Elucidation of transcription factor controlled development of dendritic cells

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This work is dedicated especially to a special, rare and amazing woman,

my mom

Smt. Vasantha Rathinam

Zusammenfassung

Bei den dendritischen Zellen (DCs) handelt es sich um eine heterogene Population von antigenpräsentierenden Zellen, die zahlreiche Funktionen im Organismus übernehmen. Die genauen Mechanismen und Signalwege zur Entwicklung von dendritischen Zellen (DCs) sind jedoch bis heute weitestgehend unbekannt.

Durch Untersuchungen der Expression von Transkriptionsfaktoren unter Anwendung eines Micro-Arrays konnte im Rahmen meiner Promotionsarbeit Gfi1 als ein entscheidender Transkriptionsfaktor in der GM-CSF-abhängigen Differenzierung von dendritischen Zellen (DCs) identifiziert werden. Wie Experimente mit $Gfi1^{+/GFP}$-Knock-in-Mäusen, bei denen ein Gfi1-Allel durch die cDNA von GFP ersetzt ist, darlegten, wird Gfi1 sowohl in dendritischen Vorläuferzellen als auch in ausgereiften dendritischen Zellen (DCs) exprimiert. Im Vergleich dazu konnte in $Gfi1^{-/-}$-Knock-out-Mäusen eine globale Reduzierung von myeloiden und lymphoiden dendritischen Zellen (DCs) in allen lymphatischen Organen nachgewiesen werden, wohingegen die Anzahl an epidermalen Langerhans-Zellen erhöht war. Darüber hinaus zeigten die $Gfi1^{-/-}$-dendritischen Zellen (DCs) eine ausgeprägte Veränderung bezüglich des Phänotypes und der Funktion, was durch eine verringerte Expression von MHC-Molekülen der Klasse II, eine ausbleibende Hochregulation von Kostimulationsfaktoren als Reaktion auf Stimulation und eine reduzierte Fähigkeit zur Stimulierung von spezifischen T-Zell–Immunreaktionen veranschaulicht wurde. Im Gegensatz dazu wiesen die $Gfi1^{-/-}$-dendritischen Zellen (DCs) ein erhöhtes Aktivierungprofil auf, welches sich in einer erhöhten Sekretion von IL-12 widerspiegelte.

Desweiteren gelang es bei Untersuchungen zur Entwicklung von dendritischen Zellen (DCs) nicht, hämatopoetische $Gfi1^{-/-}$-Progenitorzellen in vitro durch Stimulierung mit den Zytokinen GM-CSF und Flt3L in dendritische Zellen (DCs) zu differenzieren. Dagegen konnte in diesen Experimenten eine Differenzierung zu Makrophagen beobachtet werden, welche durch morphologische Untersuchungen, Expressionsanalysen der Zelloberflächenmarker und funktionelle Analysen bestätigt werden konnte. Diese gewonnenen Erkenntnisse deuten darauf hin, daß der Transkriptionsfaktor Gfi1 eine entscheidende Rolle in der Modulation der Entwicklung von dendritischen Zellen (DCs) bzw. Makrophagen spielt.

Untersuchungen des hämatopoetischen Chimärismus in bestrahlten kongenen Rezipienten im Rahmen von murinen Transplantationsversuchen konnten einen autonomen Zelleffekt und eine unersetzliche Funktion von Gfi1 in der Entwicklung von dendritischen Zellen belegen. Ferner konnte durch Überexpression von Gfi1 in $Gfi1^{-/-}$-Progenitorzellen mittels Durchführung eines retroviralen Gentransfers der Defekt einer ausbleibenden Entwicklung von dendritischen Zellen sowohl in vitro als auch in vivo behoben werden.

Durch Proteinnachweise im Western blot und EMSA-Assay konnte nachgewiesen werden, daß die Unfähigkeit der hämatopoetischen $Gfi1^{-/-}$-Progenitorzellen zur Differenzierung in dendritische Zellen (DCs) mit einer Abnahme der STAT3-Aktivierung assoziiert ist.
Zusammenfassend läßt sich festhalten, daß im Rahmen meiner Promotionsarbeit Gfi1 als entscheidender Transkriptionsfaktor für die Modulation der Entwicklung von dendritischen Zellen (DCs) bzw. Makrophagen identifiziert wurde und Gfi1 eine Schlüsselrolle in der Ausreifung sowie Aktivierung von dendritischen Zellen einnimmt.

Schlagworte: dendritische Zellen, hämatopoetische Stammzellen, Transkriptionsfaktoren

Synopsis

Dendritic cells (DCs) comprise heterogeneous and functionally diverse populations of antigen presenting cells. Their developmental pathways remain largely unknown. Using a transcriptional profiling approach, the present study identifies Gfi1 as a novel critical transcription factor in GM-CSF-dependent DC differentiation. Gfi1 is expressed in precursor and mature DCs. Gfi1$^{-/-}$ mice show a global reduction of myeloid and lymphoid DCs in all lymphoid organs whereas epidermal Langerhans cells are enhanced in number. Gfi1$^{-/-}$ DCs showed marked phenotypic and functional alterations, as exemplified by decreased MHC class II expression, absent upregulation of costimulatory molecules upon stimulation and reduced ability to stimulate specific T-cell responses. In contrast, Gfi1$^{-/-}$ DCs exhibited an increased activation profile as assessed by enhanced secretion of IL12. In vitro, Gfi1$^{-/-}$ hematopoietic progenitor cells were unable to develop into DCs in the presence of GM-CSF or Flt3L. Instead, they differentiated into macrophages, suggesting that Gfi1 is a critical modulator of DC versus macrophage development. Analysis of hematopoietic chimeras and retrovirus-reconstituted hematopoietic progenitor cells established a cell autonomous and non-redundant role for Gfi1 in DC development. The developmental defect was associated with decreased STAT3 activation in hematopoietic progenitor cells. In conclusion, the present study for the first time reports Gfi1 as a critical transcription factor that controls DC versus macrophage development and dissociates DC maturation and –activation.

Key words: Hematopoiesis, Dendritic cells, Transcription factors

Abbreviations

APCs	Antigen presenting cells
APC	Allo phycocyanin
APS	Ammonium persulphate
BM	Bone marrow
C	Celcius
CD	Clusters of differentiation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CR1mix	Chozhavendan Rathinam1 mix
DCs	Dendritic cells
DTT	Dithiothreitol
EDTA	Ethylene diamine tetrasodium acetate
ELISA	Enzyme linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FDCP	Factor dependent cell paterson
FITC	Flourescein iso thiocyanate
Flt3L	Fms like tyrosine kinase 4 Ligand
FSc	Forward scatter
Gfi1	Growth factor independent 1
GFP	Green fluorescence protein
GM-CSF	Granulocyte macrophage colony stimulating factors
GMFI	Geo mean fluorescence intensity
GMFIi	Geo mean fluorescence intensity index
Hr	Hour
HSC	Hematopoietic stem cells
IL	Interleukin
IRES	Internal ribosome entry site
ICN	Intra cell domain region of Notch 1
KDa	kilo daltons
LCs	Langerhans cells
Lin	Lineage

LPS	Lipo polysaccharide
MHC	Major histo-compatibility complex
Min	Minute
MOI	Multiplicity of infection
MLN	Mesenteric lymph node
NaCl	Sodium chloride
NH_4Cl	Ammonium Chloride
OT	Ova specific T cells
PB	Peripheral Blood
PBS	Phosphate buffered Saline
PE	Phycoerythrin
Per CP	Peridinium chlorophyll protein
PLN	Peripheral lymphnode
PIAS	Protein inhibitor of activated STAT
RBC	Red blood cells
Sca	Stem cell antigen
SCF	Stem cell factor
SDS	Sodium do-decylsulphate
SP	Spleen
STAT	Signal transducers of activated transcripts
TEMED	N,N,N’N’-Tetramethylenediamine
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Tris	Tris-(hydroxymethyl)-aminomethane
VSVG	Vesicular Stomatitis Virus Glycoprotein

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1.Introduction

1.1. The Immune system

The immune system is complex, intricate and interesting. It is composed of many interdependent cell types and specialized organs that collectively protect the body from bacterial, parasititic, fungal, viral infections and from the growth of tumor cells. Most of the cells of the immune system are derived from hematopoietic stem cells residing in the bone marrow. Bone marrow-derived stem cells differentiate into either mature cells of the immune system or into precursors of cells that migrate out of the bone marrow to continue their maturation in other anatomic compartments. Cells of the immune system include T cells, B cells, NK cells, mast cells, granulocytes, macrophages and dendritic cells. Many of these cell types have specialized functions.

The organs of the immune system (Fig 1.1) have been categorized into 2 major subtypes;

Primary organs include bone marrow and the thymus gland.

Secondary organs include adenoids, tonsils, spleen, lymph nodes, Peyer's patches, and the appendix.

Immunity is mediated by humoral and cellular effectors.

The immune responses elicited by this complex network have been classified into innate immunity and adaptive immunity.

Innate immunity refers to antigen-nonspecific defense mechanisms that a host uses immediately or within several hours after exposure to an antigen.

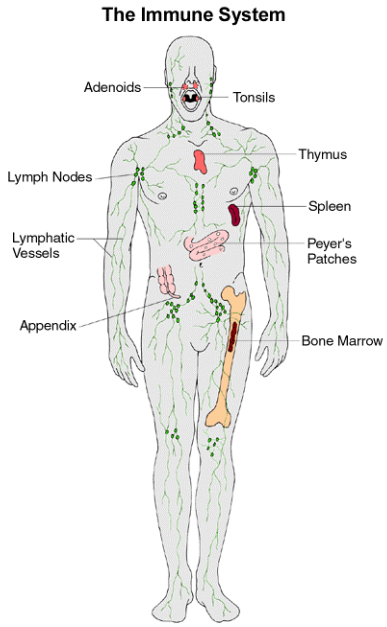

Figure 1.1. Primary and secondary lymphoid organs of the immune system (Source: Steinman lab, Rockefeller university)

Adaptive (acquired) immunity refers to antigen-specific defense mechanisms that take several days to become protective and are designed to react with and remove a specific antigen.

1.2 Hematopoietic stem cells

Hematopoietic stem cells (HSCs) sustain blood production throughout life (Orkin SH, 2004). They are capable of self-renewal to maintain the HSC pool and have the ability for multilineage differentiation (Weissman IL, 2000). Multilineage hematopoiesis is maintained by a pool of hematopoietic stem cells (HSCs). HSCs comprise phenotypically and functionally defined long-term HSCs (LT-HSCs), short-term HSCs (ST-HSCs) and multipotent progenitors (MPPs)(Adolfsson et al, 2001; Weissman IL, 2001). This pool of cells can give rise to a series of intermediate lineage committed progenitors such as common lymphoid progenitors (CLPs) and common myeloid progenitor (CMPs) (Kondo et al, 1997; Akashi et al, 2000; Adolfsson et al, 2001; Christensen and Weissman, 2001).

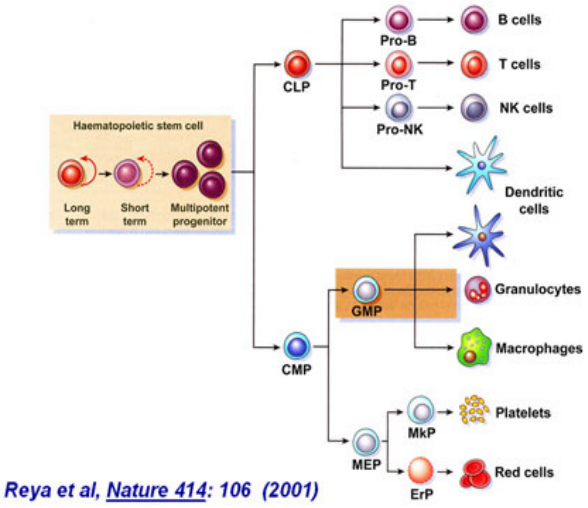

Figure 1.2. The paradigm of hematopoiesis

CLPs give rise to all the different cell types of lymphoid origin including T, B, and NK cells (Kondo, 1997) plus dendritic cells (Reya et al, 2001). CMPs are capable of generating large numbers of all the different types of myeloid cells. Especially, they either give rise to megakaryocytes/erythrocytes or granuocyte/macrophage progenitors (Akashi, 2000).

Further steps give rise to progenitors committed to the production of just one cell type (Fig. 1.2). The steps of commitment can be correlated with changes in the expression of specific gene regulatory proteins, needed for the production of different subsets of blood cells.

1.3 Dendritic cells

DCs represent a key cell type of white blood cells that initiate and control both innate and adaptive immunity (Steinman RM, 1998). As sentinels, dendritic cells patrol the body seeking out foreign invaders, whether these invaders are bacteria, viruses, or dangerous toxins (Steinman RM, 1998) and hence they are referred as "conductors, pacemakers, or gatekeepers of the immune system". DCs were first seen as Langerhans cells (LCs) in the skin in 1868. In 1973 Steinman and Cohn recognized them as major cells of the immune system. Dendritic cells originate in the bone marrow and function as antigen presenting cells (APC). These cells are usually found in the structural compartment of the lymphoid organs such as the thymus, lymph nodes, spleen and the bone marrow. However, they are also found in the bloodstream, skin and other tissues of the body.

Proliferating DC progenitors in the bone marrow give rise to precursor DCs that circulate in the blood. The precursors DCs in the blood reach the non-lymphoid tissue, where they develop into immature DCs (Cella M, 1997; Banchereau et al, 2000) through a process called 'migration'. This migration process is tightly controlled by chemokines produced upon local inflammation.

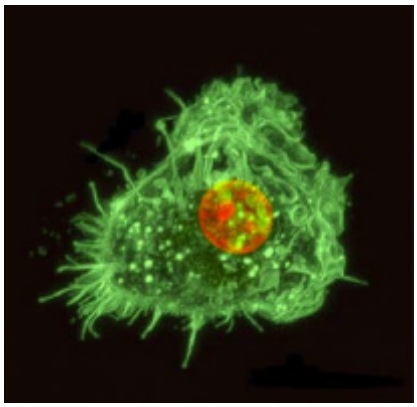

Figure 1.3. Confocal microscope image of a human DC (Source: Karla Daniel, uiowa)

Different DC subsets display unique sensitivity to certain chemokines (Banchereau J, 2000). Immature dendritic cells express a wide range of chemokine receptors such as CCR1, CCR2, CCR4, CCR5, CCR6, CXCR1, and CXCR4 in contrast to the mature dendritic cells that express only CCR7. The chemokines to which DCs respond include MIP-1α, MIP1-β, RANTES, MCP-3, MIP-5, MCPs, TARC, MDC, MIP-3α, MIP-3β, IL-8, SDF-1 and SLC.

During their migration, DCs are involved in several adhesion events. For instance, E-cadherin, uniquely expressed by LCs, permits through homotypic interactions, the residence of LCs in epidermis (Bell D, 1999; Jakob T, 1998). Ag encounter results in down regulation of E-Cadherin that allows LC migration out of the skin (Tang A, 1993). The release of collagenase of type IV by LCs may facilitate their migration though the basement membranes (Kobayashi Y, 1997). Likewise, human macrophage elastase is highly expressed by DCs and may thus contribute to their migration (Bancheraeu J, 2000).

Immature DCs are very efficient in Ag capture and can use several pathways, such as macropinocytosis, receptor mediated endocytosis via C-type lectin receptors (mannose receptor, DEC205)(Engering AJ, 1997; jiang W, 1995; Sallusto F, 1995) or Fcγ receptors types I and type II (Fanger, N.A., 1996), phagocytosis of particles such as latex beads (Matsuno, K., 1996); apoptotic and necrotic cell fragments (Albert, M.L., 1998); viruses and bacteria (Inaba, K., 1993) as well as intracellular parasites such as *Leishmania major* (Moll, H. 1993). DCs can also internalize the peptide loaded heatshock proteins gp96 and Hsp 70 through presently unknown mechanisms (Arnold-Schild, D., 1999).

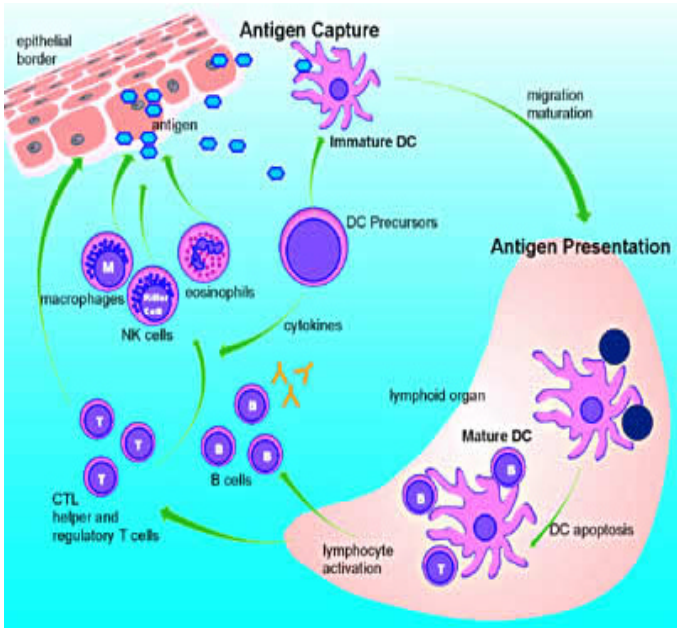

Figure 1.4. The life cycle of dendritic cells. Circulating Precursor DCs enter tissues as immature DCs. They can also directly encounter pathogens (e.g. viruses) that induce secretion of cytokines (e.g. IFNα), which in turn can activate eosinophils, macrophages (MF), and natural killer (NK) cells. After antigen capture, immature DCs migrate to lymphoid organs to allow selection of rare circulating antigen-specific lymphocytes. These activated T cells help DCs in terminal maturation, which allows lymphocyte expansion and differentiation. Helper T cells secrete cytokines, which permit activation of macrophages, NK cells, and eosinophils. B cells become activated after contact with T cells and DCs. It is believed that, after interaction with lymphocytes, DCs die by apoptosis.
(Source : SRI biosciences division; Bancheraeu J, 2000)

The antigen/pathogen induces the immature DC to undergo phenotypic and functional changes that culminate in the complete transition from Ag-capturing cell to APC. DC maturation is intimately linked with their migration from peripheral tissue to the draining lymphoid organs. Several molecules including CD40, TNF-R, and IL-1R have been shown to activate DCs and to trigger their transition from immature to mature DCs. DC maturation is a continuous process initiated in the periphery upon Ag encounter and/or inflammatory cytokines and completed during the DC-T cell interaction. Numerous factors induce or regulate DC maturation including pathogen related molecules (LPS, bacterial DNA, dsRNA), the balance between proinflammatory and anti-inflammatory signals in the local micro environment (TNF, IL-1, IL-6, IL-10, TGF-β and prosaglandins) and T cell derived signals (Rescigno M, 1999; Akbari O, N., 1999; Hartmann G, 1999; Cella M, 1999).

The maturation process is associated with several coordinated events such as loss of endocytic/phagocytic receptors, up regulation of co stimulatory molecules CD40, CD58, CD80 and CD86, change in morphology, shift in lysosomal compartments with down regulation of CD68 and upregulation of DC-lysosome associated proteins and most importantly, a change in class II MHC compartments.

Morphological changes accompanying DC maturation include a loss of adhesive structures, cytoskeleton reorganization, and acquisition of high cellular motility (Winzler C, 1997).

Even in the absence of invading pathogens, a fraction of the DCs seem to move around. The DCs that migrate in the steady state may replenish immature populations or may be on patrol to identify invaders. Not every pathogen or antigen induces a strong T cell response, but those that do can induce the mobilization and maturation of DCs (Steinman, 1998).

1.4 Functions of dendritic cells

Dendritic œlls are efficient stimulators and modulators of T (Steinman RM, 1998), NK and NK T cells (Ardavin C, 2003). In addition to stimulating responses against antigens, dendritic cells also produce tolerance to self antigens (Steinman RM, 2003).

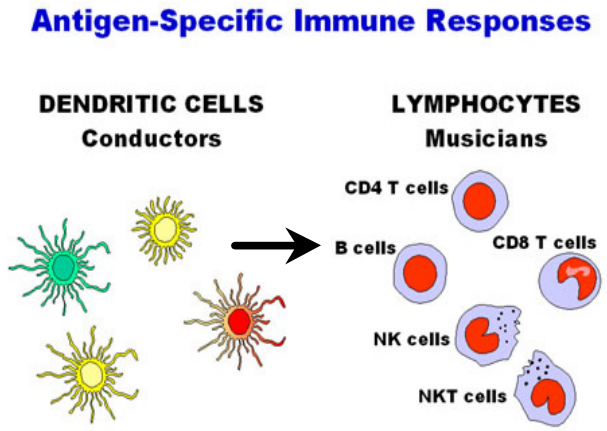

Figure 1.5. Antigen specific immune responses of lymphocytes are primarily initiated and governed by DCs. (Source: Steinman lab, Rockefeller university)

DCs use a variety of membrane receptors such as DEC205, MMR, FcR, Langerin, BDCA-2, DC-SIGN, ASGP-R to capture protein antigens (Steinman R 2003), digest the antigens, and express these antigens on the surface membranes that are bound to MHC antigens to attract T cells (Steinman RM, 1998).

Formation of this MHC-peptide complex is critical to the activation of T cells. The DCs, with their antigen tags, travel to the lymph nodes, which are rich in T cells and present the antigen to the T cell, hence the term *antigen-presenting cells (APCs).* Once the resting, "naïve" T cells are activated, they trigger a complex immune response to either fight or tolerate these antigens.

DCs also induce tolerance to self-antigens by T cells during its development. This occurs in the thymus (central tolerance) by deletion of developing T cells and in lymphoid organs (peripheral tolerance) by induction of anergy or deletion of mature T cells.

Dendritic cells are known to have major effects on B-cell growth and immunoglobulin secretion (Steinman RM, 1998). DCs activate and expand T –helper cells, which in turn induce B cell growth and antibody production.

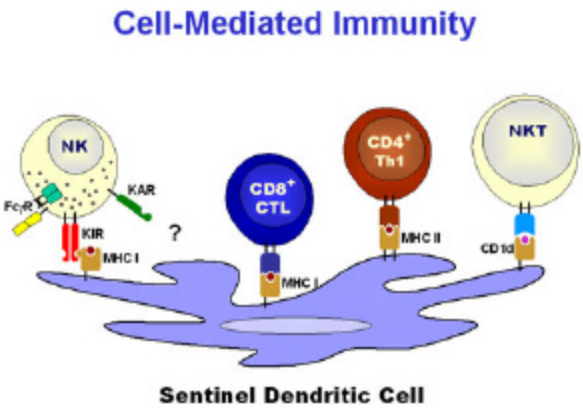

Figure 1.6. Cell mediated immunity is an outcome of an interaction between DCs and other lymphocytes. (Source: Steinman lab, Rockefeller university)

Naïve B cells respond uniquely to the interstitial non-LC type of DCs (Caux C et al, 1997; Dubios B et al, 1997), and by secretion of soluble factors (Dubios., 1997), including IL-12, DCs also orchestrate immunoglobulin class switching of T cell-activated B cells.

Expression of IgA2 appears to be strictly dependent on a direct interaction between B cells and DCs (Fayette j et al, 1997). In addition, Follicular dendritic cells (FDCs) organize the primary B cell follicles. In the germinal centers the proliferating B cells (Centro blasts) undergo somatic mutation after which they stop dividing and wait to be triggered by an immune complex on FDCs. B cells that recognize this immune complex with high affinity process the antigen and present it as peptide-MHC complexes to antigen-specific T cells. This T-B cell interaction ensures the survival of high affinity B cells. In addition DCs also enhance differentiation of CD40-activated memory B cell towards IgG secreting cells (Dubois B, 1998; Dubois B, 1997). DCs also help the differentiation of activated-naïve B cells into plasma cells (Banchereau J, 2000).

DCs at different stages of differentiation can regulate effectors of immunity such as NK cells and NK T cells by both direct cell-cell interactions and indirect cytokine mediated interactions. Precursors of CD11c DCs may activate NK cells through the release of IFN-α; thereby leading to enhanced anti-viral and anti-tumor activity of NK cells (Cella M., 1997; Siegal FP, 1999;). DCs at later stages of development may regulate the activity of NK /NKT cells through the release of IL-12, IL-15, and IL-18 (Geldhof AB, 1998).

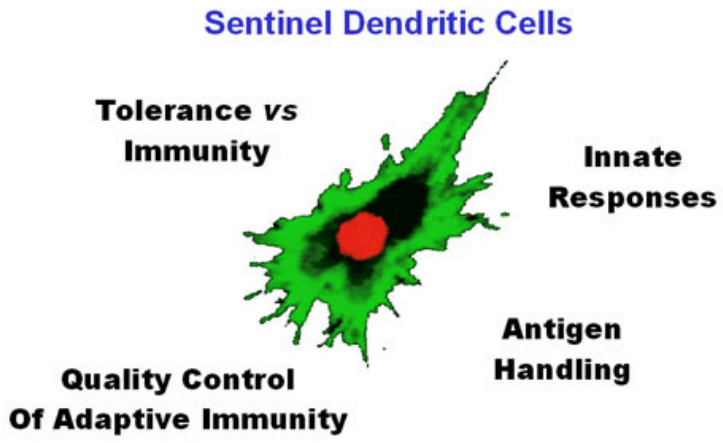

Figure 1.7. Multifarious functions of dendritic cells.
(Source: Steinman lab, Rockefeller university)

1.5 Subtypes of dendritic cells

To date several subpopulations of DCs have been identified and described based on the expression of cell surface markers, functional characteristics and anatomical localisation. Historically, both human and mouse DCs have been named according to their appearance and distribution in the body as: Langerhans, Interstitial, Blood, Veiled, Lymphoid or Interdigitating dendritic cells that belong to Epidermis, Dermis or Interstitium, Circulatory System, Afferent Lymph and Lymph Nodes respectively. During the past decade, DCs were further classified by lineage, by maturation stage, by functional and phenotypic characteristics of these stages, and by mechanisms involved in migration and function (Cella M et al, 1997; Austyn J, 1996). More recently dendritic cells are classified into various subcategories based on the expression of defined cell surface molecules.

Human: In contrast to the many studies on mouse DCs, there are relatively few studies on mature human DCs freshly isolated from tissue (Shortman K, 2002). Blood is the only readily available source and is a major source of immature dendritic cells (iDCs) and pDCs. Human blood dendritic cells are heterogeneous in their expression of a range of markers, but many of these reflect differences in the maturation and activation states of DCs rather than separate sublineages. (Hart DN, 1997). In few cases human DCs have been isolated from lymphoid

tissues and analysed for the presence of different subtypes. Splenic and tonsilar DCs show heterogeneity in the expression of CD4, CD11b and CD11c indicating a level of complexity resembling mouse splenic DCs (Shortman K, 2002). However the relationship between these subtypes is not clear. Most human thymic DCs are $CD11c^{+}$ $CD11b^{-}CD45R^{low}$ and lack myeloid markers and hence resemble mouse thymic $CD8^{+}$ DCs. A minority of human thymic DCs are $CD11c^{hi}CD11b^{+}CD45R^{hi}$ and express many myeloid markers and thus resemble mouse $CD8a^{-}$ DCs (Vandenabeele S et al, 2001; Bendriss VN et al, 2001). In addition most of the insights about human DC subsets have come from in vitro studies (Shortman K, 2003). The in vitro differentiation of human $CD34^{+}$ stem cells gives rise to 3 independent lineages of DCs (Caux C et al, 1996; Caux C et al, 1996). One of the lineages, originating in the presence of TGFβ, resembles Langerhans cells; in that they have Birbeck granules and expresses the Langerhans-associated antigens (Lag) langerin and E-Cadherin. The second lineage resembles intestinal DCs, lacking birbeck granules but expresses CD9, CD68 and coagulation factor XIIIa. The third lineage produces dendritic cells from the lymphoid-restricted precursors with in the CD34 population (Galy A et al, 1995). Additional studies on human monocytes have revealed two more intermediate subtypes of DCs; pDC1 and pDC2. pDC1 develop in the presence of GM-CSF and IL-4 (Sallusto F et al, 1994; Bender A et al, 1996; Romani N et al, 1996). They can be identified by expression of surface markers $CD14^{-}CD38^{+}$ $CD86^{+}$MHC-II^{hi}. In contrast, pDC2 precursors, which develop in the presence of IL-3 and CD40L, differentiate into interferon a/ß–producing plasmocytoid cells (Rissoan MC et al, 1999; Grouard G et al, 1997) that can be recognised by their plasma-cell-like morphology and their unique surface phenotype $CD4^{+}$IL-3-$receptor^{+}CD11c^{-}$. In spite of scattered reports on DC subtypes, a comprehensive classification system of human dendritic cell subtypes remains to be established.

Mouse: Murine DCs can be classified into many subtypes based on their expression of specific surface markers. Irrespective of the subtypes all mature dendritic cells express

CD11c- 'the hallmark of dendritic cells'. Ken Shortman and (2002) Yong-Jun-Liu have classified murine dendritic cells into 5 major categories based on their expression for surface markers CD4, CD8α, CD11b and CD205 in addition to CD11c in the lymphoid tissues of uninfected laboratory mice. According to this system of categorisation, the mouse spleen possesses three subtypes of CD11c positive dendritic cells: $CD4^{-}CD8\alpha^{+}$, $CD4^{-}CD8\alpha^{-}$ and $CD4^{-}CD8\alpha^{-}$DCs. The $CD4^{-}CD8\alpha^{+}$ DC subtype, which is $CD205^{+}CD11b^{-}$, is also found in moderate levels in LNs, but is the dominant subtype of thymic DCs. In addition, lymph nodes harbour another two novel DC subtypes that are defined by $CD4^{-}CD8\alpha^{-}CD11b^{+}CD205^{int}$, which is believed to be the mature form of tissue interstitial DCs, and $CD4^{-}CD8\alpha^{low}CD205^{high}CD11b^{+}$ $langerin^{high}$, which is believed to be the mature form of Langerhans cells.

Based on an alternative system of categorisation proposed by Ardavin (Ardavin C, 2003), mouse dendritic cells are classified into six main subpopulations. In this framework, murine DCs in the lymphoid tissues can be divided into $CD8\alpha^{-}$ and $CD8\alpha^{+}$ subpopulations. $CD8\alpha^{-}$ DCs can further be subdivided into $CD4^{-}CD8\alpha^{-}$ and $CD4^{+}CD8\alpha^{-}$ subsets. In addition, DCs that express intermediate levels of CD8α ($CD8\alpha^{int}$) constitute a lymph node specific subset of DCs. In peripheral lymph nodes, $CD8\alpha^{int}$ DCs seem to derive from epidermal Langerhans cells and in mesenteric lymph node they seem to originate in the intestinal lamina propria. Finally, a peculiar DC subtype that is been defined by the expression of B220 (a counterpart of human plasmacytoid DCs) is found in all lymphoid organs of the mouse. In spite of the phenomenological description of DC categories based on the expression of cell surface markers, a uniform and unequivocally acccepted classification system of murine dendritic cells is still lacking.

1.6 Developmental origin of Dendritic Cells

Despite the phenotypic characterisation of diverse DC subpopulations, their developmental pathways of differentiation from hematopoietic stem cells (HSCs) remain poorly defined (Ardavin C, 2003). DCs were originally thought to be derived from myeloid precursors due to their functional, phenotypic and morphological similarities with macrophages. Early concepts postulated the existence of $CD8\alpha^+$ lymphoid DCs and $CD8\alpha^-$ myeloid DCs, originating from common myeloid progenitors and common lymphoid progenitors respectively (Vremec, 1992; Anjuere, 1999; Wu, 1996). However, this conceptual dichotomy has been challenged when it was shown that myeloid progenitor cells can give rise to "lymphoid" DCs and that lymphoid progenitor cells can give rise to "myeloid" DCs (Martin, 2000; Manz, 2001; Traver, 2000). However, the finding that the differentiation of $CD8\alpha^+$ and $CD8\alpha^-$ DCs require different cytokines and involves different transcription factors indicates that their developmental pathways might differ (Wu, 1998; Guerriero, 2000; Saunders, 1996). Recent findings that are derived mainly from in vivo studies of DC development have shown that differentiation of DC is more complex than expected.

1.7 Dendritic cell precursors

The definition of DC committed precursors has remained elusive in both human and mice.

Human peripheral blood monocytes harbour at least 2 distinct DC precursors; pDC1 ($CD14^-$ $CD38^+$ $CD86^+$ MHC-II ^{high}) which differentiate into DC1, and pDC2 ($CD45Ra^{hi}CD11c^{lo}IL\text{-}3R^{hi}$) that differentiate into DC2 or plasmocytoid dendritic cells (Rissoan MC et al, 1999; Grouard G et al, 1997; Res P.C. et al, 1999; Spits H et al, 2000). The first description of a DC restricted precursor population in mouse blood was reported by Ardavin et al (2000). These cells were described as $CD11c^+$ $MHCClassII^-$ and were able to completely reconstitute splenic

CD8α$^-$ and CD8α$^+$ and plasmocytoid B220$^+$ DC subpopulations. However, these cells are devoid of lymphoid or myeloid differentiation potential (Martinez et al, 2002).

Recently, another mouse DC progenitor population has been discovered in the bone marrow (Amico et al, 2003). These progenitors are defined by the cell surface expression profile Lin$^-$ Sca1$^-$c-kit$^+$Flt3$^+$ and appear to be the earliest precursors of all DC subtypes irrespective of their myeloid or lymphoid orientation. These studies are important since it is now possible to identify distinct precursor cells of the DC lineage, a prerequisite to define distinct developmental steps of DC development.

1.8 Cytokine regulation of dendritic cell development

Cytokines are diverse and potent chemical messengers secreted by specialized cells such as T cells, B cells, macrophages, dendritic cells, epithelial cells, mesenchymal cells and neuronal cells. Cytokines and chemokines are pleiotropic, redundant, and multifunctional in nature. Upon binding to specific receptors on target cells, cytokines recruit many other cells and substances to the field of action. Cytokines encourage cell growth, promote cell activation, direct cellular traffic, and destroy target cells—including cancer cells. Because they serve as a messenger between leukocytes, many cytokines are also known as interleukins.

Hematopoiesis is largely controlled by the effects of specific cytokines in vitro. Accordingly, DC differentiation from different hematopoietic precursors requires a number of defined cytokines (Ardavin C, 2001). Initially, these cytokine combinations (see below) were defined on the basis of in vitro differentiation studies using hematopoietic precursors cells. Additional information concerning the involvement of cytokines in the differentiation of DCs arises from genetically deficient mice. These experimental data indicate that some cytokines appear to have a dispensable role, whereas others are strictly required for the generation of DCs. The most relevant cytokines involved in the differentiation of DCs are considered.

GM-CSF appears to be required for the in vitro differentiation of DCs from BM and blood progenitors, but not from thymic progenitors (Inaba K et al, 1992). Nevertheless, the generation of DCs from BM Lin^- cells in the absence of GM-CSF was reported (Brasel K et al, 2000). It should be noted that GM-CSF or GM-CSFR deficient mice display normal development of DCs (Vermac D et al, 1997).

TNF-a employed in most mouse and human DC and LC differentiation assays from BM precursors, and has been proposed to be crucial cytokine for the generation of DCs from human $CD34^+$ precursors (Caux C et al, 1992). However no defects in the differentiation of DCs have been reported in TNF-α or TNFRI deficient mice (Zhang Y et al, 1997) suggesting that TNF-α is not a necessary factor in DC development in vivo.

Flt3L has been demonstrated to have strong differentiation-promoting potential for mouse and human DCs, both in vivo and in vitro (Brasel K et al, 2000; Maraskovsky E et al, 1996; Maraskovsky E et al, 2000; Curti A et al, 2001). Consequently Flt3L deficient mice displayed important defects in the differentiation of DCs (Mc kenna HJ et al 2000). In addition, Flt3L has been claimed to increase the in vitro survival of DC precursors and selectively favor their differentiation (Curti A et al, 2001). Thus, Flt3L appears to play an essential role in the differentiation of DCs under both in vitro and in vivo conditions.

IL-4 has been shown to be a key cytokine for inducing the differentiation of DCs from human monocytes, and interestingly, it has been shown that IL-4 exerts an inhibitory function on macrophage differentiation (Romani N et al, 1994). It has been shown that IL-4 allows the generation of murine DCs from bone marrow cells, in combination with GM-CSF (Mayordomo JI et al, 1995).

TGF-ß is an important cytokine for in vitro differentiation of LCs and required to achieve a complete and/or more physiological differentiation of Langerhans cells. Importantly, the skin of TGF-β deficient mice is devoid of epidermal LCs (Borkowski T A et al, 1996).

In addition, CD40L and IL-3 have shown to promote differentiation of DC2 cells from pDC2 precursors (Rissoan MC et al, 1999; Grouard G et al, 1997). Different cytokines or combinations of cytokines are necessary for the development of the various subsets or phenotypes of DCs. Some cytokines, such as GM-CSF, have been identified to promote growth, maturation and migration of DCs both in vitro and in vivo. Ultimately, cytokine induced signalling leads to a change in the transcriptional profile of progenitor cells, including a modified expression pattern of transcription factors.

1.9 Transcription factors and dendritic cell development

The lineage fate of hematopoietic progenitor cells is controlled by an orchestrated expression pattern of transcription factors (Orkin S, 2000), yet the molecular mechanisms governing the diversification of dendritic cell progenitor cells remain largely unknown. The analysis of gene-targeted mice has revealed the functional importance of a few critical transcription factors for DC development. Moreover the analysis of DC subpopulations in mice deficient for transcription factors involved in myeloid and lymphoid development was undertaken to address the derivation of DC lineages. Few critical transcription factors controlling DC development have been identified and will be discussed in some detail:

Ikaros: Ikaros represents a transcription factor of the zinc finger family and was originally identified as a factor critical for T cell development (Kaufmann et al, 2003) In addition to the T cell phenotype, Mice homozygous for a dominant negative mutation in the Ikaros gene (Ikaros $DN^{-/-}$) displayed a complete lack of all thymic and splenic DCs (Wu et al., 1997). Even though a profound deficiency is observed in the lymphoid DC lineages, epidermal LCs are being generated in these knockout mice. Mice with an Ikaros null mutation (Ikaros $C^{-/-}$) display less severe defects in lymphoid development, having reduced differentiation of $CD8\alpha^+$ DCs in the thymus and spleen, more surprisingly the $8\alpha^-$ DCs were completely absent in the spleen.

