tgg att ctt ctg gtg tct tcc
			TLR3-rev	agt tct tca ctt cgc aac gc
TLR4	59°C	30	TLR4-for	ctg gca tca tct tca ttg tcc
			TLR4-rev	gct tag cag cca tgt gtt cc
TLR5	57°C	35	TLR5-for	ctc atc tca ctg cat acc t
			TLR5-rev	agc gag cta atg aat ata ga
TLR6	62°C	28	TLR6-for	ata tct gag ctt cgg atg cc
			TLR6-rev	atg gag aac ggt ggt att gg
TLR7	62°C	28	TLR7-for	cca cca gac ctc ttg att cc
			TLR7-rev	tcc aga tgg ttc agc cta cg
TLR8	62°C	28	TLR8-for	tgg caa cca tca act tgg gc
			TLR8-rev	tgg ctc agg tcc agc act tc
TLR9	58°C	30	TLR9-for	caa cct ctc ggt gct gga cc
			TLR9-rev	cat cat ctg cct ctt cag ggg
TLR11	58°C	30	TLR11-for	tca ttt tcc ctc cct gcg tc
			TLR11-rev	gtg tag ccc aag act cca gcc
TLR12	62°C	32	TLR12-for	caa ctg cct ggt gac cga ag
			TLR12-rev	cga aac cct gga gga gga acc
TLR13	62°C	28	TLR13-for	tca cct gct cgg aaa cct acc
			TLR13-rev	gct cgg aac aac ctg aat ccc

TLR stimulation of DCs and Flt3$^+$ progenitor cells

DCs at day 7 of differentiation or Flt3$^+$ progenitor cells were used for experiments. Cells were seeded in 12 well plates at a concentration of 2x10^6 cells/ml. Cells were stimulated with respective TLR ligands for 24h. Concentrations of TLR ligands were used as listed in Table 2-1. Supernatant was harvested and used for cytometric bead assay to determine secreted cytokine concentration.

Cytometric bead assay (CBA)

Cytokine secretion of DCs and Flt3$^+$ progenitor cells was assessed after stimulation of cells for 24h with various TLR ligands. Culture supernatants were harvested and cytokine concentration was determined by Cytometric bead assay (BD Bioscience) according to the manufacturer's instructions. In brief, for each single bead or multiplex assay a standard curve was prepared by pooling all lyophilized standard spheres into one tube, reconstituting them with 2ml of Assay Diluent. A standard curve of 1:1 (undiluted), 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256 dilutions and only Assay Diluent (zero control) was prepared.

The number of samples and standards were calculated prior to sample preparation. 50µl of previously mixed Capture Beads were added to each assay tube. Subsequently 50µl of standard or sample were added to the respective assay tube, mixed gently and incubated for 1 hour at room temperature. Then 50µl of the previously mixed PE Detection Reagent was added to each assay tube, mixed gently and incubate for another hour at room temperature in the dark. Finally, 1ml of Wash Buffer were added to each assay tube and centrifuged at 200 × g for 5min. The supernatant was carefully aspirated from each assay tube. 300µl of Wash Buffer were added to each assay tube and samples were analysed on a FACS Canto-II. Cytokine concentration was calculated by FCAP (Soft Flow Inc.) software and values of untreated cells were subtracted from stimulated samples.

Fluorescence microscopy

To determine LC density and morphology, epidermal sheets were subjected to immunofluorescence microscopy. First, ears were cut off at the base and incubated for 5min in veet depilation crème sensitive (veet) to remove hair. Ears were washed twice in PBS and separated into dorsal and ventral halves by tearing them apart. Each half was fixed on tape (crystal clear, Tesa) and floated on 20mM EDTA for 90 min at 37°C to allow

separation of the epidermal sheets from dermis. Epidermal sheets were fixed in 100% ice-cold acetone for 20min, rinsed in PBS and blocked with staining buffer containing 3% mouse serum for 10 min at room temperature before staining with MHC class II-FITC, and langerin-Alexa546 overnight at 4°C. Sheets were counterstained with DAPI (Vector Laboratories) before being mounted in ProLong Gold mounting medium (Molecular Probes). Images were acquired using phase contrast or epifluorescence microscopy with an Axiovert 200 microscope (Carl Zeiss, Germany) equipped with a Plan Neofluar 40x/oil immersion objective and 1x optovar optics. Images were recorded with a cooled, back-illuminated charge-coupled device camera (Cascade 512B, Roper Scientific Inc., USA) driven by IPLab Spectrum software (Scananalytics, USA). Digital processing of the images was done using IPLab Spectrum and Adobe Photoshop (Adobe Systems, USA)

Confocal microscopy and live cell imaging

Confocal imaging of epidermal sheets was carried out on a Zeiss LSM 510Meta confocal microscope (Zeiss, Jena, Germany) with a 63x 1.2 NA Zeiss water immersion objective. Alexa546-fluorescence was excited using the 543nm emission line of the helium-neon laser, a 543nm dichroic mirror and a 560-600nm bandpass filter. FITC-fluorescence was detected using the 488nm emission line of the argon laser, a 488nm dichroic mirror and a 505-530nm bandpass filter. Image processing was done with the Zeiss LSM image browser 4.2 software. The images shown represent confocal slices of ~1µm.

Tissue preparation for transmission electron microscopy

Samples were fixed in 3% glutaraldehyde in 0.1M B2 buffer for 14h, washed with 0.1M B2 buffer overnight, followed by 1h in 1% OsO_4 in 17% sucrose buffer, rinsed with water and successively dehydrated with ethanol (30% - 100%) and propylenoxide (100%). Finally tissues were processed for embedding in Epon, polymerized 48h at 60°C, cut into 80-100nm thick slices and contrasted with uranyl acetate and lead citrate. The samples were analyzed with a Philips EM 400 T at 60kV and micrographs were taken by an Olympus CCD-Camera Morada.

Statistics

All numerical data were analyzed for significance by a two-tailed and unpaired Student's *t* test with GraphPad Prism software (GraphPad Software Inc.). P values below 0.05 were considered to be significant.

Microarray preparation

Total RNA was isolated with the RNeasy Midi Kit including DNase digestion (Qiagen). 8µg of total RNA was used to generate cRNA using a modified protocol from the Expression Analysis Technical Manual (Affymetrix). RNA was concentrated to >0.5µg/µl by acid-ethanol precipitation. For the first-strand cDNA synthesis a T7-oligo(dT) primer was firstly hybridised to 8µg of total RNA by denaturation for 10min at 70°C, quick-chilled on ice, followed by incubation for 2min at 42°C. The reverse transcription reaction was performed using 200U SuperScript-RT (Invitrogen) per sample for 1h at 42°C. The second-strand cDNA synthesis reaction was carried out by incubating the single-stranded cDNA with 10U of DNA ligase, 40U of DNA Polymerase I and 2U of RNaseH for 2h at 16°C, and additionally for 5min at 16°C with T4 DNA Polymerase in order to enhance the efficiency of the second-strand synthesis. cDNA was cleaned-up using the QIAquick Spin PCR Purification Kit (Qiagen). Buffer PB was used for binding: this allows removal of fragments smaller than 100bp, i.e. T7-oligo(dT) primers. DNA was eluted with elution buffer. Double-stranded cDNA was then concentrated again by acid-ethanol precipitation. Synthesis of biotin-labelled cRNA was conducted using T7 Mega Script Kit (Ambion). The cDNA template was incubated with dNTPs in the presence of Bio-11-CTP and Bio-11-UTP (Perkin Elmer) with the T7 RNA Polymerase and the reaction was incubated for 6h at 37°C. cRNA was purified using RNeasy Mini Kit (Qiagen). cRNA was then fragmented using the fragmentation buffer, and 8µg of cRNA per sample was hybridised to Affymetrix mouse MOE430A arrays. DNA chips were stained, washed, and scanned according to the manufacturer's protocol.

All microarray preparation as well as bioinfomatic analysis was performed by Drs. Thomas Hieronymus and David Ruau.

3 Results

3.1 Characterization of an in vitro amplified Flt3$^+$ precursor

Many studies in the past few years have addressed the lineage relationships of DCs with other haematopoietic lineages. Different DC subsets have been identified both in lymphoid and non-lymphoid organs, but their relationship and developmental origins remain controversial [23, 81, 163, 164]. Although few DCs in lymphoid organs might divide over prolonged time *in situ* [165], data on the separation of parabiotic mice have confirmed previous conclusions from non–self-renewing progenitor cell–transfer experiments: most spleen and lymph node DCs must be continuously replaced through blood or lymph, most probably by cells originating from haematopoietic stem and progenitor cells in the bone marrow [70, 72, 77, 166].

Haematopoietic differentiation is a regulated, continuous, and multi-linear process in which haematopoietic stem cells (HSCs) give rise to cells of different lineages. This multi-lineage differentiation is attended by progressively limited developmental options [167] and a successive decline in self-renewal potential [168]. The HSC compartment is comprised of longterm reconstituting HSCs, which are able to generate all haematopoietic lineages for life, and short-term HSCs and their multipotent progenitor (MPP) progeny [69, 70, 169, 170], which give rise to all haematopoietic lineages, but with diminished self-renewal capacity [171-173]. Flt3 is one of the known factors to distinguish between longterm and short-term HSCs. In the primitive Lin$^-$Sca-1$^+$c-kit$^+$ (LSK) HSC compartment approximately 60% of cells express Flt3 and those cells exhibit short-term reconstituting activity, whereas LSK HSC that lack Flt3 expression comprise longterm HSC activity. These findings associate Flt3 expression in the HSC compartment with loss of sustained self-renewal capacity [168, 174, 175].

This section continuatively examines the previously described Flt3$^+$ DC precursor from mouse bone marrow [160]. This precursor is amplified *in vitro* from mouse bone marrow with a cocktail of specific cytokines and was shown to differentiate into fully functional DCs *in vitro* in the presence of GM-CSF. This two-step culture system yields homogeneous populations of Flt3$^+$ precursor cells in high numbers, thereby overcoming the limitations in number, purity, and homogeneity for investigation of such precursors *in vivo*.

Furthermore, it allows detailed analysis of the consecutive steps of DC development *in vitro* under well-defined conditions.

3.1.1 Surface marker profile of the Flt3$^+$ precursor

Haematopoietic cells are frequently characterized by their surface antigen expression. The initial phenotypic characterization of the Flt3$^+$ precursor as a precursor for DCs revealed the expression of the tyrosine kinase Flt3, along with the stem cell antigens CD49f (integrin α_6), CD133 (prominin) as well as CD24. Cells were reported to be negative for lineage markers, such as CD3ε, CD4, CD8α, CD14, B220, and Ter119, but positive for the lineage markers CD11b and Gr-1 (Ly6C/G). As already described, Flt3$^+$ precursor cells express neither MHC-II nor CD11c; two commonly used cell surface antigens to determine mouse DCs (Figure 3-1). Importantly, expression of these cell surface proteins increased during DC differentiation [160, 168].

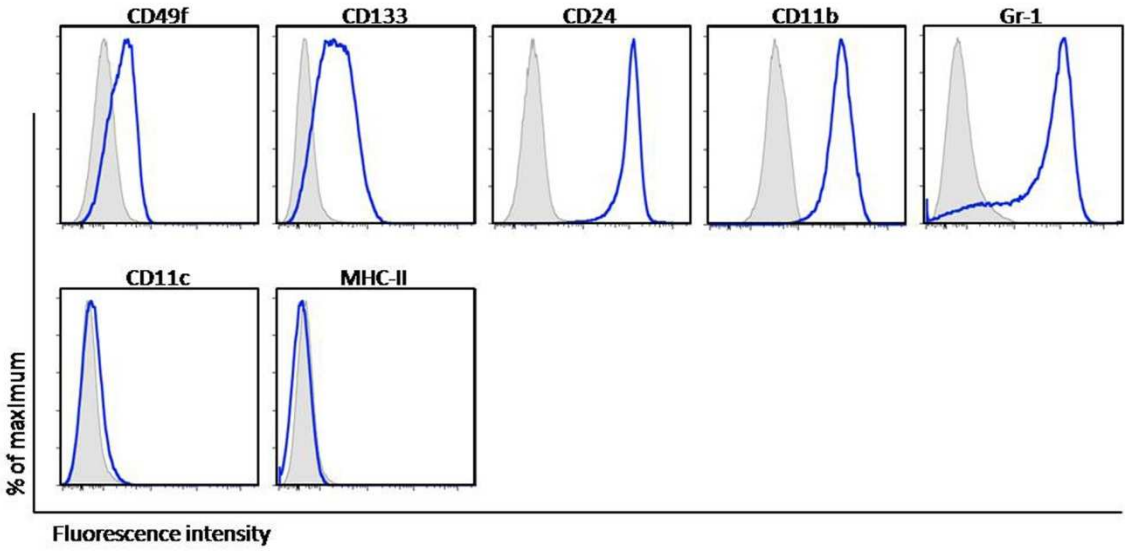


Figure 3-1: Surface marker expression on Flt3$^+$ precursor cells.
After 7 days of amplification, Flt3$^+$ precursor cells were stained for indicated markers and their expression was analyzed by flow cytometry (blue line). Grey areas represent staining with respective isotype controls. Data shown are representative of at least n = 10 independent experiments.

3.1.2 TLR repertoire of the Flt3$^+$ precursor cells

One of the main features of DCs is the recognition of invading pathogens and the resulting antigen presentation to naïve T cells to initiate an immune response. TLRs are responsible for much of that recognition and consequently have vital roles in the defence against pathogens [132].

Besides several well-studied examples of TLRs on mature immune cells, there have been some hints that TLRs might influence haematopoiesis. In addition, it would be interesting to know when maturing cells in bone marrow acquire functional TLRs.

Nagai *et al.* recently reported on early haematopoietic progenitor cells that expressed functional TLR2 and TLR4-MD-2. When stimulated with TLR ligands in culture, myeloid progenitors gave rise to monocytes and/or macrophages in the absence of exogenous growth and differentiation factors, and lymphoid progenitors became DCs [176].

If it is assumed that early haematopoietic stem cells already express TLRs, it seems to be more than likely that also other types of progenitor cells express TLRs, in particular DC precursors. Therefore, Flt3$^+$ DC precursors from *in vitro* cultures were analyzed for their expression repertoire of TLRs.

Comparative gene expression analysis for TLR pathway components

Gene expression in Flt3$^+$ precursors, differentiated DCs, and TNFα treated (100ng/ml) DCs was assessed by DNA microarray analysis. Hierarchical cluster analysis of genes encoding for TLRs and respective pathway components was performed, and a representative result is shown (Figure 3-2). Rows represent different developmental steps in DC differentiation. Cluster I contains genes which are effectively induced during DC differentiation and are not further upregulated by TNFα. Genes in cluster II are high in response to TNFα. Cluster III comprises genes that are high during DC differentiation and low after activation by TNFα. Cluster IV contains genes showing highest expression in Flt3$^+$ precursor cells. Finally, Cluster V encloses genes that show a high expression in Flt3$^+$ precursor cells and in TNFα stimulated DCs. The heatmap in Figure 3-2 is based on data shown in Table 3-1.

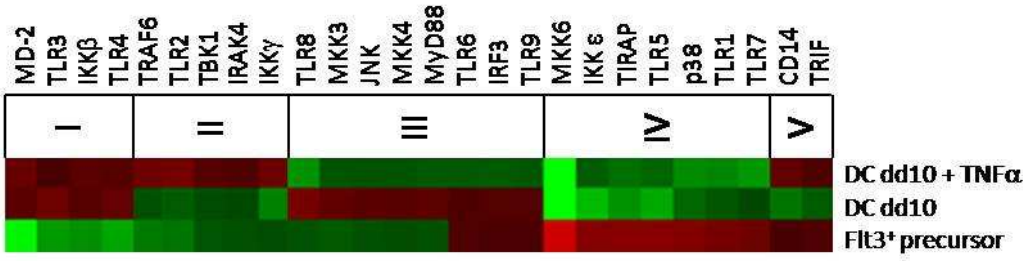


Figure 3-2: Hierarchical cluster analysis of TLR pathway components.

The Flt3$^+$ precursor cells along with differentiated DCs dd10 and DCs dd10 treated with TNFα were subjected to transcriptional profiling by DNA microarrays to analyse differentially regulated genes involved in TLR pathways. The colour of the respective box in one column represents the expression value of the gene transcript in one sample compared to the median expression level of the gene's transcript for all samples shown. *Green*, transcript levels below median; *black*, transcript levels equal to median; *red*, transcript levels higher than median. dd, day of differentiation.

		Flt3+ precursor		DCs dd10			DCs dd10 + TNF α		
Name	probe set	Signal	Detection	Signal	Detection	Change	Signal	Detection	Change
TLR1	1449049_at	59,9	P	30,4	A	D	19,7	A	D
TLR2	1419132_at	1657,9	P	2127,3	P	I	4331,9	P	I
TLR3	1422781_at	9,9	A	31,7	P	I	20,9	P	NC
TLR4	1418162_at	268	P	832,2	P	I	733,1	P	I
TLR5	1450242_at	57,6	P	13,4	A	NC	21,7	A	NC
TLR6	1421352_at	157,1	P	154,3	P	NC	117	P	NC
TLR7	1422010_at	633,1	P	406,2	P	D	196,8	P	D
TLR8	1450267_at	86,2	P	159,8	P	I	48,9	A	NC
TLR9	1422083_at	47,9	P	53,9	A	NC	39,3	A	NC
MyD88	1419272_at	1141,3	P	1655,3	P	NC	1055,6	P	NC
TIRAP	1418685_at	101,3	P	26,9	A	NC	35,4	A	MD
IRAK4	1451749_at	158,2	P	169,6	P	NC	194,6	P	NC
TRAF6	1421376_at	99,9	P	154,1	P	NC	254,7	P	NC
IKKβ	1432275_at	27,4	A	66,2	A	NC	69	A	NC
IKKγ	1421208_at	101,9	P	60,2	A	NC	160,2	A	NC
IKKε	1417813_at	343,3	P	72,7	A	D	146,2	P	D
MKK3	1451714_a_at	635,8	P	1077,4	P	NC	672,6	P	NC
MKK4	1451982_at	718,3	P	912,9	P	NC	664,9	P	NC
MKK6	1426850_a_at	98,4	P	2,6	A	D	3,6	A	D
p38	1416703_at	2517,9	P	983,4	P	D	738,1	P	D
JNK	1421876_at	207,8	P	292,4	P	I	205,3	P	NC
TRIF	1419368_a_at	100,1	P	72,3	P	D	103,1	P	NC
TBK1	1422469_at	540,5	P	599,9	P	NC	759	P	I
IRF3	1416898_a_at	152,5	P	163,9	A	NC	115,2	A	NC
MD-2	1449874_at	115,7	P	491	P	I	565,4	P	I
CD14	1417268_at	438	P	265,8	P	D	592,2	P	NC

Table 3-1: RNA microarray data of TLRs and related pathway components in Flt3$^+$ precursors and DCs.
A, absent; P, present; D, decreased; I, increased; NC, no change

Expression and up-regulation of TLR mRNA in Flt3$^+$ precursors and differentiated DCs

To validate the DNA microarray experiments and to determine the regulation of TLR gene expression during DC differentiation RT-PCR analysis was employed. Flt3$^+$ precursors and DCs were cultured according to the two-step protocol and total mRNA was isolated for RT-PCR analysis. This revealed that TLRs 1 to 9 and TLRs 12 and 13 are already expressed in the Flt3$^+$ precursor cells and get progressively upregulated during DC differentiation. However, expression levels of TLRs 1, 3, 7 and especially TLR12 are rather low in the Flt3$^+$ precursors (Figure 3-3). TLR11 is not expressed in the Flt3$^+$ precursor, but gets also expressed and upregulated during DC differentiation. Although TLR10 is present in human, the mouse homologue is so far not identified.

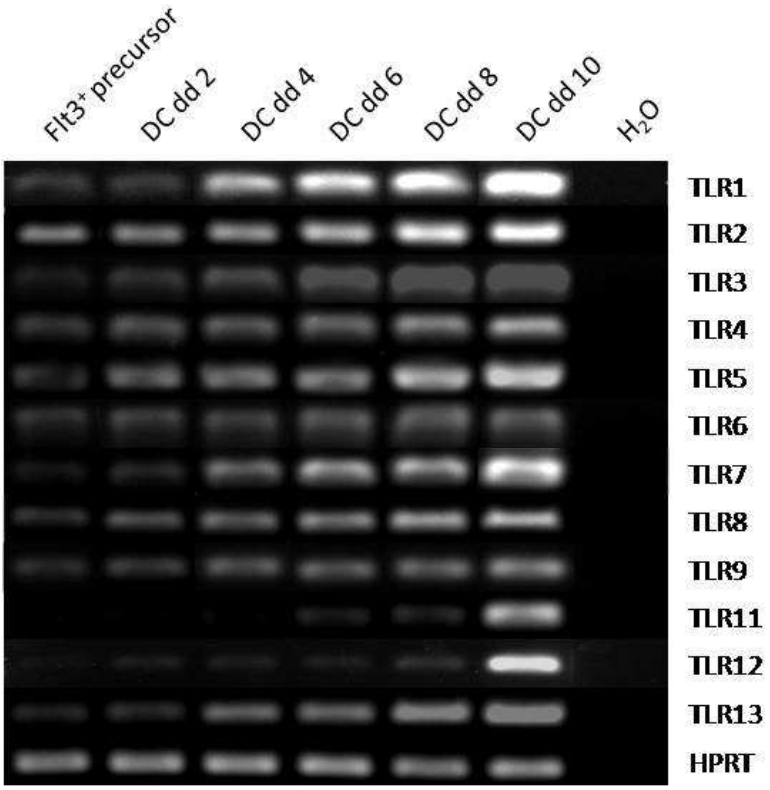


Figure 3-3: Expression and upregulation of TLRs in Flt3$^+$ precursors and differentiating DCs.
mRNA levels of TLRs in Flt3$^+$ precursors and during DC differentiation were determined by RT-PCR. Expression levels were investigated at indicated time points. HPRT was used as loading control. dd, day of differentiation. Data shown are representative of at least n = 3 independent experiments.

Protein expression of TLRs in Flt3$^+$ precursors and differentiated DCs

To asses protein levels of TLRs in Flt3$^+$ precursors and differentiated DCs, Flt3$^+$ precursors and DCs were cultured according to the two-step protocol and protein expression of TLRs was determined by flow cytometry (Figure 3-4).

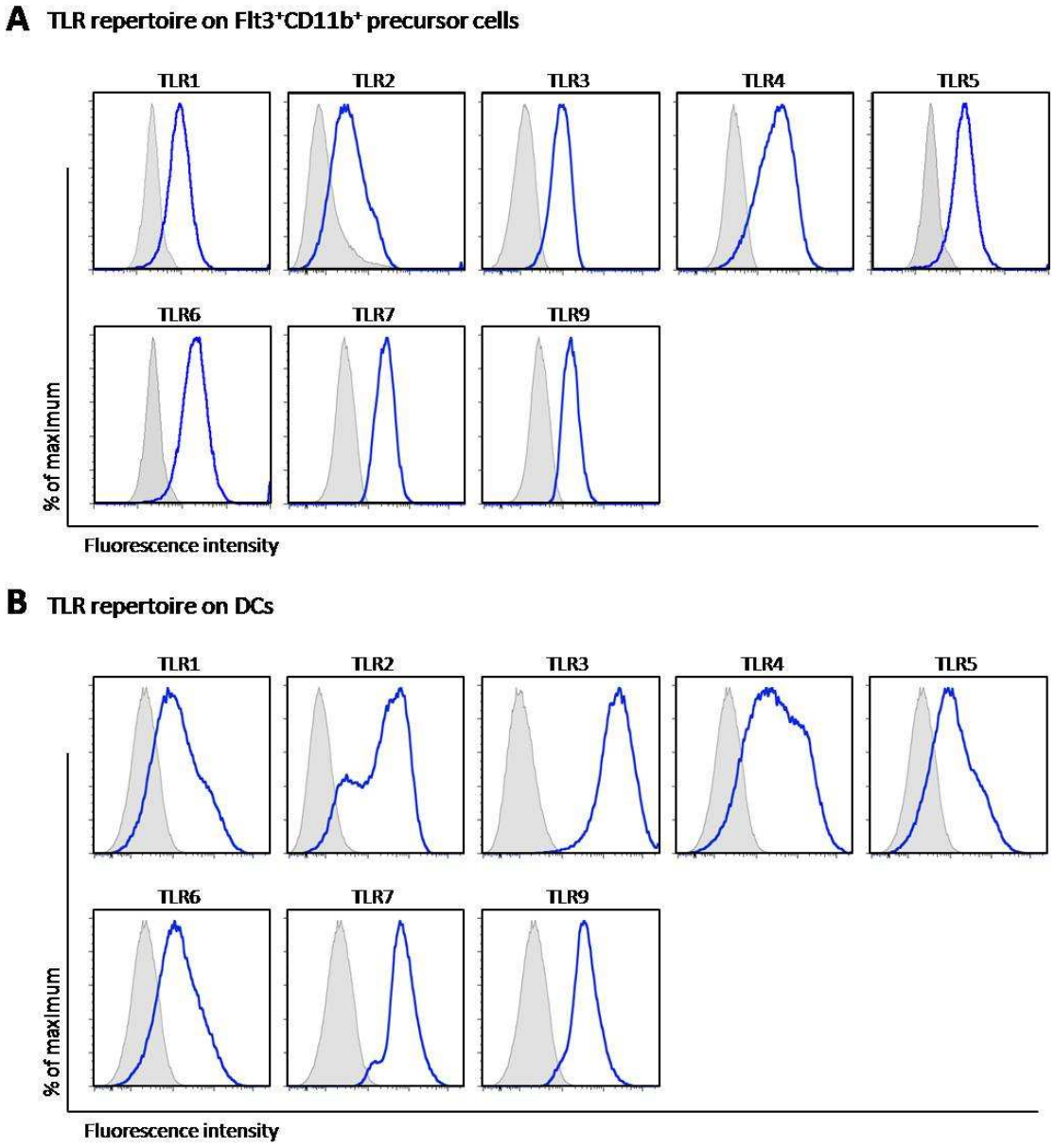


Figure 3-4: Protein expression of TLRs in Flt3$^+$ precursors and DCs.
Flt3$^+$ precursors (A) and differentiated DCs (B) were cultured according to the two-step protocol, stained for the respective TLRs and their expression was analyzed by flow cytometry (blue line). Grey areas represent staining with respective isotype controls. Data shown are representative of at least n = 5 independent experiments.

