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Development and plasticity of murine plasmacytoid dendritic cells

Inaugural-Dissertation zur Erlangung
des Doktorgrades der Naturwissenschaften
dem Fachbereich Medizin der Philipps-Universität Marburg
vorgelegt von

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Marburg, 2012

Angenommen vom Fachbereich Medizin der Philipps-Universität Marburg am: 06.02.2012

Gedruckt mit Genehmigung des Fachbereichs.

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List of Abbreviations

BATF3	Basic leucine zipper transcriptional factor ATF-like 3
BST2	Bone marrow stromal cell antigen 2
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
cDC	Conventional dendritic cell
CDP	Common dendritic cell progenitor
CpG	CpG containing oligonucleotide
CXCR	Chemokine (C-X-C motif) receptor
DC	Dendritic cell
DNA	Deoxyribonucleic acid
E2-2	E-protein 2-2
EAE	Experimental autoimmune encephalomyelitis
EpCAM	Epithelial cell adhesion molecule
FLT3	FMS-like tyrosine kinase 3
FLT3-L	FMS-like tyrosine kinase 3 ligand
Foxp	Forkhead box protein
Gfi	Growth factor independent 1
GM-CSF	Granulocyte macrophage colony stimulating factor
GM-CSF	Granulocyte macrophage stimulating factor
GR-1	Ly6g; Lymphocyte antigen 6 complex, locus G
ID2	DNA-binding protein inhibitor 2
IEC-SN	Intestinal epithelial supernatant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

IRF	Interferon regulatory factor
ITGAX	Integrin alpha X
LCMV	Lymphocytic choriomeningitis virus
Lin	Lineage
LPS	Lipopolysaccharide
Ly	Lectin-type killer cell inhibitory receptor
M-CSF	Macrophage colony stimulating factor
M-CSF-R	Macrophage colony stimulating factor receptor
MDP	Macrophage dendritic cell progenitor
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
NFkb	Nuclear factor kappa-light-chain-enhancer of activated B cells
pDC	Plasmacytoid dendritic cell
Pre-cDC	Pre conventional dendritic cell
PU.1	spleen focus forming virus proviral integration oncogene spi1
RelB	Relb avian reticuloendotheliosis viral (v-rel) oncogene related B
RIG	Retinoic acid inducible gene
RLR	RIG-1 like receptor
RNA	Ribonucleic acid
Sca-1	Stem cell antigen 1
Siglec H	Sialic acid binding Ig-like lectin H
SLE	Systemic lupus erythematodes
SPL	Spleen
BM	Bone marrow
LN	Lymph node
PP	Peyer’s patch
SI	Small intestine

CSF2r	Colony stimulating factor 2 receptor
Pre-pDC	Pre plasmacytoid dendritic cell
LI	Liver
LG	Lung
STAT	Signal transducers and activators of transcription protein
TCF4	Transcription factor 4
TGF	Transforming growth factor
Th	T helper cell type
TLR	Toll like receptor
TNF	Tumour necrosis factor
XBP-1	X-box binding protein 1

1. Introduction

The immune system has evolved to defend the body from various threats such as bacterial, viral, fungal and parasitic infection while preventing the induction of autoimmunity. This defense system is split into two very different but closely interconnected branches - the innate and the adaptive immune system. Interaction between innate and adaptive immunity is necessary to provide optimal defense against various pathogens. One of the most important connecting parts in this system are dendritic cells (DC), which I will focus on in the following chapters of this introduction.

1.1 Dendritic cells – linking innate and adaptive immunity

1.1.1 Subsets of dendritic cells

Intensive research identified two broad categories of DCs, conventional DCs (cDCs) and plasmacytoid DCs (pDCs), which can be distinguished by their morphology and the expression of certain unique surface molecules. In addition, they differ in their capacities to produce cytokines and induce T cell activation in response to various stimuli. Depending on the subset and activation state, DCs are able to shape different kinds of immune responses such as cytotoxic T cell responses and T-helper cell type 1 (Th1, IFN-γ-producing), Th2 (IL-4 producing) and Th17 (IL-17 producing). Differences in their capacity to capture and process antigen and to present this antigen to T cells, to co-stimulate T cells and to secrete various combinations of cytokines and chemokines, lead to different types of effector T cell responses.

These two categories of DCs, will be described below in detail. Because this study was conducted in mice, this introduction will be limited to recent work done in the mouse experimental system, however similar or equivalent populations in humans do exist and largely have similar functions.

1.1.1.1 Conventional dendritic cells

CDCs can be found in every organ in mice and can be identified by their specific DC morphology and with the help of certain uniquely expressed surface molecules. In mice, cDCs in lymphoid and non-lymphoid organs such as the spleen or the lung express high levels of the integrin CD11c and high levels of major histocompatibility complex (MHC) class II (Figure 1) (Steinman and