Rel-B: Rel-B is a member of NF-kB/Rel family. Rel-B gene targeted mutant mice were reported to lack DC in thymus and spleen and to have impaired antigen presenting cell function (Wu L et al, 1998). In these mice the $CD8\alpha^+$ DC appear to be normal. However, there is a lack of $CD8\alpha^-$ DCs in the spleen. The number of epidermal Langerhans cells remains unaffected.

PU.1: PU.1 is a member of the ets family of DNA binding proteins and is expressed only in the hematopoietic cells. PU.1 deficient mice (Guerriero A et al, 2000) produce functional $CD8\alpha^+$ DCs but lack $CD8\alpha^-$ DCs. PU.1 deficient mouse hematopoietic progenitor cells fail to generate DCs in culture. Thymic DCs couldn't be detected in the PU.1 deficient mice. However the dendritic cells appear to be functional.

ICSBP: Interferon consensus sequence binding protein (ICSBP) is a transcription factor that belongs to the interferon regulatory factors (IRF) family. The expression of ICSBP is restricted to myeloid and lymphoid cell lineages. ICSBP deficient mice lack interferon-producing cells (IPC) in all lymphoid organs (Schiavoni G et al, 2002). A marked reduction of $CD8\alpha^+$ cells in all lymphoid organs was noticed. Moreover, an altered response of $CD8\alpha^+$ cells to activation signals is noticed. ICSBP deficient mice exhibited a reduced frequency of LCs and a delayed mobility of DCs from skin to lymph nodes. Bone marrow derived DCs exhibited an immature phenotype and showed a severe reduction of IL-12 production.

Id2: Id proteins are a group of proteins (1-4) that possess a highly conserved helix-loop-helix (HLH) domain. Id2 knockout mice were deficient in $CD8\alpha^+$ DCs and showed a complete absence of epidermal Langerhans cells (Hacker C et al, 2003).

STAT3: STAT3 is a cytoplasmic transcription factor that is a key mediator of cytokine and growth factor signalling pathways. Mice carrying a conditional deletion of the STAT3 gene in the hematopoietic cells showed profound defects in the DC compartments (Laouar Y et al, 2003). STAT3 deficient mice showed a complete lack of $CD8\alpha^+$ DCs and an abrogated DC

development in all the lymphoid organs. Flt3L-mediated in vitro DC differentiation of STAT3 deficient bone marrow cells failed to generate plasmocytoid ($CD11c^+$ $CD11b^-$) DCs although myeloid DCs ($CD11c^+$ $CD11b^+$) DCs could be generated.

P50 and RelA: P50 and RelA are the subunits of the transcription factor NFkB that play an important role in inflammatory and immune response genes. Mice deficient for both P50 and RelA showed dramatically impaired DC development (Quaaz F et al, 2002). They showed a complete lack of splenic DCs. Both $CD8\alpha^+$ and $CD8\alpha^-$ subtypes are reduced and cells that exhibit DC morphology were completely absent.

IRF-2: IRF-2 is a transcriptional repressor that was identified as a regulator of the type I interferon system. Severe reduction of $CD4^+CD11b^+$ DCs and epidermal langerhans cells was noticed even though the CD8α+ DC compartment remained unaffected (Ichikawa E et al, 2004). Furthermore, reduced frequencies of the generation of mature DCs from IRF-2 deficient BM in vitro were noticed.

IRF-4: IFN regulatory factor 4 is a transcription factors that plays an essential role in the homeostasis and function of immune systems. Mice deficient for IRF4 show a severe reduction of $CD4^+CD8\alpha^-$ DCs in spleen and the generation of $CD11b^+$ DCs from bone marrow of IRF-4 $^{-/-}$ mice was severely impaired (Suzuki S et al, 2004).

Although the availability of the gene knockout mice mentioned above has greatly enhanced our understanding of transcription factor controlled DC development, a comprehensive view integrating all decisive factors is still lacking.

1.10 Growth-factor-independent-1

The gene locus encoding the Growth-factor-independent-1 (Gfi1) protein was discovered in a screen for moloney murine leukaemia virus (MoMuLV) proviral integration site in NB2 rat lymphoma cells and in T-lymphoid tumors (Gilks et al, 1993; Schmidt et al, 1996; Zöring et al, 1996; Scheijen et al, 1997). The Gfi1 gene encodes a 55-kDa nuclear transcription factor,

which harbours six carboxy-terminal C2-H2 zinc-finger domains and a characteristic N-terminal 20 amino acid stretch termed ‘SNAG’ domain, which is well conserved between Gfi1 and the proteins Snail and Slug (Grimes et al, 1996; Zweidler-Mckay et al, 1996). Reporter gene experiments suggested a transcriptional repressor activity of Gfi1 that depends on the DNA binding activity and on intact SNAG domain (Grimes et al, 1996; Zwidler-McKay et al 1996). An alternative activity of Gfi1 has been discovered through its interaction with PIAS (Protein inhibitor of activated STAT) 3, which is an inhibitor of signal transducers and activators of transcription (STAT) 3 suggesting a role of Gfi1 in a set of specific cytokine signalling pathways (Rodel et al 2000).

Early studies revealed a key role of Gfi1 in lymphomagenesis and lymphopoiesis (Gilks et al 1993; Grimes et al, 1996; Schmidt et al, 1998). Gfi1 can act as a dominant oncogene when over expressed, and cooperates strongly with other oncoproteins such as Pim1 (a cytoplasmic serine/threonine kinase) or Myc (an HLH-LZ transcription factor) in accelerating progression of T-cell lymphomagenesis (Zoring et al, 1996; Scheijen et al, 1997; Schmidt et al, 1998). Moreover Gfi1 regulates IL-4/STAT6-dependent Th2 proliferation (Zhu et al, 2002), and IL-6/STAT3-mediated proliferative responses to antigenic stimulation (Rodel et al, 2000).

Later studies showed that Gfi1 is also expressed in granulocytes and in activated macrophages (Karsunky et al, 2002) and in distinct areas of nervous system, most prominently in the inner ear hair cells (Wallis et al, 2003). In the immune system, ablation of Gfi1 by gene targeting in mice caused defects in early T-cell maturation (Yücel et al, 2003) and led to severe neutropenia and a profound monocytosis (Karsunky et al, 2002; Hock et al, 2003). Recently it has been shown that the loss of Gfi1 affects the frequencies of HSCs and progenitors, in particular ST- and LT-HSCs and CLPs but leaves MEPs unaltered and suggested a role of Gfi1 in regulating the development of distinct myeloid and lymphoid progenitor cell populations and HSCs (Zeng et al, 2004). In additon, the importance of Gfi1 in restricting

HSC proliferation and in preserving HSC functional integrity has also been reported (Hock et al, 2004).

The current study aimed to use an in vitro DC differentiation system for transcriptional profiling using microarray analysis and to determine the transcription factor repertoire expressed during the differentiation program of the DCs. The transcriptional repressor Gfi1 was strongly upregulated in DCs both in vitro and in vivo. Characterisation of $Gfi1^{-/-}$ mice demonstrated that Gfi1 was crucial for the development of distinct subtypes of dendritic cells. $Gfi1^{-/-}$ mice showed both quantitative and qualitative abnormalities in almost all dendritic cell compartments with an exception of langerhans cells. The ablation of DC development in vitro by the Gfi1 deficient cells is correlated with the defective STAT3 signaling.

Hence, the results of this study unravel a previously unrecognized important role of Gfi1 in the regulation of development and activation of dendritic cells and in the lineage decision between dendritic cells and macrophages.

2. Aims of the proposed study

Hypothesis:

The fundamental hypothesis guiding my studies was that an analysis of upregulated transcription factors during early steps of GM-CSF-dependent DC maturation might help to elucidate critical master regulators controlling DC differentiation.

My thesis project had the following specific aims:

1. To analyze murine hematopoietic progenitor cell lines for their potential to differentiate into dendritic cells.

2. To undertake a genome wide screen to identify differentially expressed genes during early steps of GM-CSF mediated DC differentiation in vitro.

3. To study the role of upregulated transcription factors in available transgenic mice.

3. Materials and Methods

3.1 Buffers and Media

PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO4, 1.5 mM KH_2PO_4, pH 7.2

PBS, BSA(0.5%)

PBS, FCS(2%)

RPMI-medium: RPMI, containing 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine, and 10 μM 2- Mercaptoethanol

IMDM-medium: IMDM, containing 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine, and 10 μM 2-Mercaptoethanol

DMEM-medium: DMEM, containing 10% FCS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine

RBC lysis buffer: 7 mM Tris, 140 mM NH_4Cl, pH 7.65

Agarose gel buffer: 89 mM Tris base, 89 mM boric acid, 2 mM EDTA

RPIA buffer: 10 mM Tris (pH 7.5), 150 mM Nacl, 1 mM EDTA, 1% NP-40, 0.5% Na-Deoxycholate, 0.1% SDS, PIC (70 uL/mL)

Protease inhibitor cocktail (PIC): 104 mM AEBSF, 0.08 mM Aprotinin, 2 mM leupeptin, 4 mM Bestatin, 1.5 mM Pepstatin A, 1.4 mM E-64

Laemmli electrophoresis buffer: 50 mM Tris-HCl, 0.196 M glycine (pH 8.3), 20% methanol

SDS PAGE buffer: 25 mM Tris, 192 mM glycine, 0.1% SDS

Transfer buffer (Western): 192 mM glycine, 25 mM Tris

Blocking buffers (Western): PBS-T, 5% non-fat milk

Wash buffer (Western): PBS, 0.1% Tween-20

Coomassie stain solution: 50% Methanol, 7% Acetic acid, 0.1% Coomassie

Destain solution(Coomassie): 50% Methanol, 7% Acetic acid

Ponceau stain solution: 0.1% Ponceau S, 5% Acetic acid

Hypotonic buffer (EMSA): 20 mM HEPES (pH 7.6), 10 mM KCl, 1 mM MgCl2, 20% Glycerin, 0.1% Triton X-100, 0.5 mM DTT, 1 mM Na-Orthovanadate, PIC (70 uL/mL)

Hypertonic buffer (EMSA): 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 20% Glycerin, 0.1% Triton X-100, 1 mM DTT, 1 mM Na-Orthovanadate, PIC (70 uL/mL)

Shift buffer (EMSA): 10 mM HEPES (pH 7.9), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 5% Glycerine, 0.1% NP-40, 5 mM Na-Orthovanadate

EMSA running buffer: 44 mM Tris base, 44 mM Boric acid, 1 mM EDTA

ELISA coating buffer: 0.2 M NaPo4 (pH6.5)

Wash buffer(ELISA): 0.05% Tween20 in PBS, pH 7.2 - 7.4.

Reagent diluent(ELISA): 1% BSA in PBS, pH 7.2 - 7.4, 0.2 µm filtered.

Stop solution(ELISA): 2 N H_2SO4.

3.2 Instruments

FACS Calibur (BD-Pharmingen, SD, CA)

FACScan (BD-Pharmingen, SD, CA)

Cell sorter Moflow (DAKO Cytomation, Denmark)

AutoMACS (Miltenyi-Biotec, Germany)

ELISA reader (Molecular Devices, MWG-Biotech, Germany)

Phospho imager (Fuji Image, japan)

Kodak imaging station (Kodak, Germany)

Microscope Zeiss (zeiss, Germany)

Agarose gel chamber (Biorad, Germany)

Spectrophotometer (Eppendorf, Germany)

Mastercycler gradient (Eppendorf, Germany)

Light cycler (Roche, Germany)

Western blot apparatus (Biorad, Germany)

Centrifuge (Sorval; Eppendorf, Germany)

3.3 Methods

Mice: All mice were maintained under specific pathogen free conditions in the central animal facility at Hannover Medical School. Age and sex matched transgenic Gfi1$^{-/-}$ and Gfi1$^{GFP/+}$ mice (Karsunky et al., 2002b; Yucel et al., 2004) were used at four to eight weeks of age. C57BL/6 Ly5.1-Pep3b (CD45.1) mice were purchased from Jackson Labs (Bar Harbor, Maine, USA) and used at 8-12 weeks of age. OT-I and OT-II Rag$^{-/-}$ mice were kindly provided by T. Greten, Hannover Medical School.

Cells and cell culture: FDCP-mix cells were differentiated into DCs according to a previously published protocol (Schroeder et al., 2000). In brief, FDCP-mix cells were cultured in the presence of r-mGM-CSF (5 ng/mL) and 1% of WEHI supernatant for 7 days (as a source of m-IL3) in IMDM medium supplemented with 20% horse serum, 2 mM L-Glutamine, 1% Penicillin and Streptomycin (all from Gibco, Karlsruhe, Germany). To generate bone marrow derived dendritic cells, bone marrow cells were depleted of Gr1^{+}, Mac1^{+}, CD3^{+}, TER119^{+} and B220^{+} cells using magnetic beads (Miltenyi Biotech, Bergisch Gladbach Germany). Lineage-depleted progenitor cells were cultured in IMDM, 10% FCS, 2 mM L-Glutamine, 1% Penicillin-Streptomycin, 1 mM Non-essential amino acids, 5 x 10^{-5} M 2-mercaptoethanol for 7 days in the presence of recombinant murine GM-CSF and IL-4 (Sigma, Munich, Germany), or recombinant human Flt3L (Peprotech, Rocky Hill, NJ). In some confirmatory experiments, supernatants of the engineered cell lines EL4-GM-CSF and EL4-IL4 were used as cytokine source.

For in vivo DC analysis, lymphoid organs were cut into small pieces, treated with collagenase D (Boehringer, Mannheim,Germany) for 30 min at 37 °C, gently meshed and washed with PBS containing 50 µg/mL Dnase I (Roche, Mannheim, Germany) and 2 mM EDTA. In some experiments, splenic DCs were purified by labelling splenic single cell suspensions with anti-CD11c microbeads (N418) and subsequent enrichment by immunomagnetic columns (Miltenyi Biotech). For retroviral gene transfer, Sca-1^{+}lin^{-} cells were cultured in IMDM containing 10% FCS, 2 mM L-Glutamine, 1% Penicillin-Streptomycin, 1 mM Non-essential amino acids, 10 ng/ml rm-IL3, 10 ng/ml rm-IL6, 50 ng/ml rm-SCF, 50 ng/ml rh-Flt3L and 25 ng/ml h-TPO (all from Peprotech, Rocky Hill, NJ).

For T cell proliferation studies, Gfi$1^{-/-}$ and Gfi$1^{+/+}$ splenic DCs were pulsed with 1 µg/ml Ova peptide SIINFEKL and Ova$^{323-339}$, respectively, irradiated (30 Gy) and incubated with 10^5 lymphocytes from either OT-I or OT-II mice in a 96 well plate. Allogeneic T cell proliferation was performed using 10^5 lymph node cells obtained from Balb/C mice as responder cells and irradiated (30 Gy) bone-marrow-derived cells as stimulator cells. On day 3 of culture, the cells were pulsed with 1 µCi ^{3}H-Thymidine for 16 hours. Incorporated ^{3}H-Thymidine was quantified by scintillation counting.

Flow cytometry, immunofluorescence and ELISA: Single cell suspensions were analysed by flow cytometry using a FACS-Calibur and CELLQuest software (BD Biosciences, San Jose, CA). In some experiments, cell sorting of Sca-1^{+}lin^{-} and Lin^{-}Sca-1^{+}c-kit^{+} cells was performed using a Moflo cell sorter (DAKO Cytomation, Glostrup, Denmark).

The following monoclonal antibodies (all from BD Pharmingen, San Diego, CA except noted otherwise) were used: CD3ε-FITC & -biotin, CD4-FITC & -PE, CD8α-FITC, -PE & -PerCP, CD11b-FITC & -biotin, CD11c-PE & -APC, CD34-FITC, CD40-FITC & -PE, CD45.1-biotin, CD45.2-FITC, CD80-FITC & -PE , CD86-FITC & -PE, CD117-PE & -APC, B220-FITC, -PE & -biotin, H2-k^{b}-FITC, I-A^{b}-PE, TER119-biotin, GR-1-FITC & -biotin, IL7Rα-

biotin, FcRγ-PE, Sca-1-PE, Flt3-PE, CD45.2-FITC, $V_{\alpha}2$(B20.1)-biotin, anti-mouse-STAT3, goat-anti-mouse-IgG-HRP, F4/80-FITC (Zymed laboratories, South San Francisco, CA), I-A^b-APC (eBiosciences, San Diego, CA). In all experiments, cells were also stained with corresponding isotype-matched monoclonal antibodies. Cells reacted with biotinylated monoclonal antibodies were incubated with fluorochrome-conjugated streptavidin-PerCP or streptavidin-APC (BD Pharmingen). For in vivo DC maturation studies, α-CD40 (FGK-45) monoclonal antibodies were purified from the hybridoma cell line FGK-45 (kindly provided by A. Rolink). All fluorescence intensity plots are shown in log scales.

To enumerate epidermal Langerhans cells, ears were split into dorsal and ventral halves and incubated in PBS with 20 mM EDTA for 2.5 hours. Epidermal sheets were fixed, blocked, and stained with APC-conjugated anti-I-A^b monoclonal antibodies. LC density was determined by fluorescence microscopy using a micrometer grid (field size: 0.25 mm^2).

For cytokine assays, purified splenic DCs were stimulated in a 96 well plate with 10 ng/ml LPS, 100 ng/ml TNF-α (both from Sigma Aldrich) or 10 µM CpG-DNA (TibMolBiol, Berlin, Germany) for 48 hrs. The supernatants were harvested and IL-12p70 levels were measured using mouse IL-12p70 ELISA set (BD Pharmingen).

Microscopy: Phase contrast images of DCs were taken using a Zeiss Axiovert 200 microscope at an original magnification of x 100. Photodocumentation was performed using an ORCA-2 camera system and Open lab^T software. Giemsa-stained DCs were observed using a Zeiss Axioplan 2 microscope (original magnification x 100).

Protein assays: Extraction of nuclear and cytoplasmic proteins was carried out using standard protocols. Briefly, cells were harvested and lysed in hypotonic buffer containing 20 mM Hepes (pH 7.6), 10 mM KCl, 1 mM $MgCl_2$, 2% glycerin, 0.1% Triton-X100, 0.5 mM DTT (Roche), 1 mM Pefabloc (Roche), 1 mM sodium-orthovandate (Sigma), 5 µg/mL protease

inhibitor cocktail (Sigma). Nuclear and cytoplasmic protein fractions were separated by centrifugation (x 1800g). The pellet containing nuclear proteins was re-suspended in hypertonic buffer containing 20 mM Hepes (pH 7.9), 400 mM NaCl, 1 mM EDTA, 20% Glycerin, 0.1% Triton-X, 0.5 mM DTT (Roche), 1 mM Pefabloc, 1 mM sodium-orthovandate, 5 µg/mL protease inhibitor cocktail and incubated for 15 min at 4 °C. The lysate was subjected to centrifugation (x 16,000g) and the supernatant containing the nuclear proteins was collected. Protein quantification was performed using Bradford reagent (Bio-rad, Munich, Germany). For Western blot analysis, 20 µg of protein was loaded on an 8% SDS gel, separated by electrophoresis and blotted onto nylon membrane. The membrane was exposed to anti-STAT-3 primary antibodies and mouse-IgG secondary antibodies conjugated to horse radish peroxidase. The enzymatic reaction was visualized using ECL reagents (ECL kit, Amersham Biosciences, Freiburg, Germany).

For electrophoretic mobility shift assays, 5 µg of nuclear protein was incubated with ^{32}P-labelled synthetic complementary oligonucleotides. SIE-probe 5'-AGCTTCATT TCCCGTAAATCCCTA binds to STAT3 and STAT1, OCT1 octamer probe 5'-GATCCTTAATAATTTGCATACCCTCA was used as a control. Protein-DNA interactions were performed in EMSA buffer containing 10 mM HEPES (pH 7.9), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT (Roche), 5% glycerine, 0.1% NP-40 (Sigma), 1 mM Pefabloc, and 5 mM sodium-orthovandate. Supershift analysis was performed by preincubating the nuclear proteins with a monoclonal antibody recognizing STAT3 or a mouse IgG1 isotype monoclonal antibody, respectively. Protein-DNA complexes were resolved on a 5% native PAGE using 0.25 x TBE. Autoradiography was performed using a Fujix BAS 1000 (Fuji photo film co., LTD, Japan) imaging analyzer.

RNA isolation and Real Time PCR: Total RNA was isolated with "Absolutely RNA mini prep kit" (Stratagene, La Jolla, CA). cDNA was synthesised by using oligo dT primer and

expand reverse transcriptase (Roche). Gfi1 expression was determined by Real Time PCR using the specific forward primer 5'-TCCAGTGTGCAAAGCTCATC and reverse primer 5'-TCCACAGCTTCACCTCCTCT. GAPDH specific primers were used as internal controls (forward primer 5' GTCAGTGGTGGACCTGACC; reverse primer 5'-TGAGCTTGACAAAGTGGTCG). The PCR reaction was performed in duplicates using a LightCycler–FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions.

Retroviral gene transfer: The murine Gfi1 cDNA was cloned into the retroviral vector SFβ-91-IRES-EGFP, kindly provided by C. Baum, Hannover. Recombinant VSV-G pseudotyped retroviruses were generated using transient transfection into the packaging cell line 293GPG (Klein et al., 2000). For retroviral gene transfer, Sca-1^{+}lin^{-} progenitor cells were stimulated for 48 hours in the presence of a stem cell cytokine cocktail (see above) and transduced at a multiplicity of infection (MOI) of 10 in the presence of 8 µg/ml polybrene (Sigma). In brief, cells were exposed to recombinant retrovirus for 1 hour at 37 °C, followed by spinoculation for 2 hours at x 700 g and further incubation at 37 °C in 5% $C0_2$. Subsequently, cells were washed, cultured for additional 48 hours in the presence of the stem cell cytokine cocktail and used for in vitro and in vivo experiments. The average transduction efficiency was 50-60%.

In vivo studies: To generate hematopoietic chimeras, red blood cell depleted bone marrow cells (10^6) from CD45.2^{+} Gfi$1^{+/+}$ or Gfi$1^{-/-}$ mice were intravenously injected into irradiated (9 Gy) CD45.1^{+} recipient mice. For transplantation of retrovirus transduced hematopoietic stem cells, 0.5-1 x 10^5 transduced progenitor cells were transferred into irradiated (4.5 Gy) CD45.1^{+} recipient mice. DC reconstitution was assessed 4-7 weeks after transplantation of stem cells. To assess DC maturation in vivo, 4 week old Gfi$1^{-/-}$ and Gfi$1^{+/+}$ control mice were injected i.p. with either LPS (100 ng/g body weight) or α-CD40 monoclonal antibodies (50

µg/mouse). 24 hour after injection mice were sacrificed and single cell suspensions were prepared from spleen and stained for DC markers and analyzed by flow cytometry.

DNA Microarray Hybridization and Analysis: Quality and integrity of the total RNA isolated from 2-10 x 10^6 FDCP mix cells was controlled by running all samples on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). For biotin-labelled target synthesis starting from 3 µg of total RNA, reactions were performed using standard protocols supplied by the manufacturer (Affymetrix, Santa Clara, CA). Briefly, 5 µg total RNA was converted to dsDNA using 100 pmol of a T7T23V primer (Eurogentec; Seraing, Belgium) containing a T7 promotor. The cDNA was then used directly in an in vitro transcription reaction in the presence of biotinylated nucleotides.

The concentration of biotin-labelled cRNA was determined by UV absorbance. In all cases, 12.5 µg of each biotinylated cRNA preparation were fragmented and placed in a hybridization cocktail containing four biotinylated hybridization controls (BioB, BioC, BioD, and Cre) as recommended by the manufacturer. Samples were hybridized to an identical lot of Affymetrix MG-U74Av2 for 16 hours. After hybridization, the GeneChips were washed, stained with SA-PE and read using an Affymetrix GeneChip fluidic station and scanner.

Bioinformatics: Gene expression levels were determined by means of Affymetrix's Microarray Suite 5.0 (MAS 5.0). MAS 5.0 software algorithms allow quantitative estimation of gene expression and a p-value to establish a confidence level concerning the accuracy of measurement of an mRNA of interest (detection p-value) and changes in gene expression (change p-value). Concerning the measured p-values the criteria of present (P) or absent (A) define the quality of signal measurement, and increase (I) or decrease (D) define the signal change, respectively. For normalization all array experiments were scaled to a target intensity of 150, otherwise using the default values of the Microarray suite. Filtering of the results was

done as follows: Genes are considered as regulated when their fold change is greater than or equal 2 or less than or equal –2, the change p-value not "NC" (No Change) and at least one of the two compared signals was detected by high accuracy (absent call for both signals were not allowed).

4. Results

4.1.1 DC differentiation from well-defined and available hematopoietic progenitor cell lines

The first goal of this thesis was to identify a cell line permissive for DC differentiation in vitro in the presence of recombinant cytokines that promote DC differentiation (GM-CSF and IL-4). In this regard the following cells were tested,

1. 32D cells
2. FDCP- mix cells

In a first series of experiments, 32D cells were incubated in the presence of recombinant murine GM-CSF and IL-4 at 50 ng/ml and analyzed for expression of the cell surface markers CD11c, CD40, CD80, CD86, and MHC class II. However, the cells did not differentiate into DCs, as assessed by FACS analysis. Even upon permutation of multiple experimental variables such as treatment with GM-CSF, TNF-α, LPS (lipopolysaccharide) the results were negative (data not shown).

In contrast, a systematic analysis of in vitro differentiation properties of FDCP-mix cells yielded more promising results. Multiple experimental variables were tested to optimise differentiation conditions such as such as in the presence of horse serum or fetal calf serum, various concentrations of IL-3, GM-CSF and TNF-α, and various exposure periods of cells with the mentioned cytokines. The capacity of the differentiation of the FDCP- mix cells into dendritic cells was tested by FACS analysis after staining the in vitro differentiated cells with dendritic cell specific surface markers (MHC class I and II, CD80, CD86, CD40 and CD11c). For optimizing the differentiation condition, FDCP- mix cells were cultured in the presence of 0.5 %, 1 % and 10 % of WEHI supernatant (source of mIL-3), with and without GM-CSF. After culturing the cells for 3-7 days in the presence of GM-CSF (cytokine that influences myeloid dendritic cell differentiation), cells were taken for FACS analysis to study the expression of surface markers. FACS data indicate that at low concentrations (0.5 % and 1 %)

IL-3 favors the differentiation of FDCP- mix cells into dendritic cells (Fig 4.1.1.1) upon GM-CSF treatment.

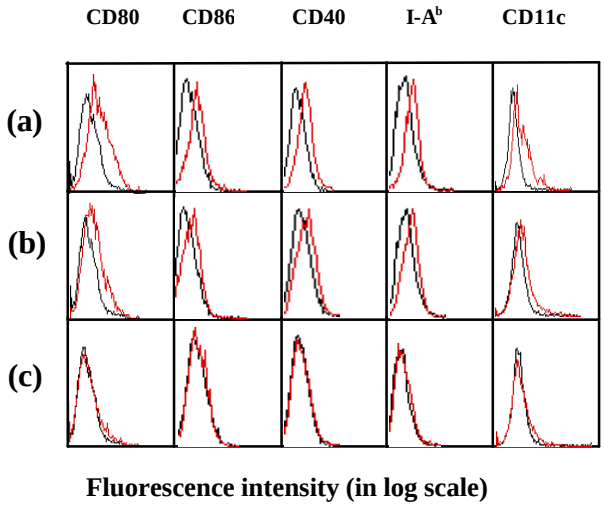

Figure 4.1.1.1. Effect of IL-3 concentration in the differentiation of FDCP- mix cells into dendritic cells.
(a) cells were cultured in the presence of 0.5 % of WEHI supernatant (source of mIL-3) with GM-CSF. (b) Cells cultured in 1 % of WEHI supernatant with GM-CSF. (c) Cells cultured in 10 % WEHI supernatant with GM-CSF. Cells were cultured in presence of GM-CSF for 3 days.
Cells were treated with CD80, CD86, CD40, CD11c and MHC class II monoclonal antibodies and with the respective isotype controls. Histograms with black line represent the isotype controls and histograms with gray line represent the expression of the indicated surface marker.

From the above experiment it was inferred that low concentrations IL-3 favors the differentiation of FDCP- mix cells into DCs. However previous studies report that FDCP- mix cells can survive only in an appropriate concentration of IL-3, as they are basically IL-3 dependent cells. The definition of an optimal concentration of IL-3 for both survival and differentiation was crucial for further differentiation studies. To find the minimal concentration of IL-3 necessary for survival, the FDCP- mix cells were cultured in the presence of 0.5 %, 1 % and 10 % of WEHI supernatant (as a source of mIL-3). After 3 days of culture the cells were stained with propidium iodide to distinguish the viable cells from dead cells and taken for FACS analysis. FACS data indicate that survival of FDCP- mix cells is proportional to IL-3 concentration. It was observed that in 0.5 % of IL-3 supernatant only 60 % of the cells were viable, in 1 % IL-3 supernatant 91 % of the cells were viable and in 10 % IL-3 supernatant 99 % of the cells were viable (Fig 4.1.1.2). 1 % WEHI supernatant was determined as the optimal concentration for the DC differentiation experiments, since viability and differentiation were best.

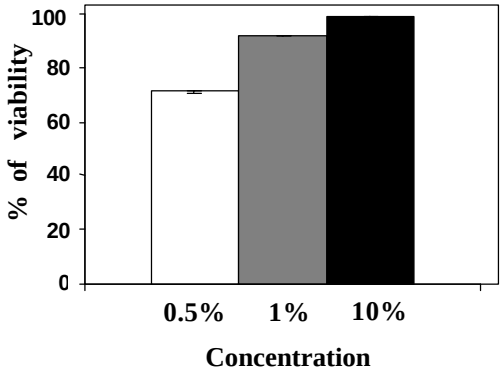

Figure. 4.1.1.2. Viability of FDCP-mix cells at increasing IL-3 concentrations. Bar diagram represents the percentage of viable cells in the y axis and the corresponding IL-3(WEHI supernatants concentration in the X axis.

For differentiating FDCP- mix cells into dendritic cells, the cells were cultured in the presence of 1% of WEHI supernatant (as a source of IL-3) and recombinant murine GM-CSF (100u/mL) for 8 days. Fig 4.1.1.3 indicates the DC specific surface marker expression in differentiated FDCP- mix cells. The results of the FACS analysis suggest that FDCP- mix cells can be differentiated into dendritic cells.

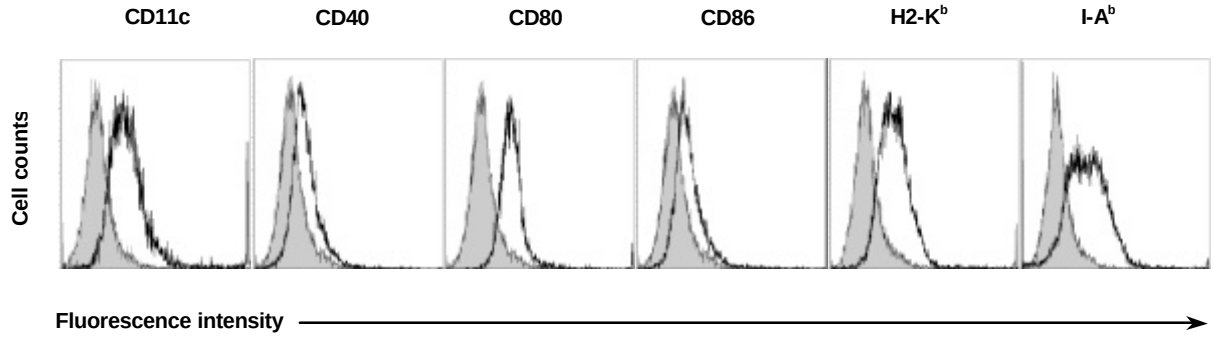

Figure. 4.1.1.3. Differentiation of FDCP-mix cells into DCs.
Cell surface marker analysis of FDCP-mix cells after differentiation into DCs, open histograms represent expression of indicated markers, shaded histograms represent isotype controls.

These studies suggest that FDCP- mix cells are permissive for DC differentiation in vitro. However, the following caveats had to be taken into consideration:

1- Atypical dendritic cell morphology

2- Incomplete DC differentiation (persistent contamination of granulocytes and macrophages).

4.1.2.1 Generation of a novel hematopoietic progenitor cell line-CR1-mix

Given the inherent limitations of 32D and FDCP- mix cells, an effort was made to create a progenitor cell line that allows an optimised DC differentiation system. Various defined transgenes have been used to immortalise HSC (Bunting KD et al., 1999; Just U et a1, 1995; Audet j et al 2001; Morrow M et al., 2004; Ye M et al., 2003; Varnum F et al., 2000; Antonchuk J et al., 2002; Krosl J et al., 2003; Reya T et al., 2003; Pinto O P et al., 2002).

We have made use of a retrovirus encoding the transcription factor Notch1. The strategy followed for making CR1 was initially described by Pears WS et al (2000). This approach utilized the retroviral mediated expression of intra cellular domain region of the transcription factor notch1 (ICN) to immortalize hematopoietic stem cells. However the generation of a hematopoietic cell line differentiating into DCs has not been reported earlier. The retroviral particles for the study were produced by transfecting the packaging cell line 293GPG (Klein et al, 2000) with the retroviral transfer vectors (kindly provided by David Scadden, Boston) encoding either intracellular domain of Notch1(ICN) along with IRES EGFP (MSCV-ICN-GFP) or EGFP (MSCV-GFP) only (Fig 4.1.2.1.).

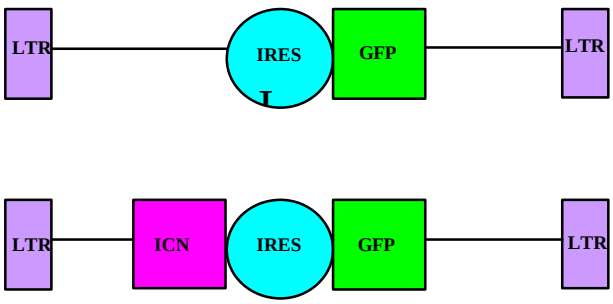

Figure. 4.1.2.1. Retroviral constructs. cDNAs encoding either GFP(top panel) or ICN IRES GFP(bottom panel) were cloned into Murine Stem Cell Virus backbone plasmids.

The VSVG pseudotyped viral particles were collected for 5 days and the viral supernatant was filtered and concentrated by spinning the soup at (16,000 X g) for three and half hours. The viral titer of the concentrated virus was determined by transducing 3T3 fibroblasts cells. Then murine HSC(Sca-1^{+} Lin^{-}) cells from the total bone marrow cells were sorted with moflow cell

sorter (DAKO cytomation, Sweden). The sorted cells were pre-stimulated with a stem cell cytokine cocktail (that contains recombinant murine cytokines; IL-3, IL-6 and SCF, and recombinant human cytokines; Flt3L and TPO) for 36 hours and transduced with a multiplicity of infection (MOI) 10 with retroviruses either encoding ICN-IRES-GFP or GFP only. The transduction efficiency was determined on the third day of transduction by measuring the GFP positive cells by flow cytometry. The transduction efficiencies were 41% and 61% in the cells transduced with either ICN-GFP or GFP respectively (Fig 4.1.2.2.a). To check the growth potential and dependency of the ICN transduced cells in the presence of different cytokines, cells were cultured in the presence of indicated cytokine combinations. As Fig 4.1.2.2.b suggests, only the notch transduced Sca-1^{+}lin^{-} cells could survive (~80%) in the presence of the complete cytokine cocktail (mentioned above), where as the viability of the GFP-transduced Sca-1^{+}lin^{-}

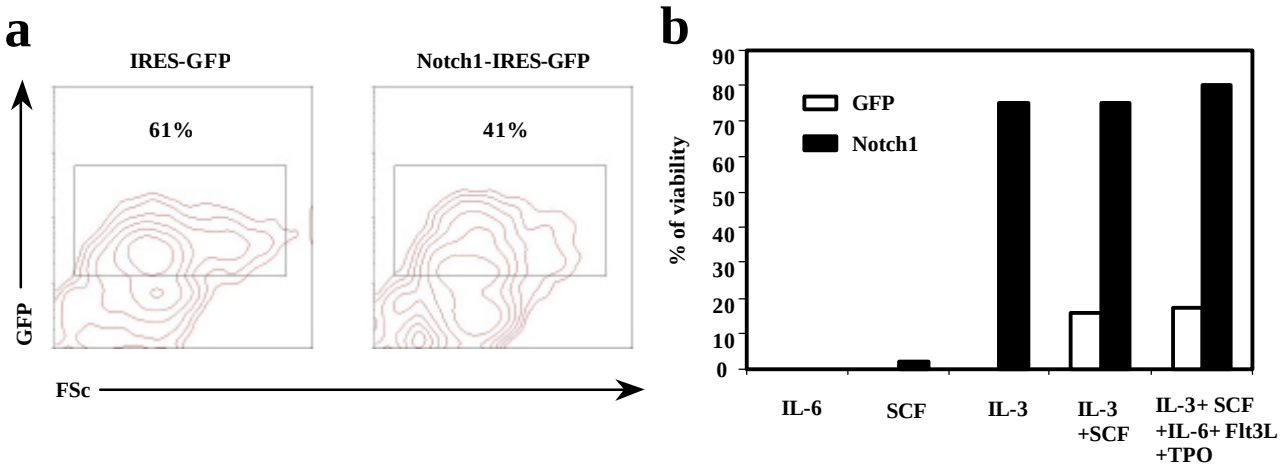

Figure. 4.1.2.2 Generattion of a novel IL-3 dependent cell line, CR1-mix cells.
a. Sca-1+Lin- cells were transduced (MOI 10) with retroviruses encoding either Notch1-IRES-GFP(right) or GFP(left) followed by a 36 hour pre-stimulation with stem cell cytokine cocktail. Transduced cells were analysed for GFP expression by flowcytometer after 78 hours of transduction.
b. The ICN IRES GFP(black bars) and GFP(white bars) transduced cells were cultured under mentioned cytokine combinations to check their dependence on individual cytokines. Cells were harvested 30 days after culture and their viability was calculated after staining the cells with propidium iodide and subsequent flowcytometric analysis.

cells was almost lost (~10%) on day 30 of culture. Next, the dependency and the viability of the ICN transduced Sca-1^{+}lin^{-} cells on individual cytokines of the cytokine cocktail were determined. As the Fig 4.1.2.2.b suggests, the viability of the cells that were cultured only in

the presence of IL-3 or in a combination of IL-3 and SCF was comparable with the viability of the cells that were cultured in a complete cytokine cocktail, however viability was almost lost in the cells that were cultured in the presence of either IL-6 or SCF alone.