As already established by RT-PCR analysis, TLRs 1 to 9, as well as TLRs 12 and 13 mRNAs were expressed in Flt3$^+$ precursor cells. Flow cytometry analysis revealed that TLRs 1 to 7 and TLR 9 were also expressed on protein levels in Flt3$^+$ precursors. In DCs all these TLRs were also expressed and were even further upregulated during differentiation compared to Flt3$^+$ precursors (Figure 3-4). TLRs 8, 11, 12, and 13 are so far not well studied and reagents and antibodies are difficult to obtain. Thus, the examination of these TLRs was not continued.

3.1.3 Functional analysis of TLRs on Flt3$^+$ precursor and differentiated DCs

To further investigate functionality of the expressed TLRs in Flt3$^+$ precursors and differentiated DCs, cells were stimulated with respective ligands to selected TLRs. Supernatants of *in vitro* cultures were harvested 24h after TLR stimulation and cytokine secretion of IL-6, IL-10, IL-12p70, MCP-1, MIG, and TNFα was analyzed by Cytometric bead assay (CBA) (Figure 3-5). Cytokine concentration was calculated using FCAP software and values of untreated cells were subtracted.

IL-6, MCP-1, and TNFα are rather unspecific pro-inflammatory cytokines, secreted by DCs after TLR activation via the MyD88-dependent pathway, whereas MIG is only secreted in response to autocrine/ paracrine secretion of IFNβ, which is activated by stimulation of TLR3, 7 or 9. IL-12p70, also a pro-inflammatory cytokine, drives polarization of naïve CD4$^+$ T cells towards Th1. IL-10 on the other hand is involved in the inhibition of Th1 response and differentiation of regulatory T cells, and therefore a rather anti-inflammatory cytokine [177-180].

The analysis of cytokine secretion after TLR stimulation shows that DCs respond effectively to TLR stimulation by secreting the examined cytokines. Particularly the activation of TLRs 2, 4, 7, and 9 led to a strong cytokine response of DCs, whereas activation of TLRs 1/2 and 5 results in only marginal activation of DCs. On the contrary, Flt3$^+$ precursor cells were not able to respond properly to any of the TLR ligands, and their cytokine secretion remained below 20pg/ml in every case. The low secretion of some cytokines in the Flt3$^+$ precursor samples is likely due to a small percentage of spontaneously differentiated cells within the culture, which is normally between 3 and 5%.

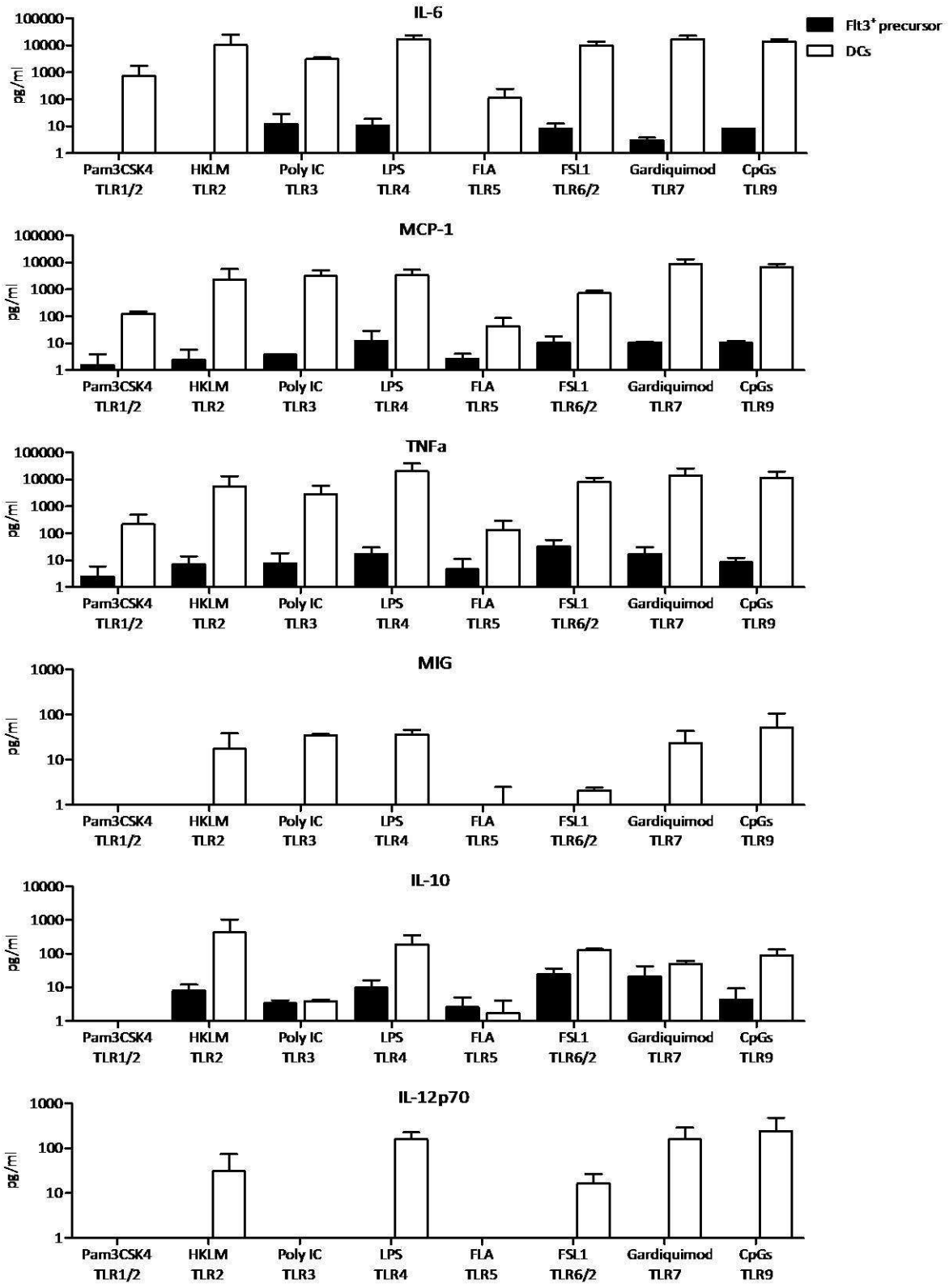


Figure 3-5: Cytokine production by Flt3$^+$ precursor cells and DCs upon TLR stimulation.
Supernatants of Flt3$^+$ precursors (black) and differentiated DCs (white) were harvested 24h after TLR stimulation and cytokine secretion was analyzed by CBA. Data shown are means of at least n = 3 independent experiments ± SD.

3.1.4 Analysis of the *in vivo* developmental potential of the Flt3$^+$ precursor

Several studies demonstrated the developmental plasticity inherent to the DC system, showing that both common lymphoid and common myeloid progenitors are able to differentiate into all types of DCs [77, 181]. Previous studies already revealed that Flt3$^+$ precursor cells give rise to both spleen CD8α^+ and CD8α^- DCs *in vivo* [160]. Furthermore, the work of Nagai *et al.* indicates that TLR signalling might not only activate immune responses in APCs, but also influences lineage decision of early haematopoietic progenitors [176].

Thus, the developmental potential of the Flt3$^+$ precursor was further examined *in vivo* in cell transfer experiments. Flt3$^+$ precursors of GFP$^+$ mice were amplified *in vitro* for seven days and different concentrations were injected intravenously (i.v.) into lethally irradiated mice together with a radio protective dose of 2x10^4 GFP$^-$ bone marrow cells to assure mouse survival. Transplantation of unfractionated GFP$^+$ bone marrow was used as control. Blood samples were analyzed for GFP$^+$ cells 2, 6, and 10 weeks after transplantation and GFP$^+$ cells were further analyzed for expression of linage markers for B cells (CD19), T cells (CD3ε), myeloid cells (CD11b), and granulocytes (Gr-1) (Figure 3-6). In addition, bone marrow, spleen, and thymus were analyzed at the end of the experiment for GFP$^+$ cells, which were further analyzed for expression of linage markers for B cells (CD19), T cells (CD3ε), myeloid cells (CD11b), granulocytes (Gr-1), DCs (CD11c), macrophages (F4/80), and erythrocytes (Ter119) (Figure 3-7).

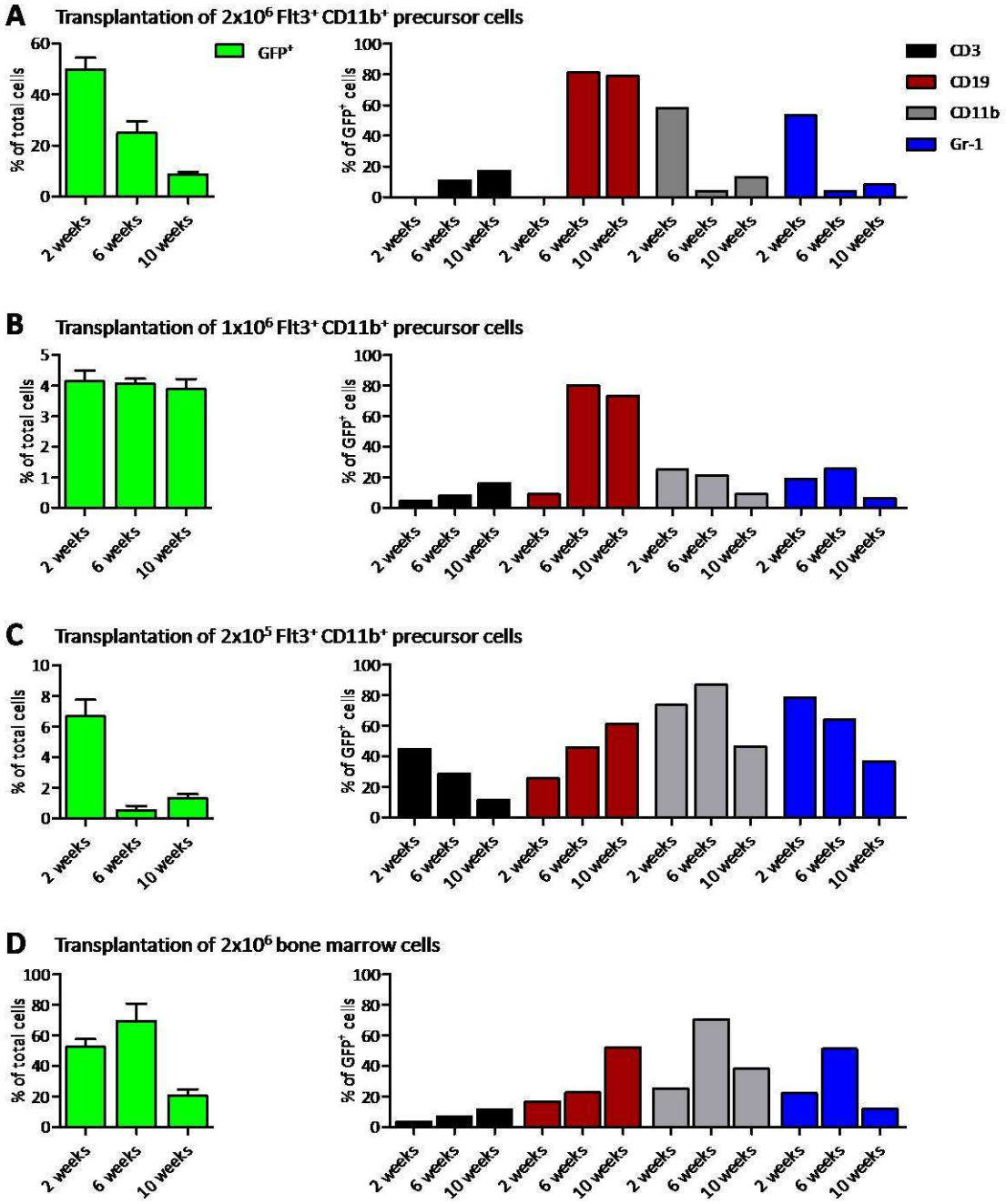


Figure 3-6: Repopulation efficiency of the Flt3$^+$ precursor in blood after adoptive transfer.
Flt3$^+$ precursors of GFP$^+$ mice were amplified *in vitro* for seven days. 2x10^6 (A), 1x10^6 (B), or 2x10^5 (C) cells were injected intravenously into lethally irradiated mice together with a radio protective dose of 2x10^4 GFP$^-$ bone marrow cells. Blood samples were analyzed at indicated time points for GFP$^+$ cells (light green) and lineage marker for T cells (black), B cells (dark red), myeloid cells (grey), and granulocytes (blue). Injection of 2x10^6 cells of unfractionated bone marrow (D) was used as control. One representative experiment out of 4 is shown.

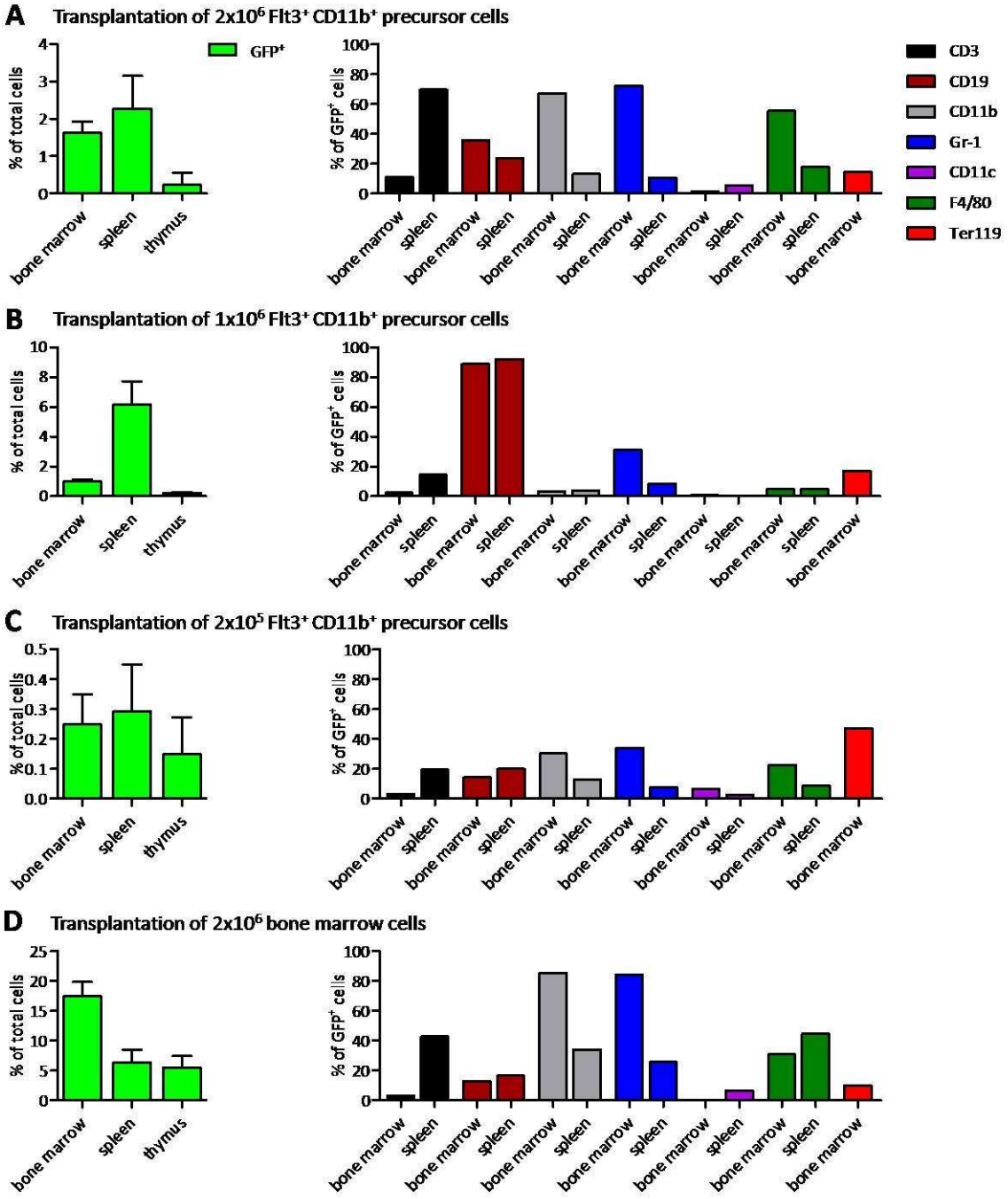


Figure 3-7: Repopulation efficiency of the Flt3$^+$ precursor in different organs after adoptive transfer.
Flt3$^+$ precursors of GFP$^+$ mice were amplified as described. 2x10^6 (A), 1x10^6 (B), or 2x10^5 (C) cells were injected intravenously into lethally irradiated mice together with a radio protective dose of GFP$^-$ bone marrow cells. 10 weeks after transfer bone marrow spleen and thymus were analyzed for GFP$^+$ cells (light green) and lineage marker for T cells (black), B cells (dark red), myeloid cells (grey), granulocytes (blue), DCs (purple), macrophages (green), and erythrocytes (red). Injection of 2x10^6 cells of unfractionated bone marrow (D) was used as control. One representative experiment out of 4 is shown.

Preliminary transplantation experiments revealed that the Flt3$^+$ precursor does not contain any bone marrow reconstitution capacity, as all mice that were injected with Flt3$^+$ precursor cells only died within 5 days. Therefore it was essential to inject a radio protective dose of bone marrow cells concomitantly. Irrespective of that the

transplantation experiments showed GFP$^+$ cells for at least 10 weeks in blood, bone marrow, spleen, and thymus. However, percentages of GFP$^+$ cells in thymus were too low to be further analyzed. Furthermore, the data demonstrate that the Flt3$^+$ precursor can give rise to all cell lineages tested. It preferentially develops into B cells and myeloid cells, which are probably mainly monocytes, but it can also generate T cells, granulocytes, DCs, macrophages, and erythrocytes; Table 3-2 is summarising these data.

Table 3-2: Summary of the repopulation efficiency of the Flt3$^+$ precursor.

	Blood	Bone marrow	Spleen
Myeloid cells (CD11b)	√	√	√
DCs (CD11c)	n.d.	n.d.	√
B cells (CD19)	√	√	√
T cells (CD3)	√	√	√
Granulocytes (Gr-1)	√	√	√
Erythrocytes (Ter119)	n.d.	√	n.d.
Macrophages (F4/80)	n.d.	√	√

n.d., not determined

Several studies already reported that GFP expression in GFP transgenic mice is not ubiquitously, but highly variegated. Many cells from GFP mice produce intense GFP fluorescence, while cells of the same tissue showed minimal or undetectable GFP expression [182]. Unfortunately, that made a reliable quantitative analysis of the donor cells impossible, especially as GFP expression is decreasing in many of the cells over time [182]. Therefore, the conclusion that can be drawn from these experiments is that the Flt3$^+$ precursor does not contain any bone marrow repopulation potential, but does give rise to cells of both myeloid and lymphoid origin upon transplantation. As a result, the Flt3$^+$ precursor is not only a DC precursor, as already reported, but rather a multipotent progenitor for all haematopoietic cell types.

3.2 Development of two distinct types of Langerhans cells in steady state and inflammation

Due to their specialized location in skin, LCs constitute the first immune barrier for invading pathogens, but have also been implicated in tolerance induction [183, 184]. LCs express CD11c, MHC-II, DEC-205 (CD205) and the endocytic receptor langerin (CD207) [35]. Langerin is involved in specific intracellular or membrane-bound structures, referred to as Birbeck granules that represent a hallmark of LCs [28, 183]. The function of these organelles has yet to be fully resolved, although it has been proposed that they are involved in antigen uptake and/or processing [28, 185].

While much is known about LC activation and trafficking towards the skin-draining lymph nodes, the developmental origin of LCs and the differentiation factors involved remained elusive [131]. Recent studies showed that LCs are maintained by local long-lived precursors that seed skin during embryonic development and self-renew under steady state conditions [83, 186]. During inflammation, LCs emigrate from skin and are replaced by circulating Gr-1^{hi} monocytic LC precursors that are recruited from blood [63].
Several gene-deficient mouse models provided novel insights into the molecular pathways that are important for LC development [131]. Mice deficient for the transcription regulator Id2 (inhibitor of differentiation/DNA binding 2) lack LCs and have a severe reduction in splenic $CD8\alpha^{+}$ DCs [156, 187]. Furthermore, it was demonstrated before that TGFβ1 induces expression of Id2 [156] and $TGF\beta1^{-/-}$ mice also lack LCs [101, 188].

This section analyses the importance of Id2 for the development of LCs and other skin DCs. It examines whether adoptive transfer of $Id2^{+/+}$ bone marrow or $Id2^{+/+}$ $Flt3^{+}$ progenitor cells is able to repopulate the missing LCs in epidermis of $Id2^{-/-}$ mice. Moreover, this section questions whether Gr-1^{hi} monocytes that were reported to give rise to LCs under inflammatory conditions [63] are able to repopulate the epidermis of $Id2^{-/-}$ mice after an inflammatory stimulus.

3.2.1 Id2 deficiency results in lack of LCs and langerin$^+$ dDCs

Id2$^{-/-}$ mice lack LCs as determined by immunohistochemistry of epidermal sheets with MHC-II and langerin (CD207) specific antibodies (Figure 3-8 A) [156].

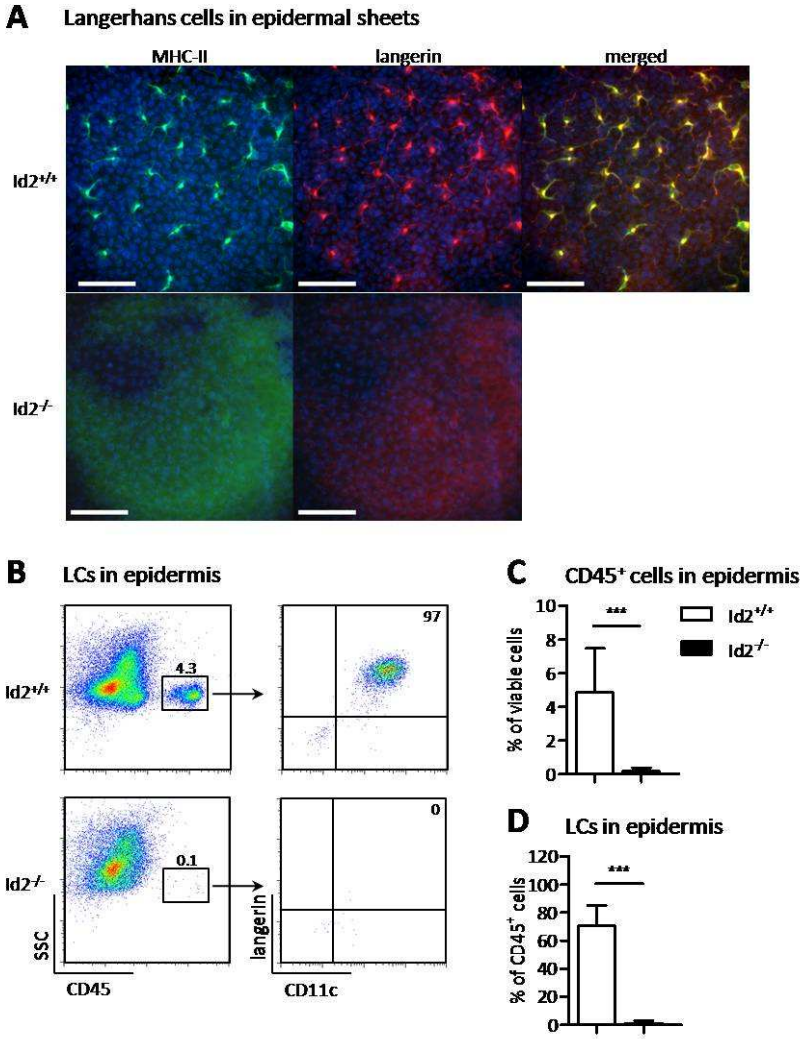


Figure 3-8: Id2$^{-/-}$ LC phenotype in epidermis.
(A) Immunofluorescence staining of skin epidermis from Id2$^{-/-}$ and Id2$^{+/+}$ mice for MHC-II (green) and langerin expression (red); nuclear staining with DAPI (blue). (B) Flow cytometry of single-cell suspensions of epidermis from Id2$^{-/-}$ and Id2$^{+/+}$ mice. For detection of LCs, cells from epidermal sheets were stained for CD45, CD11c and langerin; SSC, Side scatter. (C) Frequency of CD45$^+$ cells among total epidermal cells. (D) Frequency of LCs among CD45$^+$ leukocytes. Data shown are representative of at least n = 4 independent experiments ± SD. Scale bar 50µm. *** $p < 0.001$.