These data suggest that the transduction of $Sca1^{+}lin^{-}$ cells with ICN immortalises the cells and that IL-3 is the most important and crucial cytokine for the viability of the ICN transduced $Sca\text{-}1^{+}lin^{-}$ cells. This is in line with the available data that most of the hematopoietic progenitor cells are IL-3 dependent. To check the proliferation potential of the IL-3 dependent cells over a longer time period, the cells were continuously cultured in the presence of IL-3 (10ng/mL). The cells continued to proliferate for more than 6 months. It was concluded that the cells were immortalised and emerged as a cell line which was named as 'CR1-mix'.

4.1.2.2 Genetic and immunophenotypic characterization of CR1-mix

In many immortalized cell lines the "immortal" phenotype is associated with karyotypic abnormalities that could hamper molecular genetic and cell biological investigations of developmental pathways.

To investigate whether the cell line 'CR1- mix' has undergone some detectable chromosome changes such as chromosomal translocation or rearrangement, the cells were subjected to SKY analysis (in collaboration with Prof. Schlegelberger, Institute of Cellular and Molecular Pathology, MHH, Hannover). Spectral karyotyping of 10 metaphases revealed a normal diploid chromosome pattern without any evidence of clonal numerical or structural aberrations (Fig 4.1.2.3). This finding suggests that the karyotype of CR1- mix cells remains stable even 12 months after in vitro propagation.

It was of paramount importance to investigate whether CR1- mix has undergone spontaneous differentiation as a consequence of continuous culture in vitro for more than 12 months. To this end, cells were checked for the expression of surface markers of myeloid (CD11b, GR-1,

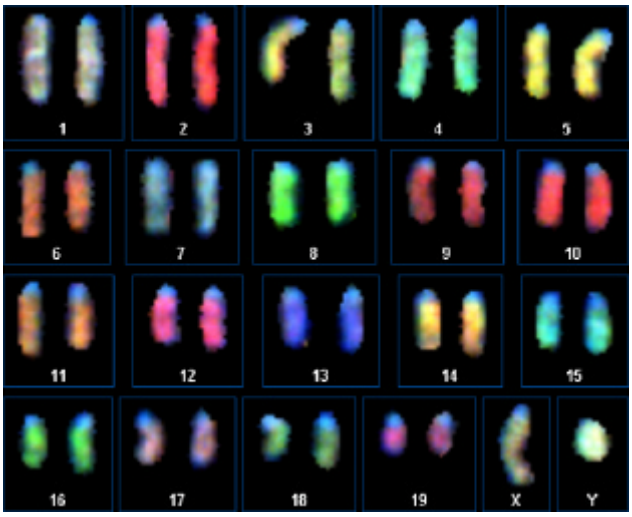

Figure. 4.1.2.3. SKY analysis of CR1-mix cells.
CR1- mix cells were harvested and their metaphase chromosomes were assessed by SKY technique for abnormal chromosome features and genetic recombinations.

CD11c), erythroid (TER119) and lymphoid (B220, CD3e, NK1.1, CD11c) lineages. As inferred from the figure 4.1.2.4, CR1- mix cells were clearly negative for all the lineage markers.

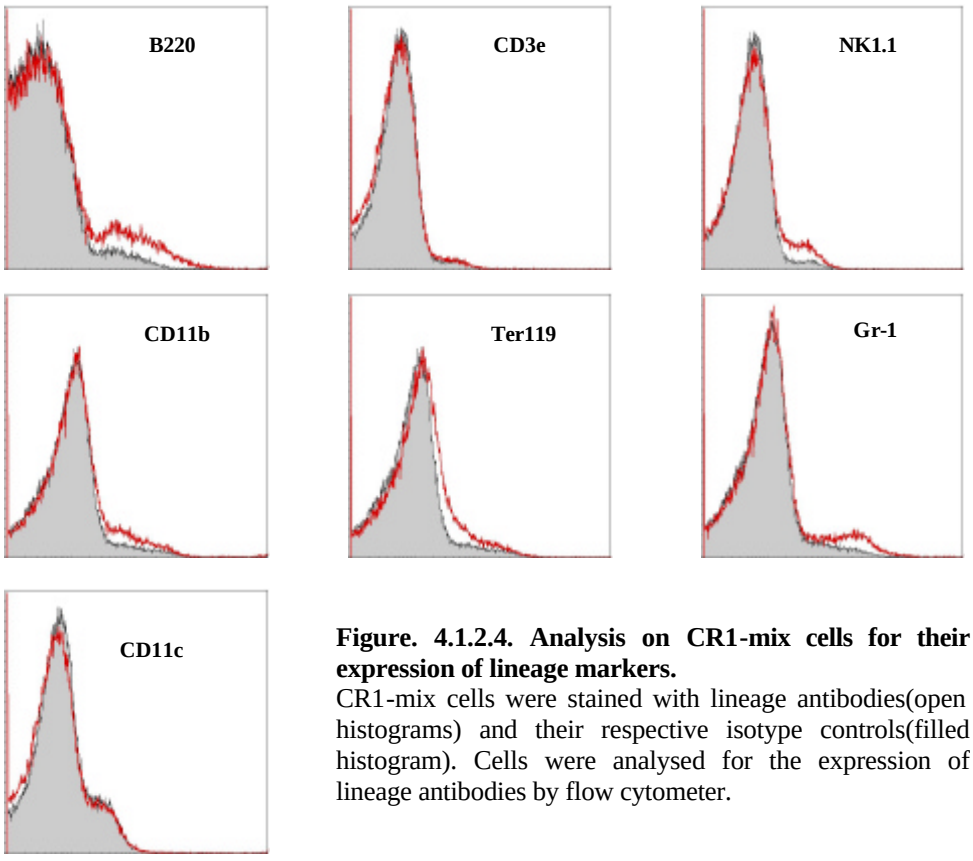

Figure. 4.1.2.4. Analysis on CR1-mix cells for their expression of lineage markers.
CR1-mix cells were stained with lineage antibodies(open histograms) and their respective isotype controls(filled histogram). Cells were analysed for the expression of lineage antibodies by flow cytometer.

In addition, to check whether the CR1- mix cells were maintained in a progenitor state, cells were analysed for their expression of various hematopoietic stem/ progenitor cell markers such as Sca-1, c-kit, Thy1.2, IL-7Rα and Flt3. It was noticed that CR1- mix cells do express hematopoietic stem cell markers Sca-1 (73%), c-kit (92%) Thy1.2 (32%), and lymphoid progenitor markers IL-7Rα(4%) and Thy1.2 (32%) (Fig 4.1.2.5). Moreover, it was noticed that CR1- mix cells also express dendritic and NK cells progenitor marker-Flt3 (10%) in a minor proportion.

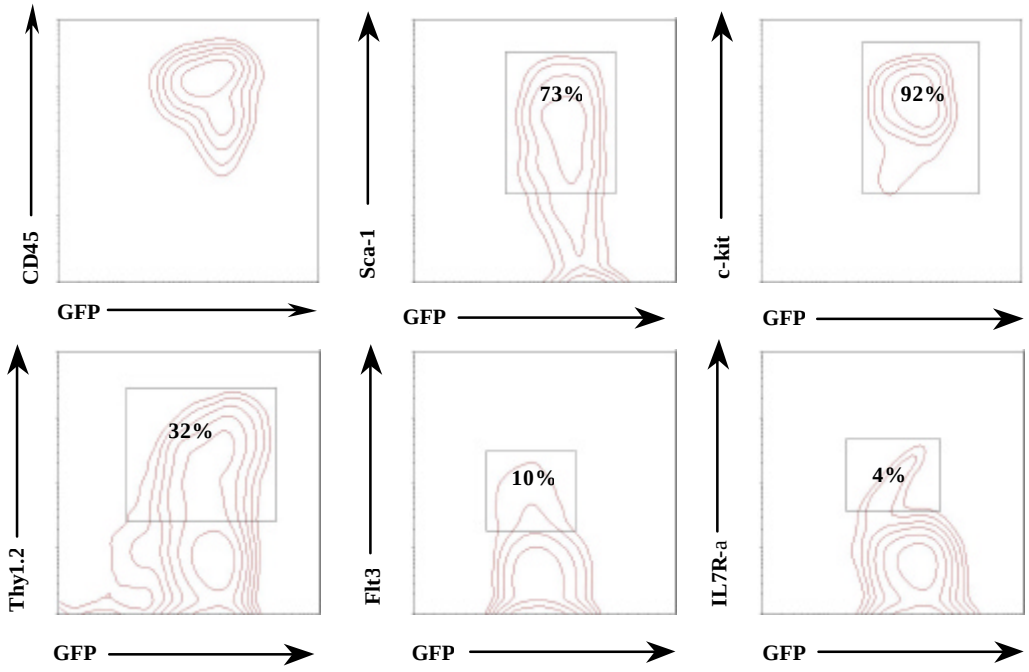

Figure. 4.1.2.5. Immunophenotypic analysis of CR1-mix cells.
Cells were stained with antibodies that recognise mentioned surface markers and analysed by flow cytometer. The gated regions indicate the frequencies of cells expressing the respective marker.

Interestingly, from the above studies, it was noticed that CR1-mix cells represent a heterogeneous cell population that comprises progenitor cells of more than one lineage. Especially the expression level of Sca-1, an antigen that is being primarily expressed by all the hematopoietic stem and progenitor cells, was quite heterogeneous. Based on this observation, it was speculated that this variation in Sca-1 expression could represent a

heterogeneous precursor populations derived from independent clones. In an effort to address this hypothesis, CR1-mix cells were stained with Lineage, Sca-1, c-Kit, and either Thy1.2 or IL-7Rα antibodies and analysed by flow cytometry. Lin^- CR1-mix cells were gated (G1) and the Sca-1, c-kit expression of the gated cells was analysed (Fig 4.1.2.6). Three distinct subsets (G2, G3 and G4) were discriminated based on Sca-1/c-kit expression and the expression levels of IL7Rα and Thy1.2 in each of these subsets was analysed. Data of these experiments

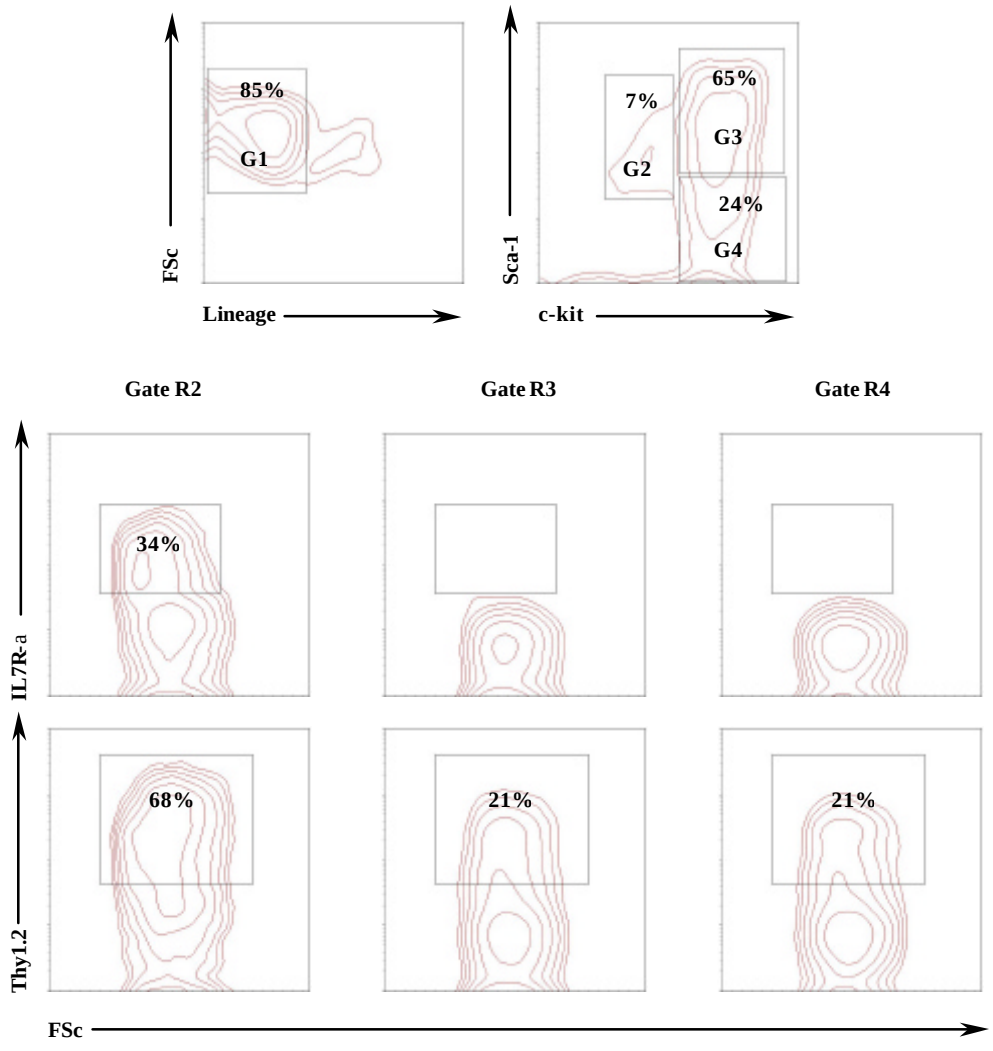

Figure. 4.1.2.6. Analysis of CR1-mix cells for their expression of stem cells/progenitor cell markers.

Cells were stained with lineage, Sca-1, c-kit, IL 7R-a and Thy1.2 antibodies and analysed by flow cytometer. Lineage negative cells were gated (G1) and analysed for Sca-1 c-kit expression. $Sca\text{-}1^{low}$ $c\text{-}kit^{low}$, $Sca\text{-}1^{high}$ $c\text{-}kit^{high}$, $Sca\text{-}1^{neg}$ $c\text{-}kit^{high}$ cells were gated (G1, G2 and G3 respectively) and their expression levels of IL7R-a and Thy1.2 markers were analysed.

identify at least 3 distinct progenitor subsets that can be discriminated as $Lin^{-}Sca\text{-}1^{high}c\text{-}kit^{low}IL7R\alpha^{+}$ $Thy1.2^{high}$, $Lin^{-}Sca\text{-}1^{high}c\text{-}kit^{high}IL7R\alpha^{-}Thy1.2^{+}$ and $Lin^{-}Sca\text{-}1^{low}c\text{-}kit^{high}IL7R\alpha^{-}$ $Thy1.2^{high}$ in the pool of CR1-mix and thus represent a heterogeneous progenitor cell population comprising various maturation stages. Furthermore, the clonality of this cell line is currently being investigated by southern blot analysis.

To check the differentiation potential of CR1-mix in vivo into committed hematopoietic lineages, the CR1-mix cells were transplanted into lethally (9.5 Gy) and sub-lethally (4.5Gy) irradiated CD45.1 congenic recipient mice. During the first three weeks of transplantation, most of the mice from the lethally irradiated group died, presumably due to the inability of CR1-mix cells to provide radioprotection. The sublethally irradiated group was sacrificed after five weeks of transplantation and the cells of bone marrow, spleen and thymus were stained with defined antibodies to recognize the differentiation of CR1-mix into various lineages and analyzed by flow cytometry for the presence of donor derived hematopoietic cells. Interestingly, CR1-mix derived cells (CD45.2) were found (Fig 4.1.2.7) in bone marrow (7-12%), spleen (11-13%), and thymus (32-35%).

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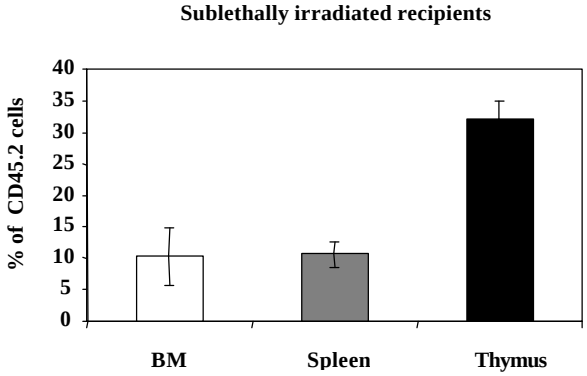


Figure. 4.1.2.7. In vivo proliferation capacity of CR1-mix cells.
CR1-mix cells(1 x10^6) were transplanted(N=3) i.v. into sublethally (4.5Gy) irradiated Ly5.1 recipient mice. Mice were analysed on 6(sublethally irradiated) weeks after transplantation. Bonamarrow(White bars), Spleen(gray bars), and Thymus(black bars) of transplanted mice were sacrificed, single cell suspensions were made, stained with CD45.2 antibody and analysed by flow cytometer. CD45.2 positive cells were gated and their relative frequencies were determined.

The differentiation potential of CR1-mix cells was studied by gating the CD45.2 cells and analyzing the surface marker expression. As inferred from the Fig 4.1.2.8, CR1-mix cells could differentiate into T-cells (CD3ε), B cells (B220), (NK1.1), DCs (CD11c), macrophages

(CD11b, F4/80) and granulocytes (GR-1), however none of the CD45.2 positive cells were found to express erythroid markers, suggesting that ICN-transduced cells fail to differentiate into the erythroid lineage.

In summary, CR1-mix cells represent a genomically stable, pluripotent cell line that is permissive for DC differentiation in vivo.

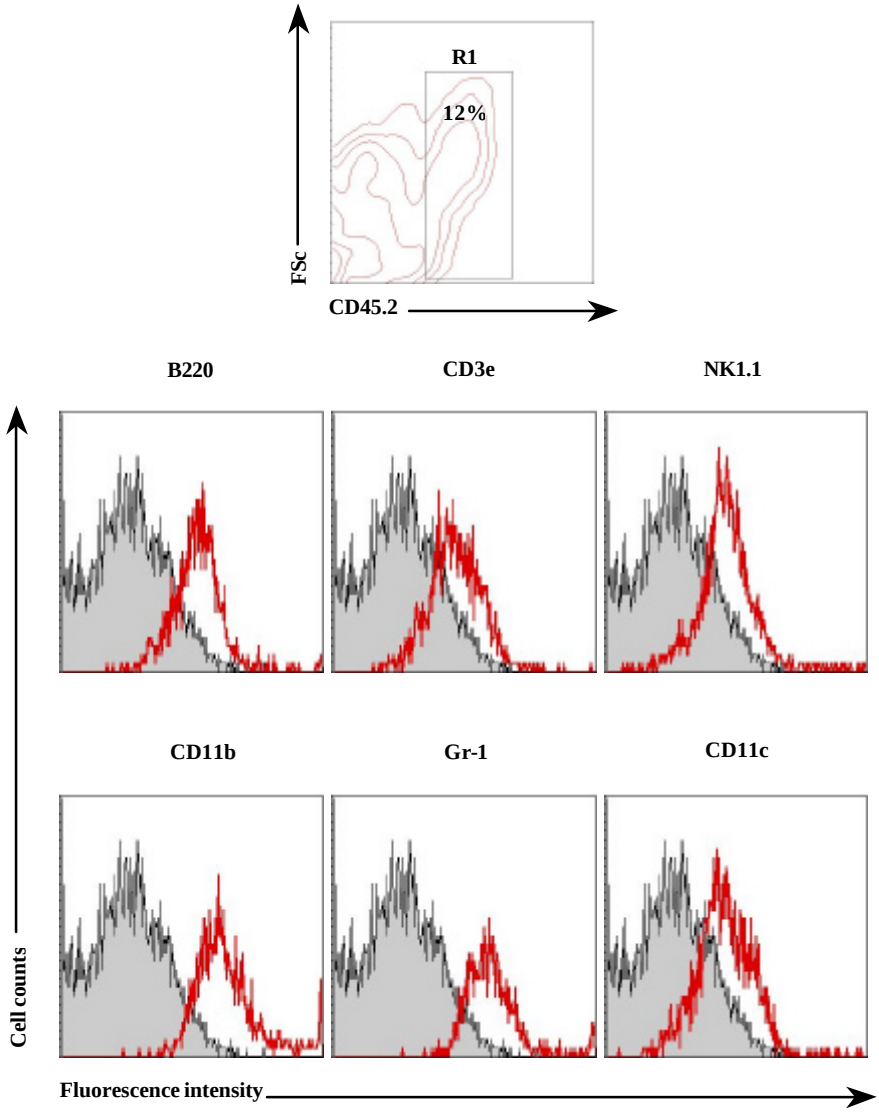


Figure. 4.1.2.8. In vivo differentiation potential of CR1-mix cells into defined hematopoietic lineages.
CR1-mix cells (1 x10^6) were transplanted i.v. into sublethally (4.5Gy) irradiated Ly5.1 recipient mice. Mice were analysed 6 weeks after transplantation. Spleens of transplanted mice were harvested(n=3), single cell suspensions were made, stained with antibodies recognising either defined hematopoietic lineage (open histograms) or their corresponding isotype control (filled histograms) and analysed by flow cytometry.

4.1.2.3 Differentiation of CR1-mix cells into dendritic cells

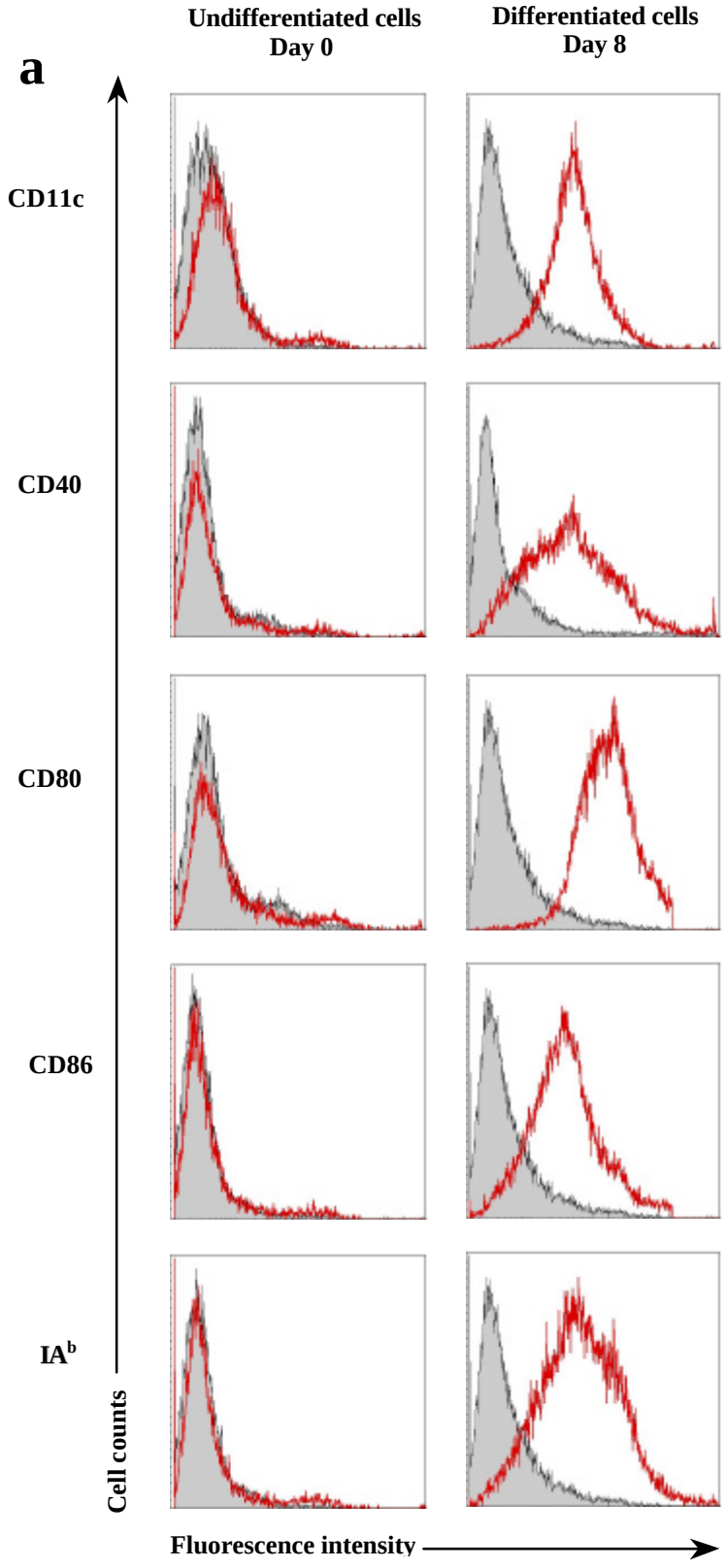
Next, I determined whether CR1-mix cells could be differentiated in DCs in vitro. As the primary aim of making the CR1-mix cell line was to differentiate them into functionally mature dendritic cells, these cells were subjected to various differentiation conditions such as GM-CSF concentration, presence or absence of IL-3, with and without addition of TNF-α.

As CR1-mix cells are IL-3 dependent cells, it was reasonable to check whether the concentration of IL-3 plays crucial role in DC differentiation. It was noticed that at higher concentration of IL-3, CR1-mix cells don't differentiate into dendritic cells, whereas at decreasing concentrations of IL-3, they could clearly differentiate into DCs (data not shown) as documented by the expression of DC specific surface markes (CD11c, CD40, CD80, CD86, MHC Class II, MHC Class I upregulation).

In contrast to FDCP-mix cells, CR1-mix cells could efficiently differentiate into DCs in the absence of IL-3. Therefore, in all subsequent experiments the DC differentiation experiments CR1- mix cells were cultured in the absence of IL-3. Fig 4.1.2.9.a compares the DC specific surface marker expression in undifferentiated CR1-mix (left panels) with CR1-mix derived dendritic cells (right panels) after culturing them in the culture medium containing GM-CSF in absence of IL3 for 8 days. Fig 4.1.2.9.b compares the morphology of undifferentiated CR1-mix with CR1-mix derived dendritic cells. Taken together, these results suggest that CR1-mix cells could be differentiated into dendritic cells upon exposure to GM-CSF in vitro and thus represent an ideal cell line to study molecular changes governing DC differentiation.

An alternative differentiation pathway into DCs is controlled by the cytokine Flt3L. Flt3L induces differentiation of plasmacytoid DCs in vitro. Interestingly, it was noticed that CR1-mix cells could also efficiently differentiate into plasmacytoid DCs in vitro. More than 95% of the DCs generated in the presence of GM-CSF and IL-4 showed morphological and

immunophenotypical features of myeloid DCs (expression of the cell surface markers MHC-II, CD11c, CD40, CD80 and CD86), whereas 70% of DCs selectively grown in the presence



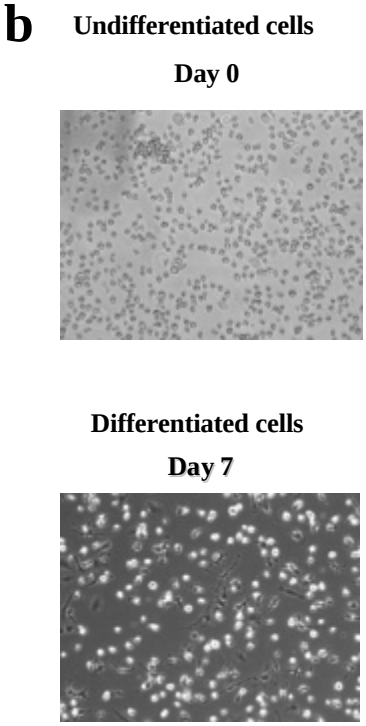


Figure. 4.1.2.9. In vitro differentiation of CR1-mix cells into DCs.
a. CR1-mix cells were cultured in the presence of GM-CSF for 7 days. On day 8, cells were harvested and stained with DC specific antibodies and analysed flow cytometer. Undifferentiated CR1-mix cells were used as controls.
b. Morphological studies of in vitro differentiated DCs. CR1-mix derived DCs (bottom panels) were visualized under the microscope (100x magnification). As controls undifferentiated CR1-mix cells (top panels) were used.

of Flt3L co-expressed CD11c and B220, a phenotype that characterises plasmocytoid DCs (Fig 4.1.2.10).

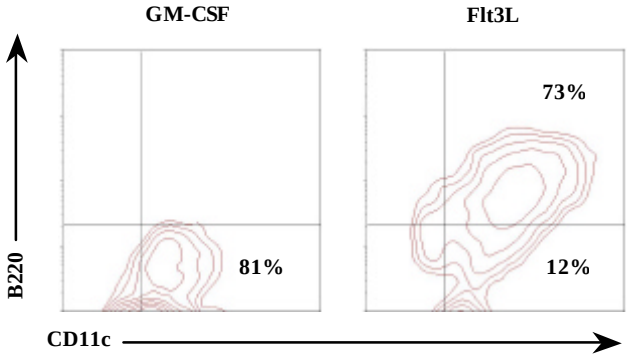


Figure. 4.1.2.10. In vitro differentiation of CR1-mix cells into pDCs. CR1-mix cells were cultured in the presence of either GM-CSF (left panels) or Flt3L (right panels) for 10 days. Cells were harvested on day 11, stained with pDC specific antibodies and analysed by flow cytometer.

To functionally characterise the in vitro generated CR1-mix derived DCs and to compare them with the bone marrow derived primary DCs, the ability of CR1-mix derived myeloid dendritic cells to stimulate allogenic T cells was assessed. CR1-mix derived DCs and BM derived DCs were irradiated (30 Gy) and cultured with allogenic T cells in an increasing ratio of stimulators (DCs) to effectors (T cells) for 48 hours. Cells were further incubated for 12 hours after adding the radio labelled thymidine(^{3}H). The proliferation of T cells was studied by measuring the incorporated radioactive thymidine by scintillation counting. Fig 4.1.2.11 indicates that CR1-mix derived DCs exhibit high stimulatory potential to induce allogenic T cell proliferation and the T cell stimulation capacity of both bone marrow derived DCs and CR1- mix derived DCs are comparable (data not shown).

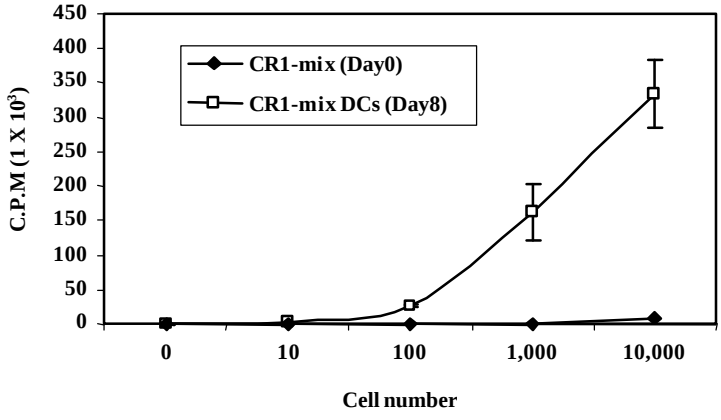


Figure. 4.1.2.11 Allogenic T cell stimulation capacity of CR1- mix derived DCs.
CR1-mix cells were differentiated in the presence of GM-CSF for 7 days. Increasing numbers of irradiated DCs were incubated with allogenic lymph node cells (1 x 10^5) for 48 hours. Following a 12 hour pulse with ^{3}H thymidine, the proliferating T cells were measured. Un stimulated CR1-mix cells were used as controls.

Furthermore, the ability of CR1-mix derived dendritic cells to secrete IL12p70 upon TNF-α exposure was examined. Both CR1-mix derived dendritic cells and BM derived dendritic cells were differentiated in vitro and stimulated for 48 hours in the presence of TNF-α. Supernatant of the cells was collected and ELISA was performed. As represented in the Fig 4.1.2.12, there is a 10-fold induction of IL12p70-secretion by CR1-mix cells upon stimulation with TNF-α, moreover the quantity of IL-12p70 was comparable in both primary and CR1-mix-derived DCs.

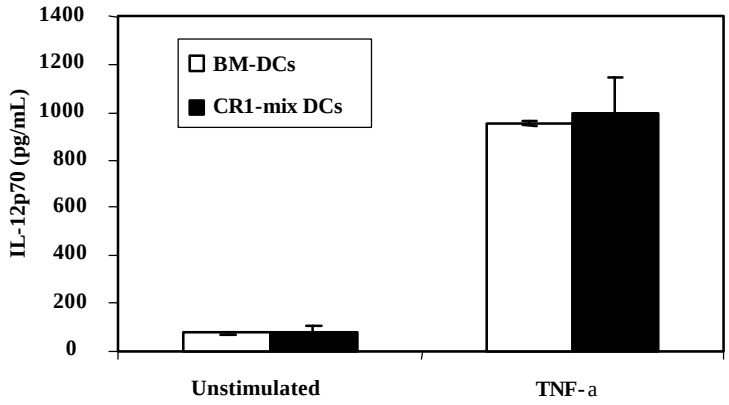


Figure. 4.1.2.12. IL12 secretion by CR1-mix derived DCs. CR1-mix cells were differentiated in the presence of GM-CSF for 7 days. On day 8 cells were stimulated with TNF-α for 48 hours and the supernatant was collected. ELISA was performed to quantify the IL12 secretion. The IL12 secretion profile of CR1- mix derived DCs was compared with bone marrow derived DCs. Unstimulated cells were used as controls.

Hence it was concluded that constitutive notch 1 expression immortalises hematopoietic cells with a DC differentiation potential. Additionally, the thus obtained DCs are morphologically, immunophenotypically and functionally comparable with the primary bone marrow derived DCs.

In summary, the present studies yielded both a conventional cell line (FDCP-mix) and a novel and optimised cell line (CR1-mix) that were available for the study of GM-CSF dependent transcription factor up regulation during DC differentiation in vitro.

4.2.1.1 Microarray analyses to identify transcription factor controlled early DC development in FDCP-mix cells.

To identify the master regulators that control dendritic cell development, microarray analyses were performed with the murine hematopoietic progenitor cell line FDCP-mix that is permissive for DC differentiation in the presence of GM-CSF. It was reasoned that GM-CSF modulates a defined set of decisive transcription factors governing DC differentiation during the early stages. To this end, total RNA from FDCP- mix was isolated in duplicates at hours 0, 6, 24 and 48 of the in-vitro differentiation system after addition of GM-CSF (100 u/ml). The extracted RNA was hybridised in duplicates to Affymetrix GeneChips® and analysed by K-means cluster analysis. Fig 4.2.1.1 represents cluster analysis of the genes that were upregulated during DC differentiation at early time points.

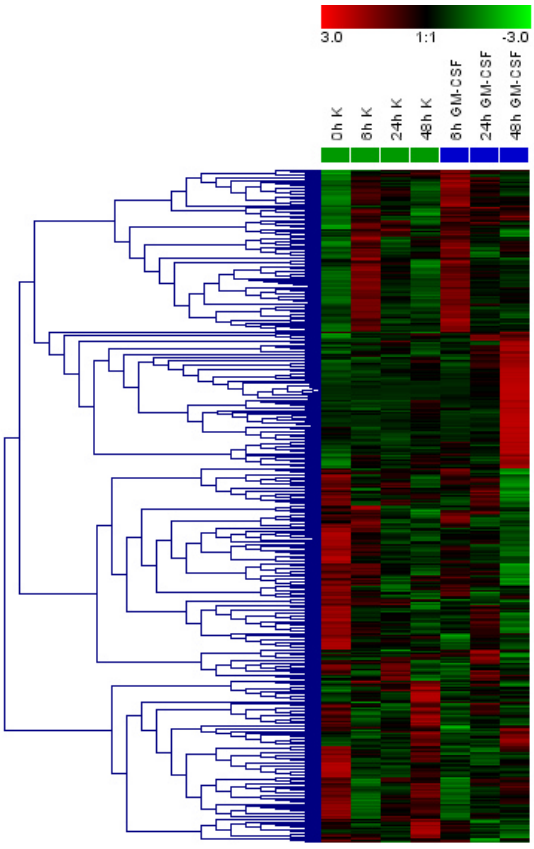


Figure. 4.2.1.1. Cluster analysis of differentially expressed genes.
Graphic representation of expression profile analysis of selected transcription factors up regulated in GM-CSF-treated FDCP-mix cells. Relative expression (normalized to the median) is displayed as color (green = normalized expression level *below*, black = *near to*, and red = *above the median*). Fold Change (hour 48 versus hour 0) is calculated by Affymetrix MAS 5.0 and p-values are based on statistical parameters as described for MAS 5.0 software.

A certain discrepancy has been noticed between the results of the duplicates at all chosen time points except at hour 48. It was believed that this discrepancy could be due to the

presence of contaminating granulocytes and macrophages in addition to DCs. However, taken together at 6 hours 17 genes were upregulated and 10 genes were downregulated, at 24 hours 57 genes were upregulated and 15 genes were downregulated, and at 48 hours 263 genes were upregulated and 116 genes were downregulated.

The goal of the study was centered the question to identify the transcription factors controling of DC differentiation. Therefore, a special focus was given to transcription factors that were upregulated at the given time points. A total of 28 transcription factors, (3 at 6 hours, 7 at 24 hours and 18 at 48 hours) were found to be upregulated (table 4.2.1.1) from the total list of 337 upregulated genes at all time points.