To further extend this observation, single cell suspensions of epidermis and dermis from Id2$^{-/-}$ and Id2$^{+/+}$ mice were analyzed for LCs and dDCs by flow cytometry. This revealed that LCs, characterized by CD45, CD11c, MHC-II, and langerin expression, were detected in epidermis of Id2$^{+/+}$ mice, whereas these cells were absent in Id2$^{-/-}$ mice (Figure 3-8, B

and D). Furthermore, the flow cytometry data showed that Id2$^{-/-}$ mice not only lack LCs, but the entire CD45^{+} cell population in the epidermis (Figure 3-8, B and C).

Recent studies identified a subpopulation of dDCs that also express langerin [189-191]. Thus, Id2$^{-/-}$ and Id2$^{+/+}$ mice were analyzed for the presence of langerin^{+} dDCs using a panel of surface markers including CD11c, MHC-II, and langerin (Figure 3-9 A). The CD45^{+} population was decreased in Id2$^{-/-}$ dermis (Figure 3-9 B). Langerin^{+} dDCs were detected in dermis of Id2$^{+/+}$ mice, whereas these cells were absent in Id2$^{-/-}$ mice. However, the population of CD11c^{+} MHC-II^{+} langerin^{-} dDCs was comparable in Id2$^{-/-}$ and Id2$^{+/+}$ mice (Figure 3-9, C and D).

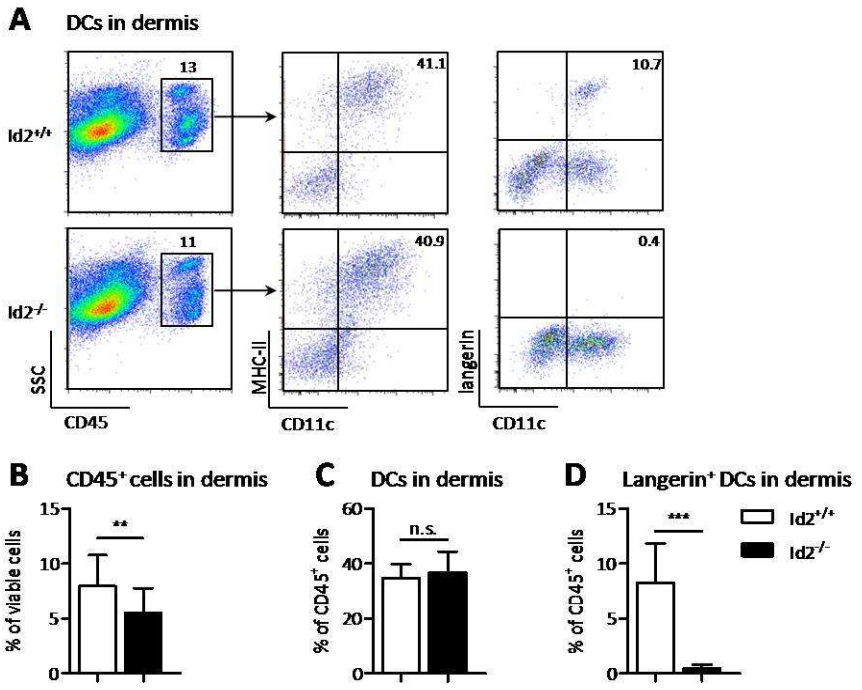


Figure 3-9: Id2$^{-/-}$ dermal DC phenotype.
(A) Langerin^{+} dDCs were identified by staining for CD45, CD11c, and langerin; SSC, Side scatter. (B) Frequency of CD45^{+} cells among total dermal cells. (C) Frequency of DCs among CD45^{+} cells. (D) Frequency of langerin^{+} dDCs among total dDCs. Data shown are representative of at least n = 4 independent experiments ± SD. n.s. not significant; ** $p < 0.01$; *** $p < 0.001$.

3.2.2 Flt3^{+} bone marrow progenitor cells rescue LC deficiency in Id2$^{-/-}$ mice

Next, it was explored whether the LC deficiency in Id2$^{-/-}$ mice results from impairment of the haematopoietic compartment. Therefore, bone marrow cells of congenic Id2$^{+/+}$ donor mice were used for the reconstitution of lethally irradiated Id2$^{-/-}$ mice. Analysis of epidermal sheets showed that within one week after transfer, Id2$^{+/+}$ bone marrow cells were able to repopulate MHC-II^{+} LCs in epidermis of Id2$^{-/-}$ mice to the full extent as

observed in WT mice (Figure 3-10 A). However, it took three weeks until these cells became langerin positive (Figure 3-10 B). Injection of $Id2^{+/+}$ bone marrow cells into non-irradiated $Id2^{-/-}$ mice also restored LCs, indicating that LC repopulation was not dependent on irradiation-associated events. Bone marrow-derived LCs remained in skin for more than 3 months. Injection of $Id2^{-/-}$ bone marrow cells, used as a negative control, did not repopulate LCs (Figure 3-10 A), demonstrating that LC development from bone marrow cells does require Id2.

Analysis of dermis showed that $Id2^{+/+}$ bone marrow cells were able to repopulate $langerin^{+}$ dDCs in $Id2^{-/-}$ mice within two to three weeks. However, repopulation did not reach the full extent of $langerin^{+}$ dDCs observed in untreated WT mice (Figure 3-10 A and Figure 3-9).

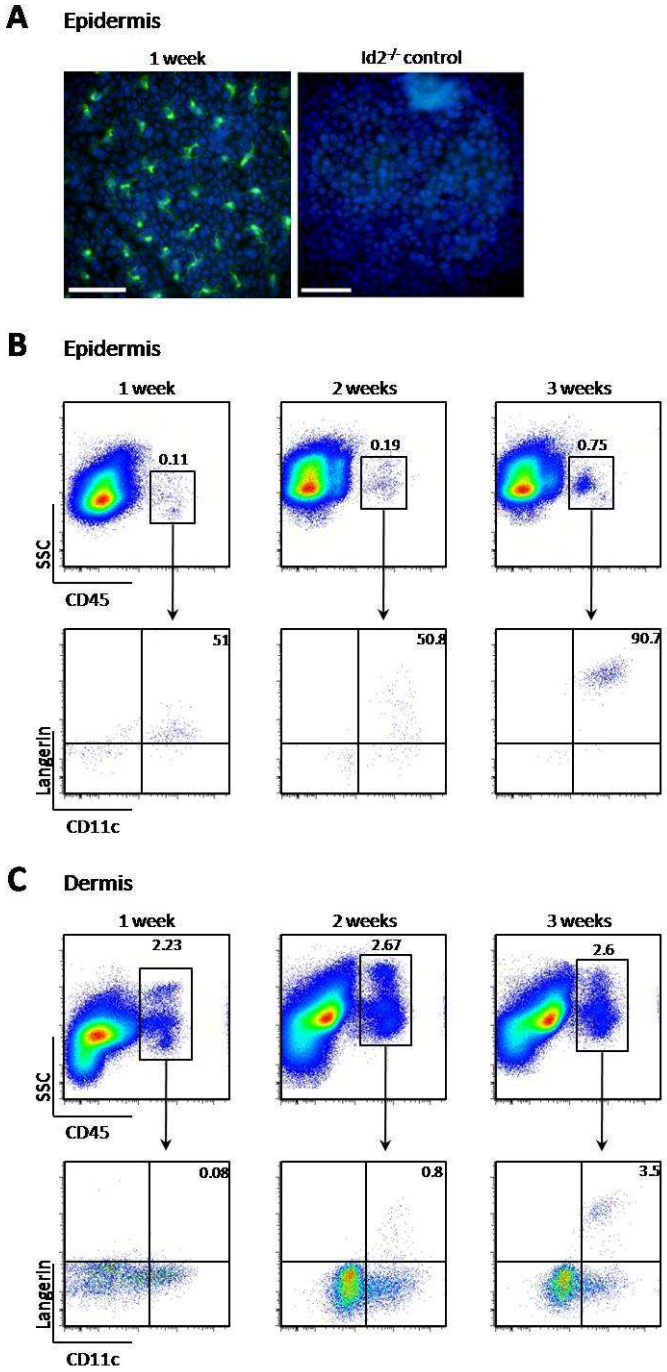


Figure 3-10: Repopulation of skin DCs in Id2$^{-/-}$ mice after adoptive transfer of bone marrow.
(A) Repopulation of LCs in Id2$^{-/-}$ mice in epidermal sheets after transplantation of Id2$^{+/+}$ bone marrow cells. Epidermal sheets were stained for MHC-II (green) and DAPI (blue) 1 week after transplantation. Transplantation of Id2$^{-/-}$ bone marrow was used as negative control. (B and C) Flow cytometry of single-cell suspensions of epidermis (B) and dermis (C) from Id2$^{-/-}$ mice after transplantation of Id2$^{+/+}$ bone marrow cells at indicated timepoints. SSC, Side scatter. Data shown are representative of at least n = 2 independent experiments. Scale bar 50µm.

Mende *et al*. showed that Flt3^{+} progenitor cells in bone marrow contain LC precursors. However these studies were done under inflammatory conditions [192]. In order to characterize the Id2-dependent bone marrow LC precursor in steady state, Flt3^{+} progenitor cells that were described in chapter 3.1 were isolated, amplified *in vitro* and

used in reconstitution experiments of lethally irradiated $Id2^{-/-}$ mice. *In vitro* amplified $Flt3^{+}$ progenitor cells repopulated MHC-II^{+} LCs in the epidermis of $Id2^{-/-}$ mice, although with slower kinetics compared to bone marrow cells (Figure 3-11, A and B). Additionally, kinetic studies showed a patchy distribution of emerging LCs, suggesting that LC repopulation might occur from immigrated precursors. Comparable to the $Id2^{+/+}$ bone marrow transplantation it took about two to three weeks for the cells to become positive for langerin (Figure 3-11 B). With respect to morphology and distribution, the newly emerged LCs were a little different from endogenous LCs of untreated mice. They appear slightly bigger, display more dendrites and express lower langerin, even 3 weeks after transfer, compared to LCs of untreated $Id2^{+/+}$ mice.

On the contrary, $langerin^{+}$ dDCs could not be repopulated by transplantation of $Id2^{+/+}$ $Flt3^{+}$ progenitor cells (Figure 3-11 C). The population of $CD45^{+}$ cells in dermis increased only three weeks after transplantation, but $langerin^{+}$ dDCs could not be detected at any given time point (Figure 3-11 C).

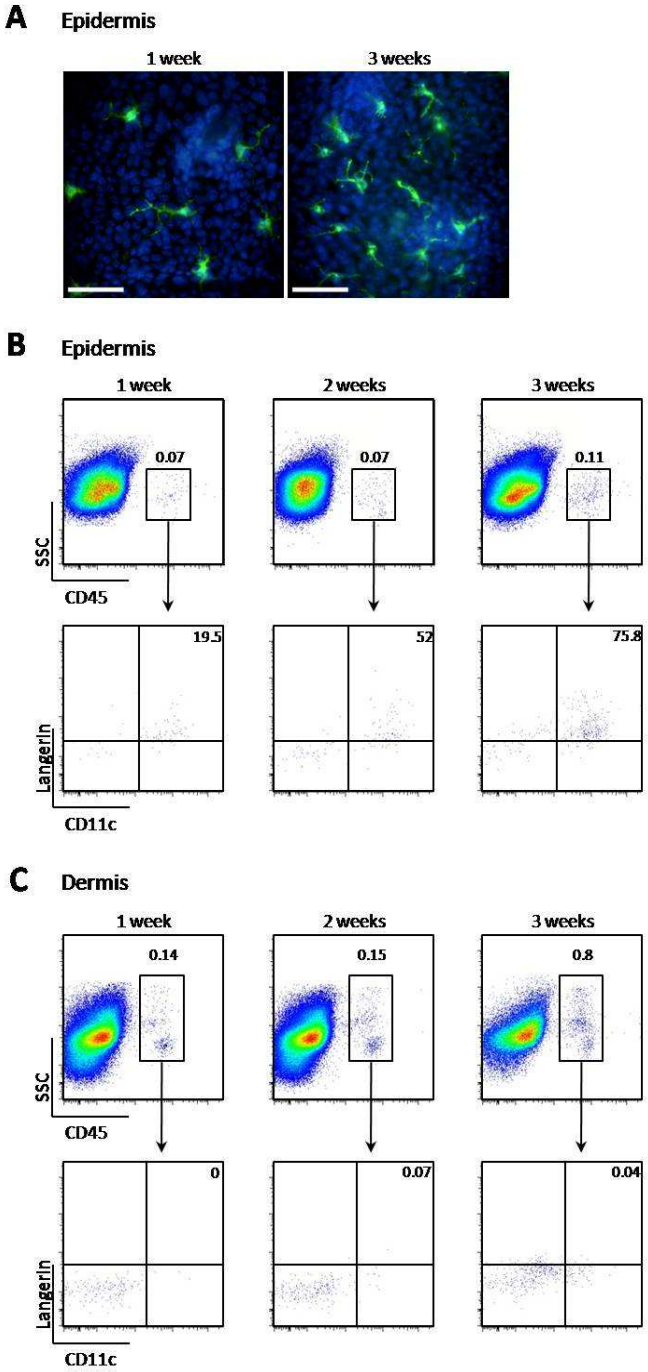


Figure 3-11: Repopulation of skin DCs in Id2$^{-/-}$ mice after adoptive transfer of Flt3^{+} progenitor cells.
(A) Repopulation of LCs in Id2$^{-/-}$ mice in epidermal sheets after transplantation of Id2$^{+/+}$ Flt3^{+} progenitor cells. Epidermal sheets were stained for MHC-II (green) and DAPI (blue) 1 and 3 weeks after transplantation as indicated. (B and C) Flow cytometry of single-cell suspensions of epidermis (B) and dermis (C) from Id2$^{-/-}$ mice after transplantation of Id2$^{+/+}$ Flt3^{+} progenitor cells at indicated timepoints; SSC, Side scatter. Data shown are representative of at least n = 2 independent experiments.

These data clearly demonstrate that (i) the deficiency of LCs in Id2$^{-/-}$ mice results from impairment in the haematopoietic compartment, (ii) in adult mice LC precursors do not only exist in skin, but also in bone marrow and (iii) bone marrow Flt3^{+} progenitor cells can give rise to LCs under steady state conditions; although not langerin^{+} dDCs.

3.2.3 Id2$^{-/-}$ Gr-1^{hi} monocytes infiltrate inflamed skin

Previously, Ginhoux *et al*. [63] demonstrated that blood Gr-1^{hi} monocytes are recruited to skin and develop into LCs under inflammatory conditions. To examine the possibility that LC deficiency in Id2$^{-/-}$ mice might be due to the absence of Gr-1^{hi} monocytes as the potential circulating LC precursor in these mice, monocyte subsets in peripheral blood of Id2$^{-/-}$ and Id2$^{+/+}$ mice were analyzed. Blood monocytes were identified as leukocytes with low side scatter profile that co-express CD11b and CD115, and were divided into two subpopulations according to their Gr-1 expression (Gr-1^{lo}, Gr-1^{hi}; Figure 3-12) [62]. Surprisingly, no significant differences in the Gr-1^{hi} monocyte population were observed in Id2$^{-/-}$ mice compared to the corresponding WT littermates (Figure 3-12 B).

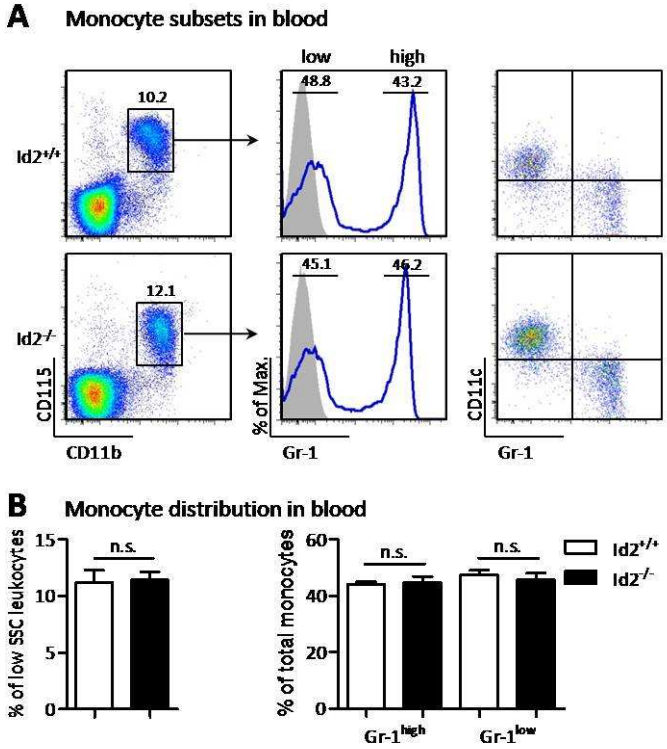


Figure 3-12: Blood monocyte subsets in Id2$^{-/-}$ mice.
Monocyte subsets in peripheral blood from Id2$^{-/-}$ and Id2$^{+/+}$ mice were evaluated by flow cytometry. Cells were stained for CD115, CD11b, CD11c, and Gr-1. Monocytes were identified by low side scatter and co-expression of CD115 and CD11b. (A) Subsets were defined by surface Gr-1 levels on CD115^{+} CD11b^{+} monocytes. (B) Distribution of CD115^{+} CD11b^{+} monocytes and Gr-1 expression on monocytes in Id2$^{-/-}$ (black) and Id2$^{+/+}$ (open) mice. Data shown are representative of at least n = 7 independent experiments ± SD. n.s.= not significant.

Next it was analyzed whether Id2$^{-/-}$ Gr-1^{hi} monocytes are capable of migrating into inflamed epidermis. Therefore, Gr-1^{hi} monocytes were selectively labelled with fluorescent latex beads using an established protocol [62]. Approximately 7% of Gr-1^{hi} monocytes in peripheral blood were found to be labelled with FITC-latex beads 24h after labelling and there was no difference seen for Id2$^{-/-}$ and Id2$^{+/+}$ mice (Figure 3-13).

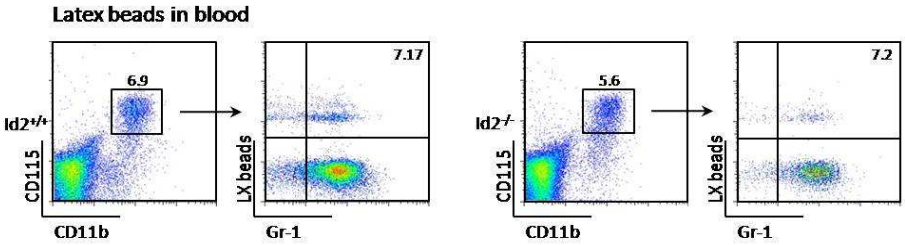


Figure 3-13: Latex beads in peripheral blood monocytes 24h after injection.
Gr-1^{hi} monocytes from Id2$^{-/-}$ and Id2$^{+/+}$ mice were labelled with FITC-conjugated latex-beads *in vivo*. The percentage of FITC-latex beads contained within monocytes is shown. SSC, Side scatter. Data shown are representative of at least n = 2 independent experiments.

Labelling of Gr-1^{hi} monocytes was followed by epicutaneous UV exposure as a stimulus for acute skin injury [83]. At day 5 after UV exposure epidermis and dermis were analyzed by flow cytometry for influx of FITC-latex bead-labelled Gr-1^{hi} monocytes (Figure 3-14, A and B).

A high influx of CD45^{+} cells at the site of inflammation for both Id2$^{-/-}$ and Id2$^{+/+}$ mice was observed compared to steady state conditions (Figure 3-14, C and D). In epidermis the majority of infiltrating CD45^{+} cells was Gr-1^{hi} expressing cells with a fraction being FITC-latex bead-positive Gr-1^{hi} monocytes. The proportion of latex bead-positive and negative Gr-1^{hi} monocytes was the same in both Id2$^{-/-}$ and Id2$^{+/+}$ mice (Figure 3-14 A). Dermis of both Id2$^{-/-}$ and Id2$^{+/+}$ mice also contained Gr-1^{hi} and Gr-1^{lo} cells with minor populations being latex bead-positive (Figure 3-14 B). In other parts of the body, where the skin was protected from UV light (*i.e.* at the trunk), no infiltration of CD45^{+} cells and no latex bead-positive cells were detected. Thus, Id2 is not required for development of the Gr-1^{hi} monocytic LC precursor and its UV-induced migration into epidermis.

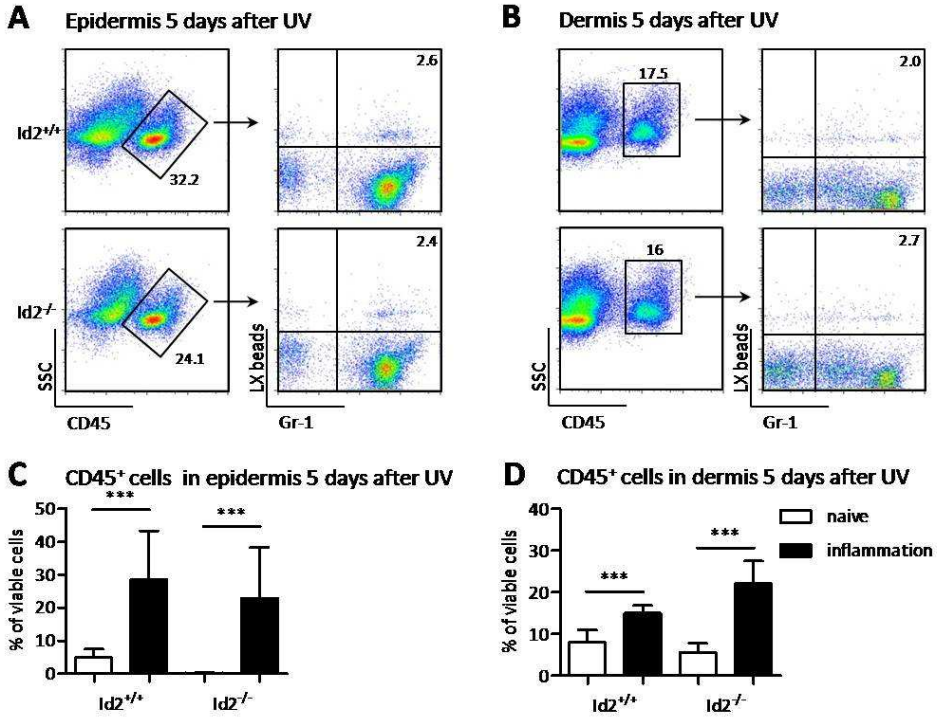


Figure 3-14: Influx of FITC-latex bead-labelled Gr-1^{hi} monocytes into inflamed skin of Id2$^{-/-}$ mice.
Gr-1^{hi} monocytes from Id2$^{-/-}$ and Id2$^{+/+}$ mice were labelled with FITC-conjugated latex-beads *in vivo* and exposed to UV light 4h later. Epidermis (A) and dermis (B) were examined 5 days after UV treatment for the presence of CD45^{+} cells and latex^{+} Gr-1^{hi} monocytes by flow cytometry. The percentage of FITC-latex bead-labelled Gr-1^{hi} monocytes contained within CD45^{+} cells is shown. The fraction of latex^{+} Gr-1^{hi} monocytes corresponds to their labelling efficiency in blood. SSC, Side scatter. (C and D) The number of CD45^{+} cells as percentage of total epidermal (C) and dermal (D) cell numbers before (naïve; open) and 5 days after UV treatment (inflammation; black) in Id2$^{-/-}$ and Id2$^{+/+}$ mice. Data shown are representative of at least n = 3 independent experiments ± SD. *** $p < 0.001$.

3.2.4 LC reconstitution after UV irradiation is Id2-independent

So far, it could be shown that Gr-1^{hi} monocytic LC precursors are present in Id2$^{-/-}$ mice at normal frequency and are recruited into epidermis upon UV exposure. Therefore, it was investigated whether LCs are repopulated in inflamed skin of Id2$^{-/-}$ mice. Id2$^{-/-}$ and Id2$^{+/+}$ mice were exposed to UV light, and epidermis and dermis were analyzed 4 weeks later for the presence of LCs (Figure 3-15) and langerin^{+} dDCs (Figure 3-16).

Flow cytometry analysis of single-cell suspensions revealed similar numbers of langerin^{+}CD11c^{+} LCs in UV-treated Id2$^{+/+}$ epidermis compared to untreated Id2$^{+/+}$ mice (Figure 3-15 C), consistent with the de-novo LC differentiation from Gr-1^{hi} monocytes following inflammation [63]. Remarkably, the epidermis of Id2$^{-/-}$ mice was also repopulated by langerin^{+}CD11c^{+} LCs within 4 weeks after UV exposure (Figure 3-15). However, the frequency of CD45^{+} cells and LCs in Id2$^{-/-}$ epidermis was lower compared to

Id2$^{+/+}$ mice, and langerin expression was diminished (Figure 3-15, A and B). Interestingly, langerin^{+} dDCs were not repopulated within 4 weeks after UV irradiation, although the entire population of CD11c^{+}MHC-II^{+} dDCs was not altered in both Id2$^{-/-}$ and Id2$^{+/+}$ mice (Figure 3-16). This indicates that Gr-1^{hi} infiltrating monocytes serve as precursors for LCs, but not for langerin^{+} dDCs following skin injury.

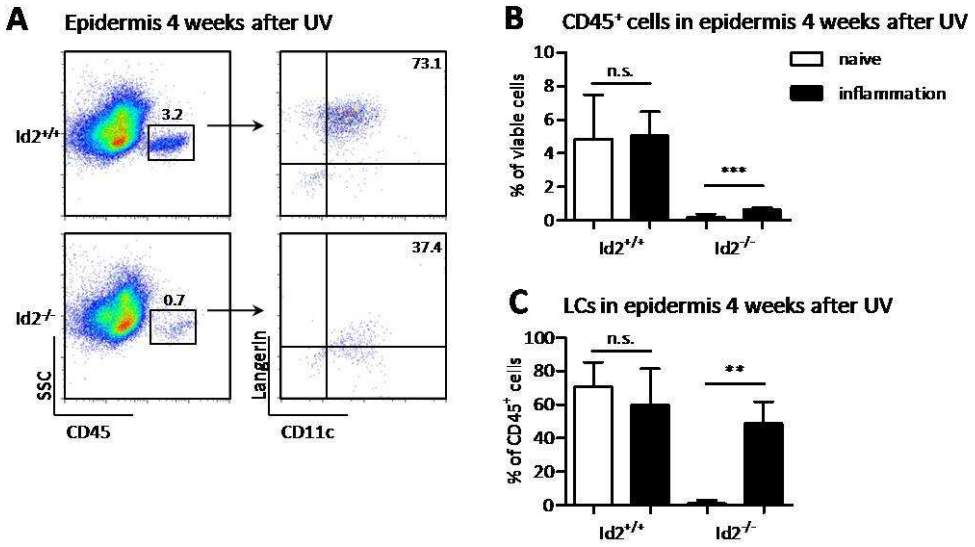


Figure 3-15: LC repopulation in epidermis in Id2$^{-/-}$ mice after UV irradiation.
Repopulation of LCs in epidermal sheets was examined by flow cytometry 4 weeks after UV irradiation. (A) Single-cell suspensions of epidermal sheets from Id2$^{-/-}$ and Id2$^{+/+}$ mice were examined for the presence of LCs (CD45^{+}, CD11c^{+}, and langerin^{+} cells). SSC, Side scatter. (B and C) Bar diagrams display the percentage of CD45^{+} cells in total cells (B) and the number of LCs in CD45^{+} cells (C) before (naïve; open) and 4 weeks after UV treatment (inflammation; black) in Id2$^{-/-}$ and Id2$^{+/+}$ mice. Data shown are representative of at least n = 3 independent experiments ± SD. n.s. not significant; ** $p < 0.01$; *** $p < 0.001$.

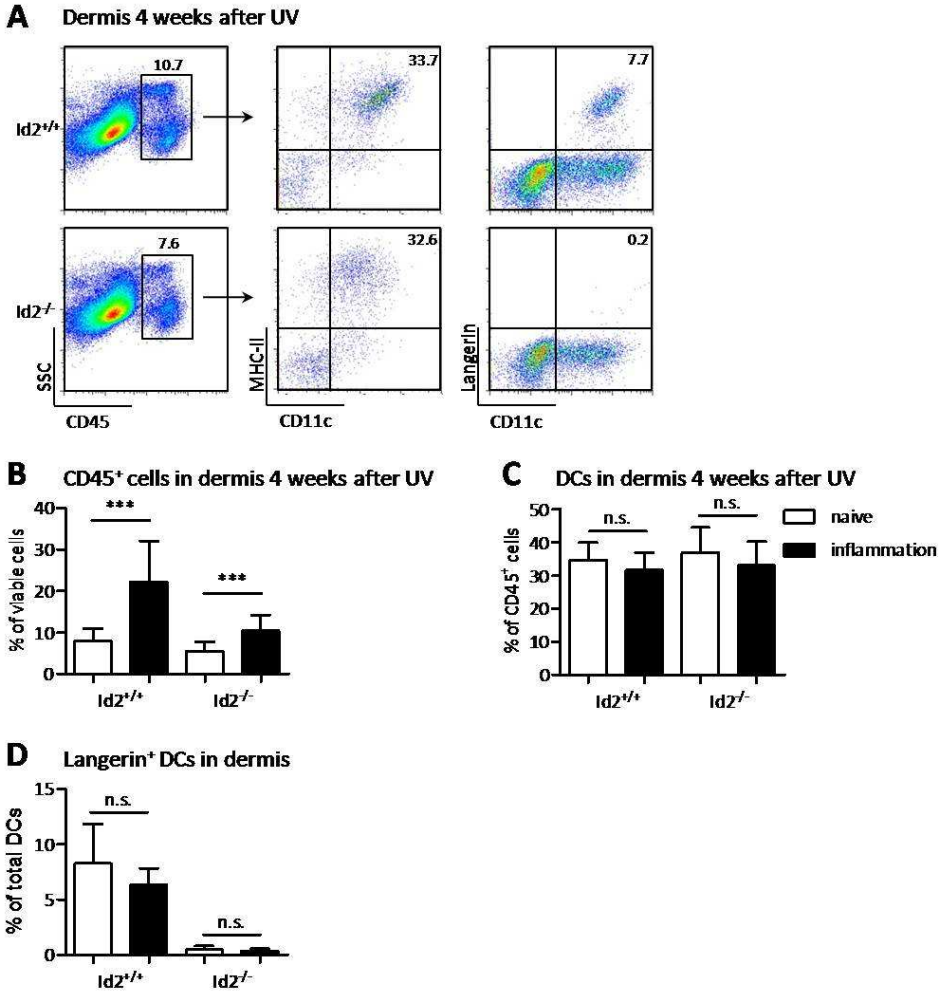


Figure 3-16: Langerin$^+$ dDC repopulation in dermis in Id2$^{-/-}$ mice after UV irradiation.
(A) Total dermal DCs and langerin$^+$ DCs in dermal sheets of Id2$^{-/-}$ and Id2$^{+/+}$ mice were examined by staining for CD45, MHC-II, CD11c, and langerin. Total dDCs were defined as CD45$^+$CD11c$^+$ and MHC-II$^+$. Langerin$^+$ dDCs were defined as CD45$^+$CD11c$^+$ and langerin$^+$. SSC, Side scatter. (B to D) Bar diagrams show the frequency of CD45$^+$ cells among total dermal cells (B), the frequency of DCs among CD45$^+$ cells (C), and the frequency of langerin$^+$ dDCs among total dDCs (D) before (naïve; open) and 4 weeks after UV treatment (inflammation; black) in Id2$^{-/-}$ and Id2$^{+/+}$ mice. Data shown are representative of at least n = 4 independent experiments ± SD. n.s. not significant; *** $p < 0.001$.

3.2.5 LC maintenance after UV irradiation is Id2-dependent

To investigate whether the monocyte derived LCs (mo-LCs) are capable of longterm repopulation of the epidermis, epidermal sheets of Id2$^{-/-}$ and Id2$^{+/+}$ were analyzed 12 weeks after UV treatment (Figure 3-17).

Interestingly, epidermal sheets of Id2$^{-/-}$ mice were devoid of MHC-II$^+$ and langerin$^+$ LCs 12 weeks after UV irradiation (Figure 3-17, A and B). The microscopic examination revealed no apparent differences in the LC compartment in Id2$^{+/+}$ mice 12 weeks after UV exposure compared to untreated Id2$^{+/+}$ mice (Figure 3-8 A and Figure 3-17 A). These data show that

Id2 is dispensable for mo-LC development under inflammatory conditions, but is an essential factor for steady state development of classical LCs in the epidermis. Moreover, it demonstrates that Gr-1^{hi} monocytes give rise to only transient mo-LCs under inflammatory conditions.

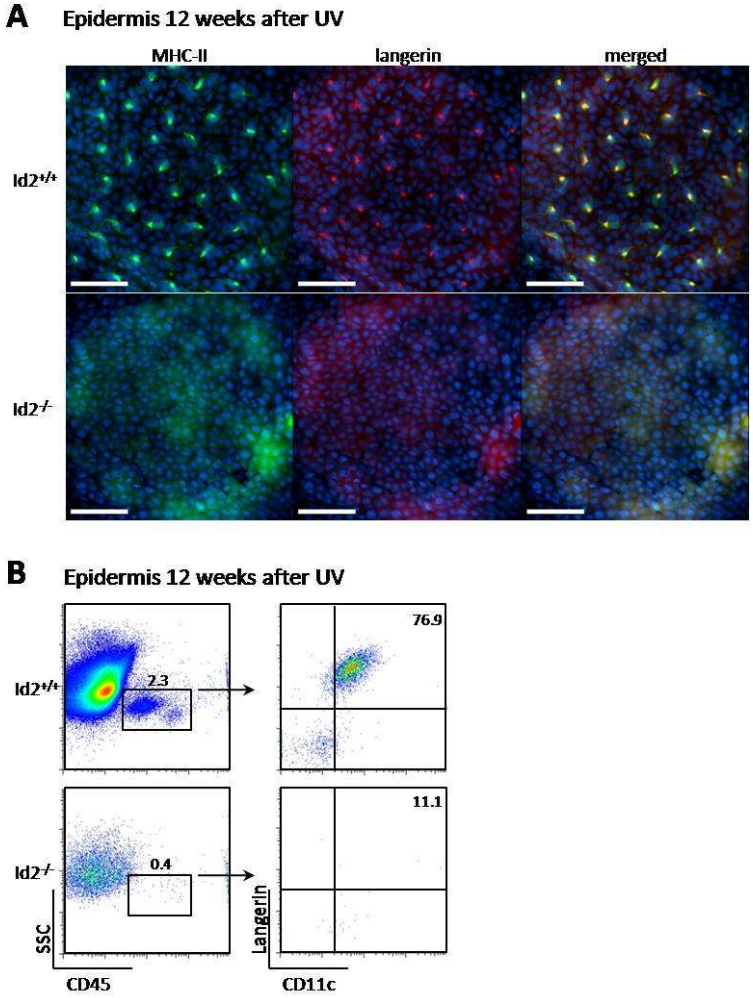


Figure 3-17: Longterm repopulation potential of LCs after UV irradiation.
Longterm repopulation potential of LCs in epidermal sheets was examined by flow cytometry and fluorescence microscopy 12 weeks after UV irradiation. (A) Epidermal sheets from Id2$^{-/-}$ and Id2$^{+/+}$ mice were stained for MHC-II (green), langerin (red), and with DAPI (blue). (B) Single-cell suspensions of epidermal sheets from Id2$^{-/-}$ and Id2$^{+/+}$ mice were examined for the presence of LCs (CD45^{+}, CD11c^{+}, and langerin^{+} cells). SSC, Side scatter. Data shown are representative of at least n = 4 independent experiments. Scale bar 50µm.

3.2.6 Reconstituted Id2$^{-/-}$ LCs differ from Id2$^{+/+}$ LCs

So far the data indicated that inflammatory, Id2-independent mo-LCs may principally differ from steady state, Id2-dependent classical LCs. To assess morphology and distribution of LCs 4 weeks after UV exposure, epidermal sheets of Id2$^{-/-}$ and Id2$^{+/+}$ mice

were double-stained with MHC-II and langerin specific antibodies. The emerging mo-LCs showed a patchy distribution as described before [63]. MHC-II^{+}langerin^{+} LCs were observed in both Id2$^{-/-}$ and Id2$^{+/+}$ mice (Figure 3-18 A). However, LC density was lower in Id2$^{-/-}$ mice compared to Id2$^{+/+}$ mice, which is consistent with the data obtained by flow cytometry (Figure 3-15 A), and the re-evolved mo-LCs showed morphological differences compared to WT control (Figure 3-18 A). To analyze this observation in more detail epidermal sheets of Id2$^{-/-}$ and Id2$^{+/+}$ mice were subjected to confocal laser-scanning microscopy. No apparent differences were observed for the expression of MHC-II in Id2$^{-/-}$ mo-LCs compared to Id2$^{+/+}$ LCs (Figure 3-18 B). Conversely, langerin expression differed in Id2$^{-/-}$ and Id2$^{+/+}$ LCs. Flow cytometry showed a diminished staining of langerin in Id2$^{-/-}$ mo-LCs (Figure 3-15 A), whereas immunofluorescence data unveiled higher expression and dissimilar distribution of langerin in Id2$^{-/-}$ mo-LCs compared to Id2$^{+/+}$ LCs (Figure 3-18 B). In Id2$^{-/-}$ mo-LCs MHC-II and langerin appear to be expressed all over the cell body, whereas in Id2$^{+/+}$ LCs MHC-II and langerin are predominantly located to the perinuclear space (Figure 3-18 B), leading to the impression that Id2$^{-/-}$ mo-LCs appear larger than Id2$^{+/+}$ LCs (Figure 3-18 A).

Co-localization analysis of MHC-II and langerin expression also revealed different pattern in Id2$^{-/-}$ mo-LCs and Id2$^{+/+}$ LCs. Id2$^{+/+}$ LCs exhibit co-localization primarily within the perinuclear space. In contrast, Id2$^{-/-}$ mo-LCs display an almost complete overlap of MHC-II and langerin not only within the perinuclear space, but also in the periphery and dendrites (Figure 3-18, B and C). Additionally, this examination revealed that Id2$^{-/-}$ mo-LCs consistently have more dendrites compared to Id2$^{+/+}$ LCs, while the entire cell dimensions were the same.

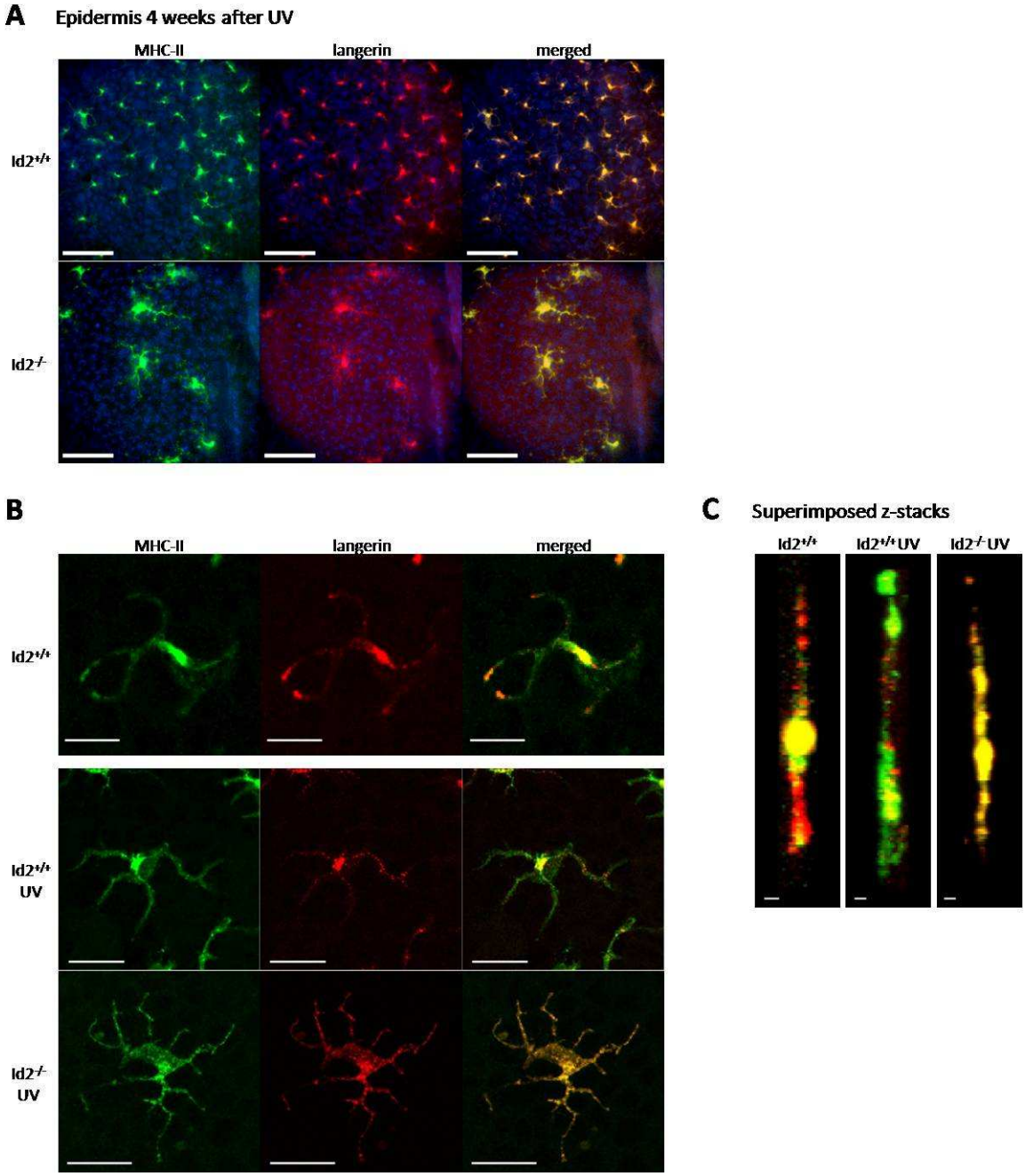


Figure 3-18: Phenotype of LC in Id2$^{-/-}$ and Id2$^{+/+}$ mice after UV irradiation.
Morphology of LCs in epidermal sheets was examined by fluorescence and confocal microscopy 4 weeks after UV irradiation. Sheets were stained for MHC-II (green), langerin (red) and with DAPI (blue). (A) Fluorescence microscopy analysis of epidermal sheets from Id2$^{-/-}$ and Id2$^{+/+}$ mice. Scale bar, 50µm. (B and C) Confocal microscopy analysis of epidermal sheets from Id2$^{-/-}$ and Id2$^{+/+}$ mice. (B) Representative LCs from untreated Id2$^{+/+}$ (upper panel), and Id2$^{+/+}$ (middle panel) and Id2$^{-/-}$ (lower panel) mice 4 weeks after UV irradiation. Scale bars, 20µm. (C) Superimposed z-stacks of LCs in Id2$^{-/-}$ and Id2$^{+/+}$ mice before and after UV treatment. Scale bars, 2µm. Data shown are representative of at least n = 4 independent experiments.

Birbeck granules (BGs) are a hallmark of classical LCs. In order to extend the morphological examination for the presence of BGs in LCs after UV irradiation, epidermal sheets from Id2$^{-/-}$ and Id2$^{+/+}$ mice were subjected to transmission electron microscopy. In epidermis from Id2$^{+/+}$ mice BGs were found in all LCs analyzed and the frequency of LCs

was comparable to the frequency determined by flow cytometry (Figure 3-19). Conversely, in ultrathin sections of $Id2^{-/-}$ epidermis no LCs could be found, presumably due to the low mo-LC frequency, and thus analysis for BGs was not possible.

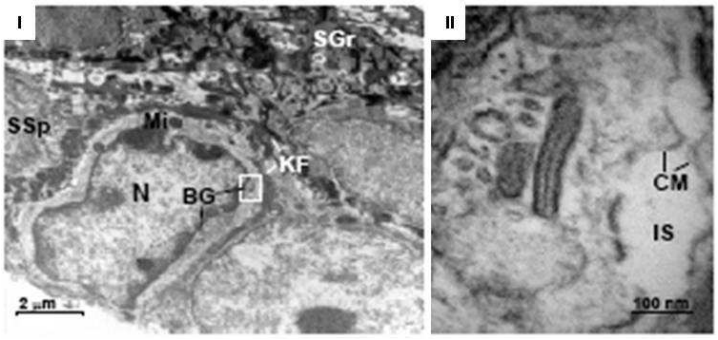


Figure 3-19: Birbeck granules in epidermal LCs of $Id2^{-/-}$ and $Id2^{+/+}$ mice after UV irradiation.
Electron micrographs of $Id2^{+/+}$ ear skin epidermis 4 weeks after UV irradiation. Magnification in (I), 8.000 x. Scale bar, 2µm. SSp, *Stratum spinosum*; SGr, *Stratum granulosum*; N, nucleus; Mi, mitochondria; KF, keratin filaments; BG, Birbeck granules. Boxed area in (I) is represented in (II) with 220.000 x magnification. Scale bar, 100nm. CM, cell membranes; IS, intercellular space. Data shown are representative of at least n = 2 independent experiments.

3.2.7 Id2 is dispensable for *in vitro* LC development

$Flt3^+$ progenitor cells develop into LCs *in vivo* under steady state conditions (Figure 3-11). To investigate underlying molecular mechanisms of LC development, different *in vitro* culture systems were employed. Cultures of $Id2^{+/+}$ and $Id2^{-/-}$ bone marrow cells with Flt3L gave cDCs and pDCs with the same efficiency, but $langerin^+$ DCs were not detected (Figure 3-20 A). When $Flt3^+$ progenitor cells were generated and differentiated into DCs with GM-CSF according to the two-step protocol [160], $langerin^+$ DCs were obtained from both $Id2^{+/+}$ and $Id2^{-/-}$ cultures, although at rather low frequency (Figure 3-20 B).

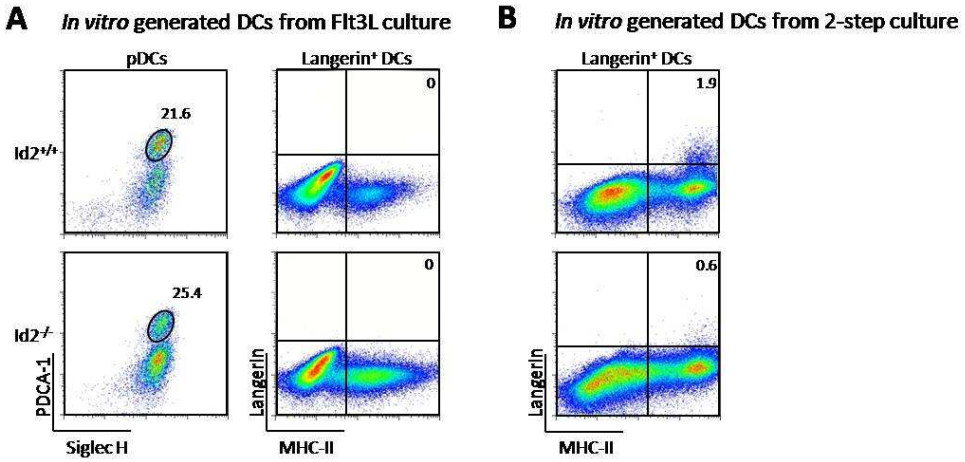


Figure 3-20: *In vitro* generation of LC-like cells in Flt3L and 2-step cultures from Id2$^{-/-}$ and Id2$^{+/+}$ mice.
(A) Flt3L cultures were examined for the presence of pDCs and langerin^{+} DCs after 8 days of differentiation. pDCs from Flt3L cultures were first gated on CD11c^{+}CD11b^{-} cells and display of PDCA-1 against Siglec H is shown (left panel). Langerin^{+} DCs were not detectable in Flt3L cultures at any time (right panel). (B) DCs from two-step cultures were first gated on CD11c^{+} cells. Staining for langerin against MHC-II is shown. Data shown are representative of at least n = 2 independent experiments.

Finally, a DC culture system where DCs were generated directly from Id2$^{+/+}$ and Id2$^{-/-}$ bone marrow with GM-CSF only was examined [161, 162]. After 8 days of culture DCs were analyzed for LC surface marker expression. Langerin^{+} DCs could be generated from both Id2$^{+/+}$ and Id2$^{-/-}$ cultures and these cells expressed E-cadherin, Gr-1, F4/80, and CCR7 and thus resembled the phenotype of classical LCs *in vivo* (Figure 3-21) [26].

This observation is in line with the previous findings that mo-LC development via the Gr-1^{hi} LC precursor occurs only under inflammatory conditions, but not in steady state. However, the percentage of MHC-II^{+}langerin^{+} DCs in Id2$^{-/-}$ cultures was found to be three to four times lower compared to Id2$^{+/+}$ cultures, which corresponds to the *in vivo* repopulation data (Figure 3-15). All this indicates that Id2 is not required for this inflammatory type of mo-LCs generated with GM-CSF *in vitro*.

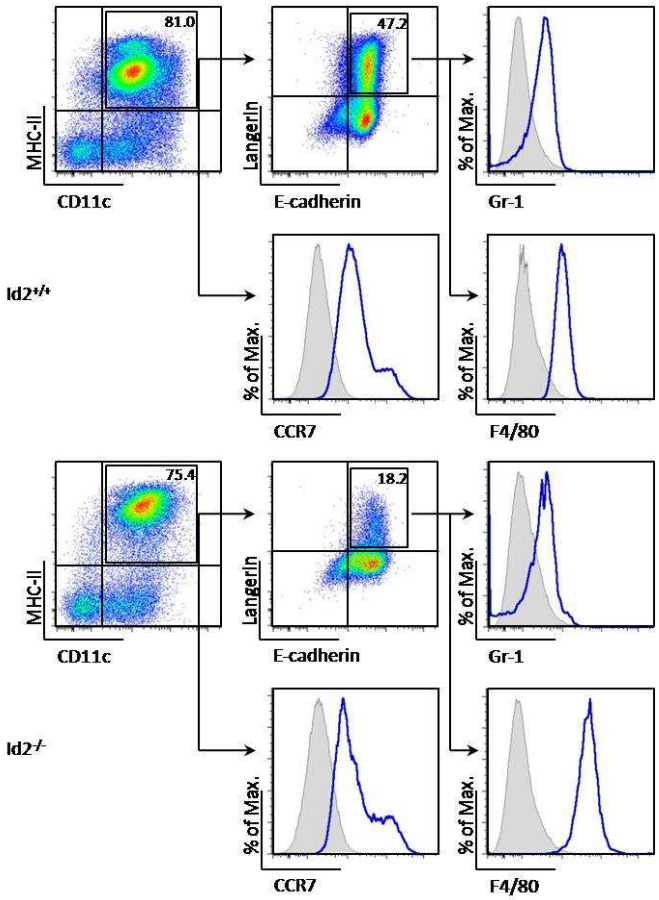


Figure 3-21: *In vitro* generation of LC-like DCs from Id2$^{-/-}$ and Id2$^{+/+}$ mice.
In vitro generated DCs were examined by flow cytometry after 8 days in culture. Single-cell suspensions from Id2$^{-/-}$ and Id2$^{+/+}$ mice were examined for the presence of LCs by expression of CD11c, MHC-II, E-cadherin, langerin, Gr-1, and F4/80. SSC, Side scatter. Data shown are representative of at least n = 3 independent experiments.