Table 4.2.1.1. List of transcription factors upregulated at chosen time points during DC differentiation

No	Name of the transcription factors	Fold change	Time points
1	Histone deacetylase 1	2.4	6 hrs
2	Hox 2.4	1.84	6 hrs
3	Zinc ring finger protein 1	2.72	6 hrs
4	Activating transcription factor 4	1.59	24hrs
5	Zinc finger protein, subfamily 1A, 1 (Ikaros)	2.5	24hrs
6	Leukemia/lymphoma related factor	7.38	24hrs
7	General transcription factor II I	2.02	24hrs
8	Pre B-cell leukemia transcription factor 3	7.8	24hrs
9	Homeo box, msh-like 1	3.36	24hrs
10	GA repeat binding protein, alpha	6.81	24hrs
11	Inhibitor of DNA binding 1	2.66	48 hrs
12	Breakpoint cluster region protein 1	1.77	48hrs
13	Recombination activating gene 2	10.8	48hrs
14	CCAAT/enhancer binding protein (C/EBP), delta	2.9	48hrs
15	Histone 4 protein	2.09	48hrs
16	POU domain, class 2, associating factor 1	3.22	48hrs
17	Friend leukemia integration 1	1.89	48hrs
18	Mini chromosome maintenance deficient 5 (S. cerevisiae)	1.54	48hrs
19	B-cell leukemia/lymphoma 2 related protein A1b(BCl2 A1b)	12.1	48hrs
20	Growth factor independent 1	2.21	48hrs
21	RNA binding motif protein, X chromosome retrogene	1.65	48hrs
22	Mini chromosome maintenance deficient (S.cerevisiae)	1.57	48hrs
23	Gene rich cluster, C2f gene	1.61	48hrs
24	RNA and export factor binding protein 1	1.55	48hrs
25	CCAAT/enhancer binding protein (C/EBP), delta	2.31	48hrs
26	Splicing factor 3a, subunit 2, 66kD	1.55	48hrs
27	CCAAT/enhancer binding protein (C/EBP), beta	1.83	48hrs
28	Cold shock domain protein A	1.81	48hrs

4.2.1.2 Real Time PCR analysis to confirm the expression status of the transcription factors in FDCP-mix cells.

In order to validate the microarray result and to confirm the expression status of the identified transcription factors, real time PCR analysis was carried out for all 28-transcription factors upregulated as by microarray analysis. In contrast to the microarray data, the real time PCR data showed an up regulation of only 6 transcription factors (Fig 4.2.1.2) out of 28 chosen candidates, namely Histone Deacetylase1 (6hours), General transcription factor II I (24 hrs),

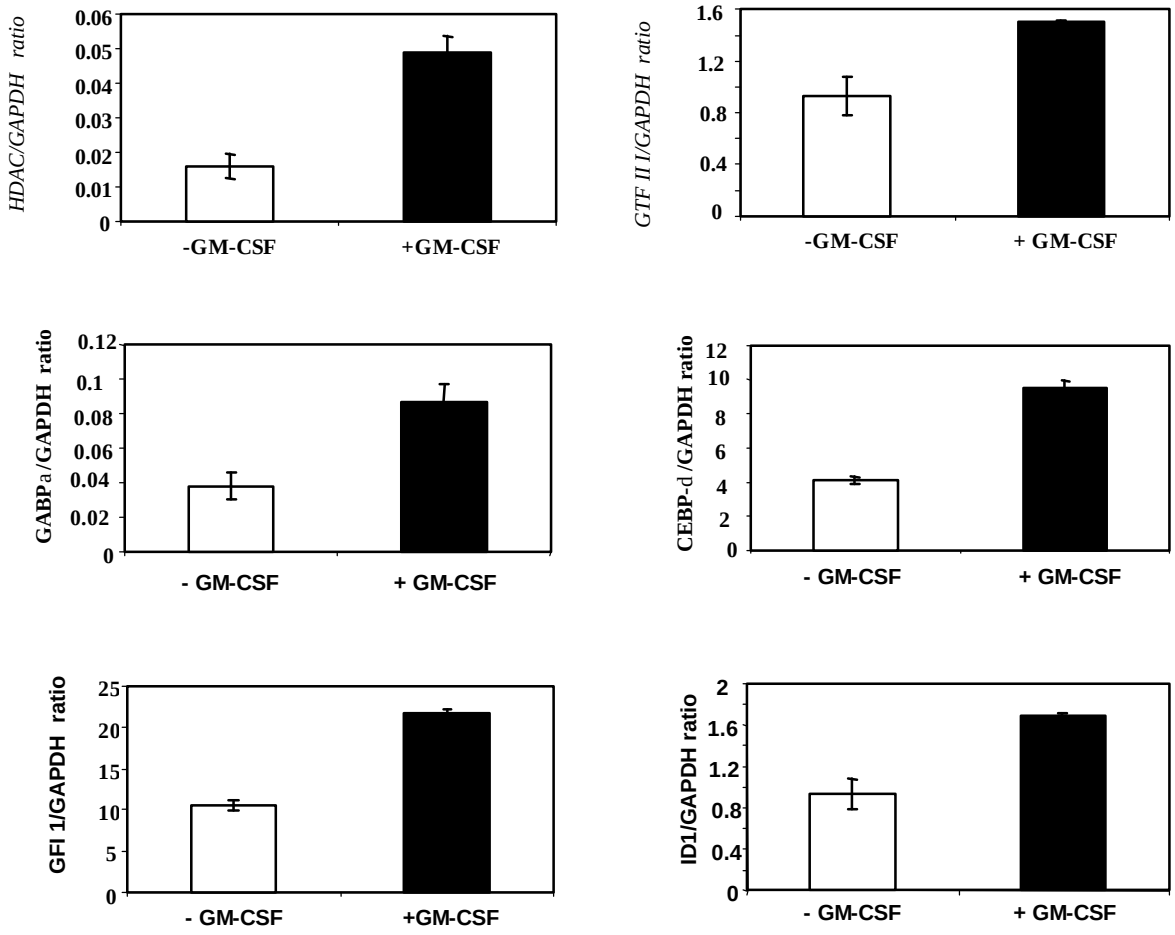


Figure. 4.2.1.2. Real time PCR analysis on candidate transcription factors upregulated during FDCPmix derived DC development.

FDCP cells were differentiated in vitro into DCs in the presence of GM-CSF and IL-4. During early time points of differentiation, cells were harvested and RNA was extracted. cDNA synthesis was followed by a semi quantification of the target transcripts using light cycler Real Time PCR approach. The expression status of the transcription factors in cells that were cultured in the presence of GM-CSF(black bars) was compared with those that were cultured in the absence of GM-CSF(white bars).

Deacetylase1 (6hours), General transcription factor II I (24 hrs), GAAT enhancer binding protein –α(24 hrs), Inhibitor of DNA binding 1(48hrs), Growth factor independent 1(48hrs), CAAT Enhancer Binding Protein δ(48 hrs).

4.2.2.1 Microarray analyses to identify transcription factor controlled early DC development of CR1-mix cells.

Due to the inconsistency of the microarray results obtained from the duplicate samples of FDCP- mix cells, the microarray analysis was repeated using the newly generated cell line CR1-mix. As mentioned above, RNA was extracted from CR1-mix cells during early stages of DC differentiation (6 hours, 24 hours and 48 hours after addition of GM-CSF). The extracted RNA was hybridized to Affymetrix Gene Chips and analyzed by K-means cluster analysis. In summary, 628 genes were up regulated at 6, 24, and 48 hours respectively. Out of 58 transcription factors identified 9 were up regulated at 6 hours (Table 4.2.1.2), 20 were up regulated at 24 hours(Table 4.2.1.3) and 29 were up regulated at 48 hours (Table 4.2.1.4).

Table 4.2.1.2. List of transcription factor upregulated after 6 hours of GM-CSF stimulation in CR1-mix cells

No	Name of the transcription factor	Gene symbol
1	RNA binding motif protein 6	Rbm6
2	Special AT-rich sequence binding protein 1	Satb1
3	SRY-box containing gene 4	Sox4
4	Transcriptional regulator, SIN3B (yeast)	Sin3b
5	RIKEN cDNA 2210412K09 gene	2210412K09Rik
6	Kruppel-like factor 7 (ubiquitous)	Klf7
7	homeo box A9	Hoxa9
8	Signal transducer and activator of transcription 5B	Stat5b
9	Hypothetical protein MGC18736	MGC18736

Table 4.2.1.3. List of transcription factor upregulated after 24 hours of GM-CSF stimulation in CR1-mix cells

No	Name of the transcription factor	Gene symbol
1	Ngfi-A binding protein 2	Nab2
2	Early growth response 1	Egr1
3	Interferon regulatory factor 7	Irf7
4	Pleiomorphic adenoma gene-like 2	Plagl2
5	Tripartite motif protein 30	Trim30
6	H2.0-like homeo box gene	Hlx
7	Promyelocytic leukemia	Pml
8	Signal transducer and activator of transcription 1	Stat1
9	Zinc finger protein 36, C3H type-like 1	Zfp36l1
10	DNA segment, Chr 16, ERATO Doi 465	D16Ertd465e
11	Avian reticuloendotheliosis viral (v-rel) oncogene related B	Relb
12	Retinoblastoma-like 2	Rbl2
13	Nucleobindin 2	Nucb2
14	Transcription factor EC	Tcfec
15	Amyloid beta (A4) precursor-like protein 2	Aplp2
16	EST AI256856	AI256856
17	DNA (cytosine-5-)-methyltransferase 3-like	Dnmt3l
18	Early growth response 2	Egr2
19	Ngfi-A binding protein 2	Nab2
20	LIM only 4	Lmo4

Table 4.2.1.4. List of transcription factor upregulated after 48 hours of GM-CSF stimulation in CR1-mix cells

No	Name of the transcription factor	Gene symbol
1	Juxn-B oncogene	Junb
2	DNA segment, Chr 16, ERATO Doi 465, expressed	D16Ertd465e
3	Nuclear receptor subfamily 4, group A, member 1	Nr4a1
4	Nuclear, factor, erythroid derived 2, like 2	Nfe2l2
5	Interferon concensus sequence binding protein	Icsbp
6	Early growth response 1	Egr1
7	Interferon regulatory factor 7	Irf7
8	Jun oncogene	Jun
9	Avian reticuloendotheliosis viral (v-rel) oncogene related B	Relb
10	SFFV proviral integration 1	Sfpi1
11	H2.0-like homeo box gene	Hlx
12	Transcription factor EC	Tcfec
13	Ngfi-A binding protein 2	Nab2
14	Tripartite motif protein 30	Trim30
15	Notch gene homolog 1, (Drosophila)	Notch1
16	CCAAT/enhancer binding protein (C/EBP), beta	Cebpb
17	cAMP responsive element binding protein 3	Creb3
18	B-cell leukemia/lymphoma 6	Bcl6
19	DNA methyltransferase 3A	Dnmt3a
20	DNA segment, Chr 16, ERATO Doi 465, expressed	D16Ertd465e
21	E26 avian leukemia oncogene 2, 3' domain	Ets2
22	Interferon activated gene 204	Ifi204
23	Forkhead box J2	Foxj2
24	AT motif binding factor 1	Atbf1
25	Nuclear factor of activated T-cells, cytoplasmic 1	Nfatc1
26	Early growth response 2	Egr2
27	Nuclear factor of activated T-cells 5	Nfat5
28	Zinc finger protein 36, C3H type-like 1	Zfp36l1
29	Transcriptional regulator, SIN3B (yeast)	Sin3b

4.2.2.2 Real Time PCR analysis to confirm the expression of the candidate transcription factors identified in CR1-mix

Real Time PCR studies were done in order to reconfirm the expression status of the transcription factors that were identified from the global screening using microarrays. An initial screen for transcription factors from the up regulated genes has listed a total of 58 transcription factors.

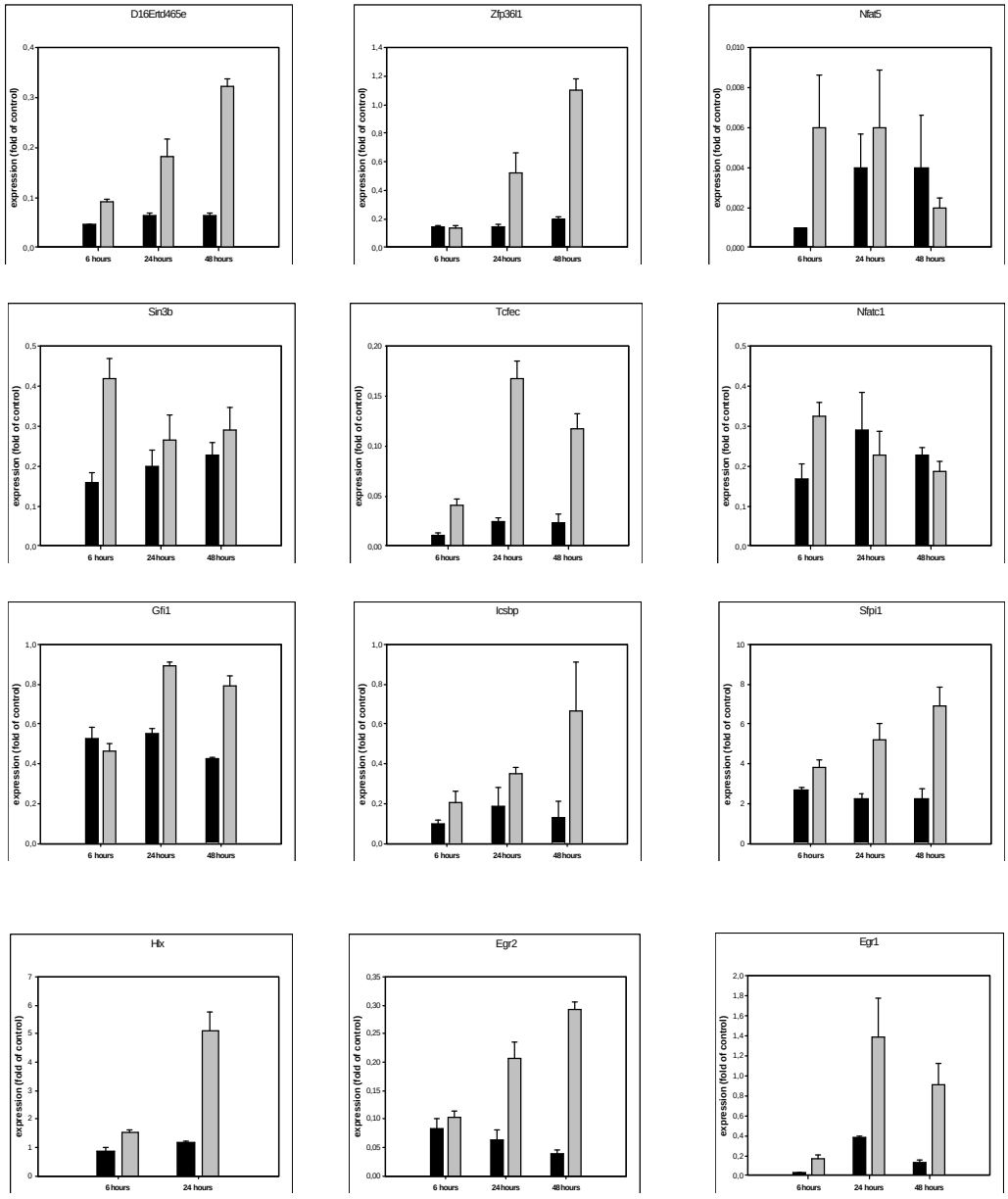


Figure. 4.2.1.3: Real time PCR analysis on candidate transcription factors upregulated during CR1-mix derived DC development.

CR1-mix cells were differentiated in vitro into DCs in the presence of GM-CSF. During early time points of differentiation cells were harvested and RNA was extracted. cDNA synthesis was followed by a semi quantification of the target transcripts using light cycler Real Time PCR approach. Black bars represent the expression of the respective transcription factors in the absence of GM-CSF, while the gray bars represent the

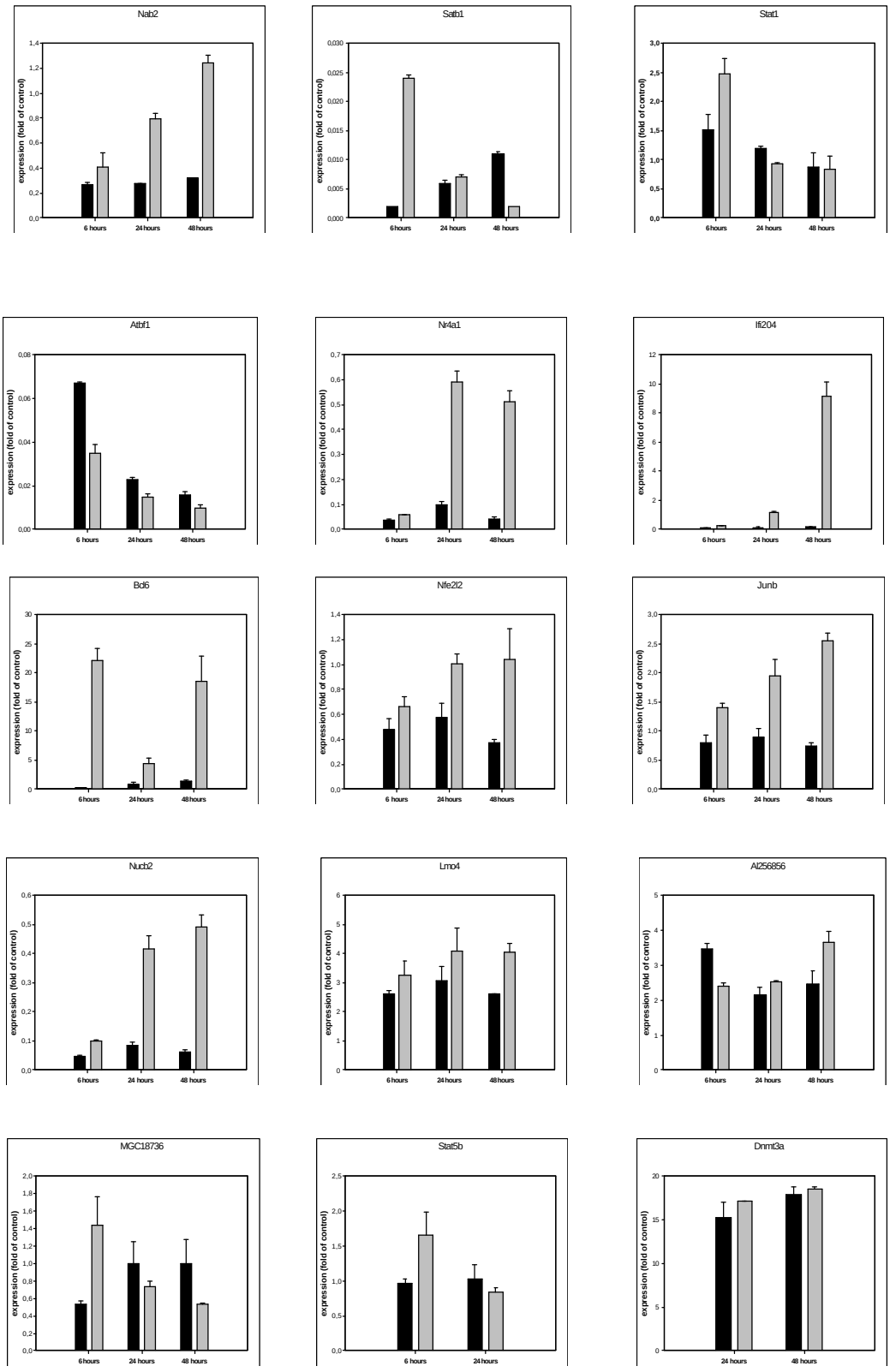


Figure. 4.2.1.3: Real time PCR analysis on candidate transcription factors upregulated during CR1- mix derived DC development.

CR1- mix cells were differentiated in vitro into DCs in the presence of GM-CSF. During early time points of differentiation cells were harvested and RNA was extracted. cDNA synthesis was followed by a semi quantification of the target transcripts using light cycler real time PCR approach. Black bars represent the expression of the respective transcription

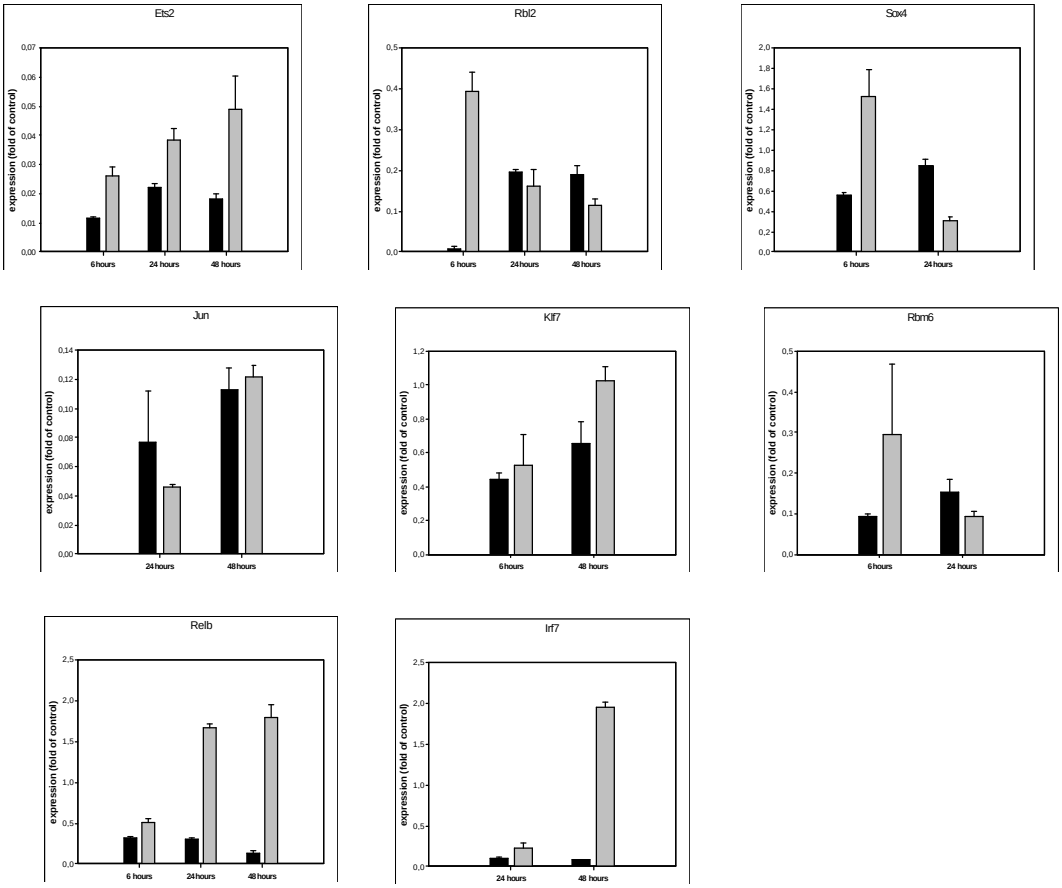


Figure. 4.2.1.3: Real time PCR analysis on candidate transcription factors upregulated during CR1- mix derived DC development.
CR1- mix cells were differentiated in vitro into DCs in the presence of GM-CSF. During early time points of differentiation cells were harvested and RNA was extracted. cDNA synthesis was followed by a semi quantification of the target transcripts using light cycler real time PCR approach. Black bars represent the expression of the respective transcription factors in the absence of GM-CSF, while the gray bars represent the expression in the

An initial pre-screening was done based on the signal intensity to choose only those genes that have a high probability of upregulation. A total of 35 transcription factors with good signal intensities (with signal filters denoted by letter A) were chosen for the Real Time PCR studies. The results of the Real Time PCR (Fig 4.2.1.3) confirmed the upregulation of 27-transcription factor at mentioned time points.

Hence, the undertaken transcriptional profiling studies in FDCP mix cell line have identified the involvement of 6 transcription factors. The analysis of CR1-mix cells yielded 27 transcription factors during the early time points of DC differentiation.

4. 3. 1. Transcriptional activity of the Gfi1 locus in precursor and mature DC

For further studies, I chose to focus on a single transcription factor and assess its role in great detail. The transcriptional repressor Gfi1 was an interesting candidate, since its role in DC development has not been reported. Gfi1 knockout mice could be obtained from Prof. Tarik Möröy, University Hospital Essen. In addition to the Gfi1 knockout mice, a novel $Gfi1^{GFP/-}$ knockin mouse has been generated by this laboratory.

The GM-CSF-dependent Gfi1 upregulation was further confirmed by RT-PCR in CR1-mix and primary $Sca1^{+}lin^{-}$ hematopoietic progenitor cells (Fig.4.3.1.1).

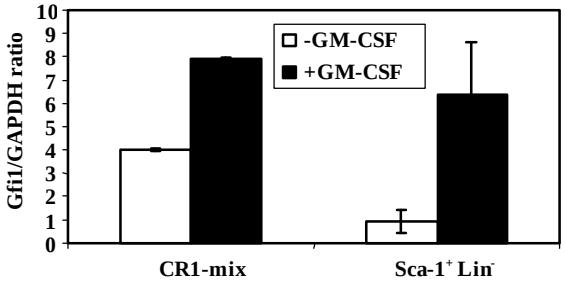


Figure 4.3.1.1. Quantification of Gfi1 expression in CR1-mix and $Sca1^{+}in^{-}$ cells.
CR1-mix and $Sca\text{-}1^{+}lin^{-}$ hematopoietic progenitor cells at hour 0(white bars) and 48 hours(black bars) after GM-CSF treatment. Shown are the mean values of duplicate samples. Data are representative of 2 independent experiments.

To assess the physiological expression pattern of Gfi1 in DC, the transgenic Gfi1: GFP knock-in mutant mouse system was used. The GFP expression profile in DC development in vitro over time was analyzed. Lineage-depleted hematopoietic progenitor cells from $Gfi1^{+/+}$, $Gfi1^{GFP/+}$ and $Gfi1^{GFP/GFP}$ mice were purified and the cells were incubated in the presence of either recombinant murine GM-CSF or recombinant human Flt3L, two cytokines controlling DC differentiation. Aliquots of cells were analyzed for GFP expression by flow cytometry every 6-12 hours.

Expression of GFP revealed transcriptional activation of the Gfi1 locus during early phases of DC development and a decline at later phases (Fig. 4.3.1.2.a**).** However, only in the case of GM-CSF a marked increase in the fluorescence intensity during hours 48-72 was observed. In homozygous $Gfi1^{GFP/GFP}$ progenitor cells, the levels of fluorescence intensity were generally

higher and followed a defined pattern over time (Fig. 4.3.1.2.b). This finding might reflect Gfi1 autoinhibition in DC development (Raif. et al., 2004), in analogy to a documented role of Gfi1 autoinhibition in T cell development.

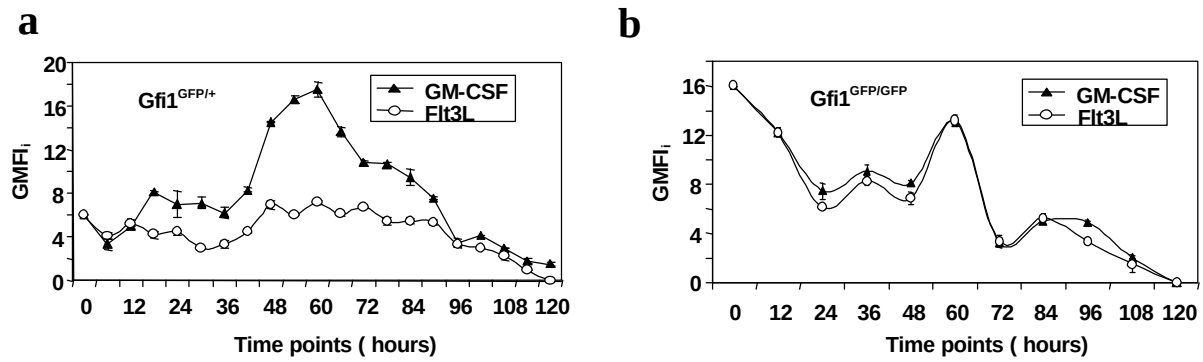


Figure4.3.1.2. Gfi1 expression in DC development in vitro.
Lineage-marker depleted bone marrow cells from $Gfi1^{+/GFP}$ mice (a) and $Gfi1^{GFP/GFP}$ mice (b) were cultured either in the presence of GM-CSF and IL-4 or Flt3L, respectively. Cells were harvested every 6-12 hours and their fluorescence was determined by FACS analysis. Shown is the specific geometric mean fluorescence intensity index calculated as follows: $GMFI_i = GMFI_{(Gfi1^{+/GFP})} - GMFI_{(Gfi1^{+/+})}$. Results represent the average values of duplicate samples. Data are representative of 3 independent experiments.

To investigate the expression pattern of Gfi1 in differentiated DCs in vivo, the fluorescence intensity of various $CD11c^+$ DC subpopulations in lymphoid organs was determined by comparing $Gfi1^{GFP/+}$ and $Gfi1^{+/+}$ mice. GFP expression was detected at various intensities in various subpopulations ($CD4^+$, $CD8^+$, $CD4^-CD8^-$, $CD11b^+$, $B220^+$) of $CD11c^+$ DCs obtained from spleen, thymus and lymph nodes (Fig. 4.3.1.3). It was hypothesized that heterogeneity in GFP expression in vivo might depend on the activation status of DCs.

To address this question, the $Gfi1^{GFP/+}$ mice were injected with LPS or stimulating anti-CD40 monoclonal antibodies in the peritoneum and the mice were sacrificed 24 hours later. Single cell suspensions were made from spleens and lymph nodes. Compared to $Gfi1^{GFP/+}$ DCs isolated from mice treated with PBS, GFP-expression was enhanced in $Gfi1^{GFP/+}$ DCs obtained from mice treated with LPS or anti-CD40 monoclonal antibodies (Fig. 4.3.1.4-top panels). Furthermore the expression of Gfi1 in DCs upon activation in vitro was assessed by isolating splenic $CD11c^+$ cells by MACS and cultured in vitro for 24 hours after the addition of specific stimuli LPS and αCD40 monoclonal antibodies.

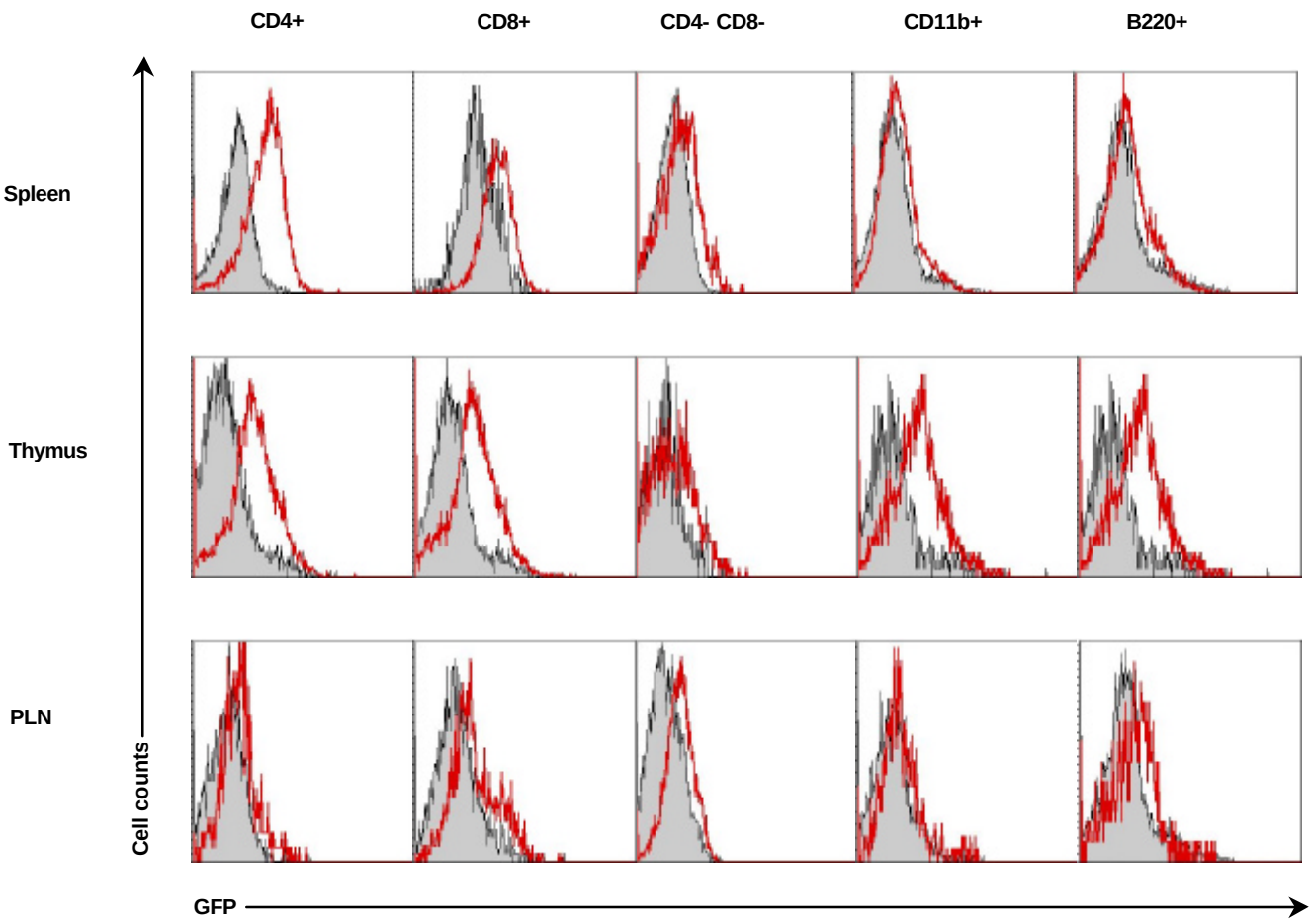


Figure4.3.1.3. Gfi1 expression in various DC compartments in vivo.
Single cell suspensions from spleen(top panels), thymus(middle panels) and lymphnodes(bottom panels) of $Gfi1^{+/GFP}$ mice were stained with antibodies recognising different DC subsets and analysed by flow cytometer. Distinct DC compartments were gated and analyzed for GFP expression (open histogram). Shaded histograms represent the autofluorescence of $Gfi1^{+/+}$ DCs used as controls. In each experiment, organs from 3-5 mice were pooled. Data are representative of 3 independent experiments.

Cells were harvested and GFP expression was measured by flowcytometry. $Gfi1^{GFP/+}$ DCs cultured in the absence of an external stimulus was taken for the baseline Gfi1 expression. Compared to unstimulated DCs, GFP-expression was enhanced in DCs cultured in the presence of either LPS or anti-CD40 monoclonal antibodies (Fig. 4.3.1.4-bottom panels). The data of these experiments suggest that Gfi1 expression is physiologically increased upon DC activation in vivo.

To validate these in vitro and in vivo findings, GFP expression in DC progenitor cells in $Gfi1^{GFP/+}$ mice in vivo was assessed. GFP expression was noticed in both progenitor DCs

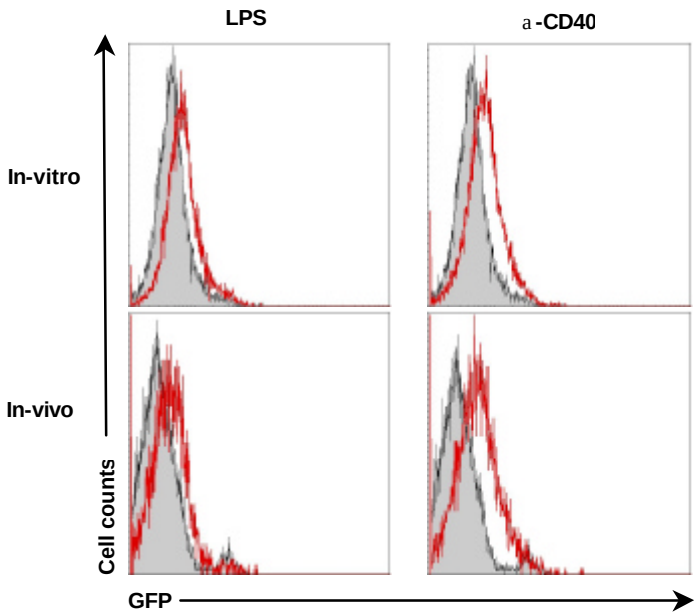


Figure 4.3.1.4. Enhanced transcriptional activity upon DC stimulation in vivo. Gfi1$^{+/GFP}$ mice were i.p. injected with LPS or anti-CD40 monoclonal antibodies and DC fluorescence was measured by flow cytometry 24 hours later (open histograms). Shaded histograms represent the DC fluorescence from PBS-treated Gfi1$^{+/GFP}$ mice.

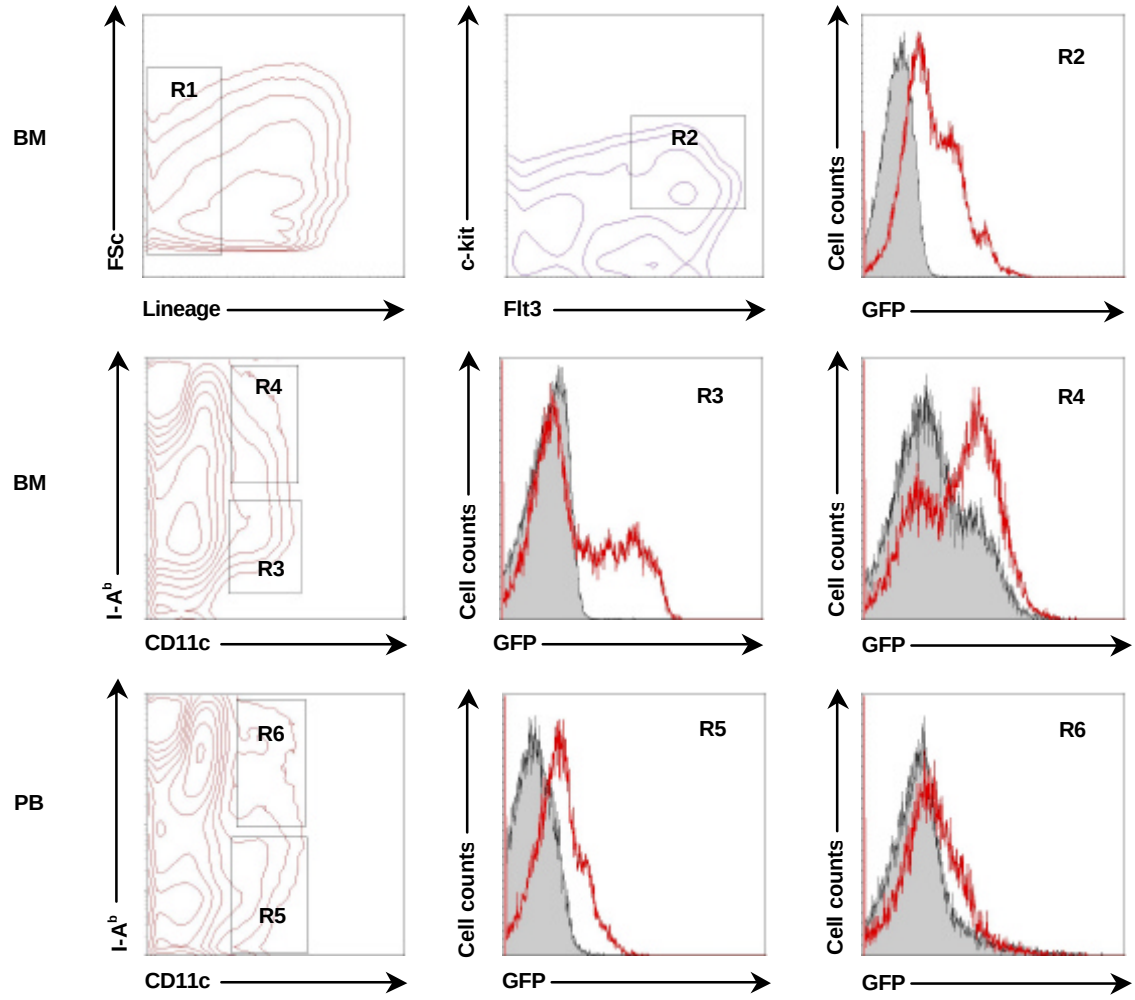


Figure4.3.1.5. Gfi1 expression in various DC compartments in vivo. Gfi1 expression in precursor DCs. Pooled bone marrow cells from 3-5 mice were analyzed for GFP expression in Lin^{-}c-kit^{+}Flt3^{+} cells (top panelss)of bone marrow and CD11c^{+}I-A^{b-}/ CD11c^{+}I-A^{b+} cells of bone marrow (middle panels) and peripheral blood (bottom panels). Open histograms represents GFP fluorescence in Gfi1$^{+/GFP}$ cells, shaded histogram represents autofluorescence of Gfi1$^{+/+}$ cells. Data are representative of 3 independent experiments.