The *in vitro* generated DCs from Id2$^{+/+}$ and Id2$^{-/-}$ mice were further examined for the presence of BGs by transmission electron microscopy. BGs were found in DCs from both Id2$^{+/+}$ and Id2$^{-/-}$ mice (Figure 3-22), revealing that Id2 is dispensable for BG formation *in vitro*. Noteworthy, only a fraction of the DCs contained BGs.

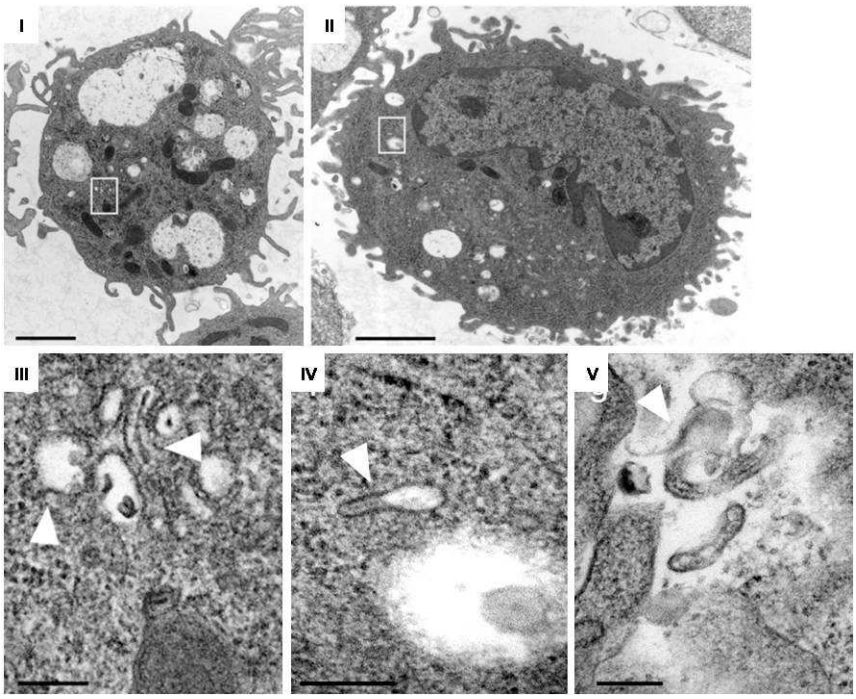


Figure 3-22: Birbeck granules in *in vitro* generated LC-like DCs from Id2$^{+/+}$ and Id2$^{-/-}$ mice.
In vitro generated LC-like DCs containing BGs from bone marrow of Id2$^{+/+}$ (I, III) and Id2$^{-/-}$ (II, IV, V) mice. Scale bars in (I) and (II), 2µm. Boxed areas in (I) and (II) locating BGs in cells displayed at higher magnification in (III) and (IV), respectively. Electron micrograph (V) shows a BG in another Id2$^{-/-}$ DC sample. BGs in (III), (IV) and (V) are indicated by arrowheads. Scale bars in (III), (IV) and (V), 200nm. Data shown are representative of at least n = 2 independent experiments.

In order to investigate the molecular mechanisms of langerin^{+} DC development *in vitro,* gene expression in Id2$^{-/-}$ and Id2$^{+/+}$ bone marrow cultures was analyzed by RT-PCR. Expression of classical LC markers, such as CD11c, langerin, M-CSFR, and E-cadherin, members of the Id-family of HLH transcriptional regulators, and inflammatory chemokine receptors important for monocyte/ DC migration and maturation were investigated (Figure 3-23).

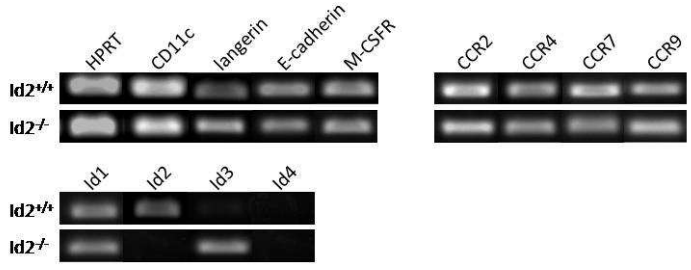


Figure 3-23: Gene expression in LC-like DCs from Id2$^{-/-}$ and Id2$^{+/+}$ mice.
RT-PCR analysis for indicated genes from *in vitro* generated bone marrow derived DCs of Id2$^{-/-}$ and Id2$^{+/+}$ mice. HPRT was used as loading control. Data shown are representative of at least n = 3 independent experiments.

CD11c, langerin, E-cadherin, M-CSFR, CCR2, CCR4, CCR7, CCR9, and Id1 were expressed at similar levels in both $Id2^{-/-}$ and $Id2^{+/+}$ cultures. Id4 was neither expressed in $Id2^{-/-}$ nor in $Id2^{+/+}$ cells, and Id2 was only expressed in $Id2^{+/+}$ cultures as expected. Interestingly, Id3 was highly expressed in $Id2^{-/-}$ cells, whereas the expression in $Id2^{+/+}$ cells was low to absent. This finding suggests that the deficiency of Id2 might be compensated by Id3 *in vitro*. The substitution of Id2 by Id3 might be sufficient for an Id2 independent development of $langerin^{+}$ DCs *in vitro* and presumably also for mo-LC development from $Id2^{-/-}$ $Gr\text{-}1^{hi}$ monocytes under inflammatory conditions *in vivo*.

3.3 The influence of Id2 in lymphoid organ DC development

In mice, secondary lymphoid organs include spleen, thymus, lymph nodes, mucosal-associated lymphoid tissues, such as nasal-associated lymphoid tissue (NALT), and Peyer's patches, as well as other gut-associated lymphoid tissue [193].

The spleen is the main organ used for studies of immunity in mice. During steady-state four functionally distinct DC subsets can be defined [53]. These include the circulating plasmacytoid DCs (pDCs), and the three subsets of tissue resident cDCs. It was believed that $CD8\alpha^-$ cDCs are located in the marginal zone, whereas the $CD8\alpha^+$ cDCs are localized to T cell areas [24, 48, 194, 195]. However, recent studies by Idoyaga *et al.* found that large numbers of $CD8\alpha^+CD207^+$ cDCs are located in the marginal zone, with smaller numbers in red pulp and the T cell area [195].

Thymic cDCs are mainly lymphoid organ resident cDCs. In contrast to the spleen, most mouse thymic cDCs are of the $CD8\alpha^+$ subtype, with only a minority population that is similar to double negative cDCs and few if any cDCs that express CD4 [196]. The thymic $CD8\alpha^+$ cDCs are generated within the thymus from the earliest intrathymic progenitors, whereas the minor $CD8\alpha^-$ cDCs originate from peripheral migratory DCs [53, 66, 165, 166, 197].

DCs show more developmental flexibility at early stages of haematopoiesis than other blood cell lineages. However, the developmental origin of these DC subtypes and their relationship has been controversial. Cell kinetic studies indicate that, at some point, the lineages leading to the different cDC subtypes branch and develop independently [67]. This is supported by studies showing that different transcription factors are required for the development of the different DC subtypes [139, 142, 156, 157].

Mice deficient in Id2 have undetectable lymphoid tissue inducer cells (LTi cells), which produce lymphotoxin. Lymphotoxin is essential for secondary lymphoid organ development and $Id2^{-/-}$ mice lack lymph nodes, Peyer's patches, and NALT [193].

This section investigates the influence of Id2 in DC development in lymphoid organs. Furthermore it analyses whether other lymphocytes, especially T cells, are also affected by the Id2 deficiency.

3.3.1 Id2 deficiency results in decreased cDC numbers in spleen

As already shown, Id2 deficient mice suffer from alymphoplasia (Figure 3-24 A), and lack Peyer's patches. Moreover, they show minor signs of splenomegaly (Figure 3-24 B), although splenic architecture is normal [198]. Conversely, the thymus of $Id2^{-/-}$ mice appeared completely normal in size and structure [198].

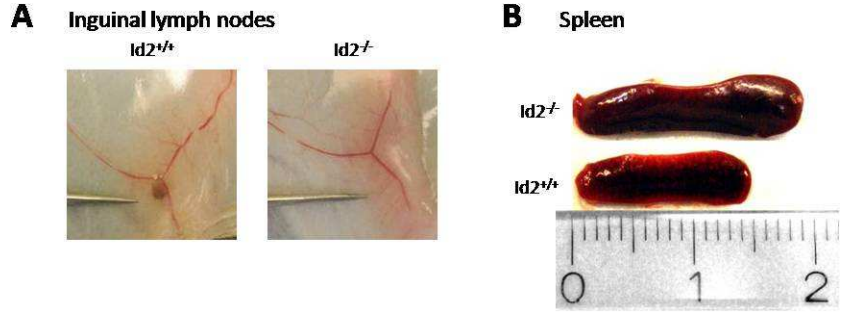


Figure 3-24: Id2 deficiency results in lack of lymph nodes and enlarged spleen.
(A) The inguinal region; a lymph node is evident in the $Id2^{+/+}$ mouse (left) but missing in the $Id2^{-/-}$ mouse (right). (B) Comparison of $Id2^{-/-}$ (upper) and $Id2^{+/+}$ (lower) spleen. The slight enlargement of the $Id2^{-/-}$ spleen is easily visible. Data shown are representative of at least n = 10 independent experiments.

In addition, previous characterization of $Id2^{-/-}$ mice demonstrated that Id2 was crucial for the development of distinct DC subsets as the splenic $CD8\alpha^+$ DCs, which are severely reduced in these mice [156]. Therefore, DCs from $Id2^{-/-}$ and $Id2^{+/+}$ spleen were prepared and analyzed by flow cytometry. In $Id2^{-/-}$ mice the percentage of total DCs, determined by MHC-II and CD11c, was found to be reduced by half compared to $Id2^{+/+}$ mice (Figure 3-25).

Furthermore, flow cytometry analysis demonstrated a marked reduction in $CD8\alpha^+$ cDCs in spleen, confirming previous studies (Figure 3-25). Thus, deletion of Id2 resulted in a severe shift in the proportion of splenic cDC subsets; $CD4^-CD8\alpha^+$ DCs were markedly reduced, and therefore $CD4^+CD8\alpha^-$ DCs were the predominant DC population in spleen (Figure 3-25).

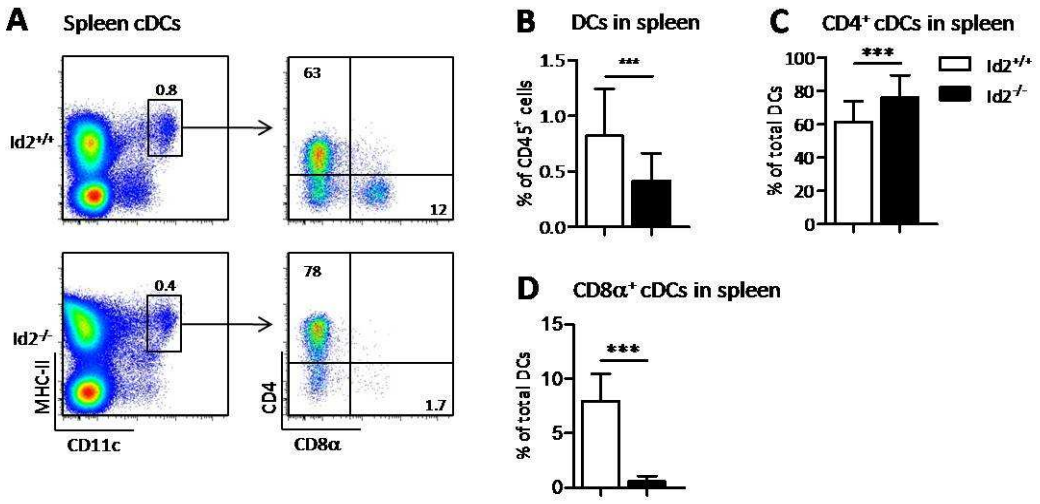


Figure 3-25: Id2 deficiency results in decreased DC numbers in spleen.
Dendritic cells were determined as $CD45^+$ cells low in side scatter and double positive for MHC-II and CD11c. Distribution of $CD4^+$ versus $CD8\alpha^+$ DCs is shown (A). Bar diagrams represent the percentage of total DCs (B), $CD4^+$ DCs (C), and $CD8\alpha^+$ DCs (D) in spleen of $Id2^{-/-}$ (black) and $Id2^{+/+}$ (open) mice. Data shown are representative of at least n = 10 independent experiments ± SD; *** $p < 0.001$.

3.3.2 Id2 mice lack $CD8\alpha^+$ and $CD8\alpha^+$langerin$^+$ DCs in spleen and thymus

Recent studies reported on a subset of $CD8a^+$ cDCs that is located to the marginal zone rather than to the T cell area and also expresses langerin [195]. Hence, spleen DCs were further analysed for this $CD8\alpha^+$langerin$^+$ subpopulation. In $Id2^{+/+}$ mice $CD8\alpha^+$langerin$^+$ cDCs were easily detected and they made up for about 40% of the $CD8\alpha^+$ cDC population in spleen (Figure 3-26). However these cells, as well as the $CD8\alpha^+$ DC subpopulation, were virtually absent in $Id2^{-/-}$ mice (Figure 3-26, A and B).

This analysis was expanded to the thymus, where langerin$^+$ DCs can also be found. Flow cytometry revealed no differences in the DC compartment in $Id2^{-/-}$ and $Id2^{+/+}$ mice (Figure 3-26 D). Moreover, $CD8\alpha^+$ cDCs were present in $Id2^{-/-}$ mice at normal frequency compared to WT control. However, the $CD8\alpha^+$langerin$^+$ DC subset was only found in $Id2^{+/+}$ mice and was absent in $Id2^{-/-}$ mice (Figure 3-26, C and E).

Interestingly, in thymus of $Id2^{-/-}$ mice $CD8\alpha^+$ cDCs are still present and develop in the absence of Id2, implying a differential requirement for Id2 in DC development in bone marrow or thymus.

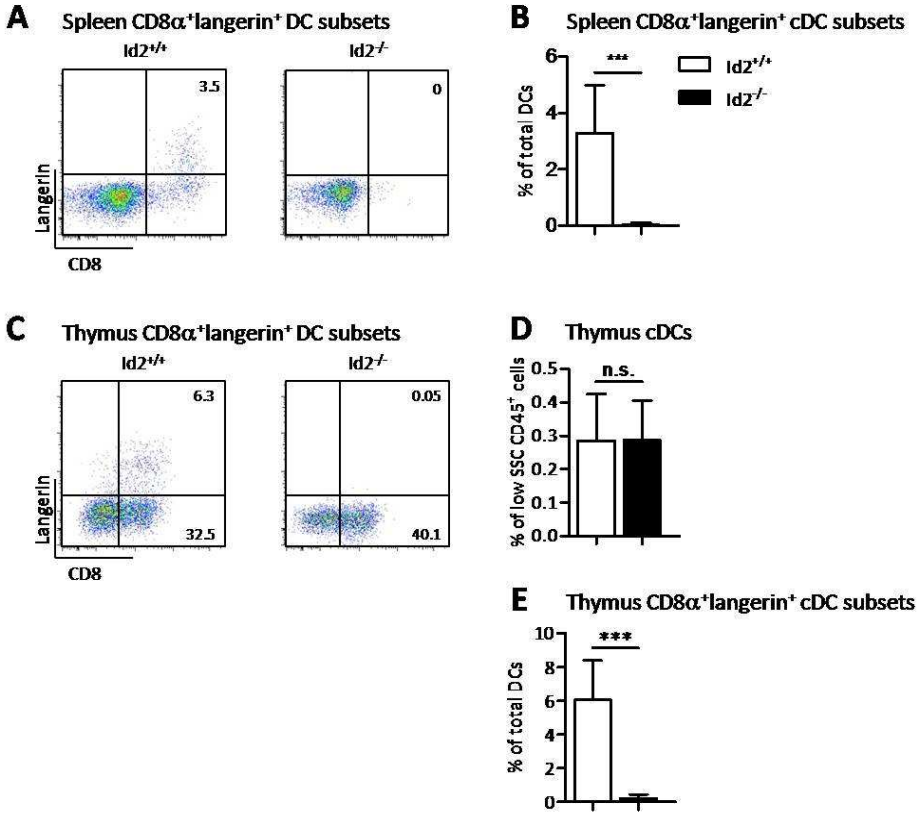


Figure 3-26: CD8α$^+$langerin$^+$ cDC subsets in spleen and thymus of Id2$^{-/-}$ and Id2$^{+/+}$ mice.
Dendritic cells were determined as CD45$^+$ cells low in side scatter, double positive for MHC-II and CD11c. (A) Distribution of langerin versus CD8α$^+$ in spleen DCs. (B) Bar diagram representing the percentage of CD8α$^+$langerin$^+$ DCs in total spleen DCs of Id2$^{-/-}$ (black) and Id2$^{+/+}$ (open) mice. (C) Distribution of langerin and CD8α on thymus DCs. (D and E) Bar diagrams represent the fraction of total DCs in thymus (D) and of CD8α$^+$langerin$^+$ DCs within total thymic DCs (E) in Id2$^{-/-}$ (black) and Id2$^{+/+}$ (open) mice. Data shown are representative of at least n = 5 independent experiments ± SD; n.s. not significant; *** $p < 0.001$.

3.3.3 Rescue of Id2$^{-/-}$ phenotype by adoptive transfer

As already described, the *in vitro* amplified Flt3$^+$ progenitors have the capacity to differentiate into all cells of the haematopoietic system upon cell transfer (section 3.1.4) and it was further demonstrated that this progenitor is able to give rise to both splenic CD8α$^+$ and CD8α$^-$ DCs *in vivo* [160]. Thus, Id2$^{+/+}$ bone marrow cells or *in vitro* amplified Flt3$^+$ progenitor cells from Id2$^{+/+}$ mice, together with a radioprotective dose of Id2$^{-/-}$ bone marrow to assure mouse survival, were transferred into lethally irradiated Id2$^{-/-}$ recipient mice. Spleen and thymus of recipients were analyzed for CD8α$^+$ and CD8α$^+$langerin$^+$ cDCs two weeks after cell transfer, because previous studies showed that CD8α$^+$ DCs reach their peak two weeks after bone marrow transfer [73].

CD8α$^+$ as well as CD8α$^+$langerin$^+$ DCs could be detected in spleen of Id2$^{-/-}$ mice two weeks after bone marrow transplantation (Figure 3-27 A). However, the proportion of each splenic DC population generated by bone marrow transplantation did not match the proportions found in the normal mouse spleen (Figure 3-25). This is not achieved until four to eight weeks after bone marrow transfer [67]. The transplantation of Flt3$^+$ progenitor cells also resulted in a repopulation of CD8α$^+$ DCs in spleen, confirming previous data [160]. Still, CD8α$^+$langerin$^+$ DCs were not detectable two weeks after transplantation (Figure 3-27 B). Analysis of the thymus two weeks after cell transfer revealed similar data. Whereas transfer of bone marrow cells repopulated CD8α$^+$langerin$^+$ cDCs in thymus, these cells remained absent after transplantation of Flt3$^+$ progenitor cells (Figure 3-27 C).

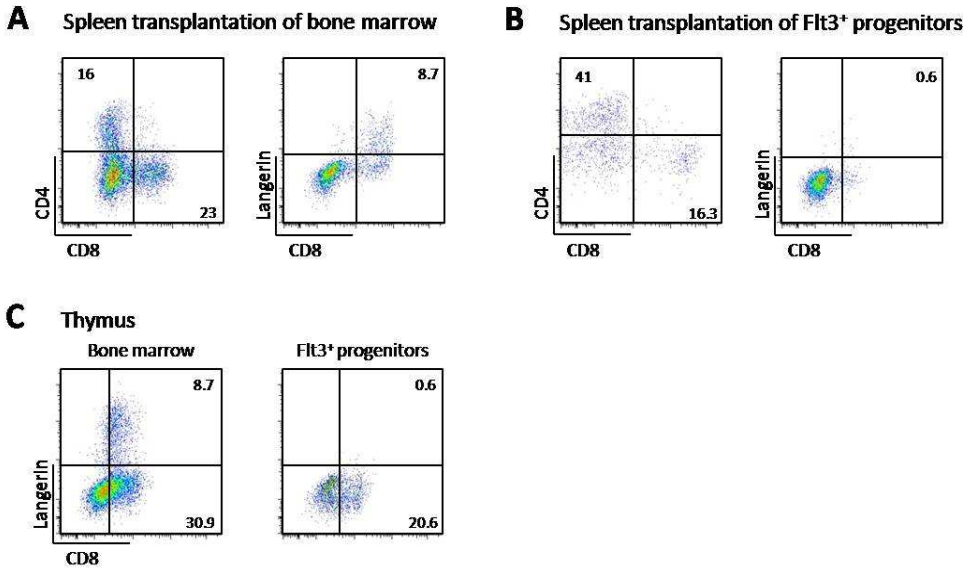


Figure 3-27: Adoptive transfer of bone marrow or Flt3$^+$ progenitor cells rescues Id2$^{-/-}$ phenotype
Id2$^{+/+}$ bone marrow cells or *in vitro* amplified Flt3$^+$ progenitor cells from Id2$^{+/+}$ mice, together with a radioprotective dose of Id2$^{-/-}$ bone marrow to assure mouse survival, were used for adoptive transfer experiments. Single-cell suspensions from spleen (A and B) and thymus (C) were analysed two weeks after transfer of unfractionated bone marrow cells (A) or Flt3$^+$ progenitor cells (B) for the presence of CD8α$^+$ and CD8α$^+$langerin$^+$ DCs respectively. Data shown are representative of at least n = 5 independent experiments.

3.3.4 Id2 deficiency leads to increased pDCs

The pDCs have many features that indicate they should arise from a developmental pathway that differs from that of cDCs [199]. In terms of gene expression, pDCs have many similarities to B cells and their development depends on the transcription factor SPI-B, which is also expressed by B cells but not by cDCs [52, 54]. Nevertheless, pDCs can

be produced from the same bone marrow precursors that give rise to cDCs [81, 82], they also depend on FLT3L for their production [93, 154], and their development involves many of the same transcription factors that are required for particular cDC subtypes.

The pDCs in spleen were defined as low side scatter cells positive for CD45 being $CD11c^{+}CD11b^{-}B220^{+}PDCA\text{-}1^{+}SiglecH^{+}$ and B cells were determined as low side scatter $CD45^{+}$ cells double positive for B220 and CD19. Comparison of single cell suspensions from spleen of $Id2^{-/-}$ and $Id2^{+/+}$ mice demonstrated that pDCs were increased by about two-fold (Figure 3-28, A and B). Conversely, the B cell population was not affected by the Id2 deficiency and remained stable (Figure 3-28, C and D).

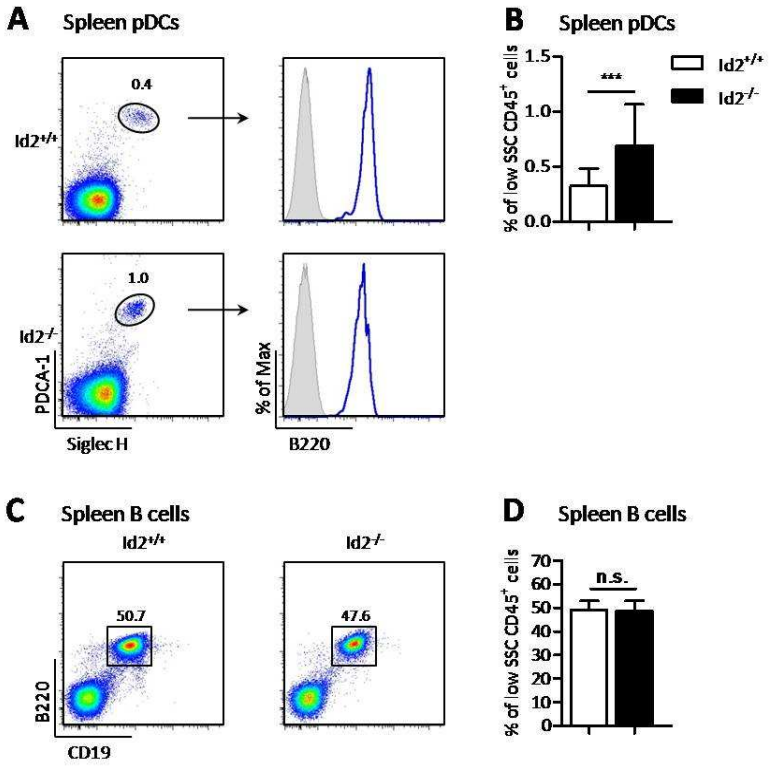


Figure 3-28: Increased pDCs in $Id2^{-/-}$ mice

Low side scatter $CD45^{+}$ cells of single-cell suspensions of $Id2^{-/-}$ and $Id2^{+/+}$ spleens were analysed for pDCs (A and B) and B cells (C and D). pDCs were gated on $CD45^{+}CD11c^{+}CD11b^{-}$ and the staining for PDCA-1 versus Siglec H and for B220 are shown (A). The percentage of pDCs in splenic $CD45^{+}$ cells of $Id2^{-/-}$ (black) and $Id2^{+/+}$ (open) mice is depicted as bar diagram (B). B cells were defined as $CD45^{+}$ cells low in side scatter and double positive for B220 and CD19 (C) and their frequency in $CD45^{+}$ cells of $Id2^{-/-}$ (black) and $Id2^{+/+}$ (open) mice is represented as bar diagram (D). Data shown are representative of at least n = 6 independent experiments ± SD; n.s. not significant; *** $p < 0.001$.

3.3.5 Id2 is essential for CD8 T cell maintenance in spleen and thymus

As shown here, $Id2^{-/-}$ mice displayed a selective and remarkable reduction of the $CD8\alpha^+$ DC subset. The $CD8\alpha^+$ DCs effectively prime naive $CD4^+$ T cells and preferentially induce T_H1 differentiation *in vivo* [200]. To investigate whether the Id2 deficiency influences the T cell compartment, spleen and thymus of $Id2^{-/-}$ and $Id2^{+/+}$ mice were analysed by flow cytometry (Figure 3-29). T cells were defined as $CD45^+$ cells low in side scatter, expressing $CD3\varepsilon$ and further separated according to their distribution of CD4 and $CD8\alpha$ (Figure 3-29). Analysis of splenocytes revealed comparable numbers of $CD3\varepsilon^+$ T cells in both $Id2^{-/-}$ and $Id2^{+/+}$ mice (Figure 3-29, A and B). However, the examination of T cell subsets showed a distinct shift in the CD4/CD8 ratio in $Id2^{-/-}$ mice. The $CD4^+$ T cell population increased from 60% in $Id2^{+/+}$ mice to 85% in $Id2^{-/-}$ mice. Conversely, the $CD8^+$ T cell population decreased from 30% in $Id2^{+/+}$ mice to 10% in $Id2^{-/-}$ mice (Figure 3-29, A and C). Analysis of the thymus revealed similar data as the results from spleen. The number of total T cells was similar in $Id2^{-/-}$ and $Id2^{+/+}$ mice (Figure 3-29, D and E), whereas the ratio of $CD4^+$ versus $CD8^+$ T cells was changed in $Id2^{-/-}$ mice. However, in thymus only the $CD8^+$ T cell population was decreased compared to $Id2^{+/+}$ mice, $CD4^+$ and double positive T cells remained unchanged (Figure 3-29, D and F). These results suggest that Id2 is profoundly involved in the development or survival of $CD8^+$ T cells.

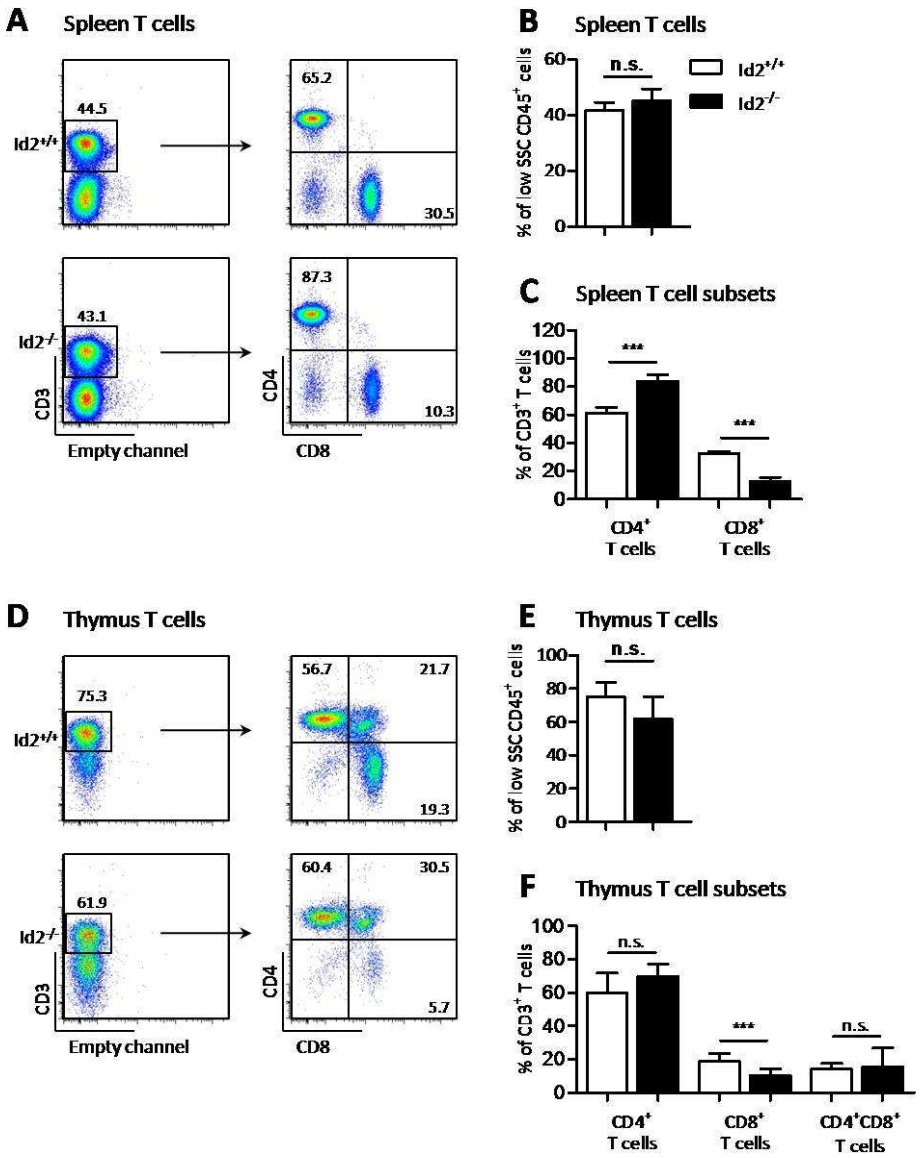


Figure 3-29: Altered T cell ratios in Id2$^{-/-}$ mice.

(A to C) Splenocytes of Id2$^{-/-}$ and Id2$^{+/+}$ mice were analysed for their T cell compartment. T cells were determined as CD45^{+} cells low in side scatter and positive for CD3ε and the distribution of CD4 versus CD8 is shown (A). The bar diagrams show the percentage of total T cells among splenocytes (B) and the distribution of CD4^{+} and CD8^{+} T cells among total T cells (C) in Id2$^{-/-}$ (black) and Id2$^{+/+}$ (open) mice. (D to F) Thymocytes of Id2$^{-/-}$ and Id2$^{+/+}$ mice were analysed for their T cell compartment. T cells were determined as CD45^{+} cells low in side scatter and positive for CD3ε and the distribution of CD4 versus CD8 is shown (D). The bar diagrams show the percentage of total T cells among thymocytes (E) and the distribution on CD4^{+} and CD8^{+} T cells among total T cells (F) in Id2$^{-/-}$ (black) and Id2$^{+/+}$ (open) mice. Data shown are representative of at least n = 4 independent experiments ± SD; n.s. not significant; *** $p < 0.001$.

3.4 Id2 antagonizes the function of activating bHLH proteins

The E protein family of transcription factors and their natural inhibitors, the "inhibitor of DNA binding" (Id) proteins, regulate many aspects of haematopoiesis, including commitment to the lymphoid lineages at several developmental stages [201, 202]. E proteins are a subclass of the basic helix-loop-helix family of transcriptional activators and repressors consisting of HEB, E2-2, and the E2A gene products, which bind specifically to DNA sequences containing the E-box consensus sequence. DNA binding by E proteins is regulated by the Id proteins, a family of highly related gene products. Id proteins, of which four (Id1–4) have been identified, contain the helix-loop-helix domain necessary for the formation of dimers, but lack the basic region that mediates DNA binding [201, 202]. Thus, Id proteins form heterodimers with E proteins and thereby prevent their association with E box motifs in target DNA sequences (Figure 3-30) [202-204].

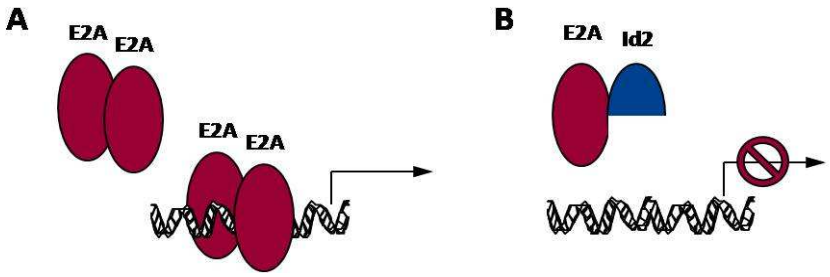


Figure 3-30: The antagonism of E2A and Id2.
(A) E2A forms homo- or heterodimer with other activating bHLH transcription factors, resulting in its binding to E-box sequences in target DNA. (B) When Id2 is present it binds to E2A and thereby preventing the complex from binding to DNA, resulting in inhibition of transcription.

The E proteins encoded by the E2A gene, E12 and E47, are known to be essential for development of committed B lymphocyte progenitors from CLPs as a result of their role in the induction of the B lineage transcription factor early B cell factor (EBF) and subsequent activation of Pax-5, which commits cells to the B lymphocyte lineage [204].

Therefore, an attractive hypothesis for the role of Id2 in DC development is that Id2 functions to inhibit E protein activity, allowing these cells to adopt non–B lineage cell fates by preventing B lymphocyte lineage commitment [157, 205]. However, Id2 has been proposed to have targets in addition to E proteins that could function in DC development, including the Ets family proteins PU.1 and the retinoblastoma protein [206, 207].

This section examines the question to what extend the antagonism of Id2 and E2A is required for the development of DC subsets, in particular for skin DCs. Furthermore, questions whether lowering E protein activity in $Id2^{-/-}$ mice by deletion of the E2A gene product E47 results in the restoration of previously absent DC subtypes.

3.4.1 E47 deletion overcomes the need for Id2 in the development of langerin^{+} skin DCs

Besides its known regulatory function in B cell development, Id2 is essential for the development of natural killer cells (NKs), LTi cells, and secondary lymphoid tissues. However, a recent study by Boos *et al*. reports that the deletion of the E protein E47 in Id2-/- mice results in the restoration of NK cells and LTi cells in the embryo, as well as the restoration of lymph nodes and Peyer's Patches [208]. Therefore, DC subsets in $E47^{+/-}$ $Id2^{+/-}$ (dhet), $E47^{-/-}Id2^{+/-}$ ($E47^{ko}$), $E47^{+/-}Id2^{-/-}$ ($Id2^{ko}$), and $E47^{-/-}Id2^{-/-}$ (dko) mice were investigated, especially those that are absent in $Id2^{-/-}$ mice.

As reported in section 3.2, $Id2^{-/-}$ mice lack LCs in the epidermis as well as a langerin expressing subpopulation of DCs in dermis (Figure 3-8). To analyse the importance of the antagonism of Id2 and its counterpart E2A for the development of skin DCs flow cytometry analysis of single cell suspensions of epidermis and dermis of dhet, $E47^{ko}$, $Id2^{ko}$, and dko mice was performed (Figure 3-31). This revealed that LCs, characterized by CD45, CD11c, MHC-II and langerin expression, were present in epidermis of dhet and $E47^{ko}$ mice as expected. Importantly, LCs were also detected in epidermis of dko mice, whereas these cells were absent in $Id2^{ko}$ mice (Figure 3-31). Furthermore, the flow cytometry data showed that the deficiency of E47 and Id2 not only restored the LC compartment in these mice, but also restored parts of the $CD45^{+}$ cell population in the epidermis (Figure 3-31, A and B).

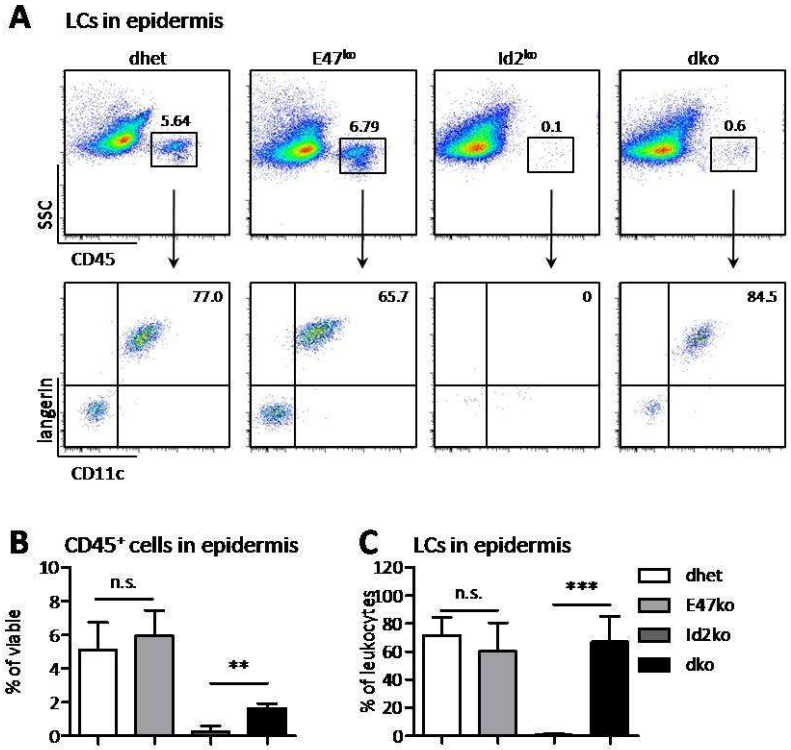


Figure 3-31: E47 deficiency overcomes the need for Id2 in LC development

(A) Flow cytometry of single-cell suspensions of epidermis from dhet, $E47^{ko}$, $Id2^{ko}$, and dko mice. For detection of LCs, cells from epidermal sheets were stained for CD45, CD11c, and langerin; SSC, Side scatter. (B) Frequency of $CD45^{+}$ cells among total epidermal cells and (C) frequency of LCs among $CD45^{+}$ cells in dhet (open), $E47^{ko}$ (light grey), $Id2^{ko}$ (grey), and dko (black) mice. Data shown are representative of at least n = 2 independent experiments ± SD. ** $p < 0.01$; *** $p < 0.001$.

Flow cytometry of dermis of dhet, $E47^{ko}$, $Id2^{ko}$, and dko mice demonstrated that $CD45^{+}$ cell frequency was comparable in dhet, and $E47^{ko}$ mice, but were decreased in $Id2^{ko}$ mice, similar to $Id2^{-/-}$ mice. The leukocyte reduction seen in $Id2^{ko}$ mice was not restored in dko mice, but remained dimished as in $Id2^{-/-}$ mice (Figure 3-32, A and B). Interestingly, the total DC number in dermis was also decreased in $Id2^{ko}$ mice, a phenotype not found in $Id2^{-/-}$ mice (Figure 3-9). The reduction in DC numbers was also not restored in dko mice and remained similar to the frequency observed in $Id2^{ko}$ mice (Figure 3-32 C).

Nevertheless, $langerin^{+}$ dDCs characterized by CD11c, MHC-II, and langerin were found in dhet, $E47^{ko}$, and dko mice, but absent in $Id2^{ko}$ mice as expected (Figure 3-32, A lower panel and D). The frequency was comparable to that of dhet and $E47^{ko}$ mice.

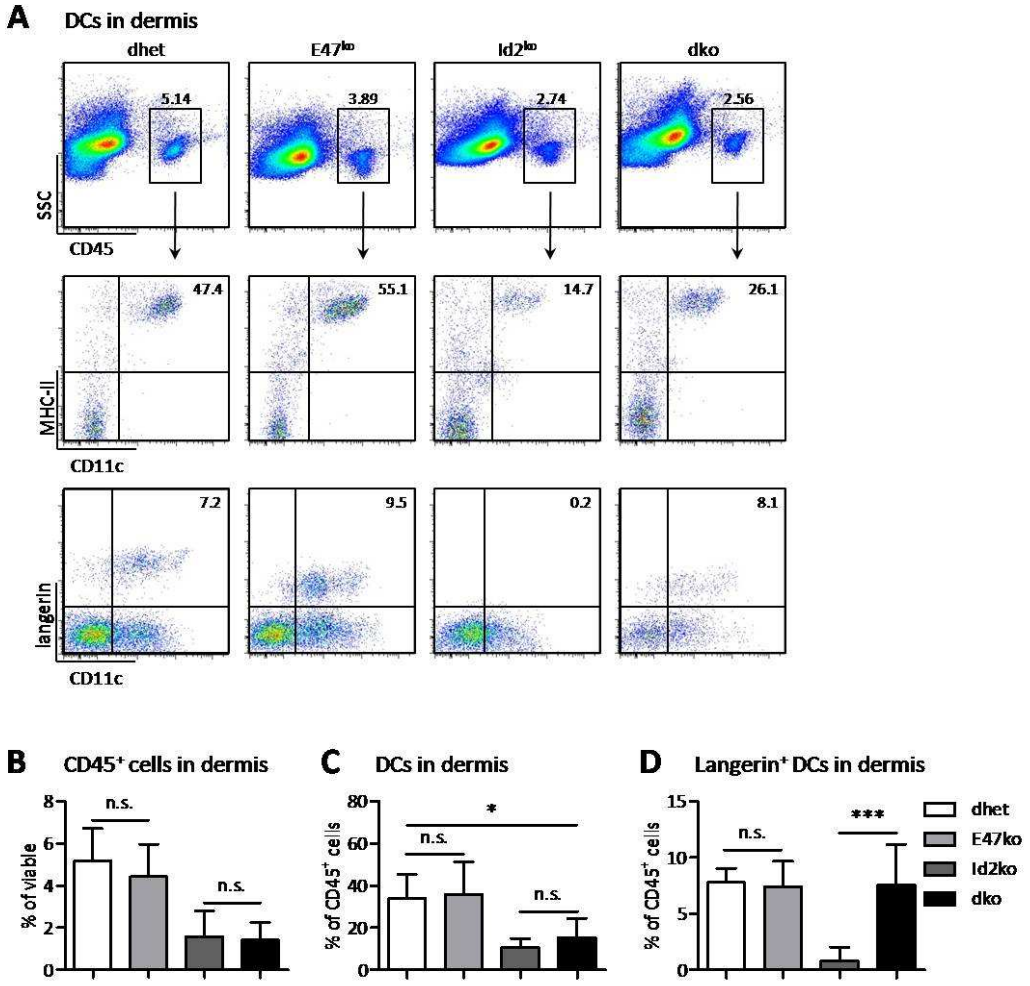


Figure 3-32: The deficiency of E47 and Id2 rescues langerin$^+$ dDCs
(A) Langerin$^+$ dDCs from dhet, E47^{ko}, Id2^{ko}, and dko mice were identified by staining for CD45, CD11c, and langerin; SSC, Side scatter. (B to D) Frequency of CD45$^+$ cells among total dermal cells (B), frequency of DCs among CD45$^+$ cells (C), and frequency of langerin$^+$ dDCs among total dDCs (D) in dhet (open), E47^{ko} (light grey), Id2^{ko} (grey), and dko (black) mice. Data shown are representative of at least n = 2 independent experiments ± SD. n.s. not significant; * $p < 0.05$; *** $p < 0.001$.

3.4.2 The double-deficiency of Id2 and E47 does not restore the Id2$^{-/-}$ phenotype in spleen DCs

Next, the impact of Id2 and E47 were analysed in spleen DCs as the data in section 3.3 demonstrate that Id2 has a strong influence on the development of certain subsets (Figure 3-25). Therefore, DCs from dhet, E47^{ko}, Id2^{ko}, and dko spleen were analyzed by flow cytometry for the different subsets. In Id2$^{-/-}$ mice the percentage of total DCs, determined by MHC-II and CD11c was found to be reduced by half compared to Id2$^{+/+}$ mice (Figure 3-25). This observation was similar for Id2^{ko} and dhet mice; Id2^{ko} mice showed a reduction in spleen DCs of about 50% compared to dhet mice (Figure 3-33, A

and B). Furthermore, flow cytometry analysis demonstrated an increase of DCs in $E47^{ko}$ and dko mice, which most probably results from the lack of B cells in E47 deficient mice. However, the increase in splenic DCs in $E47^{ko}$ and dko mice is not statistically significant when compared to dhet mice. Because deficiency of both Id2 as well as E2A results in severe difficulties during pregnancy, breeding of the E47Id2 mice resulted only in very few pups. Hence, statistical analysis in these mice is difficult. Therefore, the "normal" frequency in splenic DCs of dko mice rather due to the complete absence of B cells in mice deficient for E47 and the consequent shift in cells ratios than a regeneration of the DC population.

Further analysis of cDC subsets demonstrated a marked reduction in splenic $CD8\alpha^+$ cDCs in $Id2^{ko}$ and dko mice as already seen in $Id2^{-/-}$ mice (Figure 3-25, A and D; and Figure 3-33, A and D). This lack of $CD8\alpha^+$ cDCs resulted in a subsequent increase in percentage of $CD4^+$ cDCs in mice lacking Id2 (Figure 3-25 C and Figure 3-33 C). The proportion of $CD4^+$ and $CD8\alpha^+$ cDCs in dhet and $E47^{ko}$ mice was comparable, demonstrating that E47 deficiency has no influence on the development of these DC subsets. Nevertheless, the fact that $CD8\alpha^+$ cDCs are not restored in spleen of dko mice suggests a role for Id2 in suppression of alternative E proteins.

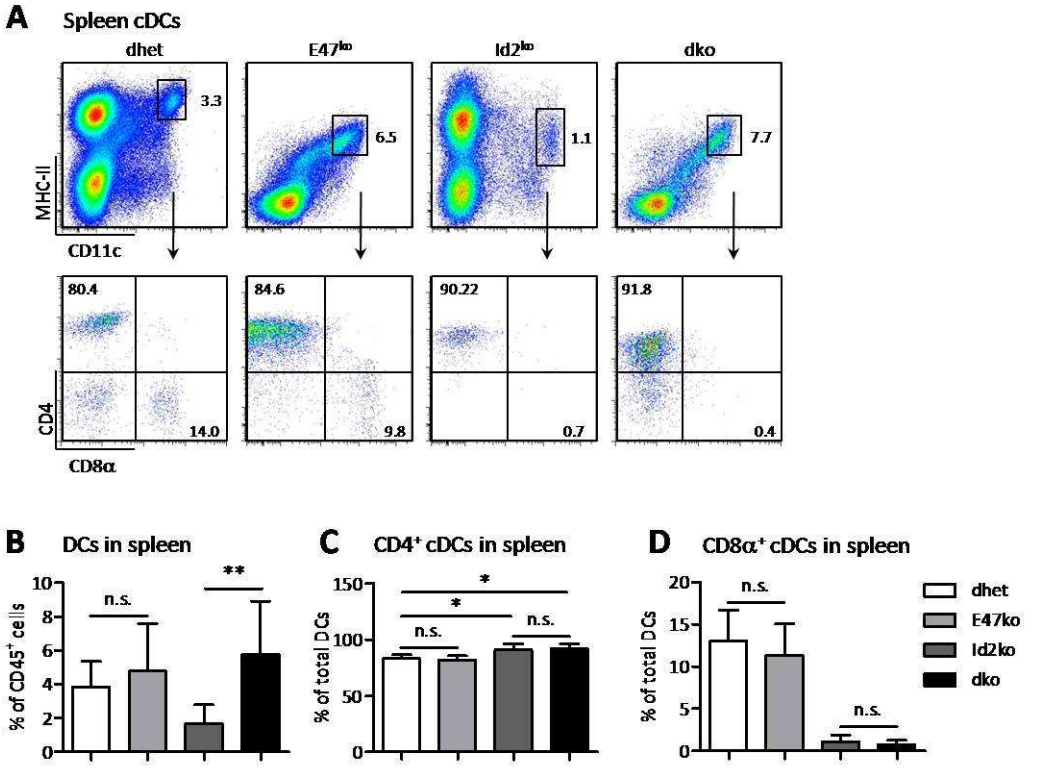


Figure 3-33: CD8α$^+$ cDCs remain absent in dko mice.
DCs of dhet, E47^{ko}, Id2^{ko}, and dko mice were determined as CD45$^+$ cells low in side scatter and double positive for MHC-II and CD11c. Distribution of CD4$^+$ versus CD8α$^+$ DCs is shown (A). Bar diagrams represent the percentage of total DCs (B), CD4$^+$ DCs (C), and CD8α$^+$ DCs (D) in spleen of dhet (open), E47^{ko} (light grey), Id2^{ko} (grey), and dko (black) mice. Data shown are representative of at least n = 2 independent experiments ± SD; n.s. not significant; ** $p < 0.01$.