(CD11c^{+}MHC-ClassII^{-}) as well as differentiated DCs (CD11c^{+}MHC-ClassII^{+}) of bonemarrow (Fig. 4.3.1.5-middle panels) and peripheral blood (Fig. 4.3.1.5-bottom panels).

GFP expression was also noticed in Flt3^{+}c-kit^{+}lin^{-} precursor DCs in the bone marrrow (Fig. 4.3.1.5-top panels). Data of the above mentioned experiments strongly suggest that Gfi1 expression is physiologically regulated in both DC progenitors and completely differentiated dendritic cells.

4. 3. 2 Gfi1$^{-/-}$ mice show decreased numbers of DCs in lymphoid organs

It was reasoned that the distinct expression profile of Gfi1 at early stages of DC development might have important implications for the control of normal DC differentiation. To investigate the importance of Gfi1 in dendritic cell development, the lymphoid organs of Gfi1$^{-/-}$ and Gfi1$^{+/+}$ mice were assessed for the presence of dendritic cells. In Gfi1$^{-/-}$ mice, the absolute numbers of CD11c^{+} DCs were significantly reduced in lymph nodes (~10fold), spleen (~2fold) and thymus (~20fold) (Fig. 4.3.2.1).

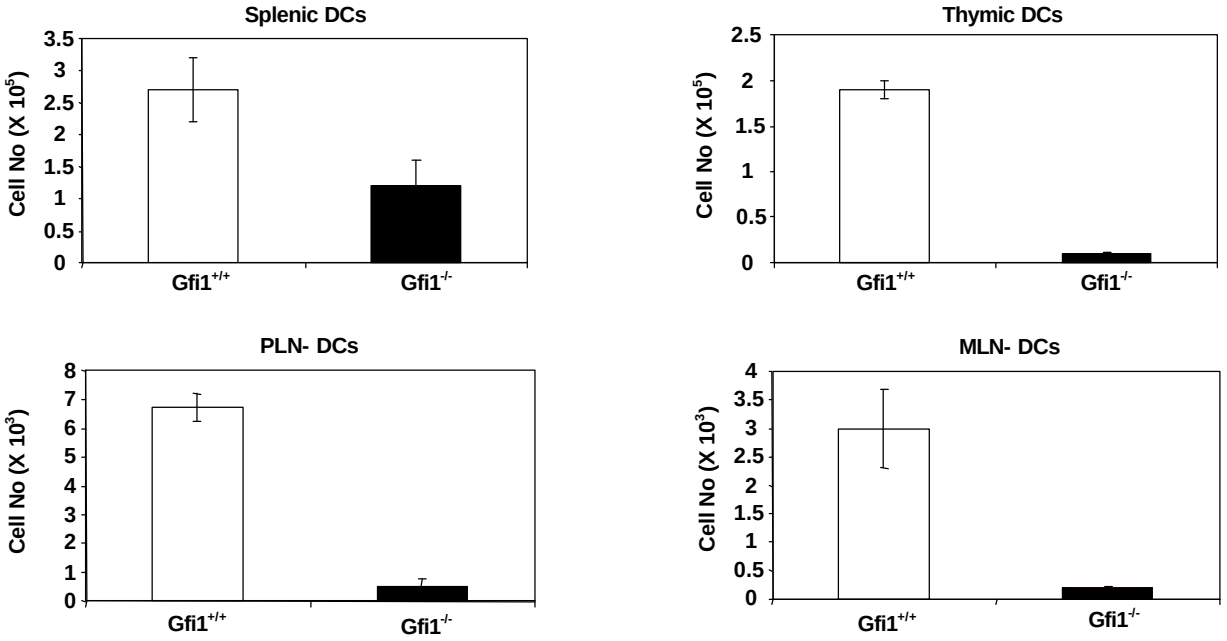
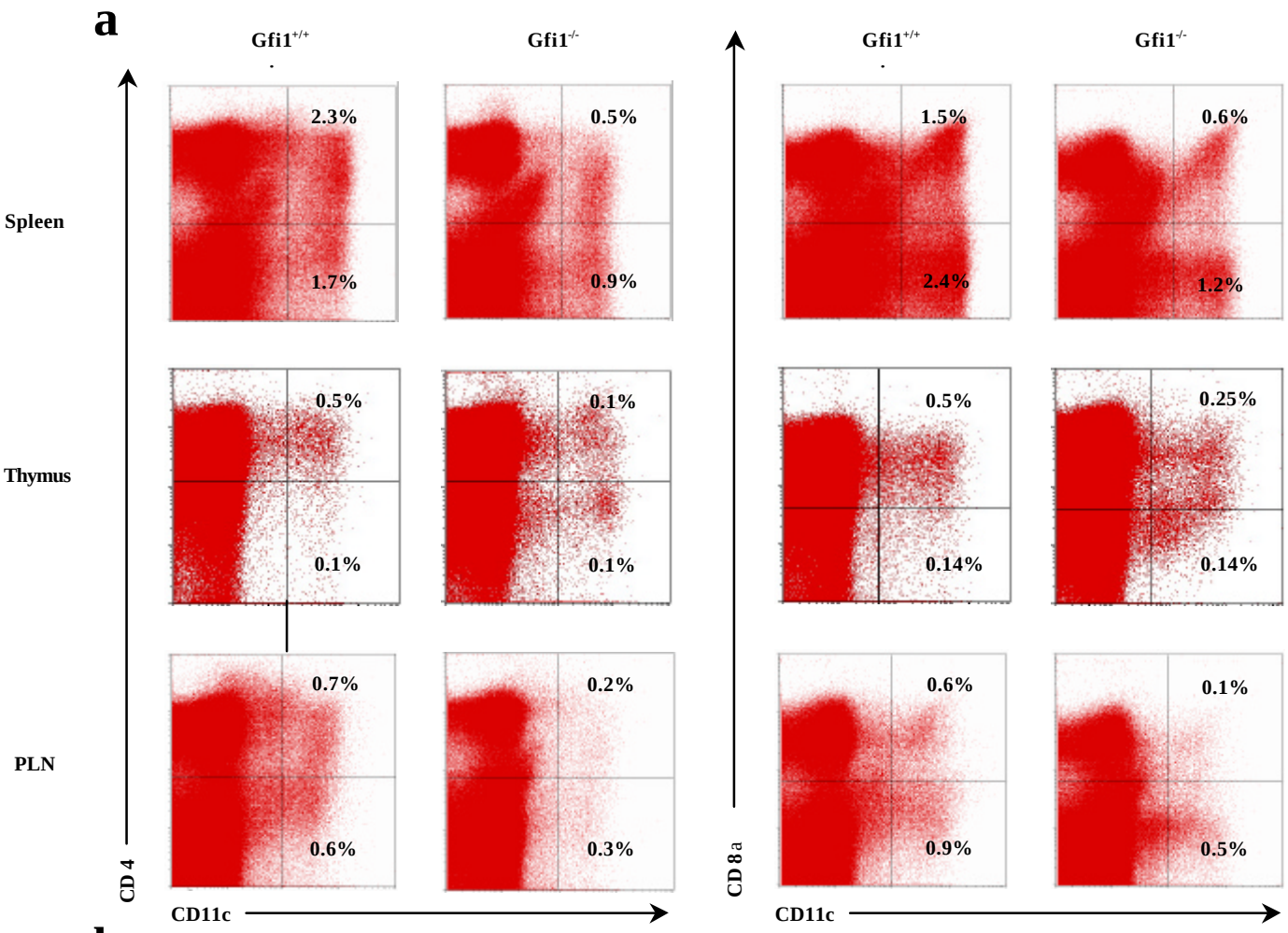


Figure 4.3.2.1. Absolute DC numbers in lymphoid organs of Gfi1$^{-/-}$ mice.
Gfi1$^{-/-}$ mice and Gfi1$^{+/+}$ mice (n=3) were sacrificed and single cell suspensions of spleens (upper left), thymi (upper right), peripheral lymph nodes (Lower left) and mesenteric lymphnodes (lower right) were pooled prior to enumeration. The absolute number was determined based on the relative FACS profile and total number of cells. Shown is the average of 3 mice.



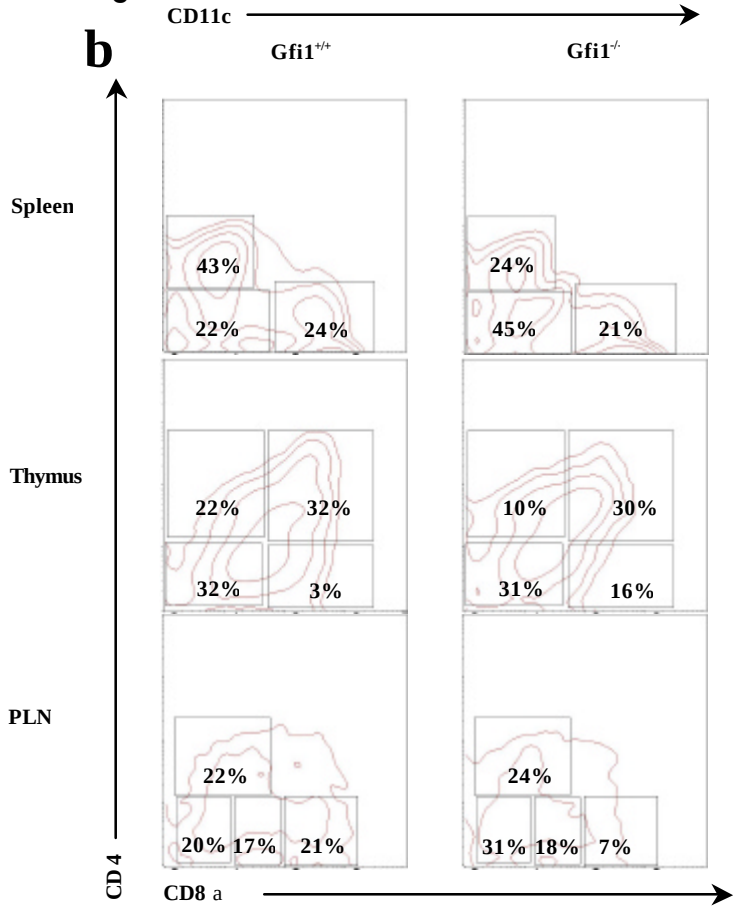


Figure 4.3.2.2 Global decrease of DCs in lymphoid organs of Gfi1$^{-/-}$ mice.
(a). Dot plots indicating relative decrease of CD11c^{+}CD4^{+} DCs (upper left) and relative decrease of CD11c^{+}CD8^{+} DCs (upper right) in speen(top panels), thymus(middle panels) and lymph nodes (lower panelss).
(b) Contour plots indicating relative decrease of selective DC subtypes, CD4^{+} in spleen (top panels), CD4^{+} (middle panels) and CD8^{high} in lymph nodes (lower panels). Analysis was done by gating on the total CD11c^{+} cells of the respective organs.

In view of the GM-CSF-specific upregulation of Gfi1 during early DC development, it was interesting to find out whether Gfi1 may act as a lineage-specific transcription factor in "myeloid" versus "lymphoid" DC development. Therefore the analysis was repeated with a specific focus on defined DC subpopulations. As shown in Figures 4. 3. 22, 4.3.2.3 and 4.3.2.4, the relative proportion of all defined DC subtypes, including myeloid, lymphoid and plasmocytoid that are described as $CD4^+$, $CD8^+$, $CD8^-$, $CD8^{high}$, $CD4^-CD8^-$, $CD11b^+$ $CD4^+CD11b^+$, $CD4^-CD11b^+$, $CD4^+CD11b^-$, $B220^+$ were drastically reduced in the spleen (top panels), thymus (middle panels), lymph nodes (bottom panels) of $Gfi1^{-/-}$ mice.

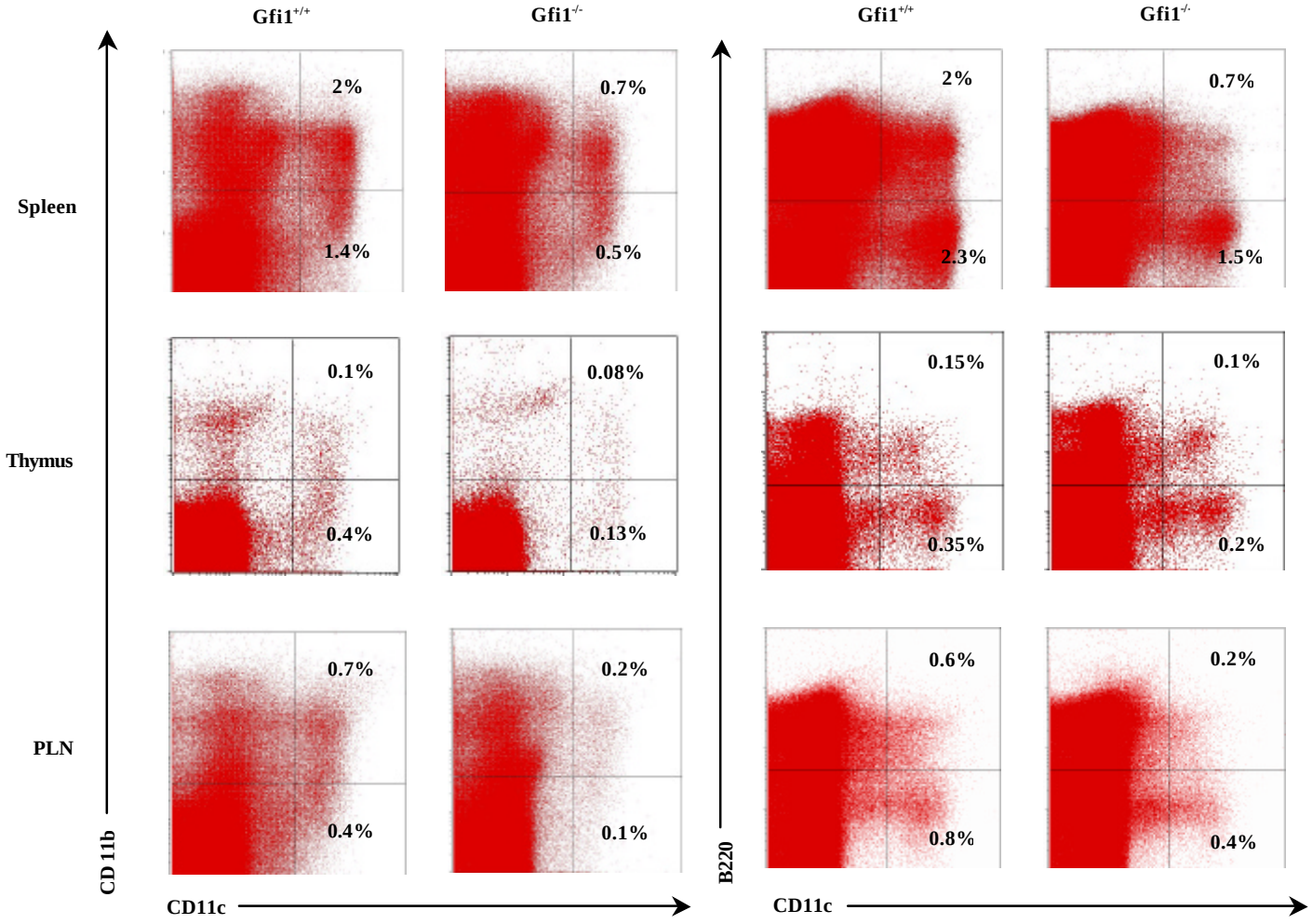


Figure 4.3.2.3. Global decrease of DCs in lymphoid organs of $Gfi1^{-/-}$ mice.
Dot plots indicating relative decrease of conventional ($CD11c^+CD11b^+$) myeloid DCs (upper left) and relative decrease of plasmocytoid $CD11c^+BB220^+$ DCs (upper right) in speen(top panels), thymus(middle panels) and lymph nodes (lower panels).

In the Gfi1$^{-/-}$ mice, relative DC deficiency affected mostly the CD4^{+} DC compartment in the spleen (21% versus 40%, Fig 4. 3. 2.2. top panels), and thymus (10% versus 22%, Fig 4. 3. 2.2. middle panels), whereas in the peripheral lymph nodes, mostly CD8^{high} expressing cells appeared reduced (7% in Gfi1$^{-/-}$ versus 21% in Gfi1$^{+/+}$, Fig 4.3. 2.2 bottom panels).

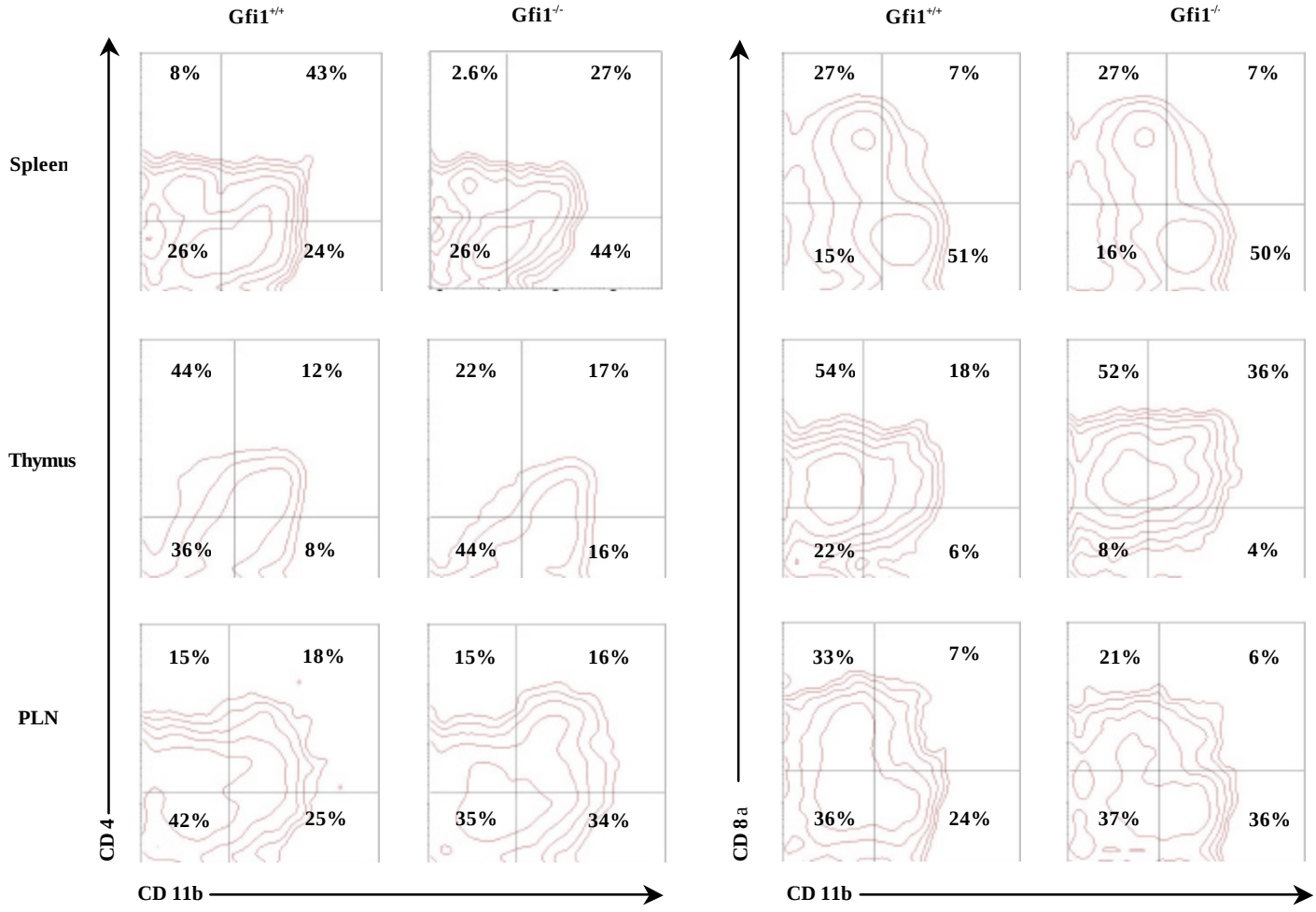


Figure 4.3.2.4. Global decrease of DCs in lymphoid organs of Gfi1$^{-/-}$ mice.
Contour plots indicating the relative profile of more specific compartments based on CD4/CD11b expression(Upper left) and CD8/CD11b expression (upper right) in speen (top panels), thymus(middle panels) and lymph nodes (bottom panels). Analysis was done after gating on the total CD11c^{+} cells of the respective organs.

Despite the variations in the relative composition of the DC compartment, the absolute numbers of all DC subpopulations were significantly reduced in spleen (Fig 4.3.2.5. top panel), thymus (Fig 4.3.2.5. middle panel) and lymph nodes (Fig 4. 3. 2.5. bottom panel).

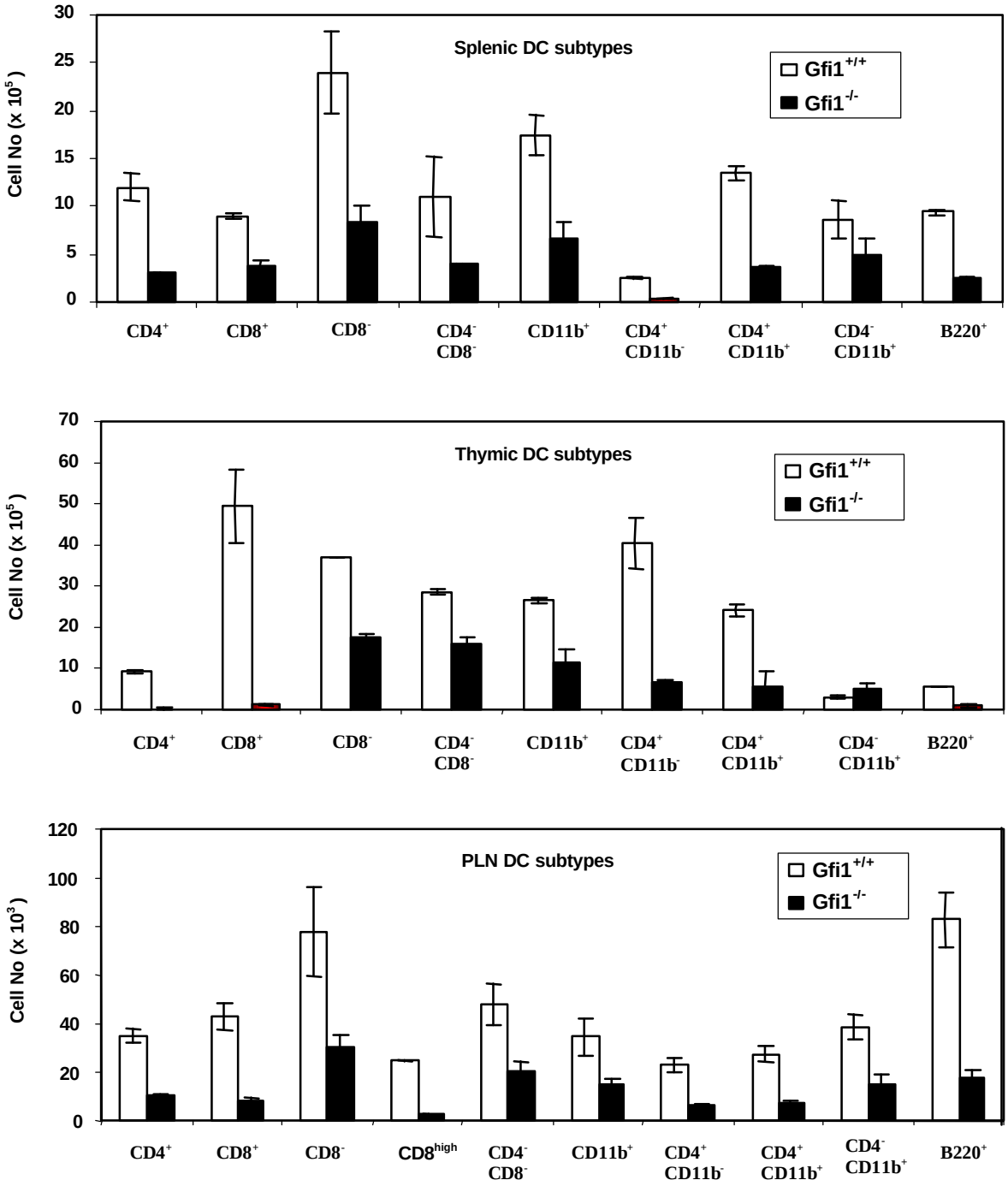


Figure 4.3.2.5 Reduced absolute numbers of DC subtypes in lymphoid organs of Gfi1$^{-/-}$mice.
Gfi1$^{-/-}$ mice and Gfi1$^{+/+}$ mice (n=5) were sacrificed and single cell suspensions of spleens (upper panels), thymi (middle panels) and peripheral lymph nodes (lower panels) were pooled prior to enumeration. The absolute number was determined based on the relative FACS profile and total number of cells. Shown is the average of 2 independent experiments.

This finding was further supported by immunofluorescence studies. Tissue sections of the lymphoid organs were stained with monoclonal antibodies that recognize T cells (Thy1.2), B cells (B220) and DCs (CD11c). Figure 4.3.2.6 depicts the reduction of dendritic cells in the

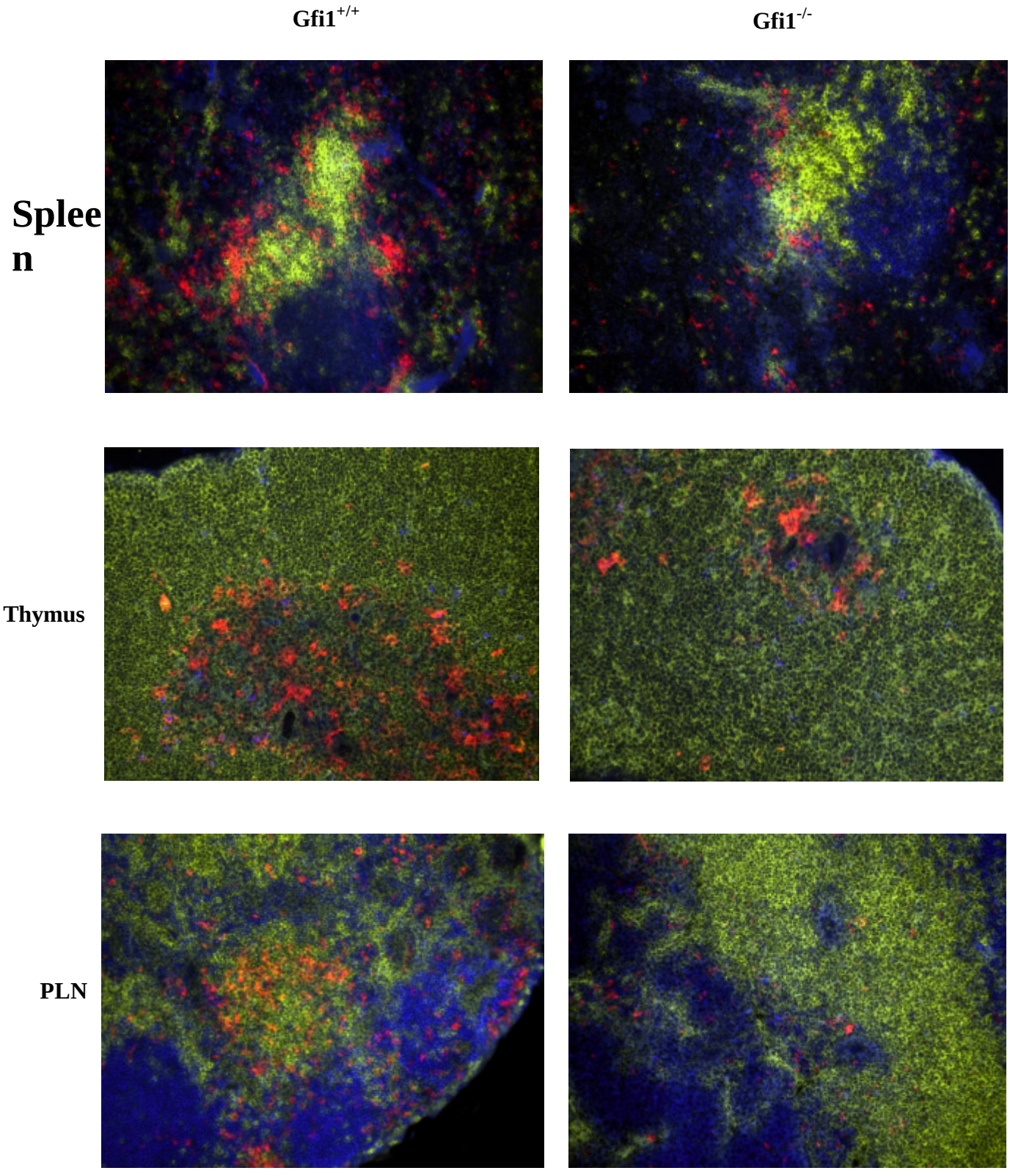


Figure 4.3.2.6. Immunofluorescence studies in lymphoid organs of Gfi1$^{-/-}$mice.
Gfi1$^{-/-}$ mice and Gfi1$^{+/+}$ mice were sacrificed and spleens (upper panels), thymi (middle panels) and peripheral lymph nodes (lower panels) were isolated. Cryosections were prepared, fixed and stained with B220-FITC, Thy1.2-CyC3, CD11c-APC monoclonal antibodes. Sections were mounted and visualised under the fluorescence microscope.

spleen (Figure 4.3.2.6-top panel), thymus (Figure 4.3.2.6-middle panel) and lymph nodes (Figure 4.3.2.6-bottom panel) of Gfi1$^{-/-}$ mice. In striking contrast to DCs in lymphoid organs, the number of epidermal Langerhans cells was increased (LCs, 92/mm^2 in Gfi1$^{-/-}$ versus 40/mm^2 in Gfi1$^{+/+}$, Fig 4.3.2.7), suggesting that Gfi1 does not play a critical role in the differentiation of LCs.

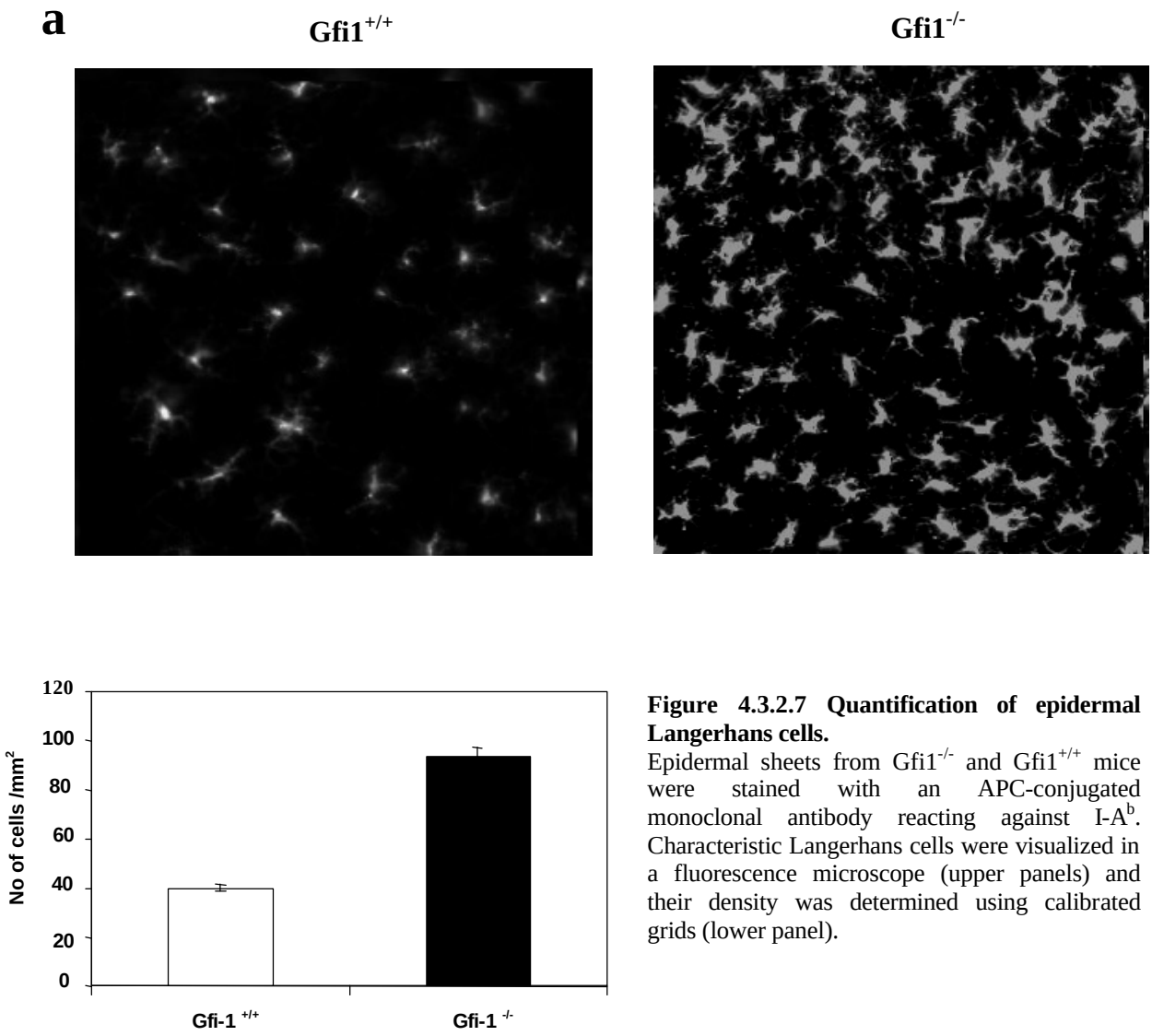


Figure 4.3.2.7 Quantification of epidermal Langerhans cells.
Epidermal sheets from Gfi1$^{-/-}$ and Gfi1$^{+/+}$ mice were stained with an APC-conjugated monoclonal antibody reacting against I-A^b. Characteristic Langerhans cells were visualized in a fluorescence microscope (upper panels) and their density was determined using calibrated grids (lower panel).

To assess whether the decreased number of DCs was due to cell-autonomous or rather extrinsic effects, hematopoietic chimeras were generated by transplanting lineage-depleted hematopoietic progenitor cells obtained from Gfi1$^{-/-}$ and Gfi1$^{+/+}$ (CD45.2) into irradiated congenic recipient mice (CD45.1). In these chimeras, DCs of donor origin developed only from Gfi1$^{+/+}$ but not from Gfi1$^{-/-}$ progenitor cells (Fig 4.3.2.8.), suggesting that the non-

hematopoietic environment did not contribute any critical factors causing deficient DC development in Gfi1$^{-/-}$ mice.

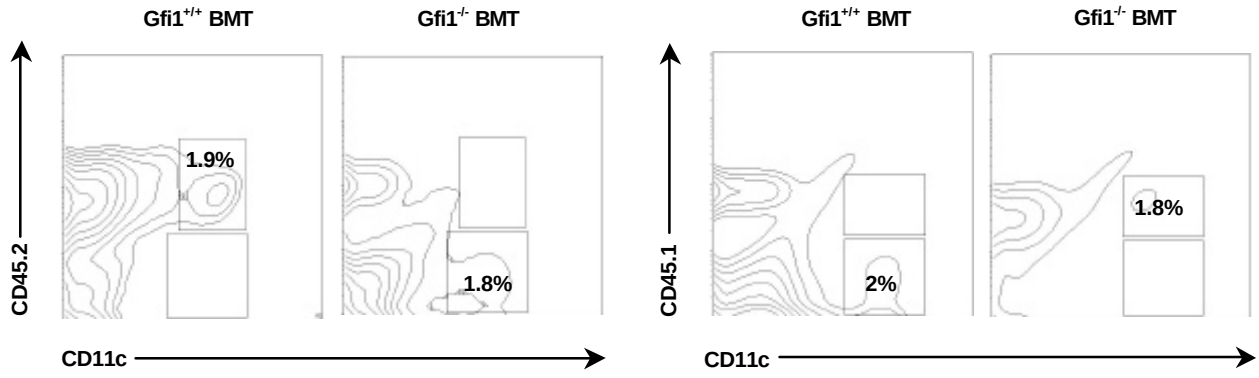


Figure4.3.2.8. Development of DC chimerism upon bone marrow transplantation.
Lineage depleted bone marrow cells from Gfi1$^{-/-}$ mice and Gfi1$^{+/+}$ mice (CD45.2) were transplanted into lethally irradiated congenic recipient mice (CD45.1). Flow cytometric analysis of splenic DCs reveals donor origin (CD45.2, upper left) in Gfi1$^{+/+}$ transplanted mice and recipient origin (CD45.1, upper right) in Gfi1$^{-/-}$ transplanted mice. Data are representative of 2 independent experiments.

4. 3. 3. Gfi1 controls DC differentiation from hematopoietic stem cells on multiple levels

In principle, decreased DC numbers in Gfi1$^{-/-}$ mice could be explained by increased turnover, aberrant distribution or decreased production of DCs. To assess whether the lack of DCs in the Gfi1$^{-/-}$ mice was due to decreased production, distinct progenitor populations were analysed. Hematopoietic stem cells (HSC), classically characterized as negative for lineage markers and positive for c-kit, Sca-1 and Thy1.2 (Lin- Sca-1+ c-kit+ Thy1.2low) were reduced by three fold in Gfi1$^{-/-}$ mice (Fig. 4.3.3.1.a). HSCs give rise to committed progenitors, named CMP (lin^{-}IL7R^{-}c-kit^{+}FcRγ^{+}CD34^{+}) and CLP (lin^{-}IL7R^{+}c-kit^{+}). Both CMP and CLP contain Flt3^{+} cells that might represent progenitor cells preceding a DC precursor cell. The percentage of CMP cells was decreased, while more committed granulocyte-monocyte progenitors (GMPs) were enhanced (36% versus 18%, Fig. 4.3.3.1.b). Similarly the percentage of CLP cells was significantly reduced in the bone marrow of Gfi1$^{-/-}$ mice (0.03% in Gfi1$^{-/-}$ versus 1% Gfi1$^{+/+}$, Fig. 4.3.3.1.c). Furthermore, DC precursor cells in bone marrow

and peripheral blood, defined as $CD11c^+$ MHC class II^- cells (0.3% in $Gfi1^{-/-}$ and 1% in $Gfi1^{+/+}$(Fig. 4.3.3.2.a) and $c\text{-}kit^+Flt^+lin^-$ (5% versus 18% of lin^- $c\text{-}kit^+$ cells, Fig. 4.3.3.2.b), were significantly reduced in numbers.