3.4.3 Loss of E47 fails to restore CD8α$^+$langerin$^+$ cDCs spleen or thymus of Id2$^{-/-}$ mice

As already reported in section 3.3, both spleen and thymus contain a small subset of cDCs that expresses CD8α as well as langerin. Hence, the analysis of DC subsets was broadened to langerin$^+$ DCs in spleen and thymus. CD8α$^+$langerin$^+$ cDCs were easily detected in spleen and thymus of dhet and E47^{ko} mice, but absent in Id2^{ko} mice, as already seen for Id2$^{-/-}$ mice (Figure 3-34 and Figure 3-26). There was no difference seen in the langerin$^+$ population between dhet and E47^{ko} mice. Interestingly, in contrast to splenic DCs, the number of thymic DCs remained stable in all mice, regardless of their genotype. No differences were seen for CD8α$^+$ cDCs among dhet, E47^{ko}, Id2^{ko}, and dko mice, similar to the observation in section 3.3 (Figure 3-34 and Figure 3-26, C to E). However, langerin$^+$ cDCs were virtually absent in spleen and thymus of Id2^{ko} and dko mice, supporting the data in section 3.3 (Figure 3-34 and Figure 3-26).

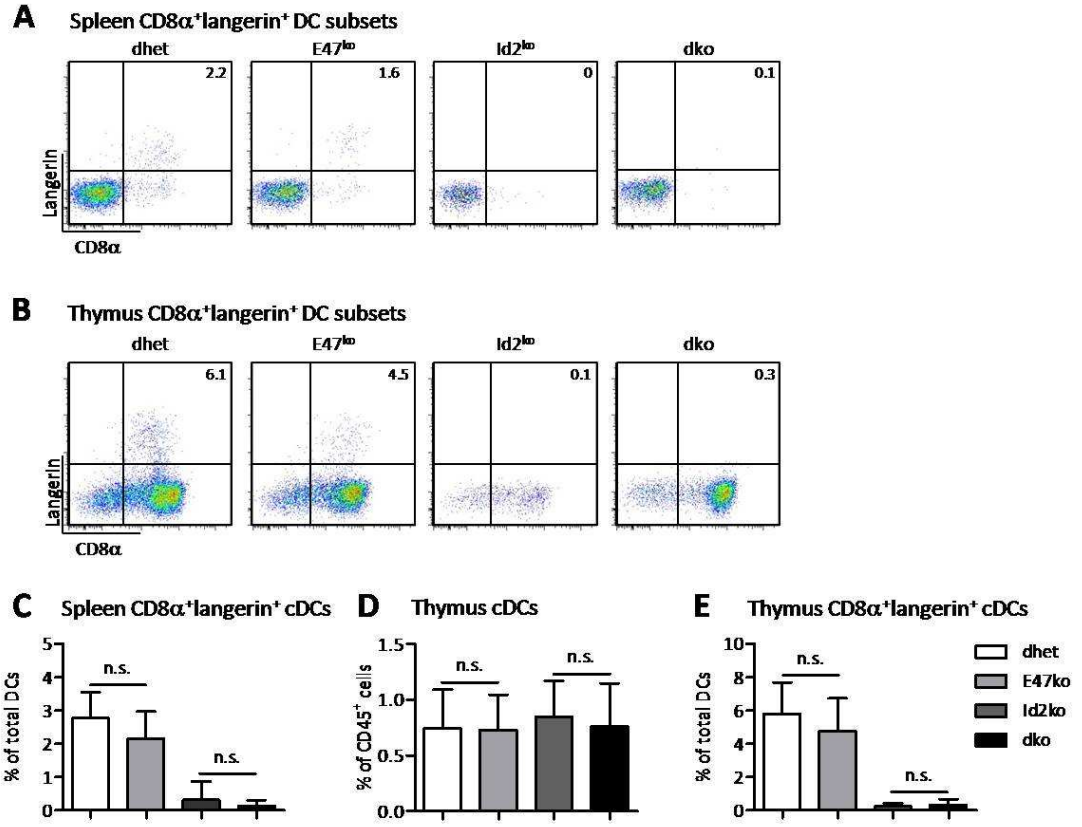


Figure 3-34: CD8α^{+}langerin^{+} cDCs cannot be found in spleen or thymus of dko mice.
DCs of dhet, E47^{ko}, Id2^{ko} , and dko mice were determined as CD45^{+} cells low in side scatter, double positive for MHC-II and CD11c. (A) Distribution of langerin versus CD8α^{+} in spleen DCs. (B) Distribution of langerin and CD8α on thymus DCs. (C) Bar diagram representing the percentage of CD8α^{+}langerin^{+} DCs in total spleen DCs. (D and E) Bar diagrams represent the fraction of total DCs in thymus (D) and of CD8α^{+}langerin^{+} DCs within total thymic DCs (E) of dhet (open), E47^{ko} (light grey), Id2^{ko} (grey), and dko (black) mice. Data shown are representative of at least n = 2 independent experiments ± SD; n.s. not significant.

3.4.4 Only E47$^{-/-}$Id2$^{-/-}$ double deficiency leads to increased pDCs

Section 3.3 demonstrated that the deficiency of Id2 resulted in an increased frequency of pDCs in spleen, whereas the B cell population remained unchanged (Figure 3-28). Moreover, specific functions of the E2A proteins have been most extensively studied in the context of B lineage development, which is completely blocked at a stage preceding the onset of immunoglobulin gene rearrangement in E2A-deficient mice and therefore are a good example for the crosstalk of HLH transcription factors/ regulators during development [204, 205].

The pDCs and B cells in spleen were analysed by flow cytometry as defined in section 3.3. Comparison of single cell suspensions from spleen of dhet, E47^{ko}, Id2^{ko}, and dko mice demonstrated that pDCs were not changed in dhet, E47^{ko}, and Id2^{ko} mice, only in dko

mice an increase in pDCs could be observed (Figure 3-35, A and C). This differs from the data obtained in section 3.3, where an increase in pDCs was already observed in $Id2^{-/-}$ mice. This discrepancy might arise from the different background of these mice. However, taken into account that breeding resulted only in very few mice and statistical analysis was not possible in this case, it might be that these data do not represent *in vivo* conditions.

Conversely, the B cell population was completely absent in mice that were deficient in E47, which is consistent with current literature. In dhet and $Id2^{ko}$ mice B cells were not affected, supporting the data of $Id2^{+/+}$ and $Id2^{-/-}$ mice in section 3.3, indicating that the role of Id2 is negligible for B cell development (Figure 3-35, B and C; Figure 3-28, C and D).

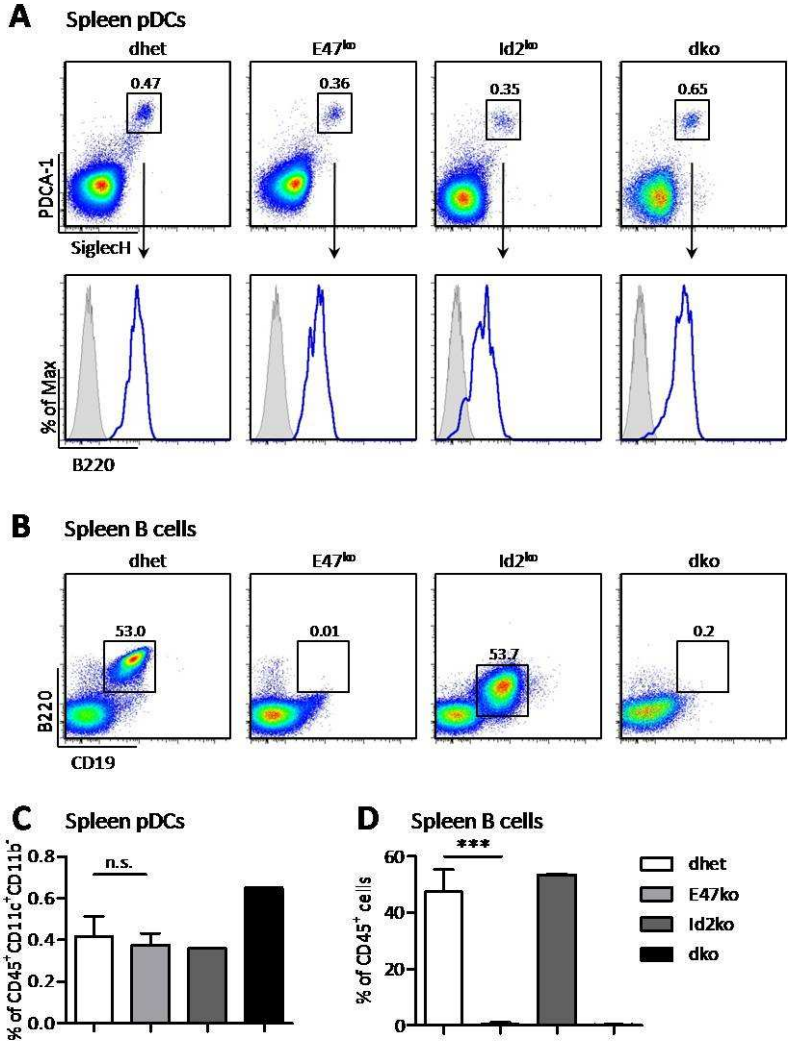


Figure 3-35: Increased pDCs in dko mice.

Low side scatter $CD45^+$ cells in spleens of dhet, $E47^{ko}$, $Id2^{ko}$, and dko mice were analysed for pDCs (A) and B cells (B). pDCs were gated on $CD45^+CD11c^+CD11b^-$ and the stainings for PDCA-1 versus SiglecH and for B220 are shown. The percentage of pDCs in $CD45^+CD11b^-$ cells (C) and the percentage of B cells (D) among low side scatter $CD45^+$ cells of dhet (open), $E47^{ko}$ (light grey), $Id2^{ko}$ (grey), and dko (black) mice are depicted as bar diagrams. Data shown are representative of at least n = 1 independent experiments ± SD.

3.4.5 Id2 remains essential for CD8$^+$ T cell maintenance in spleen and thymus

As shown in section 3.3, Id2$^{-/-}$ mice displayed a selective and remarkable reduction of the CD8$^+$ T cell subset. To investigate whether the antagonism of E47 and Id2 influences T cell development, spleen and thymus of dhet, E47^{ko}, Id2^{ko}, and dko mice were analysed by flow cytometry (Figure 3-36 and Figure 3-37). T cells were defined as described in section 3.3 and the analysis of splenocytes revealed an increase of CD3ε$^+$ T cells in E47^{ko} and dko mice. Again, this apparent increase in T cells results from the lack of B cells, caused by the E47 deficiency (Figure 3-36, A and B). Further analysis of T cell subsets showed the distinct shift in the CD4/CD8 ratio in Id2^{ko} and dko mice, already seen in Id2$^{-/-}$ mice in section 3.3 (Figure 3-36). The increase in the CD4$^+$ T cell population and simultaneous decrease in CD8$^+$ T cells was only seen in those mice lacking Id2 and remained unchanged in dhet and E47^{ko} mice. However, in dko mice a partial reconstitution of the CD8$^+$ T cell population could be observed, indicating a responsibility of Id2 for T cell maintenance (Figure 3-36, A and D).

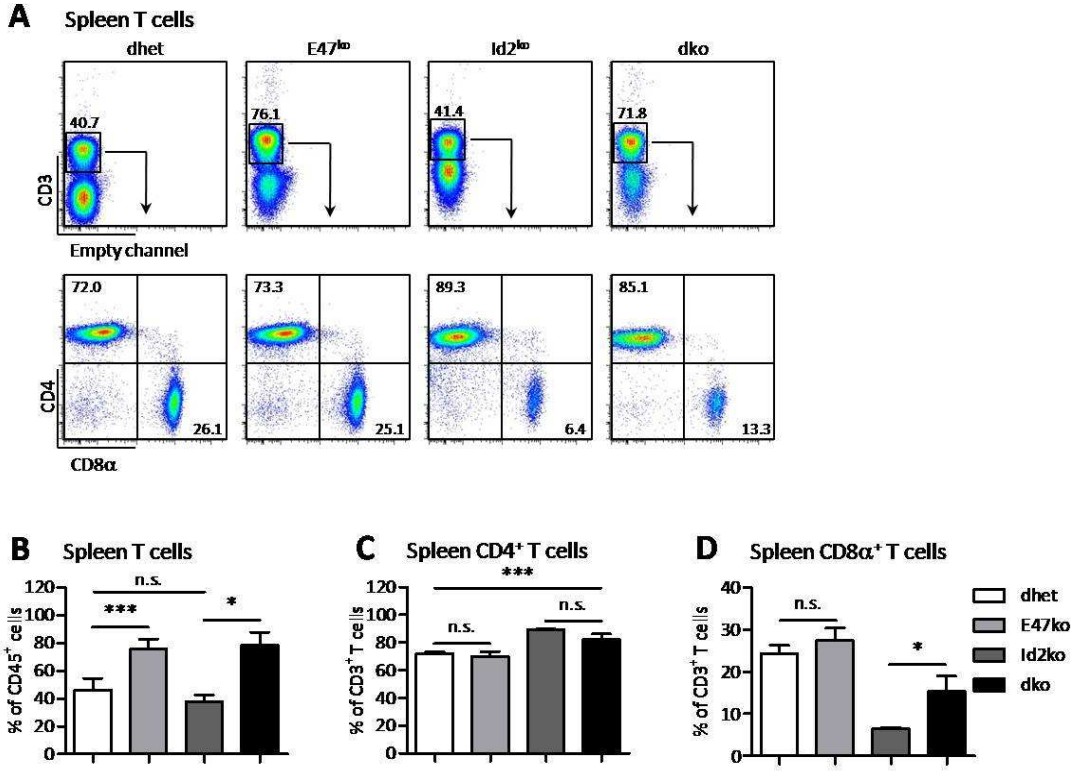


Figure 3-36: Id2 remains essential for CD8$^+$ T cell maintenance in spleen of dko mice.
(A) Splenocytes of dhet, E47^{ko}, Id2^{ko}, and dko mice were analysed for their T cell compartment. T cells were determined as low side scatter CD45$^+$ cells positive for CD3ε and the distribution of CD4 versus CD8 is shown (A, lower panel). The bar diagrams show the percentage of total T cells among splenocytes (B), the distribution of CD4$^+$ T cells among total T cells (C), and the distribution of CD8$^+$ T cells among total T cells (D) in dhet (open), E47^{ko} (light grey), Id2^{ko} (grey), and dko (black) mice. Data shown are representative of at least n = 2 independent experiments ± SD; n.s. not significant; * $p < 0.05$; *** $p < 0.001$.

Analysis of thymii of dhet, $E47^{ko}$, $Id2^{ko}$, and dko mice revealed similar data as seen in section 3.3. The number of $CD3\varepsilon^{+}$ T cells was comparable in dhet, $E47^{ko}$, $Id2^{ko}$, and dko mice (Figure 3-37, A and B). Investigations of T cell subsets showed a mild increase in $CD4^{+}$ T cells in $Id2^{ko}$, and dko mice compared to dhet mice (Figure 3-37, A and C). Furthermore, the analysis demonstrated a reduction of $CD8^{+}$ T cells in those mice that were deficient in Id2, but unchanged frequency in dhet and $E47^{ko}$ mice. These data are comparable to the results obtained from thymii of $Id2^{-/-}$ and $Id2^{+/+}$ mice and can be ascribed to the Id2 deficiency, indicating an essential role for Id2 in $CD8^{+}$ T cell maintenance (Figure 3-37, A and D). The population of $CD4^{+}CD8^{+}$ T cells remained unchanged in dhet, $E47^{ko}$, $Id2^{ko}$, and dko mice (Figure 3-37, A and E).

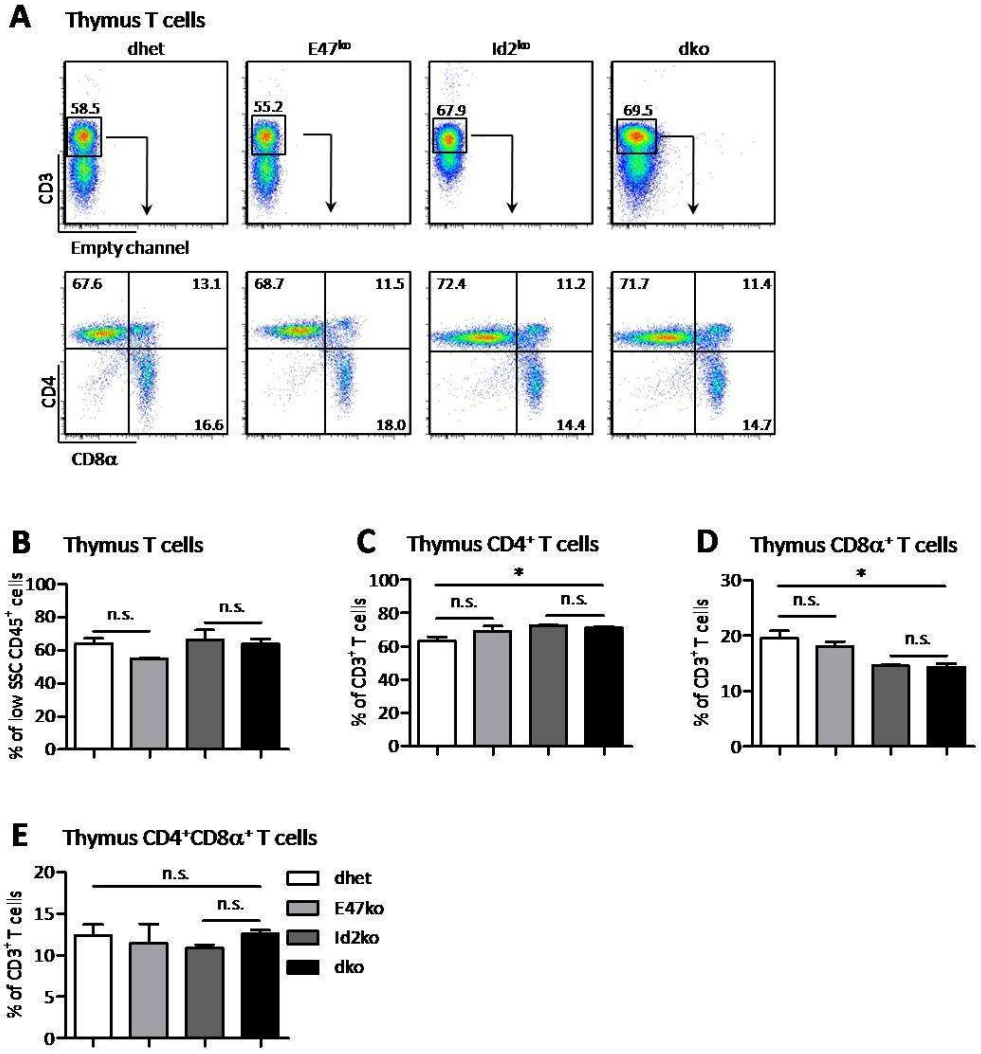


Figure 3-37: E47 deficiency in Id2$^{-/-}$ mice does not rescue the Id2$^{-/-}$ phenotype in thymic T cells

(A to E) Thymocytes of dhet, E47^{ko}, Id2^{ko}, and dko mice were analysed for their T cell compartment. T cells were determined as CD45^{+} cells low in side scatter and positive for CD3ε and the distribution of CD4 versus CD8 is shown (A). The bar diagrams show the percentage of total T cells among thymic CD45^{+} cells (B), the distribution of CD4^{+} T cells among total T cells (C), the distribution of CD8^{+} T cells among total T cells (D), and the distribution of CD4^{+}CD8^{+} T cells among total T cells (E) in dhet (open), E47^{ko} (light grey), Id2^{ko} (grey), and dko (black) mice. Data shown are representative of at least n = 2 independent experiments ± SD; n.s. not significant.

4 Discussion

By now it is evident that DCs are not a homogeneous population, but a group of discrete subpopulations. They share several features, but also differ in their location, their functional properties, and their origin. Because of this diversity and their rare frequency in steady state detailed examination of DC development remained difficult. Although a lot of effort has been made over the last years, the final steps in DC development and the underlying mechanisms are still elusive. Nevertheless, several studies have recently reported on the existence of different DC precursors, either from bone marrow or locally developing in lymphoid tissue [209-211]. To what extent these precursors overlap or emerge from each other requires further examination.

4.1 Characterization of an in vitro amplified Flt3$^+$precursor

The previously described Flt3$^+$ progenitor is also a precursor for DCs, although placed earlier in haematopoiesis than the above mentioned progenitors, as it is not restricted to the DC lineage [160, 168]. This progenitor was already determined to give rise to CD8α^+ and CD8α^- DCs *in vivo* as well as to CD11c$^+$CD11b$^+$ DCs *in vitro*, demonstrating its potential to develop into different types of DCs. Because of the results by Nagai *et al.*, indicating that undifferentiated progenitor cells can distinguish ‘‘non-self’’ from ‘‘self’’ via TLRs and that microbial components could be cues for haematopoietic cell development [176], the Flt3$^+$ progenitor was examined for its TLR repertoire *in vitro*. All TLRs, except TLR11, were found to be expressed in Flt3$^+$ precursors on mRNA levels. Furthermore, flow cytometry analysis showed that TLRs 1 to 7 and TLR9 are expressed on protein levels as well. This expression was further upregulated when the Flt3$^+$ precursors were differentiated into CD11c$^+$CD11b$^+$ DCs *in vitro*.

TLR signalling normally results in the secretion of cytokines and chemokines to initiate an immune response. Within the context of HSCs and progenitor cells, such signals may help to replace and/or increase cells that constitute a first line of defence against pathogens.
Interestingly, stimulation of Flt3$^+$ precursor cells with various TLR ligands did not result in the secretion of cytokines. Conversely, DCs differentiated from the Flt3$^+$ progenitor by GM-CSF did respond effectively to all TLR ligands used in the experiments by secreting IL-

6, IL-10, IL-12p70, MCP-1, MIG, and TNFα. The lack of cytokine secretion from the $Flt3^+$ precursor cells might be due to non-functional TLRs or to non-functional signalling cascades. Although TLR stimulation does not result in an immune response in the $Flt3^+$ precursor, the possibility that TLR signalling might have an influence on lineage decision cannot be excluded. Another possibility is that TLR signalling in the $Flt3^+$ precursor might be inhibited by dexamethasone, which is present during the amplification phase of the two-step culture. Dexamethasone is described to inhibit NFκB activity and to reduce the functionality of IRF3, which are both essential components in the TLR signalling cascades [212].

This project was continued within the diploma thesis of Julia Pabich. For further analysis of TLR signalling in the $Flt3^+$ precursor *in vitro* and *in vivo* and the influence of dexamethasone, please refer to her diploma thesis.

Furthermore, these data demonstrate that DCs differentiated with GM-CSF are not limited in their response to TLR stimulation and that they produce Th1 as well as Th2 cytokines. However, this is most probably not representing a steady state situation *in vivo* as GM-CSF levels are almost undetectable during steady state and GM-CSF deficient mice display normal levels of DCs [89, 90]. However, GM-CSF levels increase *in vivo* during inflammation, resulting in the appearance of an additional DC type, so called inflammatory DCs [92, 213]. These inflammatory DCs are also called Tip-DCs, as they were shown to produce TNFα and iNOS as well as IL-10, which is in line with the data presented here [214]. Conversely, DCs that were differentiated with Flt3L do not secrete IL-10 or TNFα, but IFNγ as well as IL-12p70 [215]. Taken together, these findings assign GM-CSF derived DCs, like the two step DCs, to the inflammatory or Tip-DCs, rather than some kind of steady state DC.

Regardless of the influence of TLR signalling, the cell transfer experiments in chapter 3.1.4 demonstrated that the $Flt3^+$ progenitor is not only a precursor for all subtypes of spleen DCs, but develops into all haematopoietic lineages *in vivo*. Although the $Flt3^+$ progenitor does not contain bone marrow reconstituting potential, it does develop into both myeloid and lymphoid cells. Therefore the $Flt3^+$ progenitor is not only a DC precursor, but rather a multipotent progenitor for all haematopoietic cell types.

4.2 Development of two distinct types of Langerhans cells in steady state and inflammation

Mende *et al.* [192] showed that the Flt3$^+$ fraction of both CMP and CLP contains LC precursor activity. However, these studies were done under inflammatory conditions and did not address the question whether skin-resident LC precursors originate from Flt3$^+$ precursors under steady state conditions. Recent studies reported on a clonal Flt3$^+$M-CSFR$^+$c-kit$^+$ common DC precursor (CDP) for cDCs and pDCs *in vivo* [210, 211], but they did not address the CDP potential to differentiate into LCs. Therefore, the Flt3$^+$ progenitor used in chapter 3.1 was used for further transfer experiments in Id2$^{-/-}$ mice to analyse LC development from bone marrow progenitors. The Flt3$^+$ progenitor is defined as Flt3$^+$M-CSFR$^+$c-kit$^+$ CD11c$^-$MHC-II$^-$, but CD11b$^+$ [160, 168] and thus is removed by the frequently used lineage depletion protocols, including the protocols by Onai *et al*. and Naik *et al.*, that contain anti-CD11b antibody during stem/progenitor cell purification [210, 211]. The results reported here now demonstrate that the Flt3$^+$ progenitor repopulates LCs without overt inflammation, indicating that under steady state conditions LCs can indeed develop, like other DC subtypes, from Flt3$^+$ haematopoietic precursors. However, the LCs that developed after Flt3$^+$ progenitor transfer show some differences compared to steady state or bone marrow derived LCs. They appear bigger in size, display more dendrites, and express less langerin. Thus, they resemble more the mo-LCs rather than classical LCs. It remains to be further analysed if this is due to the low amount of GM-CSF present in the amplification medium, which could direct the Flt3$^+$ progenitor already to the monocyte lineage or due to preparation of the Flt3$^+$ progenitor.