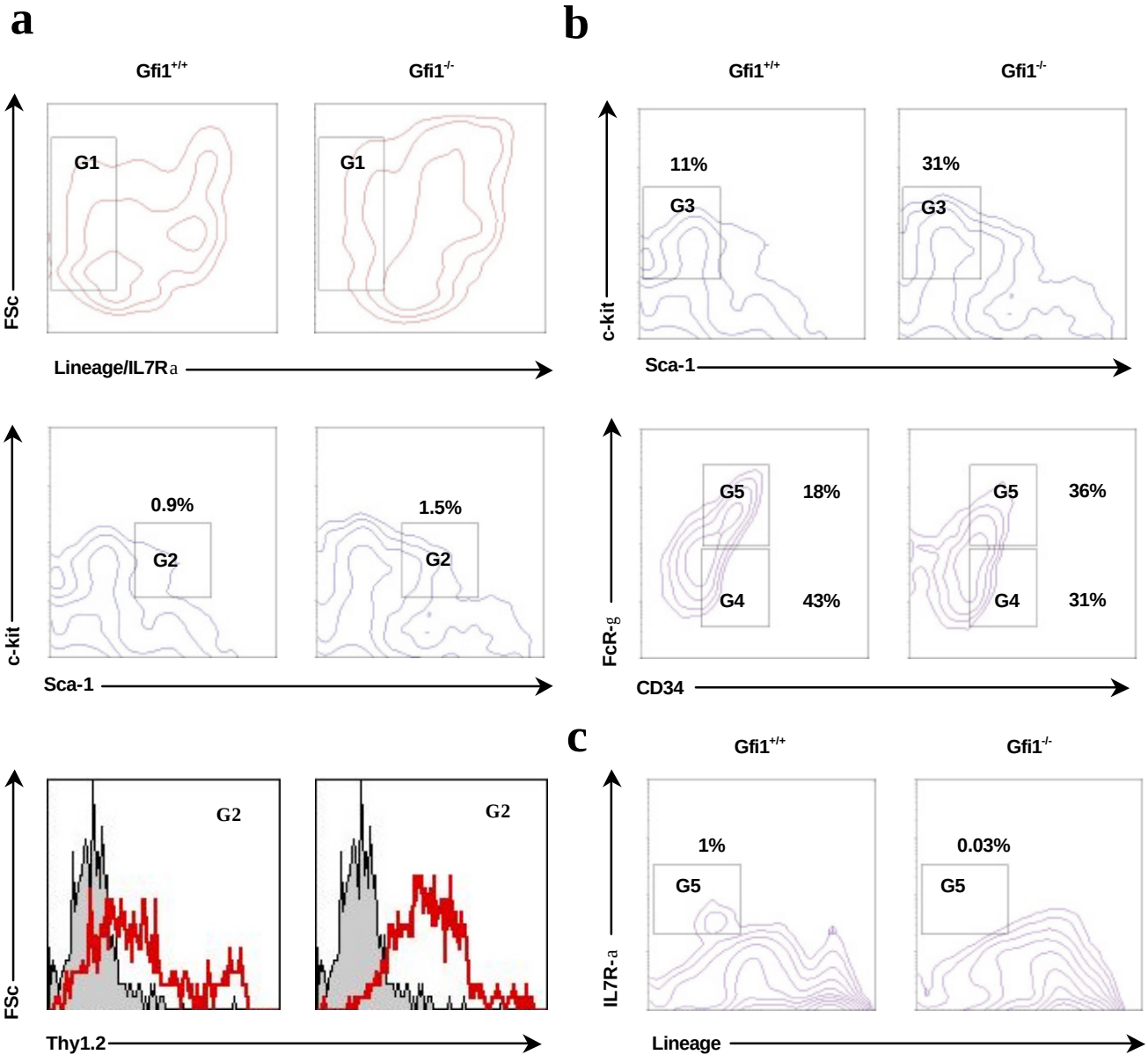


Figure. 4.3.3.1. Hematopoietic progenitor cell analysis in $Gfi1^{-/-}$ mice.
(a) Contour plot showing decreased hematopoietic stem cells (G2) in $Gfi1^{-/-}$ mice. Mononuclear bone marrow cells were stained with lineage-specific monoclonal antibodies reacting against B220, Gr-1, CD11b, CD4, CD8, Ter119, IL-7Rα, and stem cell markers c-kit, Sca-1 and Thy1.2. Lineage negative(G1) cells that are positive for both Sca-1 and c-kit (G2) were analysed for Th1.2 expression. **(b)** Contour plots showing fluorescence profile of Lineage negative(G1) bone marrow cells stained with Sca-1 and c-kit(top panels). $c\text{-}kit^+/Sca\text{-}1^-$ cells (G3) cells were analysed for FcRγ and CD34 (bottom panels). The frequencies of of $FcR\text{-}\gamma^{low}CD34^+$ common myeloid progenitors (G4) and $FcR\text{-}\gamma^+CD34^+$ granulocyte monocyte progenitors (G5) were calculated, both gated on G3. **(c)** Contour plot showing decreased common lymphoid precursor cells (G5) in $Gfi1^{-/-}$ mice. Mononuclear bone marrow cells were stained with FITC-labelled lineage-specific monoclonal antibodies reacting against B220, Gr-1, CD11b, CD4, CD8, Ter119; IL-7Rα is visualized upon staining with Streptavidin PerCP.

Although the exact lineage relations of CMP, CLP, pre-DCs and differentiated DCs are currently not yet known, these data suggest that Gfi1 plays a critical role in shaping lymphoid and myeloid hematopoiesis. In particular, a severe reduction in lymphoid and DC progenitors is contrasted by a relative increase of committed granulocyte-monocyte progenitor cells. Results from the extensive progenitor analysis suggest that the decreased numbers of DCs in Gfi1$^{-/-}$ mice is primarily due to insufficient production.

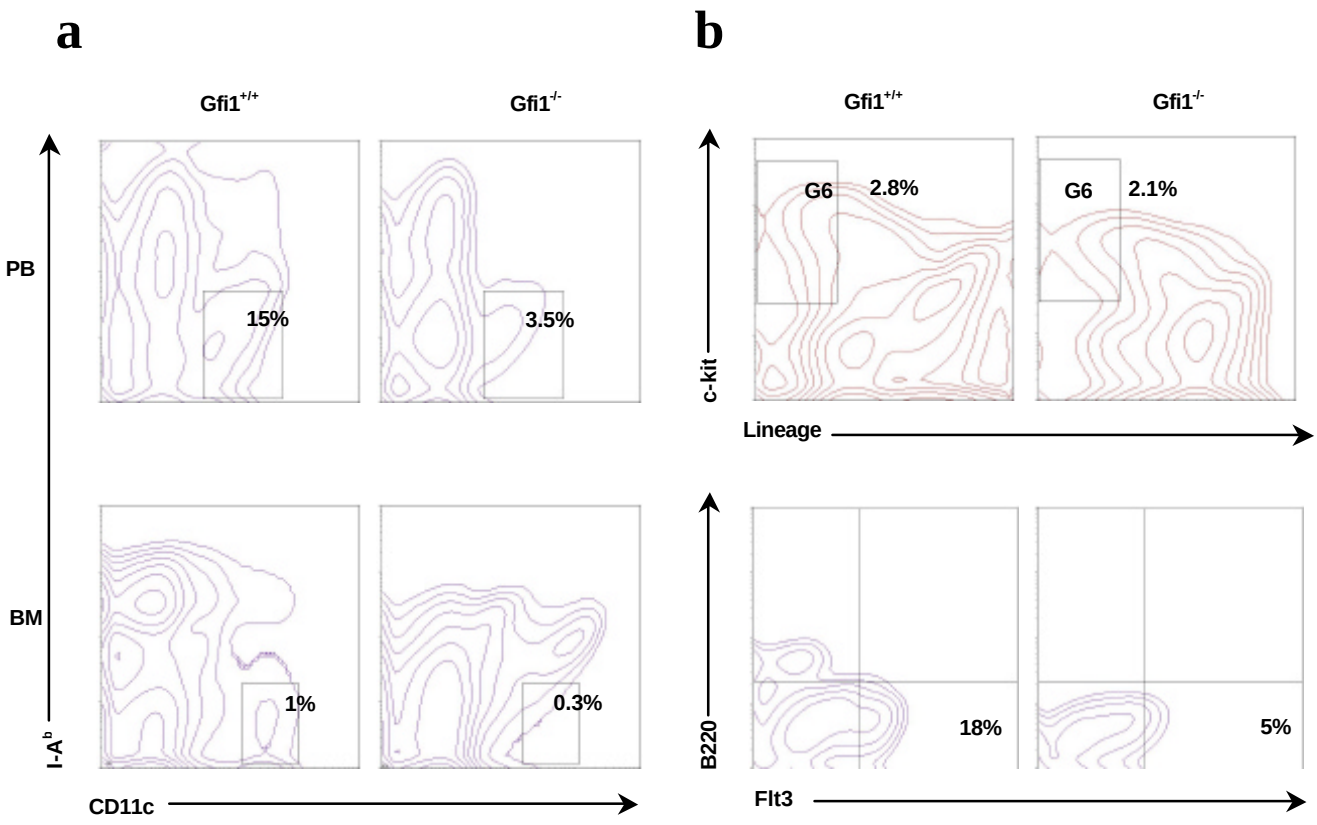


Figure4.3.3.2 Dendritic cell progenitor analysis in Gfi1$^{-/-}$ mice.
(a) Contour plot showing decreased dendritic cell progenitors (CD11c^{+} IAb^{-}) cells in peripheral blood (top panels) and bone marrow (bottom panels) of Gfi1$^{-/-}$ mice. **(b)** Contour plot showing distribution of (Lin^{-}c-kit^{+}B220^{-}Flt3^{+}) precursor DC cells. Lin^{-}c-kit positive cells (top panels) were gated (G6) and the frequency of Flt3^{+}B220^{-}cells was determined (lower panels).

4. 3. 4. Gfi1$^{-/-}$ DCs show functional abnormalities revealing distinct effects in maturation and activation profiles

DC development is not completely abrogated in Gfi1$^{-/-}$ mice. Since Gfi1 is up regulated upon DC activation in Gfi1$^{GFP/+}$ mice, it was interesting to assess whether the remaining DCs in Gfi1$^{-/-}$ mice were characterized by functional defects. First, the expression levels of MHC and co stimulatory molecules on DCs as markers of the maturation status in lymphoid organs were assessed. In contrast to normal expression levels of MHC class I, CD40, CD80 and CD86, a significant reduction in MHC class II expression in all DC subtypes in Gfi1$^{-/-}$ mice (Fig. 4.3. 4.1) was found.

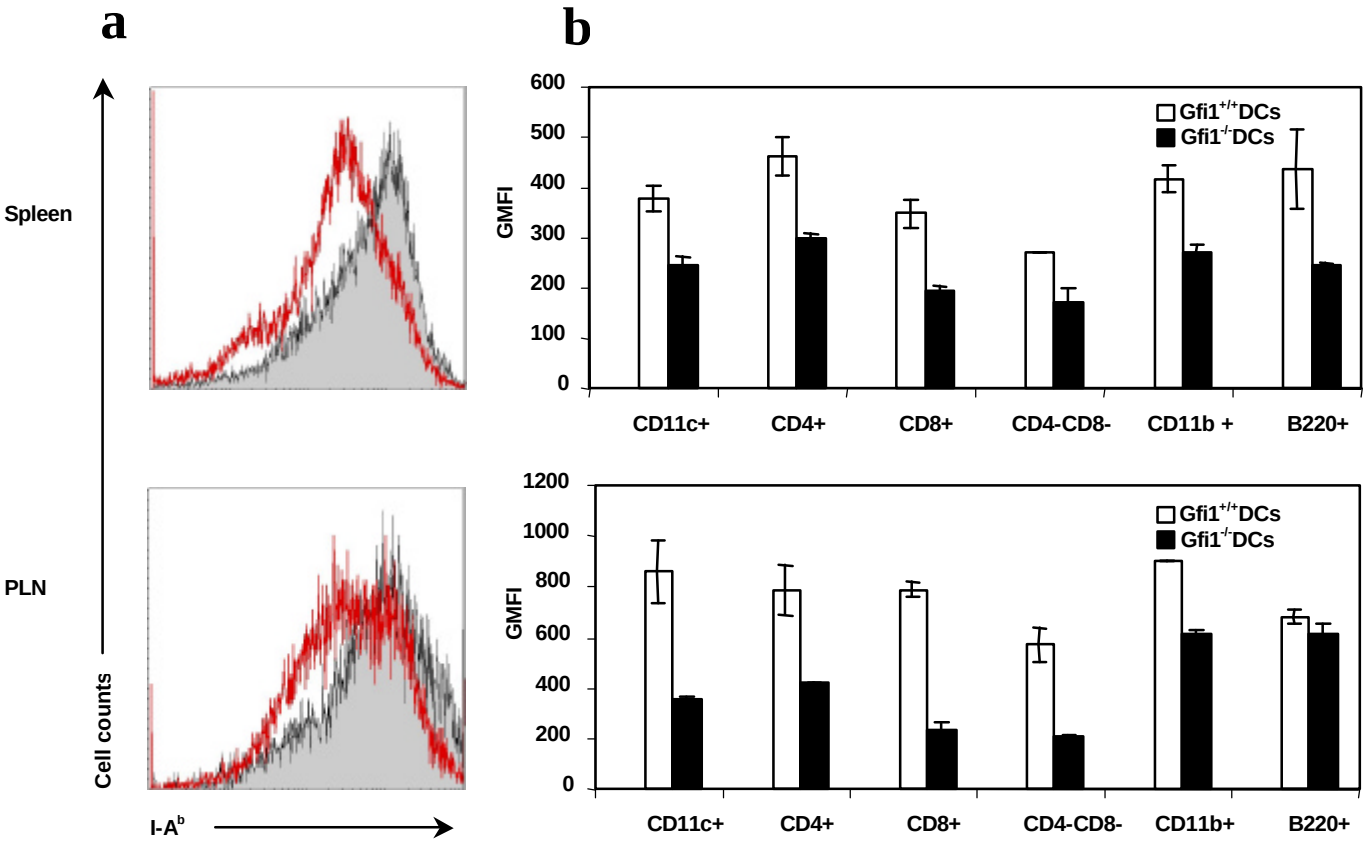


Figure4.3.4.1. Decreased MHC class II expression in Gfi1$^{-/-}$ DCs.
(a) MHC class II expression in CD11c^{+} cells of spleen (top panels) and lymph nodes (bottom panels). MHC Class II expression in DCs of Gfi1$^{+/+}$(filled histogram) and Gfi1$^{-/-}$(open histogram) mice is compared.
(b) Geomean fluorescence index (GMFI) of MHC Class II expression in various subtypes of DCs in spleen (upper panels) and lymph node (lower panels) of Gfi1$^{+/+}$ (open bars) and Gfi1$^{-/-}$ (filled bars) mice was represented. Shown is the average GMFI of two independent experiments, pooling organs from 5 mice. Data are representative of 6 independent experiments.

To further assess whether DC maturation could be triggered by a response to microbial components or inflammatory cytokines, Gfi1$^{-/-}$ and Gfi1$^{+/+}$ mice were injected with anti-CD40 monoclonal antibodies or LPS, respectively. 24 hours later, splenic DCs were harvested,

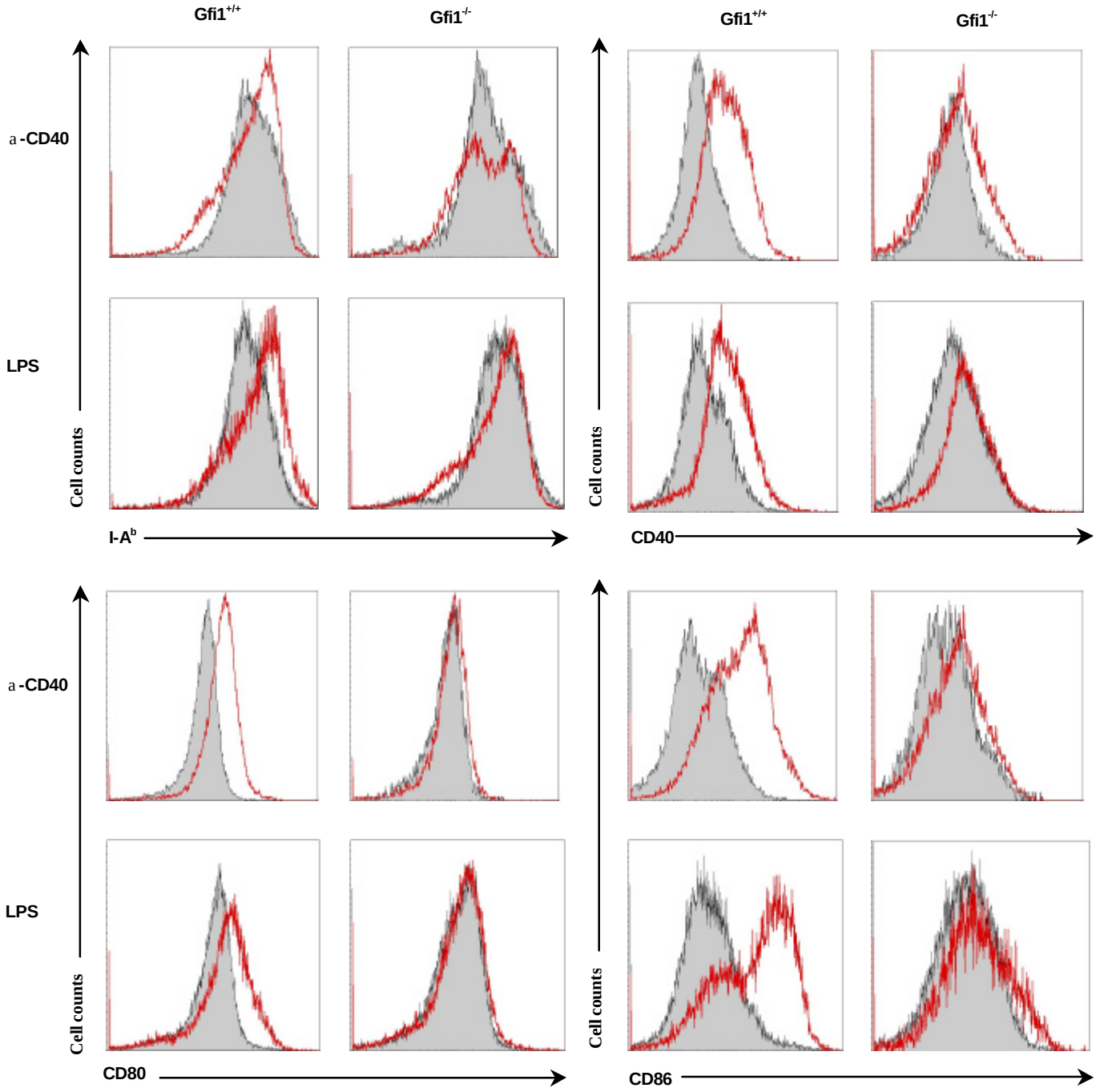


Figure 4.3.4.2 Gfi1$^{-/-}$ DCs are refractory to upregulate costimulatory molecules upon stimulation.
Histograms indicating that Gfi1$^{-/-}$ DCs are refractory to upregulate costimulatory molecules. Gfi1$^{-/-}$ and Gfi1$^{+/+}$ mice were injected with LPS or anti-CD40 monoclonal antibodies. Pooled DCs from 3-5 mice were analyzed for expression of CD40, CD80 and CD86, I-A^{b}(open histograms). Shaded histograms represent expression levels on DCs from PBS injected mice used as negative controls.

stained, and analyzed by flow cytometry. As shown in Fig. 4.3.4.2, Gfi1$^{-/-}$ DCs were refractory to up regulation of the costimulatory molecules CD40, CD80, and CD86. Similar findings were noted in bone marrow derived DCs.

To test whether a decreased level of DC maturation/activation might be associated with decreased production of inflammatory cytokines such as IL12, splenic DCs from Gfi1$^{-/-}$ and Gfi1$^{+/+}$ mice were purified and stimulated with TNFα or the Toll-like-receptors ligands LPS (TLR2, 4) and CpG (TLR9), respectively. Surprisingly, it was found that Gfi1$^{-/-}$ DCs showed

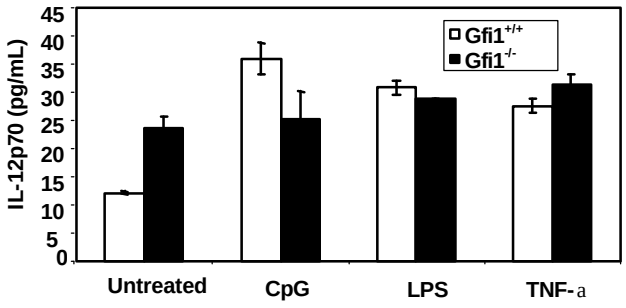


Figure4.3.4.3. Constitutive secretion of IL12 in Gfi1$^{-/-}$ DCs. Splenic DCs from Gfi1$^{-/-}$ and Gfi1$^{+/+}$ mice were purified and stimulated with TNFα and the TLR ligands CpG and LPS in duplicates. Shown is one representative experiments out of five. Error bars represent standard error in ELISA.

a higher baseline level of IL12 secretion that could not be further increased upon stimulation; suggesting that maturation (expression of MHC class II and costimulatory molecules) and activation (expression of IL12) of DCs represent the result of at least partially independent and distinct molecular events (Fig. 4.3.4.3).

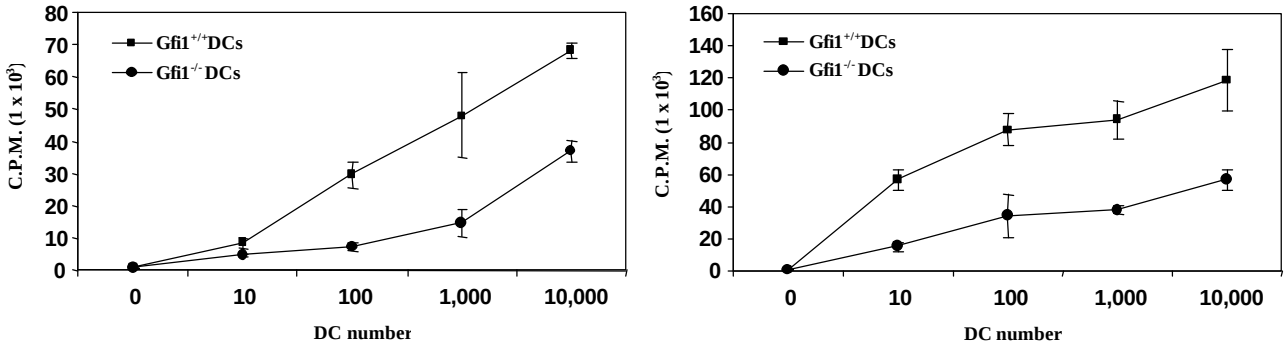


Figure4.3.4.4. Deficient antigen presentation in Gfi1$^{-/-}$ DCs in-vitro. Splenic DCs from Gfi1$^{-/-}$ and Gfi1$^{+/+}$ mice were loaded with peptides and used as antigen presenting cells to stimulate the proliferation of transgenic OT-I (upper left) and OT-II cells (upper right), respectively. T-cell proliferation was measured in triplicates by incorporation of ^{3}H-Thymidine. Data are representative of 3 independent experiments.

To further characterize the function of Gfi1$^{-/-}$ DCs, the capacity of Gfi1$^{-/-}$ DCs to present specific antigens to T-cells was studied. Splenic DCs from Gfi1$^{-/-}$ and Gfi1$^{+/+}$ mice were isolated and loaded with Ova peptides SIINFEKL and Ova$^{323-339}$, two epitopes presented by MHC class II and I, respectively.

The antigen pulsed DCs were incubated with transgenic OT-I and OT-II T-cells recognizing Ova peptides in association with MHC class I and class II. As shown in Figure. 4. 3. 4.4, Gfi1$^{-/-}$ DCs induced a significantly reduced OT-I (Fig. 4. 3. 4.4-upper left) and OT-II (Fig. 4. 3. 4.4-upper right) T-cell proliferation in vitro. In addition the capacity of Gfi1$^{-/-}$ dendritic cells to present a surrogate tumor antigen, ova-albumin, to ovaspecific T cells (OT-II) was analysed. Fig. 4.3.4.5 showst that the Gfi1 deficient DCs had defect in presenting the ova protein to OT-II cells .

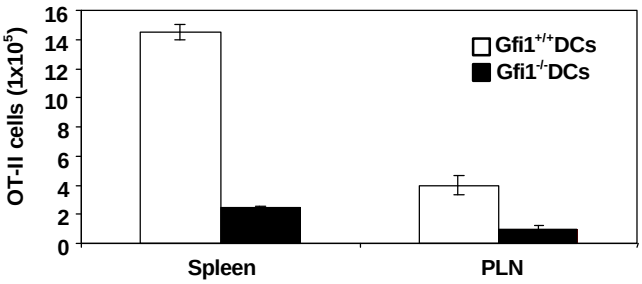


Figure 4.3.4.5. Deficient antigen presentation in Gfi1$^{-/-}$ DCs in-vivo. Gfi1$^{+/+}$ and Gfi$^{-/-}$ mice were transplanted i.v. with ova specific T cells(OT-II) and subsequent subcutaneous immunisation of ova albumin protein. Ova specific T (OT-II) cell proliferation was measured based on the relative FACS profile and total number of cells .

Taken together these data suggest that impaired antigen presentation is an intrinsic feature of Gfi1$^{-/-}$ DCs that can not be readily explained by decreased MHC class II expression levels.

4. 3. 5. Gfi1 is a critical cell-intrinsic modulator of DC versus macrophage development

To further elucidate the mechanism of decreased DC differentiation and –function, DC differentiation assays in vitro were performed. Lineage-depleted hematopoietic progenitor cells from Gfi1$^{-/-}$ and Gfi1$^{+/+}$ mice were incubated in the presence GM-CSF or Flt3L, two cytokines controlling DC differentiation. Interestingly, in contrast to Gfi1$^{+/+}$ progenitor cells, neither GM-CSF nor Flt3L induced DC differentiation in Gfi1$^{-/-}$ progenitor cells. Instead, in conditions classically permissive for DC differentiation, Gfi1$^{-/-}$ progenitor cells differentiated

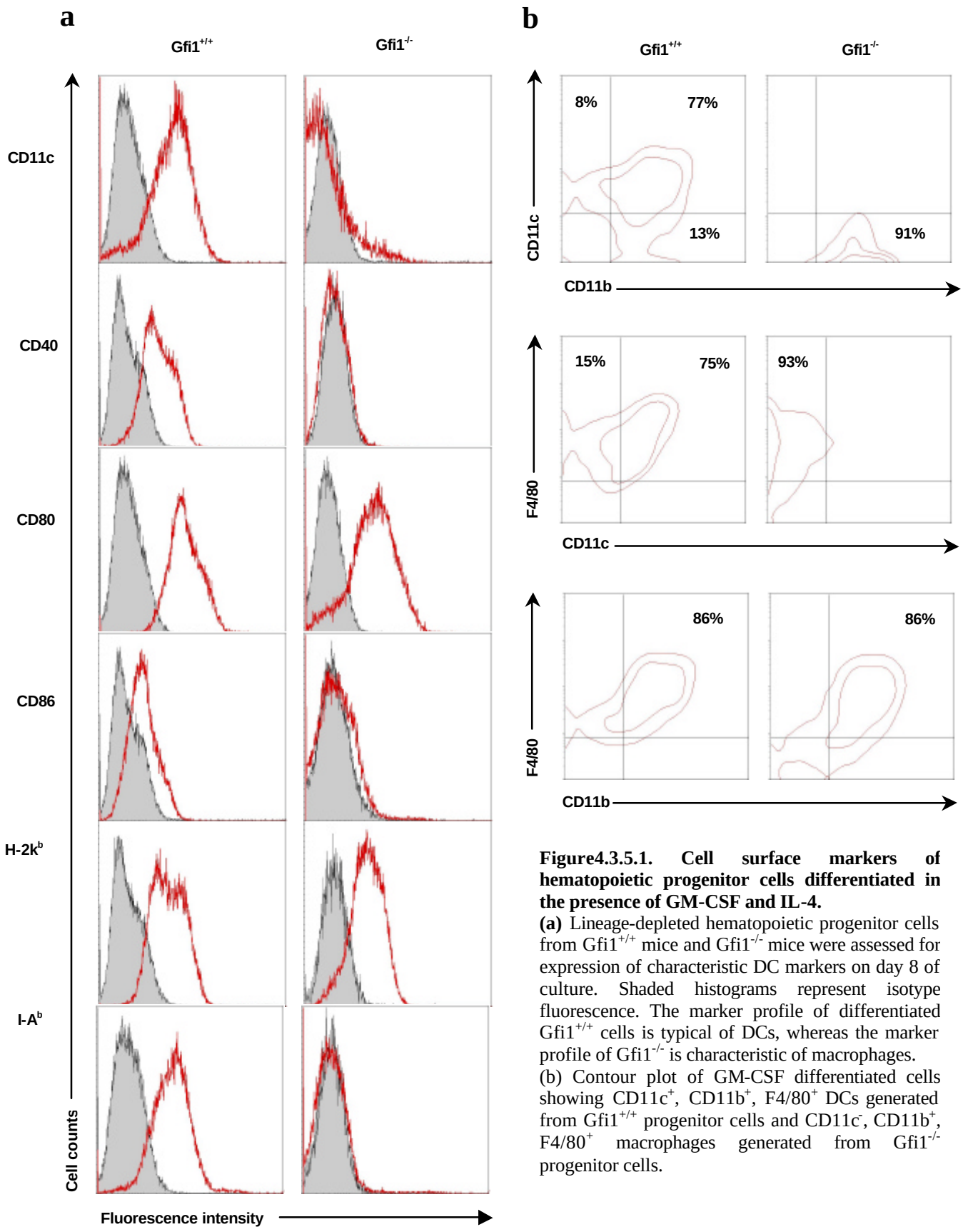


Figure4.3.5.1. Cell surface markers of hematopoietic progenitor cells differentiated in the presence of GM-CSF and IL-4.
(a) Lineage-depleted hematopoietic progenitor cells from Gfi1$^{+/+}$ mice and Gfi1$^{-/-}$ mice were assessed for expression of characteristic DC markers on day 8 of culture. Shaded histograms represent isotype fluorescence. The marker profile of differentiated Gfi1$^{+/+}$ cells is typical of DCs, whereas the marker profile of Gfi1$^{-/-}$ is characteristic of macrophages.
(b) Contour plot of GM-CSF differentiated cells showing CD11c^{+}, CD11b^{+}, F4/80^{+} DCs generated from Gfi1$^{+/+}$ progenitor cells and CD11c^{-}, CD11b^{+}, F4/80^{+} macrophages generated from Gfi1$^{-/-}$ progenitor cells.

into macrophages, as assessed by immunophenotype (Fig.4.3.5.1.a & b), morphology (Fig. 4.3.5.2.a), and capacity to stimulate allogeneic T-cell proliferation (Fig. 4.3. 5.2.b). Since Gfi1$^{-/-}$ myeloid cells show an enhanced production of cytokines and since IL6 has been described to direct macrophage over DC development in human cells, it was reasoned that this diverted development might be secondary to the cytokine milieu in culture. However, supernatants from in vitro differentiation assays did not have any effect on DC differentiation of Gfi1$^{+/+}$ progenitor cells (data not shown), suggesting that the deficiency of Gfi1$^{-/-}$ cells to develop into DC is a cell-autonomous feature. The data of these experiments suggest that Gfi1 is a crucial factor for the lineage determination of DC versus macrophages.

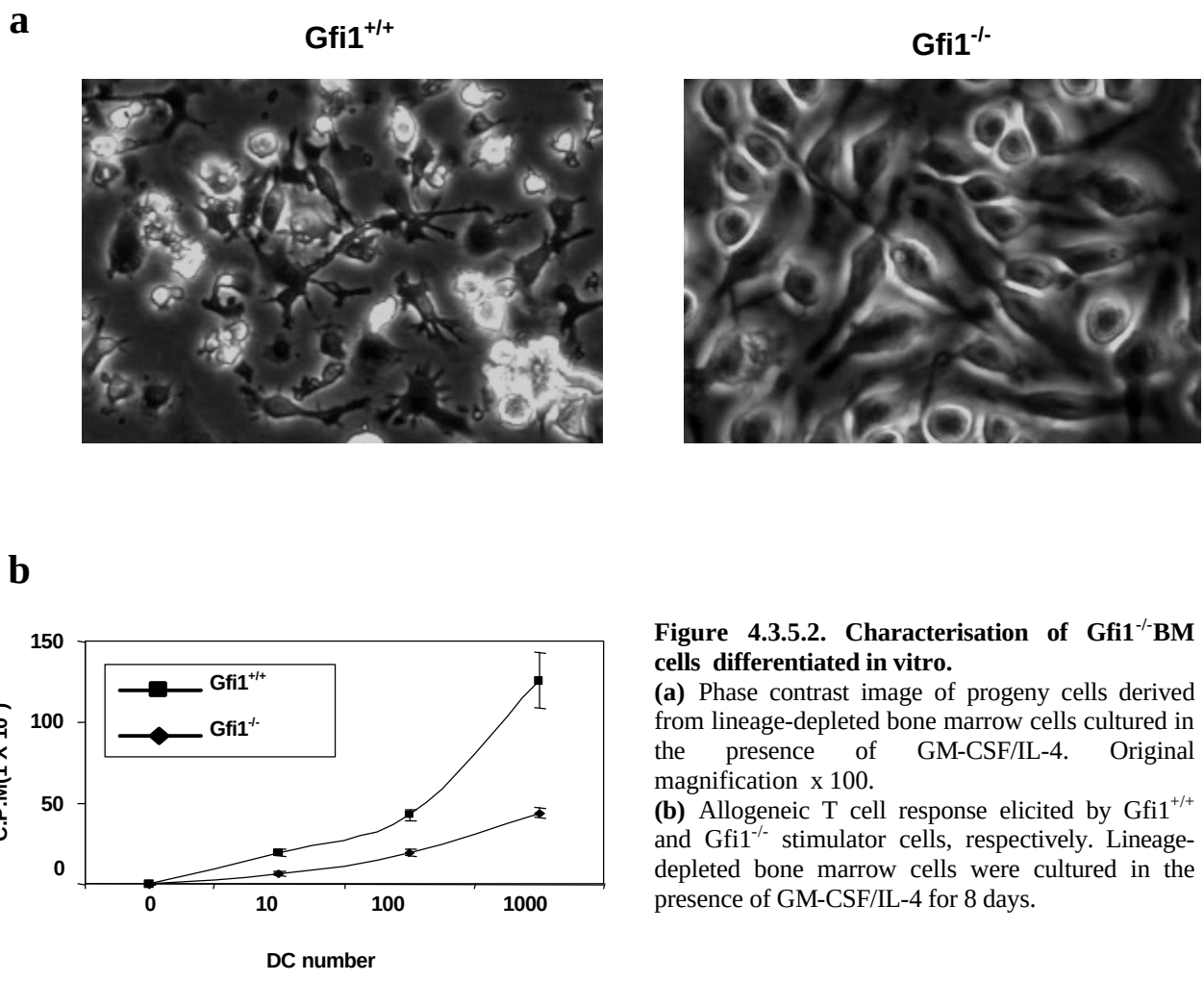


Figure 4.3.5.2. Characterisation of Gfi1$^{-/-}$BM cells differentiated in vitro.
(a) Phase contrast image of progeny cells derived from lineage-depleted bone marrow cells cultured in the presence of GM-CSF/IL-4. Original magnification x 100.
(b) Allogeneic T cell response elicited by Gfi1$^{+/+}$ and Gfi1$^{-/-}$ stimulator cells, respectively. Lineage-depleted bone marrow cells were cultured in the presence of GM-CSF/IL-4 for 8 days.

4. 3.6 Retroviral mediated complementation of Gfi1 restores DC differentiation potential of Gfi1$^{-/-}$ BM cells

In vitro differentiation studies of Gfi1$^{-/-}$ BM cells suggest that the defective DC differentiation might be a cell intrinsic feature. This notion was further confirmed by complementation experiments using Gfi1 encoding retroviruses. The retroviral plasmids encoding Gfi1 cDNA was constructed by amplifying the cDNA from the plasmid p-CMV-Gfi1 (kindly provided by Prof.Tarik Möröy) by PCR. The amplified PCR fragment was then subcloned into the Topo 2.1 T-plasmid. The Gfi1 cDNA was released by digesting the Topo-Gfi1 subcloning plasmid with Not I and cloned into the NotI site of SFβ91 –IRES-EGFP retroviral plasmid (kindly provided by Prof. Christopher Baum,). Figure 4.3.6.1 outlines the cloning strategy followed for constructing SFβ91 Gfi1–IRES-EGFP retroviral plasmid.