A growing body of data indicates that LCs are unique in their development compared to other DC subtypes, but the mechanisms that regulate LC development and homeostasis have not been fully elucidated. Several studies demonstrate that LCs can self-renew in the epidermis in steady state since about 2-3% of LCs are in cell cycle [26]. A further concept suggests a local pool of proliferating haematopoietic precursor cells [83] that populate the skin during embryonic development [186, 216]. Moreover, the data presented in this study now ascribe Id2 a key function in LC homeostasis and development from such haematopoietic precursor cells as Id2$^{-/-}$ mice lack LCs. Additionally, this study demonstrates that these LC precursors are still present in bone marrow of adult Id2$^{+/+}$

mice and reconstitute epidermal LCs as well as langerin^{+} dDCs in transplantation experiments. This suggests that Id2$^{-/-}$ mice not only lack LCs and langerin^{+} dDCs, but also show a defect in their respective precursors in bone marrow. Yet, the identity of these bone marrow precursors and the question if they are present in Id2 deficient mice at all or just have impaired differentiation potential needs to be further analyzed.

The absence of LCs in Id2$^{-/-}$ mice has allowed the comparative analysis of LC development from bone marrow and Gr-1^{hi} monocytic LC precursors. It could be demonstrated that LC development from bone marrow cells in steady state requires Id2, while their development from Gr-1^{hi} monocytes is independent of Id2, but requires an inflammatory stimulus, such as UV irradiation. This suggests two independent pathways for LC development under steady state and inflammatory conditions, respectively (Figure 4-1).

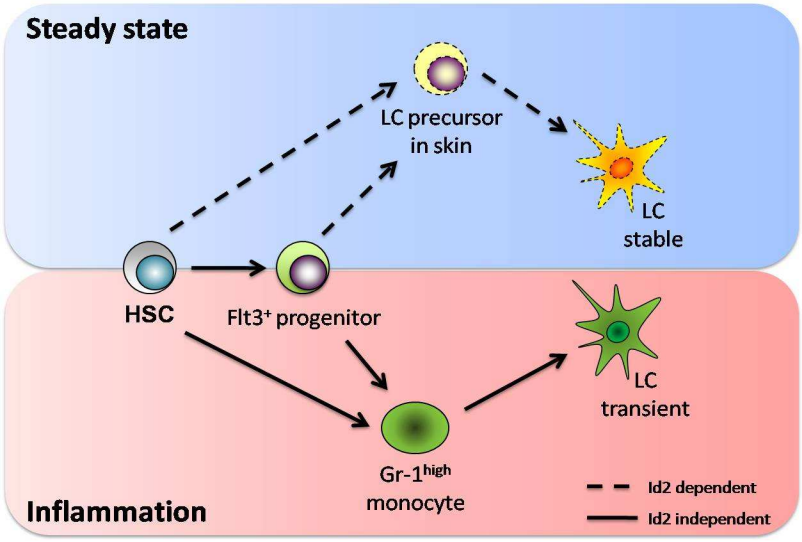


Figure 4-1: Model for LC development in steady state and under inflammatory conditions.
In steady state LCs develop from either HSC or a local precursor in skin strictly Id2-dependent (dashed line). These LCs stably populate the epidermis and self-renew throughout life. During inflammation LCs can develop from Gr-1^{hi} monocytes and this is independent of Id2 (solid line). However, Gr-1^{hi} monocytes give rise to only transient LCs. HSC, haematopoietic stem cell; MPP, multi-potent progenitor.

Furthermore, these results are in agreement with previous studies on a Gr-1^{hi} monocytic LC precursor that repopulates LCs in the epidermis under inflammatory condition [63]. This Gr-1^{hi} monocytic LC precursor is present in Id2$^{-/-}$ mice, yet does not repopulate classical LCs under steady state conditions. Importantly, this study demonstrates that Id2$^{-/-}$ Gr-1^{hi} monocytic precursors are recruited into epidermis and develop into mo-LCs following UV irradiation, indicating a strict requirement of inflammatory stimuli for this LC precursor. However, mo-LCs appear to be different from classical LCs observed under

steady state conditions. They exhibit more dendrites in epidermal sheets and lower langerin expression than classical LCs. Moreover, mo-LCs are not capable of longterm repopulation of the epidermis. All this might refer to a remnant of their monocytic origin.

What would be the advantage of having two separate LC differentiation pathways? One advantage would clearly be to allow increased numbers of LCs to be deployed quickly to communicate potential dangers and to initiate an immune response during inflammation. Another one would guarantee a quick replacement for activated and emigrated LCs to reduce further vulnerability during an infection. This is supported by the fact that DCs can be generated from monocytes *in vitro* in the presence of GM-CSF, which establish the concept that DC homeostasis is critically dependent on conditions of quiescence or inflammation [53]. The factors that govern this difference are largely unknown. However, results from this study have established that LCs can indeed develop by different pathways and that Id2 is one of the major factors for classical LC development, but not mo-LC development.

Recent studies found langerin expression also in a subset of dermal DCs, showing that langerin is not exclusively confined to epidermal LCs [189-191]. Remarkably, as demonstrated here, $Id2^{-/-}$ mice also lack these $langerin^{+}CD11c^{+}$ dDCs and upon UV-induced inflammation $Gr\text{-}1^{hi}$ monocytes failed to compensate for this deficiency. Thus, the question arises whether $langerin^{+}$ dDCs and LCs are developmentally interrelated and whether or not they share a common precursor in steady state or inflammation. It could be demonstrated that $Gr\text{-}1^{hi}$ monocytes do not develop into $langerin^{+}$ dDCs after UV irradiation in $Id2^{-/-}$ mice, but give rise exclusively to LCs. This argues against a common precursor for LCs and $langerin^{+}$ dDCs at least under inflammatory conditions. Furthermore, the data presented here demonstrate that the $Flt3^{+}$ progenitor does repopulate LCs, but not $langerin^{+}$ dDCs in transplantation experiments, also arguing against a common precursor in steady state. The experiments of Nagao *et al.* further support this conclusion showing that LCs and $langerin^{+}$ dDCs develop from different precursors by using langerin-Diphtheria toxin receptor bone marrow chimeras [217]. After the selective ablation of both LCs and $langerin^{+}$ dDCs by Diphtheria toxin the repopulated LCs remained of host origin, whereas $langerin^{+}$ dDCs developed from donor bone marrow cells.

Langerin$^+$ dDCs and LCs also differ in cell surface marker expression: (i) langerin$^+$ dDCs, but not LCs express CD103, and (ii) LCs express high levels of CD11b and are positive for EpCAM and F4/80, while langerin$^+$ dDCs are low or negative for these markers [26]. The langerin$^+$ DCs in this study obtained from bone marrow cultures with GM-CSF are CD103$^-$ and express E-cadherin, Gr-1, F4/80 and CD11b (Figure 3-21), and thus strikingly resemble LCs rather than langerin$^+$ dDCs. Intriguingly, development of langerin$^+$ DCs *in vitro* does not require Id2 and thus appears to follow a pathway similar to *in vivo* mo-LC differentiation from Gr-1^{hi} monocytes under inflammatory conditions (Figure 4-1).

Id2 is a member of the Id family of HLH transcription regulators that contain the highly conserved HLH domain but lack the basic domain required for DNA binding. The four Id proteins (Id1-Id4) can form heterodimers with activating bHLH transcription factors, such as E2A, and thus act as negative regulators of transcription [201, 202]. Several lines of evidence suggest that Id2 and Id3 have both distinct as well as overlapping activities in lymphocyte development [157, 218]. This includes DC differentiation, especially as both Id2 and Id3 are direct target genes of TGFβ1/SMAD-signalling [156, 219, 220]. Therefore, Id2 and Id3 might function in a cooperative manner in DC differentiation with both distinct and common target genes downstream of the TGFβ1 signalling pathway.
Id3 was expressed in DCs from Id2$^{-/-}$ bone marrow cultures containing GM-CSF, but much lower in GM-CSF cultures from Id2$^{+/+}$ mice. Thus, Id3 might substitute for Id2 under inflammatory conditions. This might account for the observed Id2-independent differentiation of Gr-1^{hi} monocytes into langerin$^+$ mo-LCs *in vivo* and the formation of langerin$^+$ and Birbeck granule$^+$ DCs *in vitro*.

Taken together, these data emphasize the high degree of plasticity observed in DC development and reveal distinct mechanisms for LC development under steady state and inflammatory conditions.

4.3 The influence of Id2 in lymphoid organ DC development

So far it was shown that $Id2^{-/-}$ mice have reduced numbers of DCs in spleen, and that the splenic $CD8\alpha^+$ cDCs were markedly reduced. Interestingly, thymic $CD8\alpha^+$ cDC development is not perturbed in $Id2^{-/-}$ mice, indicating a different and Id2-independent origin of these cells. Careful studies on thymic cDC development by parabiotic mice revealed two patterns of thymic cDC development. One is simultaneously to the cyclical entry of T-cell precursors into the thymus, and these precursors were found to have the potential to develop into $CD8\alpha^+$ cDCs if transferred into the thymus of irradiated recipients. The other is unrelated to T-cell developmental kinetics and indicates a more continuous input from the bloodstream [197]. Therefore, the data presented here suggest that splenic $CD8\alpha^+$ cDCs originate from bone marrow in an Id2-dependent mechanism, while thymic $CD8\alpha^+$ cDCs develop locally in thymus from an early T cell/ DC precursor, independent of Id2.

Furthermore, this study demonstrates that also $CD8\alpha^+$langerin$^+$ cDCs are absent in spleen as well as in thymus of Id2 deficient mice, whereas $CD8\alpha^-$ cDCs were apparently not affected. Hence, the common absence of $CD8\alpha^+$langerin$^+$ DCs in spleen and thymus points to a precursor for these cells that originates from bone marrow, and requires Id2 for either its development or its migration from bone marrow into lymphoid organs. However, it cannot be ruled out that also thymic T cell/DC precursor could give rise to $CD8\alpha^+$langerin$^+$ cDCs, and that this differentiation remains Id2-dependent.
In addition, this chapter shows that Id2 deficiency leads to an increase in splenic pDCs, whereas the B cell compartment remained unchanged, although it is assumed that these cell types share early events in their development [81]. Thus, Id2 has a selective impact on the development of distinct DC subsets rather than on the entire DC development.

The absence of Id2 also influences other lymphoid cell lineages, such as T cells. Analysis of spleen and thymus revealed a striking decrease in $CD8^+$ T cells and a subsequent higher proportion of $CD4^+$ T cells in $Id2^{-/-}$ mice.
An increase in the relative percentage of mature single positive thymocytes, such as the $CD4^+$ population described here, can arise through several mechanisms. For example,

either aberrant proliferation of the $CD4^+$ populations, increased frequency of apoptosis within the $CD8^+$ population, or increased maturation of the double positive cells towards the $CD4^+$ population could explain the phenotypical changes observed in the Id2 deficient mice.

Previous studies have led to the hypothesis that Id proteins function in lineage decision by inhibiting E protein activity and preventing T lymphopoiesis [205].

Furthermore, a recent study showed that Id2 is essential in regulating the survival of effector $CD8^+$ T cells and influences their differentiation to memory cells [221]. Cannarile and colleagues found that Id2 was upregulated in $CD8^+$ T cells and remained high in memory cells. $CD8^+$ T cells from $Id2^{-/-}$ mice could be activated normally by antigen, proliferated and differentiated to effector cells, but ultimately failed to accumulate in numbers and the decreased frequency of antigen-specific $CD8^+$ T cells from $Id2^{-/-}$ mice was maintained during the transition to the memory population. Although Id2 deficient $CD8^+$ T cells showed no defect in proliferation, they were much more susceptible to apoptosis, correlating with the loss of cell numbers. Thus, the $CD8^+$ T cell population of $Id2^{-/-}$ mice undergoes “premature” contraction due to increased cell death while proliferating normally, demonstrating that inhibition of E proteins by Id2 is important for sustained survival [221].

4.4 Id2 antagonizes the function of activating bHLH proteins

So far, it could be shown that Id2 is required for the development of several DC subtypes. Id2 is known to function by repressing E protein activity. Hence, the impact of the simultaneous deletion of Id2 and E47 was investigated. Loss of E47 reduced total E protein activity sufficiently to allow DC development in the absence of Id2, indicating that Id2 is the major antagonist of E proteins in these cells. Moreover, the data presented here provide genetic evidence that the interaction between an E and Id proteins *in vivo* is necessary for DC development. However, the role of Id proteins in supporting DC lineage specification requires further investigation.

In the absence of Id2, essentially no langerin$^+$ DCs are found in skin. Importantly, section 3.4 shows that lowering E protein activity in Id2$^{-/-}$ mice by deletion of E47 results in the restoration of langerin$^+$ DCs in epidermis and dermis.

CD45$^+$ cells, which are highly decreased in skin of Id2$^{-/-}$ mice are also present in epidermis and dermis of dko mice, although at reduced levels, leading to the suggestion that they may fail to emigrate from the periphery. Therefore, appropriate regulation of E protein activity may be critical for CD45$^+$ cells to exit the bone marrow and accumulate in peripheral tissues, such as the skin.

CD8α^+ cDCs in spleen as well as CD8α^+langerin$^+$ cDCs are also absent in Id2$^{-/-}$ mice. However, deletion of E47 in Id2$^{-/-}$ mice fails to restore CD8α^+ cDCs in spleen and CD8α^+langerin$^+$ cDCs in spleen or thymus, suggesting that suppression of alternative E proteins by Id2 is required for their development.

The increase in pDCs observed in Id2$^{-/-}$ mice in Figure 3-28 was not seen in Id2^{ko} mice, but in dko mice. The pDC increase in the latter is most probably not representing the *in vivo* state, but an accidental outlier, as recent studies showed that E2-2, but not E2A is important for pDC development [222].

Nevertheless, the normal frequency of pDCs in Id2^{ko} mice could indicate that the role of Id2 in pDC development may be strain dependent. Id2$^{-/-}$ mice used in section 3.3 are on the 129/SvJ background, whereas dko mice used in chapter 3.4 are on a mixed 129/SvJ FVB/NJ background. The interesting possibility that strain differences exist requires further investigation and may indicate differential regulation of Id or E proteins in different strains of mice, which has not yet been reported.

The shift of splenic and thymic T cells towards the CD4$^+$ population seen in section 3.3 was retained in dko mice, although the CD8$^+$ T cells were partially restored; at least in spleen. This is consistent with other studies showing that thymocyte development is not influenced by E2A alone, but in concert with HEB, another member of the bHLH family [201]. Collectively, these observations raise the possibility that both E and Id proteins play a role in controlling the effector functions of CD8 T cells. Additional studies will be required to determine the role of these proteins in CD8 T cells and how their expression is regulated.

In summary, these data indicate that the major function of Id2 in lymphoid differentiation is to control E protein activity and that the excess in E protein activity is the major cause of the lymphoid phenotypes in $Id2^{-/-}$ mice. These findings redefine the essential functions of Id2 in DC development and provide insight into the dynamic regulation of E and Id proteins during this process. However, future studies are necessary to address the requirement for modulation of E protein activity in DCs and whether E proteins play a positive role in any aspect of DC development or function.

Last but not least the important question remains whether langerin expression is Id2 dependent and which role do E proteins play in its regulation? So far, langerin expression is only described for DCs and this study demonstrates that $Id2^{-/-}$ mice lack exclusively the $langerin^+$ fraction of DCs in different tissues; except for the splenic $CD8\alpha^+$ cDCs where the defect is more comprehensive. The hypothesis that all these different $langerin^+$ DC types develop from a common precursor is quite unlikely, as several precursors have already been identified for the different DC subtypes. Nevertheless, it is supposable that these DC subtypes undergo certain steps during their development, where Id2 is essential to proceed in differentiation. The expression of langerin might be a good example for such a requirement. How Id2 exactly regulates langerin expression and whether E proteins have an important part in it, remains to be investigated. The data in this study suggest that Id2 acts indeed through E47 to induce langerin expression, at least in skin DCs. The absence of $langerin^+$ cDCs in dko mice also indicates that E47 might not be the only E protein that interacts with Id2 to regulate langerin expression. This is actually quite likely, as Id2 is reported to interact efficiently with all E proteins *in vitro* [208].

Two possible models could explain how the interaction of Id2 and E proteins controls langerin expression. The first one is based on the fact that E proteins belong to a group of activating HLH proteins, which are negatively regulated by Id2. In this case, an E protein, such as E47, would activate the expression of a langerin inhibitor and this activation would be prevented when Id2 is present. The second possibility assumes that E proteins can not only activate transcription, but also block it. This is a rather unusual situation, although there is evidence that exactly this happens during thymocyte development [223]. Yang *et al.* propose that E47 negatively regulates CD5 expression by interacting with the κE2 site in the CD5 regulatory promoter and that decreases in E47 in response to developmental signals are critical to the progressive increase in CD5 expression as

thymocytes mature [223]. Something similar could be true for the langerin promoter. In addition, this latter hypothesis would explain why langerin expression can occur in $Id2^{-/-}$ mice during inflammation when Id3 is substituting for Id2.

Taken together these data suggest an essential role of HLH proteins in the regulation of langerin expression. It would be exciting to understand the molecular regulation of langerin and would also provide enormous help in addressing its functional properties, which are still controversial.

5 References

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Appendix

List of tables

Table 1-1: The influence of cytokines in DC development. 11
Table 1-2: Toll like receptors 13
Table 1-3: Differential requirements for transcription factors in DC development 15
Table 2-1: TLR ligands 21
Table 2-2: Kits 22
Table 2-3: Buffer and solutions 22
Table 2-4: Antibodies for flow cytometry 23
Table 2-5: Antibodies for immunohistochemistry 23
Table 2-6: Mice used for experiments in this thesis 24
Table 2-7: Standard PCR protocol 29
Table 2-8: Primer used for RT-PCR 30
Table 3-1: RNA microarray data of TLRs and related pathway components in $Flt3^+$ precursors and DCs. 37
Table 3-2: Summary of the repopulation efficiency of the $Flt3^+$ precursor. 45

List of figures

Figure 1-1: Mouse DC populations, location, and turn-over in steady-state. 4
Figure 1-2: Scheme of mouse DC development from bone marrow HSCs and lineage-committed progenitors.... 9
Figure 2-1: Generation of Flt3^{+} progenitor cells and two-step DCs. 27
Figure 3-1: Surface marker expression on Flt3^{+} precursor cells. 35
Figure 3-2: Hierarchical cluster analysis of TLR pathway components. 36
Figure 3-3: Expression and upregulation of TLRs in Flt3^{+} precursors and differentiating DCs. 38
Figure 3-4: Protein expression of TLRs in Flt3^{+} precursors and DCs. 39
Figure 3-5: Cytokine production by Flt3^{+} precursor cells and DCs upon TLR stimulation. 41
Figure 3-6: Repopulation efficiency of the Flt3^{+} precursor in blood after adoptive transfer. 43
Figure 3-7: Repopulation efficiency of the Flt3^{+} precursor in different organs after adoptive transfer. 44
Figure 3-8: Id2$^{-/-}$ LC phenotype in epidermis. 47
Figure 3-9: Id2$^{-/-}$ dermal DC phenotype. 48
Figure 3-10: Repopulation of skin DCs in Id2$^{-/-}$ mice after adoptive transfer of bone marrow. 50
Figure 3-11: Repopulation of skin DCs in Id2$^{-/-}$ mice after adoptive transfer of Flt3^{+} progenitor cells. 52
Figure 3-12: Blood monocyte subsets in Id2$^{-/-}$ mice. 53
Figure 3-13: Latex beads in peripheral blood monocytes 24h after injection. 54
Figure 3-14: Influx of FITC-latex bead-labelled Gr-1^{hi} monocytes into inflamed skin of Id2$^{-/-}$ mice. 55
Figure 3-15: LC repopulation in epidermis in Id2$^{-/-}$ mice after UV irradiation. 56
Figure 3-16: Langerin^{+} dDC repopulation in dermis in Id2$^{-/-}$ mice after UV irradiation. 57
Figure 3-17: Longterm repopulation potential of LCs after UV irradiation. 58
Figure 3-18: Phenotype of LC in Id2$^{-/-}$ and Id2$^{+/+}$ mice after UV irradiation. 60
Figure 3-19: Birbeck granules in epidermal LCs of Id2$^{-/-}$ and Id2$^{+/+}$ mice after UV irradiation. 61
Figure 3-20: *In vitro* generation of LC-like cells in Flt3L and 2-step cultures from Id2$^{-/-}$ and Id2$^{+/+}$ mice. 62
Figure 3-21: *In vitro* generation of LC-like DCs from Id2$^{-/-}$ and Id2$^{+/+}$ mice. 63
Figure 3-22: Birbeck granules in *in vitro* generated LC-like DCs from Id2$^{+/+}$ and Id2$^{-/-}$ mice. 64
Figure 3-23: Gene expression in LC-like DCs from Id2$^{-/-}$ and Id2$^{+/+}$ mice. 64
Figure 3-24: Id2 deficiency results in lack of lymph nodes and enlarged spleen. 67
Figure 3-25: Id2 deficiency results in decreased DC numbers in spleen. 68
Figure 3-26: CD8α^{+}langerin^{+} cDC subsets in spleen and thymus of Id2$^{-/-}$ and Id2$^{+/+}$ mice. 69
Figure 3-27: Adoptive transfer of bone marrow or Flt3^{+} progenitor cells rescues Id2$^{-/-}$ phenotype 70
Figure 3-28: Increased pDCs in Id2$^{-/-}$ mice 71
Figure 3-29: Altered T cell ratios in Id2$^{-/-}$ mice. 73
Figure 3-30: The antagonism of E2A and Id2. 74
Figure 3-31: E47 deficiency overcomes the need for Id2 in LC development 76
Figure 3-32: The deficiency of E47 and Id2 rescues langerin^{+} dDCs 77
Figure 3-33: CD8α^{+} cDCs remain absent in dko mice. 79
Figure 3-34: CD8α^{+}langerin^{+} cDCs cannot be found in spleen or thymus of dko mice. 80
Figure 3-35: Increased pDCs in dko mice. 81
Figure 3-36: Id2 remains essential for CD8^{+} T cell maintenance in spleen of dko mice. 82
Figure 3-37: E47 deficiency in Id2$^{-/-}$ mice does not rescue the Id2$^{-/-}$ phenotype in thymic T cells 84
Figure 4-1: Model for LC development in steady state and under inflammatory conditions. 88

Genotyping of Id2 SV129 Mice

DNA was isolated from tailcuts by the use of the NucleoSpin Tissue kit (Macherey & Nagel) according to manufacturers' instruction.

Primer Mix B

Id2-2 for GCA TCC CCC AGA ACA AGA AG
Id2-2 rev GTA GCC CCC CTC CTC CTT TC
(at a ratio of 1:1)

PCR reaction

Reagent	Volume
10x PCR Puffer + $(NH_4)_2SO_4$	5µl
$MgCl_2$ (25mM)	3µl
dNTPs (10mM)	1µl
Primer Mix A	2µl
DMSO	2µl
DNA	3µl
Taq Polymerase (homemade)	1µl
MilliQ	33µl
	total 50µl

PCR parameter

Step	Temperature	Cycles
Initial activation	5min at 95°C	1
Denaturation	45sec at 95°C	40
Annealing	30sec at 60°C	
Extension	1min at 72°C	
Final extension	5min at 72°C	1

Product
WT Band: ~500bp

Primer Mix C

Id2S: TCT GAG CTT ATG TCG AAT GAT AGC
Id2AS: CGT GTT CTC CTG GTG AAA TGG CTG
Id2Neo: TCG TGC TTT ACG GTA TCG CCG CTC
(at a ratio of 4:2:1)

PCR reaction

Reagent	Volume
10x PCR Puffer + $(NH_4)_2SO_4$	5µl
$MgCl_2$ (25mM)	3µl
dNTPs (10mM)	1µl
Primer Mix C	3µl
DMSO	2µl
DNA	5µl
Taq Polymerase (homemade)	1µl
MilliQ	30µl
	total 50µl

PCR parameter

Step	Temperature	Cycles
Initial activation	5min at 95°C	1
Denaturation	45sec at 95°C	40
Annealing	30sec at 60°C	
Extension	1min at 72°C	
Final extension	5min at 72°C	1

Product
WT Band: ~900bp
KO Band: ~400bp

Genotyping of E47-Id2 Mice

DNA was isolated from tailcuts by the use of the NucleoSpin Tissue kit (Macherey & Nagel) according to manufacturers' instruction.

Genotyping for Id2 was similar as for Id2 SV129 mice.

Primer Mix E47

E47 bas for: CAG ATG AGG TGC TGT CCC
E47 bas rev: CAG GAT CAC CTG CAC CGC
(at a ratio of 1:1)

PCR reaction

Reagent	Volume
10x PCR Puffer + $(NH_4)_2SO_4$	5µl
$MgCl_2$ (25mM)	3µl
dNTPs (10mM)	1µl
Primer Mix E	2µl
DMSO	2µl
DNA	3µl
Taq Polymerase (homemade)	1µl
MilliQ	33µl
	total 50µl

PCR parameter

Step	Temperature	Cycles
Initial activation	5min at 95°C	1
Denaturation	1min at 95°C	38
Annealing	30sec at 61°C	
Extension	2min at 72°C	
Final extension	5min at 72°C	1

Product
WT Band: ~200bp
KO Band: ~1500bp

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