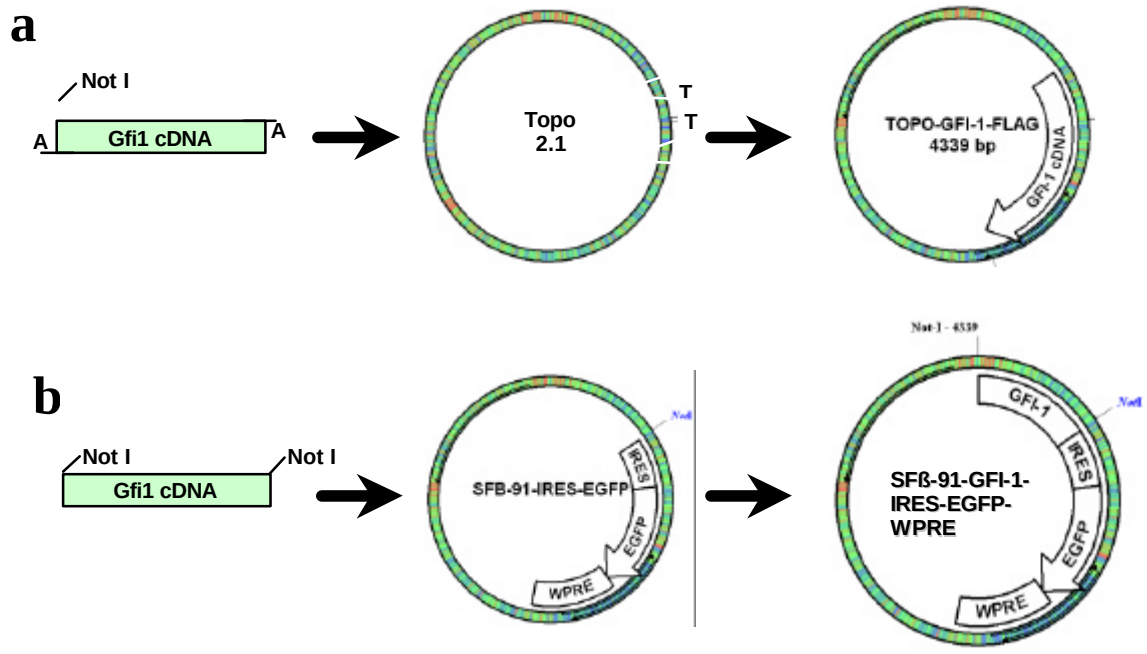


Figure4.3.6.1. Cloning stratergy of Sfb91-Gfi1-IRES-EGFP-WPRE.
(a) Gfi1 cDNA was PCR amplified using pCMV-Gfi1 plasmid as a template with a 5' NotI flanking sites. The 1272 bp amplicon was cloned into Topo 2.1 subcloning vector.
(b)Then the Gfi1 cDNA was released from Topo-Gfi1 plasmid by digesting with NotI and then cloned into the NotI site of Sfβ91 -IRES-EGFP-WPRE to get the final Sfβ91-Gfi1-IRES-EGFP-WPRE retroviral construct.

The pseudotyped retroviral particles were produced by transfecting 293 gpg packaging cells with the respective retroviral plasmids. Sca-1^{+}lin^{-} hematopoietic progenitor cells from Gfi$1^{-/-}$ mice were isolated and transduced with either a retrovirus encoding the marker gene GFP (SFβ91-GFP) or a bicistronic retrovirus encoding Gfi1 and GFP (SFβ91-Gfi1-IRES-GFP). It was noted that a significant loss of viability of the transduced cells upon Gfi1 gene transfer, presumably secondary to non-physiological levels of retrovirus-mediated Gfi1 expression. However, in the surviving cells, 18% showed expression of the DC marker CD11c, suggesting a partial reconstitution of DC development in vitro (Fig. 4.3.6.2.a). We also addressed reconstitution of the DC development upon transplantation of retrovirus-transduced HSC in vivo. Sca-1^{+}lin^{-} HSC from

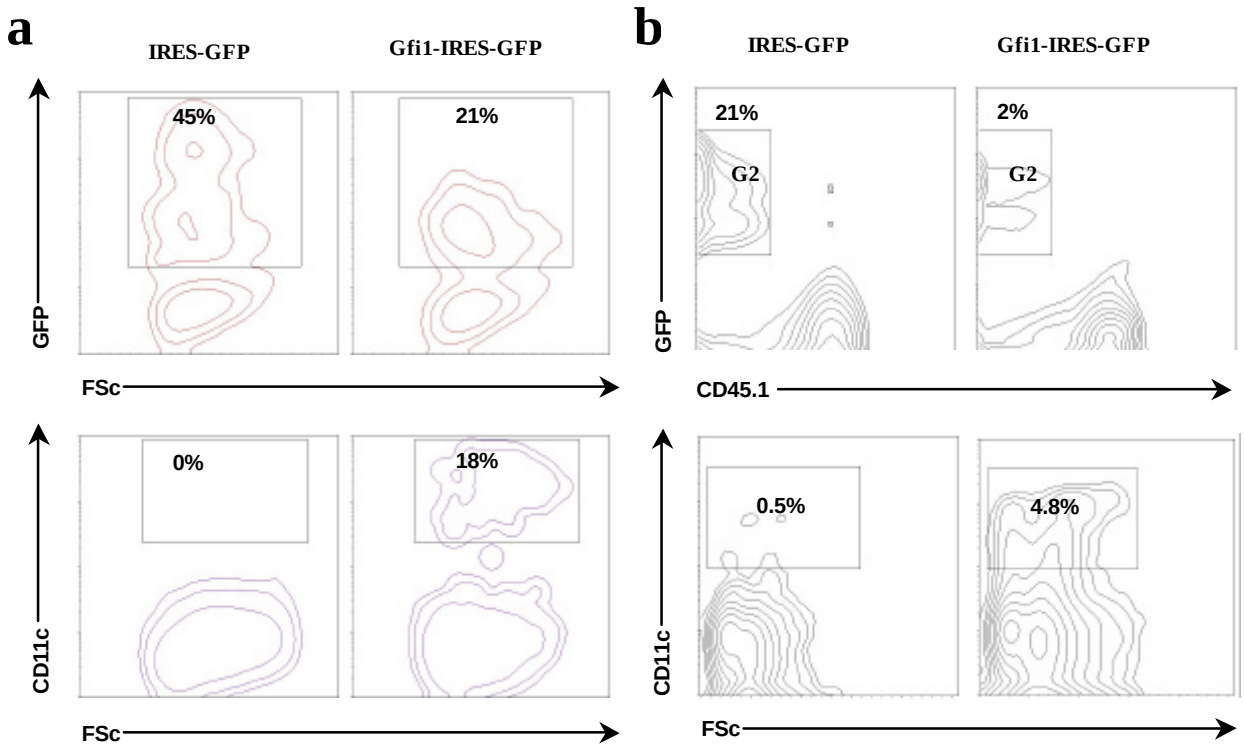


Figure 4.3.6.2 Reconstitution of DC development upon retroviral gene transfer.
(a) Bone marrow progenitor cells of Gfi$1^{-/-}$ mice were transduced with retroviruses encoding GFP (left panels) and Gfi1GFP (right panels), respectively. Cells were then differentiated in the presence of GM-CSF. On day 8 of culture, GFP positive cells (upper panels) were gated (G1) and CD11c-expression (lower panels) was analysed by flow cytometry.**(b)** For in vivo reconstitution assays, Sca-1^{+}lin^{-} cells from Gfi$1^{-/-}$ mice (CD45.2^{+}) were transduced with retroviruses encoding either GFP (left panels) or Gfi1-GFP (right panels) and transplanted into irradiated (4.5 Gy) congenic recipient mice (CD45.1^{+}). GFP-positive splenocytes of recipient mice were gated (G2) analysed for CD11c expression (lower panels).

Gfi1$^{-/-}$ mice were transduced either with SFβ91-Gfi1-IRES-GFP or SFβ91-GFP and transplanted into irradiated recipient mice. Upon reconstitution of the hematopoietic system, the mice were sacrificed and analysed for DC reconstitution. Compared to mice transplanted with GFP-expressing progenitor cells, it was determined that the percentage of CD11c^{+} cells among GFP-positive cells was significantly higher in mice that had received Gfi1-transduced progenitor cells (4.8%) compared to mice that had received GFP-transduced progenitor cells (0.2%, Fig. 4.3.6.2.b), suggesting that the retrovirus mediated expression of Gfi1 corrected the defect of DC development

4. 3. 7. Deficient DC development in Gfi1$^{-/-}$ mice is associated with decreased STAT3 activation in progenitor cells

Previous in vitro data have shown that Gfi1 interacts with PIAS3, a known inhibitor of STAT3 (Rodel et al., 2000). STAT3 has recently emerged as an important mediator of DC differentiation. Based on the previous studies it was hypothesized that altered STAT3-signalling may influence the developmental pathway of macrophage versus dendritic cell development. To investigate the activation status of STAT3 during DC differentiation, Lin^{-} hematopoietic progenitor cells from Gfi1$^{+/+}$ and Gfi1$^{-/-}$ mice were isolated and cultured in the presence of GM-CSF. Cells were harvested at hour 0, 30 min, and 4 hour after GM-CSF stimulation and the cytoplasmic and nuclear proteins were isolated. STAT3 activation upon GM-CSF stimulation in Gfi1$^{-/-}$ and Gfi1$^{+/+}$ hematopoietic cells was assessed by Western blot analysis, comparing cytosolic and nuclear protein fractions. Whereas the specific band corresponding to STAT3 protein in the cytosol was comparable in Gfi1$^{-/-}$ and Gfi1$^{+/+}$ cells (Fig. 4.3.7.1-top panel), a significant reduction of STAT3 in the nuclear protein fraction at all time points was documented (Fig. 4.3.7.1- bottom panel), suggesting that the transnuclear shift of activated STAT3 was impaired in Gfi1$^{-/-}$ cells.

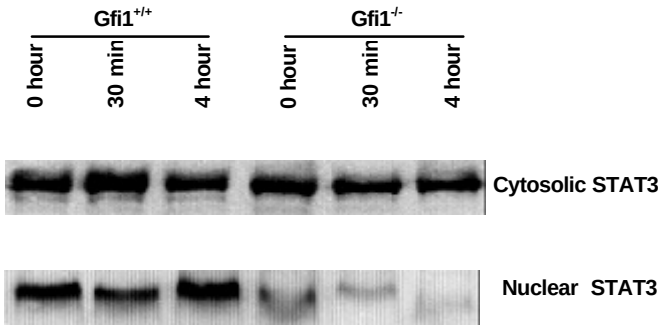


Figure 4.3.7.1. Decreased STAT3 translocation in the nucleus.
Lin-depleted bone marrow cells from Gfi1$^{+/+}$ and Gfi1$^{-/-}$ mice were incubated in the presence of GM-CSF and IL-4. Cytosolic (upper panels) and nuclear protein fractions (lower panels) were purified at indicated time points. STAT3 was detected by western blot using a STAT3-specific monoclonal antibody.

This was further confirmed by electrophoretic mobility shift assays (EMSA), which revealed a significant reduction of STAT3 homo- and heterodimers in Gfi1$^{-/-}$ cells (Fig. 4. 3.7.2.a). Oct-1 specific EMSA confirmed the equal amount of protein loaded in each lanes.

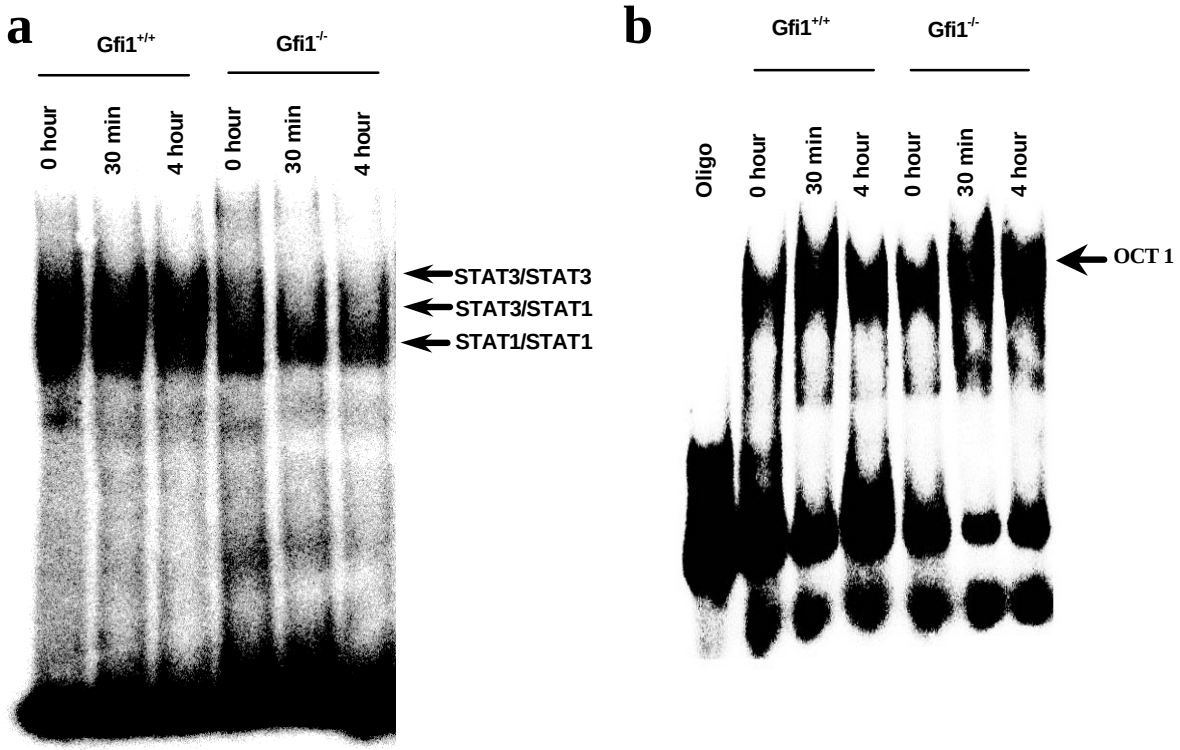


Figure 4.3.7.2. Decreased STAT3 translocation in the nucleus.
(a) Nuclear proteins were incubated with ^{32}P-labeled consensus sequence oligonucleotides (h-SIE) recognizing STAT3 protein and subjected to electrophoresis and autoradiography.
(b) To confirm equal loading, nuclear proteins were incubated with OCT1-specific oligonucleotides and subjected to electrophoresis and autoradiography.

the OCT1 (Fig. 4.3.7.2.b.) Specificity of protein-DNA interaction was proven by supershift assays (Fig. 4.3.7.3.a) and competition assays using excess of unlabelled oligonucleotides (Fig. 4.3.7.3.b). Thus it was concluded that the Gfi1 deficiency and defective DC differentiation were associated with impaired, GM-CSF induced STAT3 signalling in hematopoietic progenitor cells.

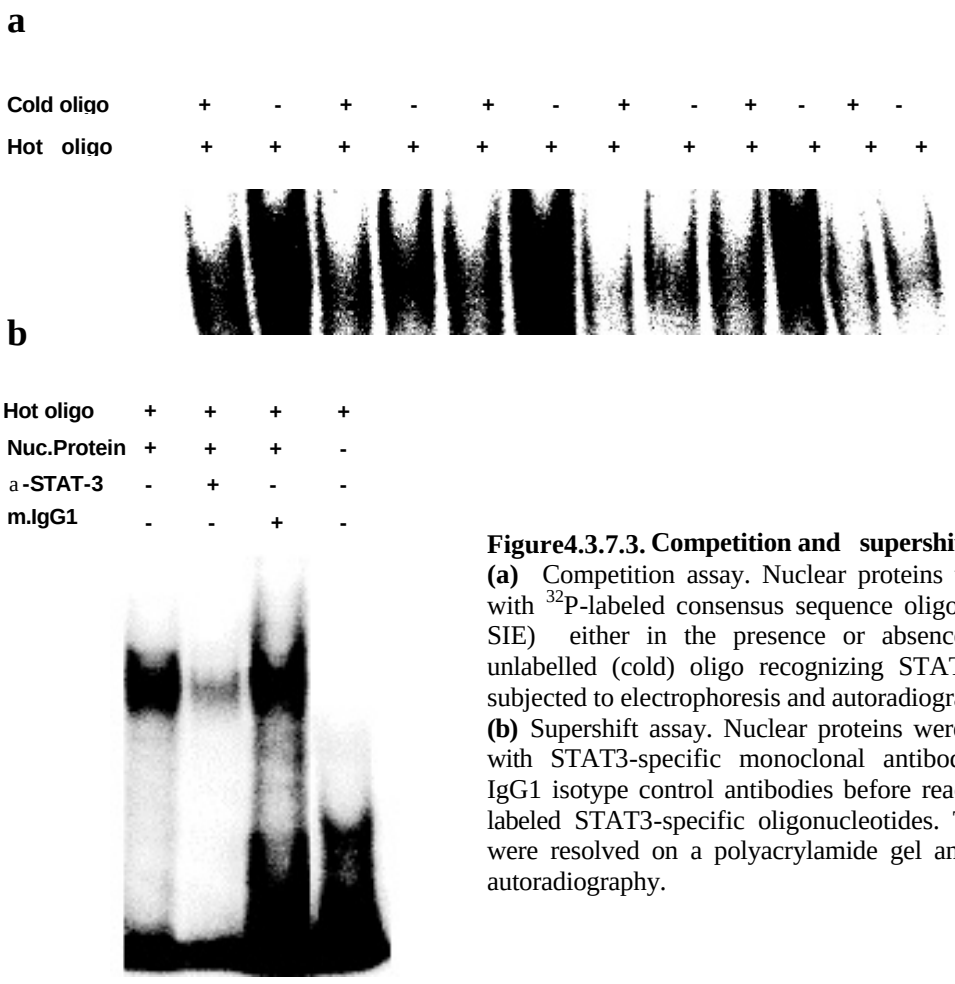


Figure4.3.7.3. Competition and supershift assays.
(a) Competition assay. Nuclear proteins were incubated with ^{32}P-labeled consensus sequence oligonucleotides (h-SIE) either in the presence or absence of 100 fold unlabelled (cold) oligo recognizing STAT3 protein and subjected to electrophoresis and autoradiography.
(b) Supershift assay. Nuclear proteins were pre-incubated with STAT3-specific monoclonal antibodies or mouse IgG1 isotype control antibodies before reaction with ^{32}P-labeled STAT3-specific oligonucleotides. The complexes were resolved on a polyacrylamide gel and subjected to autoradiography.

In summary, the results of this study convincingly demonstrate that Gfi1 plays a vital role in DC development and maturation. In the absence Gfi1, dendritic cells of all described lineages, except the Langerhans cells, are affected both qualitatively and quantitatively. Preliminary studies on the signal transduction pathways of Gfi1 suggest that Gfi1 might control DC development through STAT3 signalling.

5. Discussion

5.1 Transcription factor controlled differentiation of dendritic cells

The very aim of the current study was to increase the understanding of the molecular complexity of the differentiation program of dendritic cells from hematopoietic progenitor cells. In the past few years, a series of reports have been published regarding the gene expression profile of dendritic cells. However, most of these studies attempted to unravel the genes that are involved in the maturation process of dendritic cells either in response to various pathogens or in response to various stimuli. Several independent approaches have been adopted in these studies such as serial analysis of gene expression, (SAGE; Hashimoto SI et al., 2000), microarray studies (Granucci F et al., 2001; Matsunaga T et al., 2002; Messmer D et al., 2003), combined cDNA subtraction and microarray approaches (Ahn J H et al., 2002), and an integrated genomic and proteomic approaches (Richards J et al., 2002; La NF et al 2001).

In addition to the reports on genes involved in DC maturation, a few reports also focused on gene expression during DC differentiation from human monocytes (Angenieux C et al., 2001; Hashimoto S et al., 1999). Some pilot studies were also performed to study the differentially expressed genes in dendritic cells during the development from human $CD34^+$ cells (Fisher M et al., 1999; Ju xs et al., 2003) and from mouse spleen derived long-term cultures (LTC; Wilson H et al., 2003).

A recent report by Fohrer et. al has focused on the transcription factors, RelB and PU.1, to trace the lineage origin of human thymic dendritic cells and has categorised thymic dendritic cells into myeloid and plasmocytoid lineages based on the differences in the expression profiles of these transcription factors (Fohrer H et al., 2004).

Even though most of the reports focus on identifying the genetic control of DC differentiation and maturation on a global level, to date, a comprehensive knowledge about the role of transcription factors in DC development is still lacking. This lack of knowledge could be

explained by the difficulties in obtaining hematopoietic stem cells and to culture dendritic cells in-vitro. To circumvent these technical limitations, the present study has set out to generate an in vitro DC differentiation system based on a novel cell line CR1-mix.

A pioneering study on transcription factor based DC development has been recently reported by Hacker et al (2003). Even though their studies have identified transcription factors involved in DC differentiation and established the role of the transcription factor Id2 in DC development, their studies primarily utilised DCs derived from human $CD34^+$ cells. In addition, this study was based on a direct comparison on the gene expression profiles between the progenitor cells and differentiated dendritic cells. In contrast, the present study differs from their work in the following aspects. Firstly, the present study utilised a murine hematopoietic DC culture system for the gene expression studies. Secondly, this work focused on the transcription factors that were upregulated during the early pathways of DC differentiation. Hence, this study for the first time deciphers the role of transcription factors that control the early pathways of dendritic cells.

5.2 DCs differentiated from FDCP-mix cells have considerable proportion of 'contaminating' granulocytes and macrophages

To establish a cell system that allows the molecular analysis of DC differentiation with an emphasis on transcription factors, the initial work was done in 32D cells. 32D cells are originally described by Valtieri et al (Valtieri M et al, 1987) as a murine, hematopietic, IL3 dependent cells. Even though the differentiation potential of these cells across granulocytic and macrophage lineages (Ramos G, 2004) has been demonstrated, their ability to differentiate into DC lineage has not been explored so far. Thus the present study aimed to differentiate these cells into DC lineage. In spite of culturing these cells under various culture conditions, successful results could not be obtained. The failure of these cells to differentiate into DCs could be explained either by their loss of potential to differentiate into DCs or by the

unusual chromosome instability phenomenon (Agliano AM et al, 2000) reported previously in this cell line.

Another hematopoietic cell line, FDCP-mix was used in this study. FDCP-mix cells were established by infecting the long-term bone marrow cultures with src virus (Just U, 1991). FDCP-mix cells are IL-3 dependent, multipotent hematopietic progenitor cells. Previous studies have shown that FDCP-mix cells can be differentiated into granulocytes, macrophages (Ford AM,1992), erythrocytes, megakaryocytes, and early B-lymphoid lineage cells in vitro (Spooncer et al., 1986; Just et al, 1991; Ford et al, 1992). Recently Schroeder T, et. al (2000) have documented the differentiation potential of FDCP-mix into dendritic cells (Schroeder T., 2000). However in the present studies it was observed that the DCs that were obtained from FDCP-mix cells, in the presence of GM-CSF, were quite heterogeneous due to the 'contaminating' granulocyte and macrophage populations: Hence it was inevitable to define a novel differentiation system which might allow us to obtain synchronised and homogeneous DC population.

5.3 Retroviral mediated overexpression of the intracellular domain of Notch1 generates a novel IL3 dependent hematopoietic progenitor cell line

To date several approaches have been used to immortalize and expand hematopoietic progenitor cells. Retrovirus mediated overexpression of genes such as MDR1 (Bunting KD et al., 1999), Src (Just U et al 1991), gp130 (Audet j et al 2001), TEL-AML1 (Morrow M et al., 2004), and myeloid lysozyme gene (Ye M et al., 2003) and transcription factors such as Notch1 (Varnum F et al., 2000), Hox B4 (Antonchuk J et al., 2002;), ß-catenin (Reya T et al., 2003) and Lhx2 (Pinto O P et al., 2002) have been reported in the literature.

However, none of these cell lines has been reported to have DC differentiation potential. The present study made use of the technology initially reported by Varnum-Finney and colleagues (2000). These investigators have generated a retrovirus encoding the intracytoplasmic domain

of notch1 (ICN) to immortalize hematopoietic stem cells (Varnum F, 2000). In contrast to their approach, the present study modified the strategy in various aspects. Firstly Varnum-F al have shown that the overexpression of ICN immortalizes hematopoietic cells. However, these investigators did not check ICN transduced cells for their dependency on individual cytokines. In contrast, the present work examined the dependency of ICN transduced cells on the individual cytokines of the stem cell cytokine cocktail (IL-3, IL-6, Flt3L, SCF) and concluded that IL-3 plays a major role in the viability of these cells. Secondly, their goal was not to generate a hematopoietic cell line from the ICN transduced cells. In contrast, our purpose was to generate a hematopoietic cell line. CR1-mix cells showed a normal karyotype and could be maintained in culture for more than 2 years by repeated thawing and freezing. Thirdly, this study has shown that CR1-mix cells could be differentiated into dendritic cells. From the transplantation studies it was observed that CR1-mix cells did not provide efficient radioprotection to lethally irradiated mice. Nevertheless, their in vivo differentiation potential into most of the hematopoietic lineages such as dendritic cells, T cells, B cells, NK cells, granulocytes and macrophages except erythrocytes is documented in sublethally irradiated mice. The failure of CR1-mix cells to protect the lethally irradiated mice from radiation damage could be explained by the inability of these cells to differentiate into erythrocytes, despite its potential to generate all the other hematopoietic lineages. Moreover, immunophenotypic studies conducted in this cell line provided an evidence that this cell line expresses most of the hematopoietic stem/progenitor cell markers and lack the expression of surface markers that indicate a specific hematopoietic lineage.

Taken together, the present study suggests that CR1-mix cells may be considered as an immature multipotent progenitor cell line.

5.4 CR1-mix- a novel tool to study both myeloid and plasmocytoid dendritic cell development

In the past, several other cell lines with DC features have been described (Paglia et al., 1993;Girolomoni et al, 1995; Xu et al, 1995; Rasko et al, 1997, Schroeder T et al, 2000). However, these cell lines differ from CR1-mix in various aspects. Some of these cell lines were frozen at one stage of DC development; some others are tumorigenic or transformed by an oncogene, while the others have an 'improper' DC phenotype. Examples include the tumorigenic and factorindependent IGM36 cell line, derived from a GM-CSF transgenic mouse (Rasko et al, 1997), the immature skin-derived DC cell line- FDSC (Girolomoni et al, 1995), the immature spleen derived CB1 cell line (Paglia at al, 1993) and the FDCP-mix cells (Schroeder et al 2000). On the other hand none of these cell lines has been shown to differentiate equally into both myeloid and plasmocytoid dendritic cells in vitro. Interestingly, the data of this study indicate that CR1-mix cells can efficiently differentiate into both myeloid and lymphoid dendritic cell lineages in a directed and synchronous manner both in vitro and in vivo.

In addition, the morphology, immunophenotype and functional properties such as IL-12 secretion and allogenic T cells proliferation capacity of the CR1mix cells resemble bone marrow derived primary dendritic cells. These features along with the karyotypic stability make the cell line a versatile tool for analysing molecular and biochemical events associated with determination, differentiation and maturation of the different dendritic cell lineages.

5.5 The transcriptional repressor Gfi1 is critically important for DC development

Using a transcriptional screening approach, Gfi1 was identified as a crucial transcription factor controlling DC-development and -function. Gfi1 is a transcriptional zinc finger repressor originally identified as a target gene for proviral insertions leading to IL2

independent growth in a T cell lymphoma line (Gilks et al., 1993). Gfi1 contains six C2H2 zinc fingers and a transcriptional repressor domain (SNAG) (Grimes et al., 1996). Previous studies suggest that Gfi1 acts as a protooncogene by accelerating T-cell proliferation and inhibiting apoptosis and cell cycle arrest (Grimes et al., 1996; Karsunky et al., 2002a; Schmidt et al., 1998). Furthermore, Gfi1 regulates proliferation and differentiation of thymic T-cells (Yucel et al., 2003). More recently, an intrinsic role of Gfi1 in granulocyte development has evolved, when the analysis of $Gfi1^{-/-}$ revealed an unexpected absence of mature neutrophils (Hock et al., 2003; Karsunky et al., 2002b). Heterozygous mutations in Gfi1 have been identified in rare patients with hereditary neutropenia, suggesting that dominant negative variants may block neutrophil differentiation (Person et al., 2003). The present study provides evidence for yet another role of Gfi1 in controlling the complexity of hematopoietic stem cell differentiation: Gfi1 deficiency leads to a global reduction in the number of DC precursors and their progeny in bone marrow, thymus, spleen and lymph nodes, as well as to incomplete DC maturation and function. Furthermore, Gfi1 appears to be key regulator of DC versus macrophage development.

5.6 Gfi1 kncokout mice show reduced DC numbers of myeloid, lymphoid and plasmocytoid lineages except Langerhans cells

The initial aim of this study was to identify transcription factors controlling specific subsets of DCs, in particular in the development of “myeloid” DCs as progeny of GM-CSF treated HSC. Previous reports have implicated a role for PU. 1(Anderson et al., 2000; Guerriero et al., 2000), RelB (Wu et al., 1998), and Ikaros C (Wu et al., 1997) in the differentiation of $CD11c^{+}CD8\alpha^{-}$ “myeloid” DCs. Unexpectedly, a global reduction of all DCs in primary and secondary lymphoid organs in Gfi1 deficient mice was observed in this study, irrespective of CD8α expression. Although these data do not exclude the existence of “myeloid” DCs, the analysis of $Gfi1^{-/-}$ mice does not provide any evidence for a distinct myeloid DC lineage.

Interestingly, Gfi1 deficiency did not perturb Langerhans cell development. In that respect, Gfi1-deficient mice resemble RelB$^{-/-}$ (Wu et al., 1998) and IkarosC$^{-/-}$ mice (Wu et al., 1997) that are characterized by a deficiency of CD8α^- DCs while epidermal LC appear normal. In contrast, TGF$\beta^{-/-}$ (Borkowski et al., 1996) and Id2-knockout mice (Hacker et al., 2003) show a complete lack of epidermal LCs, while other DC subpopulations are at least partially preserved, supporting the notion of a distinct LC lineage. Thus, the analysis of Gfi1$^{-/-}$ deficient mice provides additional evidence for a dissociation of DC development in peripheral lymphoid organs and epidermal Langerhans cells.

5.7 Gfi1 plays a crucial role in determining the lineage outcome between dendritic cells and macrophages

The decisive factors controlling macrophage versus DC development remain elusive. Various cytokines, such as IL6, TNFα, and interferon-γ induce DC versus macrophage differentiation in vitro (Chomarat et al., 2000; Chomarat et al., 2003; Delneste et al., 2003). Other studies suggest that the notch ligand delta-1 inhibits macrophage differentiation while permitting DC differentiation (Ohishi et al., 2001). However, analysis of cytokine knockout mice has not yet revealed any specific factor that dissociates DC and macrophage development. Intracellular mechanisms responsible for DC versus macrophage development are currently not known. The phenotype of Notch-1$^{-/-}$ mice suggests that both DC and LC development is completely independent of Notch-1 (Wilson et al., 2001). In mice doubly deficient for two NFκB subunits (p50$^{-/-}$RelA$^{-/-}$), GM-CSF-dependent DC development is severely reduced whereas M-CSF dependent macrophage development appears normal (Ouaaz et al., 2002). In the undertaken study, both Flt3L and GM-CSF, two cytokines inducing DC development in wildtype hematopoietic stem cells, drive Gfi1$^{-/-}$ hematopoietic progenitor cells into macrophage differentiation. Thus, Gfi1 is a unique factor governing DC versus macrophage development in vitro. Previous reports have identified an excess of atypical “immature” myeloid cells in

Gfi1$^{-/-}$ mice, characterized by expression of Gr1 and Mac1 (Hock et al., 2003; Karsunky et al., 2002b). Hock et al. proposed a dual function of Gfi1, which may be required not only for neutrophil maturation but also for terminal macrophage differentiation (Hock et al., 2003). We have shown that the absence of Gfi1 leads to a significant reduction in HSC, CLP, CMP and DC progenitor cells while GMPs are increased in numbers, suggesting that Gfi1 acts on multiple levels of progenitor cell differentiation, including critical checkpoints of DC versus macrophage development (Fig. 5. 7.1).

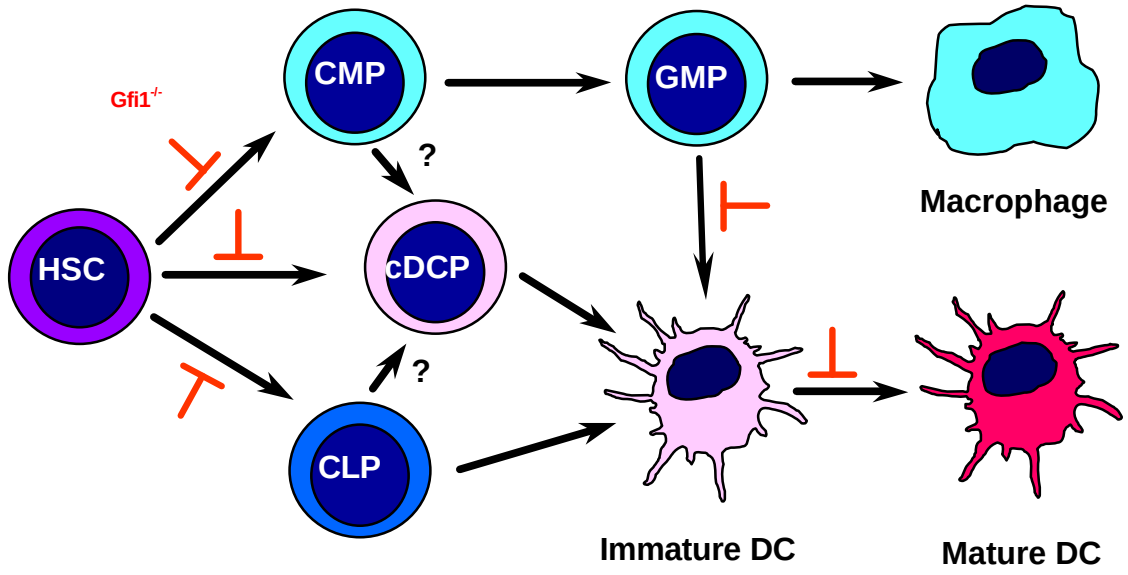


Figure 5..7.1 Proposed Gfi1 dependent checkpoint in DC development

5.8 Gfi1 controlled DC differentiation is dependent on STAT3 activation

Mechanistically, the function of Gfi1 is under active investigation. Gfi1 shares the same DNA binding- and a SNAG (Snail and Gfi1 family of proteins) repression domain with its homologue, Gfi1B. Both factors have redundant and unique biological roles in controlling hematopoiesis. As a transcription factor, Gfi1 displays activity in the nucleus. In addition there is evidence for a potential cytoplasmatic role for Gfi1 mediated by physical interaction with PIAS3 (protein inhibitor of activated STAT3), a specific inhibitor of STAT3. Using a

STAT3-dependent reporter gene assay, earlier studies have suggested that Gfi1 can increase STAT3 signalling by overcoming the inhibitory effects mediated by PIAS3 (Rodel et al., 2000). The present study confirms and extends this observation in a functional model of DC differentiation and provide unequivocal evidence that in the absence of Gfi1, STAT3 signalling is significantly reduced in early hematopoietic progenitor cells. Further evidence for a critical role of STAT3 in DC development has recently been proposed by Laouar et al. (Laouar et al., 2003). Their studies determined STAT3 activation as a critical checkpoint of Flt3L regulated DC development. In the absence of STAT3, the transition of CLP and CMP to $Flt3^{+}$ DC precursors was severely impaired, while GM-CSF-induced DC differentiation in vitro was not altered in this system. In contrast to the complete deficiency of STAT3, in the absence of Gfi1, both Flt3L and GM-CSF-dependent DC differentiation is abrogated, suggesting that deficient STAT3 signalling is not the only mechanism explaining the phenotype of $Gfi1^{-/-}$ hematopoiesis. These differences could either be explained by residual STAT3 activation in $Gfi1^{-/-}$ cells or the effects of multiple STAT3 isoforms present in DC development (Welte et al., 1997). Furthermore, Gfi1 acts as a transcriptional modulator and influences a multitude of downstream factors. The identification of downstream targets illustrates the complexity of Gfi1-dependent pathways. Gfi1 binds to functionally diverse sets of genes in myeloid cells such as JAK3, IL8, c-myc and members of the C/EBP family (Duan and Horwitz, 2003). Interestingly, Gfi1 also represses its own transcription in vitro (Doan et al., 2004; Duan and Horwitz, 2003) and in vivo (Yucel et al., 2004). The observation of fluctuating Gfi1 expression and untoward toxic effects of retrovirus-mediated Gfi1 expression in the present work, reflect the importance of tight transcriptional regulation of Gfi1 gene expression in DC precursor cells.

5.9 Gfi1 controls both development and activation of dendritic cells

Previous reports have differentiated DC maturation and DC activation as molecularly distinct events, phenotypically characterized by upregulation of surface expression of MHC class II and the costimulatory molecules CD40, CD80 and CD86 and production of inflammatory cytokines (e.g. IL12) (Kaisho and Akira, 2001; Kobayashi et al., 2003; Ouaaz et al., 2002). DC maturation is primarily induced upon signalling via Toll-like-receptors (TLR) and CD40. Gfi1$^{-/-}$ DCs show decreased expression of MHC class II and decreased upregulation of costimulatory receptors upon stimulation with LPS and anti-CD40 monoclonal antibodies, a phenotype consistent with impaired DC maturation. TRAF6 has emerged as a point of convergence for both TLR- and CD40-mediated signalling cascades, linking both pathways to NFκB activation. TRAF6$^{-/-}$ deficient DCs show decreased maturation and thus resemble Gfi1$^{-/-}$ DCs (Kobayashi et al., 2003). However, in contrast to Gfi1$^{-/-}$ DCs, TRAF6$^{-/-}$ DCs are characterized by decreased cytokine production (Kobayashi et al., 2003). The analysis of mice deficient for the expression of defined factors regulating NFκB have provided more insights into the complex network regulating DC activation and cytokine production. For example, mice doubly deficient in the NFkB subunits p50 and RelA exhibit a severe reduction in CD8+ and CD8- DCs, while mice doubly deficient in p50 and cRel show impaired CD40L-mediated survival and IL12 production (Ouaaz et al., 2002). Furthermore, DCs from mice deficient in the negative NFκB regulator NFκB2 show enhanced expression of activation markers but produce normal levels of cytokines (Speirs et al., 2004). In contrast to these models however, Gfi1$^{-/-}$ deficient DCs reveal a dissociated phenotype characterized by decreased maturation and increased activation. In view of these results documenting decreased STAT3 activation in DC progenitors, it is tempting to speculate that the increased cytokine secretion is related to deficient STAT3 activation in DCs. Targeted STAT3 deficiency in the hematopoietic system is associated with chronic enterocolitis, aberrant inflammation and lethality to septic peritonitis (Matsukawa et al., 2003; Takeda et al., 1999; Welte et al., 1997). This pathology is

associated with increased expression of inflammatory cytokines. No data has been published about maturation of STAT3$^{-/-}$ DCs, but overstimulated innate immunity is associated with enhanced NFκB activity in STAT3$^{-/-}$ hematopoietic cells (Welte et al., 2003). Thus Gfi1$^{-/-}$ mice offer a model system to further dissect the mechanisms controlling DC maturation and activation. Recent data suggest a PIAS3-dependent negative regulation of the p65 subunit (RelA) of NFkB and thus establish a potential link between STAT3 and NFkB pathways (Jang et al., 2004). Further studies are required to elucidate the significance of this potential crosstalk.

In conclusion, results of this study demonstrated a key role for the transcriptional repressor Gfi1 in DC development and function. These studies also provide insights into the complex hierarchical network controlling DC differentiation, maturation and activation, and reveal specific pathways that might ultimately be important for the design of rational DC therapies.

6. References

Adolfsson, J., Borge, O. J., Bryder, D., Theilgaard-Monch, K., Astrand-Grundstrom, I., Sitnicka, E., Sasaki, Y., and Jacobsen, S. E. (2001). Upregulation of Flt3 expression within the bone marrow Lin(-)Sca1(+)c-kit(+) stem cell compartment is accompanied by loss of self-renewal capacity. Immunity *15*, 659-669.

Agliano, A. M., Santangelo, C., Silvestri, I., Gazzaniga, P., Giuliani, L., Naso, G., Frati, L., and Castiglia, R. (2000). On chromosomal instability: what is the karyotype of your 32D CI3 cell line. Blood *95*, 3636-3637.

Ahn, J. H., Lee, Y., Jeon, C., Lee, S. J., Lee, B. H., Choi, K. D., and Bae, Y. S. (2002). Identification of the genes differentially expressed in human dendritic cell subsets by cDNA subtraction and microarray analysis. Blood *100*, 1742-1754.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I. L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature *404*, 193-197.

Akbari, O., Panjwani, N., Garcia, S., Tascon, R., Lowrie, D., and Stockinger, B. (1999). DNA vaccination: transfection and activation of dendritic cells as key events for immunity. J Exp Med *189*, 169-178.

Albert, M. L., Sauter, B., and Bhardwaj, N. (1998). Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature *392*, 86-89.

Anderson, K. L., Perkin, H., Surh, C. D., Venturini, S., Maki, R. A., and Torbett, B. E. (2000). Transcription factor PU.1 is necessary for development of thymic and myeloid progenitor-derived dendritic cells. J Immunol *164*, 1855-1861.

Angenieux, C., Fricker, D., Strub, J. M., Luche, S., Bausinger, H., Cazenave, J. P., Van Dorsselaer, A., Hanau, D., de la Salle, H., and Rabilloud, T. (2001). Gene induction during differentiation of human monocytes into dendritic cells: an integrated study at the RNA and protein levels. Funct Integr Genomics *1*, 323-329.

Anjuere, F., Martin, P., Ferrero, I., Fraga, M. L., del Hoyo, G. M., Wright, N., and Ardavin, C. (1999). Definition of dendritic cell subpopulations present in the spleen, Peyer's patches, lymph nodes, and skin of the mouse. Blood *93*, 590-598.

Antonchuk, J., Sauvageau, G., and Humphries, R. K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell *109*, 39-45.

Ardavin, C., Martinez del Hoyo, G., Martin, P., Anjuere, F., Arias, C. F., Marin, A. R., Ruiz, S., Parrillas, V., and Hernandez, H. (2001). Origin and differentiation of dendritic cells. Trends Immunol *22*, 691-700.

Ardavin, C. (2003). Origin, precursors and differentiation of mouse dendritic cells. Nat Rev Immunol *3*, 582-590.

Asselin-Paturel, C., Boonstra, A., Dalod, M., Durand, I., Yessaad, N., Dezutter-Dambuyant, C., Vicari, A., O'Garra, A., Biron, C., Briere, F., and Trinchieri, G. (2001). Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. Nat Immunol *2*, 1144-1150.

Austyn, J. M. (1996). New insights into the mobilization and phagocytic activity of dendritic cells. J Exp Med *183*, 1287-1292.

Banchereau, J., and Steinman, R. M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y. J., Pulendran, B., and Palucka, K. (2000). Immunobiology of dendritic cells. Annu Rev Immunol *18*, 767-811.

Bell, D., Young, J. W., and Banchereau, J. (1999). Dendritic cells. Adv Immunol *72*, 255-324.

Bender, A., Sapp, M., Schuler, G., Steinman, R. M., and Bhardwaj, N. (1996). Improved methods for the generation of dendritic cells from nonproliferating progenitors in human blood. J Immunol Methods *196*, 121-135.

Borkowski, T. A., Letterio, J. J., Farr, A. G., and Udey, M. C. (1996). A role for endogenous transforming growth factor beta 1 in Langerhans cell biology: the skin of transforming growth factor beta 1 null mice is devoid of epidermal Langerhans cells. J Exp Med *184*, 2417-2422.

Brasel, K., De Smedt, T., Smith, J. L., and Maliszewski, C. R. (2000). Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. Blood *96*, 3029-3039.

Bunting, K. D., Galipeau, J., Topham, D., Benaim, E., and Sorrentino, B. P. (1999). Effects of retroviral-mediated MDR1 expression on hematopoietic stem cell self-renewal and differentiation in culture. Ann N Y Acad Sci *872*, 125-140; discussion 140-121.

Caux, C., Dezutter-Dambuyant, C., Schmitt, D., and Banchereau, J. (1992). GM-CSF and TNF-alpha cooperate in the generation of dendritic Langerhans cells. Nature *360*, 258-261.

Caux, C., Vanbervliet, B., Massacrier, C., Dezutter-Dambuyant, C., de Saint-Vis, B., Jacquet, C., Yoneda, K., Imamura, S., Schmitt, D., and Banchereau, J. (1996a). CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. J Exp Med *184*, 695-706.

Caux, C., Vanbervliet, B., Massacrier, C., Dubois, B., de Saint Vis, B., Fayette, J., Rissoan, M. C., Briere, F., Lebecque, S., Rousset, F., *et al.* (1996b). In vitro regulation of development and function of dendritic cells. Hematol Cell Ther *38*, 463.

Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I., and Lanzavecchia, A. (1999). Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. J Exp Med *189*, 821-829.

Cella, M., Sallusto, F., and Lanzavecchia, A. (1997). Origin, maturation and antigen presenting function of dendritic cells. Curr Opin Immunol *9*, 10-16.

Chaperot, L., Bendriss, N., Manches, O., Gressin, R., Maynadie, M., Trimoreau, F., Orfeuvre, H., Corront, B., Feuillard, J., Sotto, J. J., *et al.* (2001). Identification of a leukemic counterpart of the plasmacytoid dendritic cells. Blood *97*, 3210-3217.

Chomarat, P., Banchereau, J., Davoust, J., and Palucka, A. K. (2000). IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. Nat Immunol *1*, 510-514.

Chomarat, P., Dantin, C., Bennett, L., Banchereau, J., and Palucka, A. K. (2003). TNF skews monocyte differentiation from macrophages to dendritic cells. J Immunol *171*, 2262-2269.

Christensen, J. L., and Weissman, I. L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: a simple method to isolate long-term stem cells. Proc Natl Acad Sci U S A *98*, 14541-14546.

Colonna, M., Navarro, F., Bellon, T., Llano, M., Garcia, P., Samaridis, J., Angman, L., Cella, M., and Lopez-Botet, M. (1997). A common inhibitory receptor for major histocompatibility complex class I molecules on human lymphoid and myelomonocytic cells. J Exp Med *186*, 1809-1818.

Curti, A., Fogli, M., Ratta, M., Tura, S., and Lemoli, R. M. (2001). Stem cell factor and FLT3-ligand are strictly required to sustain the long-term expansion of primitive CD34+DR- dendritic cell precursors. J Immunol *166*, 848-854.

Davoust, J., and Banchereau, J. (2000). Naked antigen-presenting molecules on dendritic cells. Nat Cell Biol *2*, E46-48.

D'Amico, A., and Wu, L. (2003). The early progenitors of mouse dendritic cells and plasmacytoid predendritic cells are within the bone marrow hemopoietic precursors expressing Flt3. J Exp Med *198*, 293-303.

del Hoyo, G. M., Martin, P., Vargas, H. H., Ruiz, S., Arias, C. F., and Ardavin, C. (2002). Characterization of a common precursor population for dendritic cells. Nature *415*, 1043-1047.

Delneste, Y., Charbonnier, P., Herbault, N., Magistrelli, G., Caron, G., Bonnefoy, J. Y., and Jeannin, P. (2003). Interferon-gamma switches monocyte differentiation from dendritic cells to macrophages. Blood *101*, 143-150.

Doan, L. L., Porter, S. D., Duan, Z., Flubacher, M. M., Montoya, D., Tsichlis, P. N., Horwitz, M., Gilks, C. B., Grimes, H. L., Akashi, K., *et al.* (2004). Targeted transcriptional repression of Gfi1 by GFI1 and GFI1B in lymphoid cells. Nucleic Acids Res *32*, 2508-2519.

Duan, Z., and Horwitz, M. (2003). Targets of the transcriptional repressor oncoprotein Gfi-1. Proc Natl Acad Sci U S A *100*, 5932-5937

Dubois, B., Massacrier, C., Vanbervliet, B., Fayette, J., Briere, F., Banchereau, J., and Caux, C. (1998). Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. J Immunol *161*, 2223-2231.

Dubois, B., Vanbervliet, B., Fayette, J., Massacrier, C., Briere, F., Banchereau, J., and Caux, C. (1997a). Human dendritic/Langerhans cells control growth and differentiation of CD40 activated B cells. Adv Exp Med Biol *417*, 329-334.

Dubois, B., Vanbervliet, B., Fayette, J., Massacrier, C., Van Kooten, C., Briere, F., Banchereau, J., and Caux, C. (1997b). Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes. J Exp Med *185*, 941-951.

Eaves, C., Glimm, H., Eisterer, W., Audet, J., Maguer-Satta, V., and Piret, J. (2001). Characterization of human hematopoietic cells with short-lived in vivo repopulating activity. Ann N Y Acad Sci *938*, 63-70; discussion 70-61.

Engering, A. J., Cella, M., Fluitsma, D. M., Hoefsmit, E. C., Lanzavecchia, A., and Pieters, J. (1997). Mannose receptor mediated antigen uptake and presentation in human dendritic cells. Adv Exp Med Biol *417*, 183-187.

Fanger, N. A., Wardwell, K., Shen, L., Tedder, T. F., and Guyre, P. M. (1996). Type I (CD64) and type II (CD32) Fc gamma receptor-mediated phagocytosis by human blood dendritic cells. J Immunol *157*, 541-548.

Fayette, J., Dubois, B., Vandenabeele, S., Bridon, J. M., Vanbervliet, B., Durand, I., Banchereau, J., Caux, C., and Briere, F. (1997). Human dendritic cells skew isotype switching of CD40-activated naive B cells towards IgA1 and IgA2. J Exp Med *185*, 1909-1918.

Fayette, J., Durand, I., Bridon, J. M., Arpin, C., Dubois, B., Caux, C., Liu, Y. J., Banchereau, J., and Briere, F. (1998). Dendritic cells enhance the differentiation of naive B cells into plasma cells in vitro. Scand J Immunol *48*, 563-570.

Fisher, G. M., Iqball, S., and Knight, S. C. (1999). Gene expression during differentiation of human dendritic cells from cord blood cd34 stem cells. Cytokine *11*, 111-117.

Fohrer, H., Audit, I. M., Sainz, A., Schmitt, C., Dezutter-Dambuyant, C., and Dalloul, A. H. (2004). Analysis of transcription factors in thymic and CD34+ progenitor-derived plasmacytoid and myeloid dendritic cells: evidence for distinct expression profiles. Exp Hematol *32*, 104-112.

Ford, A. M., Healy, L. E., Bennett, C. A., Navarro, E., Spooncer, E., and Greaves, M. F. (1992). Multilineage phenotypes of interleukin-3-dependent progenitor cells. Blood *79*, 1962-1971.

Galy, A., Travis, M., Cen, D., and Chen, B. (1995). Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. Immunity *3*, 459-473.

Geldhof, A. B., Moser, M., and De Baetselier, P. (1998). IL-12-activated NK cells recognize B7 costimulatory molecules on tumor cells and autologous dendritic cells. Adv Exp Med Biol *451*, 203-210.

Gilks, C. B., Bear, S. E., Grimes, H. L., and Tsichlis, P. N. (1993). Progression of interleukin-2 (IL-2)-dependent rat T cell lymphoma lines to IL-2-independent growth following activation of a gene (Gfi-1) encoding a novel zinc finger protein. Mol Cell Biol *13*, 1759-1768.

Girolomoni, G., Lutz, M. B., Pastore, S., Assmann, C. U., Cavani, A., and Ricciardi-Castagnoli, P. (1995). Establishment of a cell line with features of early dendritic cell precursors from fetal mouse skin. Eur J Immunol *25*, 2163-2169.

Granucci, F., Vizzardelli, C., Pavelka, N., Feau, S., Persico, M., Virzi, E., Rescigno, M., Moro, G., and Ricciardi-Castagnoli, P. (2001a). Inducible IL-2 production by dendritic cells revealed by global gene expression analysis. Nat Immunol *2*, 882-888.

Granucci, F., Vizzardelli, C., Virzi, E., Rescigno, M., and Ricciardi-Castagnoli, P. (2001b). Transcriptional reprogramming of dendritic cells by differentiation stimuli. Eur J Immunol *31*, 2539-2546.

Grimes, H. L., Gilks, C. B., Chan, T. O., Porter, S., and Tsichlis, P. N. (1996). The Gfi-1 protooncoprotein represses Bax expression and inhibits T-cell death. Proc Natl Acad Sci U S A *93*, 14569-14573.

Grimes, H. L., Chan, T. O., Zweidler-McKay, P. A., Tong, B., and Tsichlis, P. N. (1996). The Gfi-1 proto-oncoprotein contains a novel transcriptional repressor domain, SNAG, and inhibits G1 arrest induced by interleukin-2 withdrawal. Mol Cell Biol *16*, 6263-6272.

Grouard, G., Rissoan, M. C., Filgueira, L., Durand, I., Banchereau, J., and Liu, Y. J. (1997). The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. J Exp Med *185*, 1101-1111.

Guerriero, A., Langmuir, P. B., Spain, L. M., and Scott, E. W. (2000). PU.1 is required for myeloid-derived but not lymphoid-derived dendritic cells. Blood *95*, 879-885.

Hacker, C., Kirsch, R. D., Ju, X. S., Hieronymus, T., Gust, T. C., Kuhl, C., Jorgas, T., Kurz, S. M., Rose-John, S., Yokota, Y., and Zenke, M. (2003). Transcriptional profiling identifies Id2 function in dendritic cell development. Nat Immunol *4*, 380-386.

Hart, D. N. (1997). Dendritic cells: unique leukocyte populations which control the primary immune response. Blood *90*, 3245-3287.

Hartmann, G., Weiner, G. J., and Krieg, A. M. (1999). CpG DNA: a potent signal for growth, activation, and maturation of human dendritic cells. Proc Natl Acad Sci U S A *96*, 9305-9310.

Hashimoto, S., Suzuki, T., Dong, H. Y., Nagai, S., Yamazaki, N., and Matsushima, K. (1999). Serial analysis of gene expression in human monocyte-derived dendritic cells. Blood *94*, 845-852.

Hashimoto, S. I., Suzuki, T., Nagai, S., Yamashita, T., Toyoda, N., and Matsushima, K. (2000). Identification of genes specifically expressed in human activated and mature dendritic cells through serial analysis of gene expression. Blood *96*, 2206-2214.

Hock, H., Hamblen, M. J., Rooke, H. M., Schindler, J. W., Saleque, S., Fujiwara, Y., and Orkin, S. H. (2004). Gfi-1 restricts proliferation and preserves functional integrity of haematopoietic stem cells. Nature.

Hock, H., Hamblen, M. J., Rooke, H. M., Traver, D., Bronson, R. T., Cameron, S., and Orkin, S. H. (2003). Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation. Immunity *18*, 109-120.

Ichikawa, E., Hida, S., Omatsu, Y., Shimoyama, S., Takahara, K., Miyagawa, S., Inaba, K., and Taki, S. (2004). Defective development of splenic and epidermal CD4+ dendritic cells in mice deficient for IFN regulatory factor-2. Proc Natl Acad Sci U S A *101*, 3909-3914.

Inaba, K., Inaba, M., Naito, M., and Steinman, R. M. (1993). Dendritic cell progenitors phagocytose particulates, including bacillus Calmette-Guerin organisms, and sensitize mice to mycobacterial antigens in vivo. J Exp Med *178*, 479-488.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R. M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. J Exp Med *176*, 1693-1702.

Jakob, T., and Udey, M. C. (1998). Regulation of E-cadherin-mediated adhesion in Langerhans cell-like dendritic cells by inflammatory mediators that mobilize Langerhans cells in vivo. J Immunol *160*, 4067-4073.

Jang, H. D., Yoon, K., Shin, Y. J., Kim, J., and Lee, S. Y. (2004). PIAS3 suppresses NF-kappaB-mediated transcription by interacting with the p65/RelA subunit. J Biol Chem *279*, 24873-24880.

Jiang, W., Swiggard, W. J., Heufler, C., Peng, M., Mirza, A., Steinman, R. M., and Nussenzweig, M. C. (1995). The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature *375*, 151-155.

Ju, X. S., Hacker, C., Madruga, J., Kurz, S. M., Knespel, S., Blendinger, G., Rose-John, S., and Martin, Z. (2003). Towards determining the differentiation program of antigen-presenting dendritic cells by transcriptional profiling. Eur J Cell Biol *82*, 75-86.

Just, U., Katsuno, M., Stocking, C., Spooncer, E., and Dexter, M. (1993). Targeted in vivo infection with a retroviral vector carrying the interleukin-3 (multi-CSF) gene leads to immortalization and leukemic transformation of primitive hematopoietic progenitor cells. Growth Factors *9*, 41-55.

Kaisho, T., and Akira, S. (2001). Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. Trends Immunol *22*, 78-83.

Kampgen, E., Koch, F., Heufler, C., Eggert, A., Gill, L. L., Gillis, S., Dower, S. K., Romani, N., and Schuler, G. (1994). Understanding the dendritic cell lineage through a study of cytokine receptors. J Exp Med *179*, 1767-1776.

Karsunky, H., Mende, I., Schmidt, T., and Moroy, T. (2002a). High levels of the onco-protein Gfi-1 accelerate T-cell proliferation and inhibit activation induced T-cell death in Jurkat T-cells. Oncogene *21*, 1571-1579.

Karsunky, H., Zeng, H., Schmidt, T., Zevnik, B., Kluge, R., Schmid, K. W., Duhrsen, U., and Moroy, T. (2002b). Inflammatory reactions and severe neutropenia in mice lacking the transcriptional repressor Gfi1. Nat Genet *30*, 295-300.

Kaufmann, C., Yoshida, T., Perotti, E. A., Landhuis, E., Wu, P., and Georgopoulos, K. (2003). A complex network of regulatory elements in Ikaros and their activity during hemo-lymphopoiesis. Embo J *22*, 2211-2223.

Klein, C., Bueler, H., and Mulligan, R. C. (2000). Comparative analysis of genetically modified dendritic cells and tumor cells as therapeutic cancer vaccines. J Exp Med *191*, 1699-1708.

Kobayashi, T., Walsh, P. T., Walsh, M. C., Speirs, K. M., Chiffoleau, E., King, C. G., Hancock, W. W., Caamano, J. H., Hunter, C. A., Scott, P., *et al.* (2003). TRAF6 is a critical factor for dendritic cell maturation and development. Immunity *19*, 353-363.

Kobayashi, Y. (1997). Langerhans' cells produce type IV collagenase (MMP-9) following epicutaneous stimulation with haptens. Immunology *90*, 496-501.

Kondo, M., Weissman, I. L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell *91*, 661-672.

Kondo, M., Wagers, A. J., Manz, M. G., Prohaska, S. S., Scherer, D. C., Beilhack, G. F., Shizuru, J. A., and Weissman, I. L. (2003). Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annu Rev Immunol *21*, 759-806.

Laouar, Y., Welte, T., Fu, X. Y., and Flavell, R. A. (2003). STAT3 is required for Flt3L-dependent dendritic cell differentiation. Immunity *19*, 903-912.

Le Naour, F., Hohenkirk, L., Grolleau, A., Misek, D. E., Lescure, P., Geiger, J. D., Hanash, S., and Beretta, L. (2001). Profiling changes in gene expression during differentiation and maturation of monocyte-derived dendritic cells using both oligonucleotide microarrays and proteomics. J Biol Chem *276*, 17920-17931.

Manz, M. G., Traver, D., Miyamoto, T., Weissman, I. L., and Akashi, K. (2001). Dendritic cell potentials of early lymphoid and myeloid progenitors. Blood *97*, 3333-3341.

Maraskovsky, E., Brasel, K., Teepe, M., Roux, E. R., Lyman, S. D., Shortman, K., and McKenna, H. J. (1996). Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. J Exp Med *184*, 1953-1962.

Maraskovsky, E., Daro, E., Roux, E., Teepe, M., Maliszewski, C. R., Hoek, J., Caron, D., Lebsack, M. E., and McKenna, H. J. (2000). In vivo generation of human dendritic cell subsets by Flt3 ligand. Blood *96*, 878-884.

Martin, P., del Hoyo, G. M., Anjuere, F., Ruiz, S. R., Arias, C. F., Marin, A. R., and Ardavin, C. (2000). Concept of lymphoid versus myeloid dendritic cell lineages revisited: both CD8alpha(-) and CD8alpha(+) dendritic cells are generated from CD4(low) lymphoid-committed precursors. Blood *96*, 2511-2519.

Martinez del Hoyo, G., Martin, P., Arias, C. F., Marin, A. R., and Ardavin, C. (2002). CD8alpha+ dendritic cells originate from the CD8alpha- dendritic cell subset by a maturation process involving CD8alpha, DEC-205, and CD24 up-regulation. Blood *99*, 999-1004.

Matsukawa, A., Takeda, K., Kudo, S., Maeda, T., Kagayama, M., and Akira, S. (2003). Aberrant inflammation and lethality to septic peritonitis in mice lacking STAT3 in macrophages and neutrophils. J Immunol *171*, 6198-6205.

Matsunaga, T., Ishida, T., Takekawa, M., Nishimura, S., Adachi, M., and Imai, K. (2002). Analysis of gene expression during maturation of immature dendritic cells derived from peripheral blood monocytes. Scand J Immunol *56*, 593-601.

Matsuno, K., Ezaki, T., Kudo, S., and Uehara, Y. (1996). A life stage of particle-laden rat dendritic cells in vivo: their terminal division, active phagocytosis, and translocation from the liver to the draining lymph. J Exp Med *183*, 1865-1878.

Mayordomo, J. I., Zorina, T., Storkus, W. J., Zitvogel, L., Celluzzi, C., Falo, L. D., Melief, C. J., Ildstad, S. T., Kast, W. M., and Deleo, A. B. (1995). Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. Nat Med *1*, 1297-1302.

McKenna, H. J., Stocking, K. L., Miller, R. E., Brasel, K., De Smedt, T., Maraskovsky, E., Maliszewski, C. R., Lynch, D. H., Smith, J., Pulendran, B., *et al.* (2000). Mice lacking flt3 ligand have deficient hematopoiesis affecting hematopoietic progenitor cells, dendritic cells, and natural killer cells. Blood *95*, 3489-3497.

Mellman, I., and Steinman, R. M. (2001). Dendritic cells: specialized and regulated antigen processing machines. Cell *106*, 255-258.

Messmer, D., Messmer, B., and Chiorazzi, N. (2003). The global transcriptional maturation program and stimuli-specific gene expression profiles of human myeloid dendritic cells. Int Immunol *15*, 491-503.

Moll, H. (1993). Epidermal Langerhans cells are critical for immunoregulation of cutaneous leishmaniasis. Immunol Today *14*, 383-387.

Morrow, M., Horton, S., Kioussis, D., Brady, H. J., and Williams, O. (2004). TEL-AML1 promotes development of specific hematopoietic lineages consistent with preleukemic activity. Blood *103*, 3890-3896.

Ohishi, K., Varnum-Finney, B., Serda, R. E., Anasetti, C., and Bernstein, I. D. (2001). The Notch ligand, Delta-1, inhibits the differentiation of monocytes into macrophages but permits their differentiation into dendritic cells. Blood *98*, 1402-1407.

Orkin, S. H. (2000). Diversification of haematopoietic stem cells to specific lineages. Nat Rev Genet *1*, 57-64.

Ouaaz, F., Arron, J., Zheng, Y., Choi, Y., and Beg, A. A. (2002). Dendritic cell development and survival require distinct NF-kappaB subunits. Immunity *16*, 257-270.

Paglia, P., Girolomoni, G., Robbiati, F., Granucci, F., and Ricciardi-Castagnoli, P. (1993). Immortalized dendritic cell line fully competent in antigen presentation initiates primary T cell responses in vivo. J Exp Med *178*, 1893-1901.

Person, R. E., Li, F. Q., Duan, Z., Benson, K. F., Wechsler, J., Papadaki, H. A., Eliopoulos, G., Kaufman, C., Bertolone, S. J., Nakamoto, B., *et al.* (2003). Mutations in proto-oncogene GFI1 cause human neutropenia and target ELA2. Nat Genet *34*, 308-312.

Pinto do, O. P., Richter, K., and Carlsson, L. (2002). Hematopoietic progenitor/stem cells immortalized by Lhx2 generate functional hematopoietic cells in vivo. Blood *99*, 3939-3946.

Ramos, G., Weiss, B., Cordova, Y., Hernandez, J., Zambrano, I., and Santiago, E. (2004). Sodium caseinate induces expression and secretion of murine multipotent myeloid cell line 32D macrophage colony-stimulating factor. Arch Med Res *35*, 109-113.

Rasko, J. E., Metcalf, D., Alexander, B., Strasser, A., and Begley, C. G. (1997). Establishment of multipotential and antigen presenting cell lines derived from myeloid leukemias in GM-CSF transgenic mice. Leukemia *11*, 732-742.

Regnault, A., Lankar, D., Lacabanne, V., Rodriguez, A., Thery, C., Rescigno, M., Saito, T., Verbeek, S., Bonnerot, C., Ricciardi-Castagnoli, P., and Amigorena, S. (1999). Fcgamma receptor-mediated induction of dendritic cell maturation and major histocompatibility complex class I-restricted antigen presentation after immune complex internalization. J Exp Med *189*, 371-380.

Res, P. C., Couwenberg, F., Vyth-Dreese, F. A., and Spits, H. (1999). Expression of pTalpha mRNA in a committed dendritic cell precursor in the human thymus. Blood *94*, 2647-2657.

Reya, T., Duncan, A. W., Ailles, L., Domen, J., Scherer, D. C., Willert, K., Hintz, L., Nusse, R., and Weissman, I. L. (2003). A role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature *423*, 409-414.

Reya, T., Morrison, S. J., Clarke, M. F., and Weissman, I. L. (2001). Stem cells, cancer, and cancer stem cells. Nature *414*, 105-111.

Richards, J., Le Naour, F., Hanash, S., and Beretta, L. (2002). Integrated genomic and proteomic analysis of signaling pathways in dendritic cell differentiation and maturation. Ann N Y Acad Sci *975*, 91-100.

Rissoan, M. C., Soumelis, V., Kadowaki, N., Grouard, G., Briere, F., de Waal Malefyt, R., and Liu, Y. J. (1999). Reciprocal control of T helper cell and dendritic cell differentiation. Science *283*, 1183-1186.

Rodel, B., Tavassoli, K., Karsunky, H., Schmidt, T., Bachmann, M., Schaper, F., Heinrich, P., Shuai, K., Elsasser, H. P., and Moroy, T. (2000). The zinc finger protein Gfi-1 can enhance STAT3 signaling by interacting with the STAT3 inhibitor PIAS3. Embo J *19*, 5845-5855.

Romani, N., Reider, D., Heuer, M., Ebner, S., Kampgen, E., Eibl, B., Niederwieser, D., and Schuler, G. (1996). Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. J Immunol Methods *196*, 137-151.

Sallusto, F., Cella, M., Danieli, C., and Lanzavecchia, A. (1995). Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med *182*, 389-400.

Sallusto, F., and Lanzavecchia, A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med *179*, 1109-1118.

Saunders, D., Lucas, K., Ismaili, J., Wu, L., Maraskovsky, E., Dunn, A., and Shortman, K. (1996). Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte/macrophage colony-stimulating factor. J Exp Med *184*, 2185-2196.

Scheijen, B., Jonkers, J., Acton, D., and Berns, A. (1997). Characterization of pal-1, a common proviral insertion site in murine leukemia virus-induced lymphomas of c-myc and Pim-1 transgenic mice. J Virol *71*, 9-16.

Schiavoni, G., Mattei, F., Sestili, P., Borghi, P., Venditti, M., Morse, H. C., 3rd, Belardelli, F., and Gabriele, L. (2002). ICSBP is essential for the development of mouse type I interferon-producing cells and for the generation and activation of CD8alpha(+) dendritic cells. J Exp Med *196*, 1415-1425.

Schmidt, T., Karsunky, H., Gau, E., Zevnik, B., Elsasser, H. P., and Moroy, T. (1998). Zinc finger protein GFI-1 has low oncogenic potential but cooperates strongly with pim and myc genes in T-cell lymphomagenesis. Oncogene *17*, 2661-2667.

Schmidt, T., Zornig, M., Beneke, R., and Moroy, T. (1996). MoMuLV proviral integrations identified by Sup-F selection in tumors from infected myc/pim bitransgenic mice correlate with activation of the gfi-1 gene. Nucleic Acids Res *24*, 2528-2534.

Schroeder, T., Lange, C., Strehl, J., and Just, U. (2000). Generation of functionally mature dendritic cells from the multipotential stem cell line FDCP-mix. Br J Haematol *111*, 890-897.
Speirs, K., Lieberman, L., Caamano, J., Hunter, C. A., and Scott, P. (2004). Cutting edge: NF-kappa B2 is a negative regulator of dendritic cell function. J Immunol *172*, 752-756.

Shortman, K., and Liu, Y. J. (2002). Mouse and human dendritic cell subtypes. Nat Rev Immunol *2*, 151-161.

Siegal, F. P., Kadowaki, N., Shodell, M., Fitzgerald-Bocarsly, P. A., Shah, K., Ho, S., Antonenko, S., and Liu, Y. J. (1999). The nature of the principal type 1 interferon-producing cells in human blood. Science *284*, 1835-1837.

Spits, H., Couwenberg, F., Bakker, A. Q., Weijer, K., and Uittenbogaart, C. H. (2000). Id2 and Id3 inhibit development of CD34(+) stem cells into predendritic cell (pre-DC)2 but not into pre-DC1. Evidence for a lymphoid origin of pre-DC2. J Exp Med *192*, 1775-1784.

Spooncer, E., Heyworth, C. M., Dunn, A., and Dexter, T. M. (1986). Self-renewal and differentiation of interleukin-3-dependent multipotent stem cells are modulated by stromal cells and serum factors. Differentiation *31*, 111-118.

Steinman, R. M., Hawiger, D., and Nussenzweig, M. C. (2003). Tolerogenic dendritic cells. Annu Rev Immunol *21*, 685-711.

Steinman, R. M., Pack, M., and Inaba, K. (1997). Dendritic cells in the T-cell areas of lymphoid organs. Immunol Rev *156*, 25-37.

Suzuki, S., Honma, K., Matsuyama, T., Suzuki, K., Toriyama, K., Akitoyo, I., Yamamoto, K., Suematsu, T., Nakamura, M., Yui, K., and Kumatori, A. (2004). Critical roles of interferon regulatory factor 4 in CD11bhighCD8alpha- dendritic cell development. Proc Natl Acad Sci U S A *101*, 8981-8986.

Takeda, K., Clausen, B. E., Kaisho, T., Tsujimura, T., Terada, N., Forster, I., and Akira, S. (1999). Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity *10*, 39-49.

Tang, A., Amagai, M., Granger, L. G., Stanley, J. R., and Udey, M. C. (1993). Adhesion of epidermal Langerhans cells to keratinocytes mediated by E-cadherin. Nature *361*, 82-85.

Traver, D., Akashi, K., Manz, M., Merad, M., Miyamoto, T., Engleman, E. G., and Weissman, I. L. (2000). Development of CD8alpha-positive dendritic cells from a common myeloid progenitor. Science *290*, 2152-2154.

Valtieri, M., Tweardy, D. J., Caracciolo, D., Johnson, K., Mavilio, F., Altmann, S., Santoli, D., and Rovera, G. (1987). Cytokine-dependent granulocytic differentiation. Regulation of proliferative and differentiative responses in a murine progenitor cell line. J Immunol *138*, 3829-3835.

Vandenabeele, S., Hochrein, H., Mavaddat, N., Winkel, K., and Shortman, K. (2001). Human thymus contains 2 distinct dendritic cell populations. Blood *97*, 1733-1741.

Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W. S., and Bernstein, I. D. (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med *6*, 1278-1281.

Vremec, D., Lieschke, G. J., Dunn, A. R., Robb, L., Metcalf, D., and Shortman, K. (1997). The influence of granulocyte/macrophage colony-stimulating factor on dendritic cell levels in mouse lymphoid organs. Eur J Immunol *27*, 40-44.

Vremec, D., Zorbas, M., Scollay, R., Saunders, D. J., Ardavin, C. F., Wu, L., Shortman, K., Anjuere, F., Martin, P., Ferrero, I., *et al.* (1992). The surface phenotype of dendritic cells purified from mouse thymus and spleen: investigation of the CD8 expression by a subpopulation of dendritic cells. J Exp Med *176*, 47-58.

Wallis, D., Hamblen, M., Zhou, Y., Venken, K. J., Schumacher, A., Grimes, H. L., Zoghbi, H. Y., Orkin, S. H., and Bellen, H. J. (2003). The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. Development *130*, 221-232.

Weissman, I. L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. Cell *100*, 157-168.

Weissman, I. L., Anderson, D. J., and Gage, F. (2001). Stem and progenitor cells: origins, phenotypes, lineage commitments, and transdifferentiations. Annu Rev Cell Dev Biol *17*, 387-403.

Welte, T., Koch, F., Schuler, G., Lechner, J., Doppler, W., and Heufler, C. (1997). Granulocyte-macrophage colony-stimulating factor induces a unique set of STAT factors in murine dendritic cells. Eur J Immunol *27*, 2737-2740.

Welte, T., Zhang, S. S., Wang, T., Zhang, Z., Hesslein, D. G., Yin, Z., Kano, A., Iwamoto, Y., Li, E., Craft, J. E., *et al.* (2003). STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: a critical role of STAT3 in innate immunity. Proc Natl Acad Sci U S A *100*, 1879-1884.

Wilson, A., MacDonald, H. R., and Radtke, F. (2001). Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. J Exp Med *194*, 1003-1012.

Wilson, H. L., and O'Neill, H. C. (2003). Identification of differentially expressed genes representing dendritic cell precursors and their progeny. Blood *102*, 1661-1669.

Winzler, C., Rovere, P., Rescigno, M., Granucci, F., Penna, G., Adorini, L., Zimmermann, V. S., Davoust, J., and Ricciardi-Castagnoli, P. (1997). Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. J Exp Med *185*, 317-328.

Wu, L., Li, C. L., and Shortman, K. (1996). Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. J Exp Med *184*, 903-911.

Wu, L., Nichogiannopoulou, A., Shortman, K., and Georgopoulos, K. (1997). Cell-autonomous defects in dendritic cell populations of Ikaros mutant mice point to a developmental relationship with the lymphoid lineage. Immunity *7*, 483-492.

Wu, L., D'Amico, A., Winkel, K. D., Suter, M., Lo, D., and Shortman, K. (1998). RelB is essential for the development of myeloid-related CD8alpha- dendritic cells but not of lymphoid-related CD8alpha+ dendritic cells. Immunity *9*, 839-847.

Wu, L., Li, C. L., and Shortman, K. (1996). Thymic dendritic cell precursors: relationship to the T lymphocyte lineage and phenotype of the dendritic cell progeny. J Exp Med *184*, 903-911.

Wu, L., Nichogiannopoulou, A., Shortman, K., and Georgopoulos, K. (1997). Cell-autonomous defects in dendritic cell populations of Ikaros mutant mice point to a developmental relationship with the lymphoid lineage. Immunity *7*, 483-492.

Xu, S., Ariizumi, K., Edelbaum, D., Bergstresser, P. R., and Takashima, A. (1995). Cytokine-dependent regulation of growth and maturation in murine epidermal dendritic cell lines. Eur J Immunol *25*, 1018-1024.

Ye, M., Iwasaki, H., Laiosa, C. V., Stadtfeld, M., Xie, H., Heck, S., Clausen, B., Akashi, K., and Graf, T. (2003). Hematopoietic stem cells expressing the myeloid lysozyme gene retain long-term, multilineage repopulation potential. Immunity *19*, 689-699.

Yucel, R., Karsunky, H., Klein-Hitpass, L., and Moroy, T. (2003). The transcriptional repressor Gfi1 affects development of early, uncommitted c-Kit+ T cell progenitors and CD4/CD8 lineage decision in the thymus. J Exp Med *197*, 831-844.

Yucel, R., Kosan, C., Heyd, F., and Moroy, T. (2004). Gfi1:GFP knock-in mutant reveals differential expression and auto-regulation of the gene growth factor independence 1 (Gfi1) during lymphocyte development. J Biol Chem.

Zeng, H., Yucel, R., Kosan, C., Klein-Hitpass, L., and Moroy, T. (2004). Transcription factor Gfi1 regulates self-renewal and engraftment of hematopoietic stem cells. Embo J.

Zhang, Y., Mukaida, N., Wang, J., Harada, A., Akiyama, M., and Matsushima, K. (1997). Induction of dendritic cell differentiation by granulocyte-macrophage colony-stimulating factor, stem cell factor, and tumor necrosis factor alpha in vitro from lineage phenotypes-negative c-kit+ murine hematopoietic progenitor cells. Blood *90*, 4842-4853.

Zhu, J., Guo, L., Min, B., Watson, C. J., Hu-Li, J., Young, H. A., Tsichlis, P. N., and Paul, W. E. (2002). Growth factor independent-1 induced by IL-4 regulates Th2 cell proliferation. Immunity *16*, 733-744.

Zornig, M., Schmidt, T., Karsunky, H., Grzeschiczek, A., and Moroy, T. (1996). Zinc finger protein GFI-1 cooperates with myc and pim-1 in T-cell lymphomagenesis by reducing the requirements for IL-2. Oncogene *12*, 1789-1801.

Zweidler-Mckay, P. A., Grimes, H. L., Flubacher, M. M., and Tsichlis, P. N. (1996). Gfi-1 encodes a nuclear zinc finger protein that binds DNA and functions as a transcriptional repressor. Mol Cell Biol *16*, 4024-4034.

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