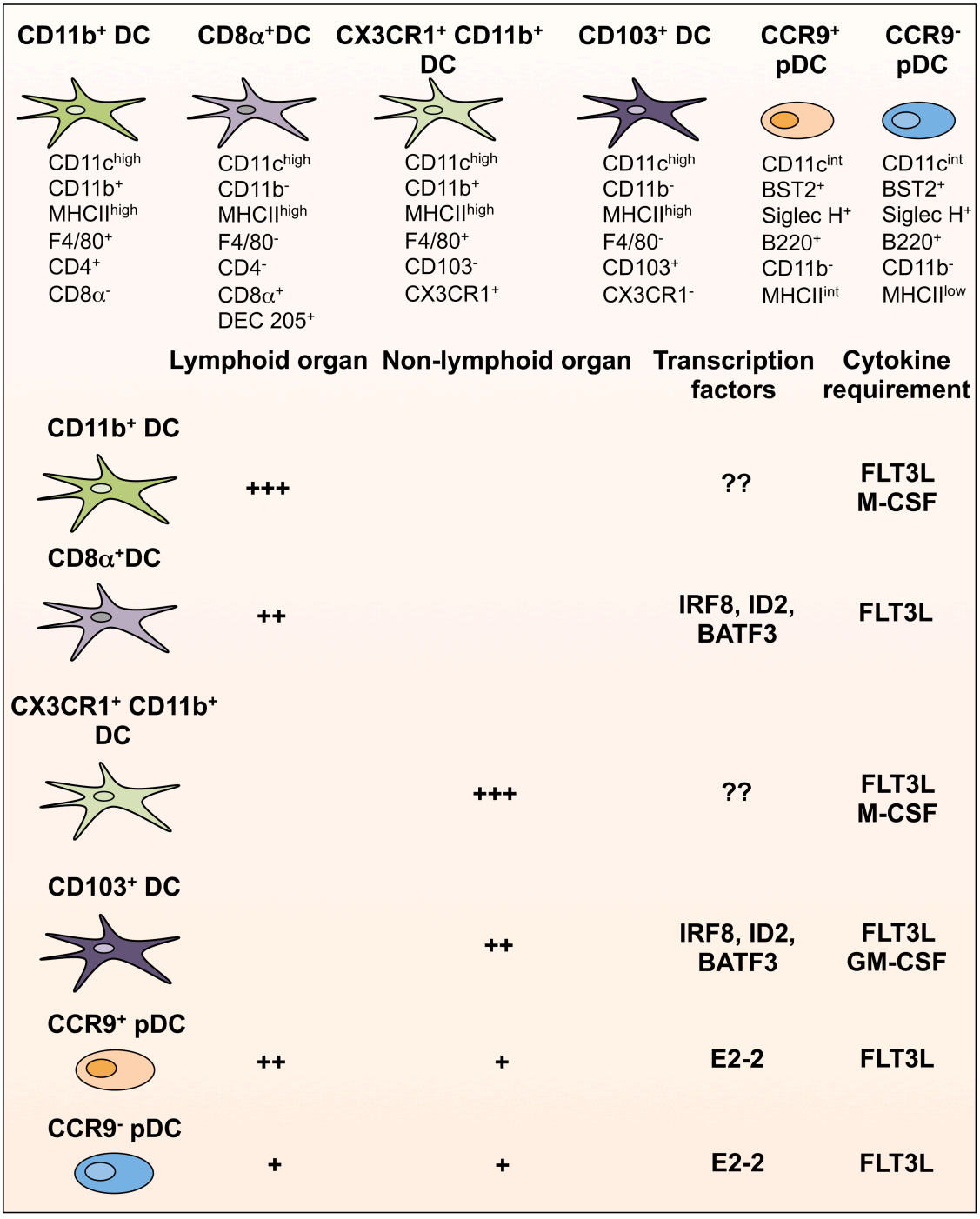

Figure 1 Subsets of mouse DCs found in lymphoid as well as non-lymphoid organs.

Inaba, 1999). Another feature of cDCs is the ability to mature in response to microbial stimuli which integrate into various pathogen recognition receptor pathways such as the toll like receptor (TLR) family pathway and the intracellular RNA receptors of the RIG-I like receptor family pathway. Maturation after stimulation of the TLR pathway is dependent on the intracellular signalling adaptors MyD88 (TLRs 1,2,4,5,6,7,8,9) and Trif (TLRs 3 & 4) and leads to the induction of pro-inflammatory cytokines and to upregulation of cell surface molecules such as CD69, CD80, CD86 and CD40, which are crucial for efficient T cell co-stimulation (Barchet *et al.*, 2005).

In lymphoid organs three types of $CD11c^{high}$ MHC class II^{high} cDCs can be distinguished, $CD4^+$ $CD11b^+$ DCs, $CD4^-$ $CD8\alpha^-$ $CD11b^+$ DCs and $CD8\alpha^+$ $CD11b^-$ DCs (Figure 1) (Shortman and Liu, 2002). $CD8\alpha^-$ $CD11b^+$ DCs make up for approximately 70 % of all DCs in the spleen. They express high levels of the TLRs 1-9 and also the cytosolic RNA receptor RIG-I. Upon stimulation with various TLR ligands $CD8\alpha^-$ $CD11b^+$ DCs are able to produce high amounts of IL-6 but only low levels of IL-12p70, which skews the T cell response induced by these cells towards the Th2 response (Moser and Murphy, 2000). $CD8\alpha^+$ $CD11b^-$ DCs, account for 20 % of DCs in lymphoid organs, such as the spleen and lymph nodes and 70 % of DCs in the thymus. $CD8\alpha^+$ DCs express all TLRs except TLR7 and do not express the intracellular RNA receptor RIG-I either (Delamarre and Mellman, 2011). In the presence of TLR ligands such as immune stimulatory CpG DNA or lipopolysaccharide (LPS) $CD8\alpha^+$ DCs produce high levels of IL12p70 (Hochrein *et al.*, 2001), which promotes the induction of Th1 immunity (Hildner *et al.*, 2008). Most importantly $CD8\alpha^+$ DCs are able to efficiently cross-present soluble or cell-bound antigen on MHC class I to induce strong $CD8^+$ T cell immunity against tumours and viruses whereas $CD8\alpha^-$ DCs fail to do so (Hildner *et al.*, 2008). Interestingly, under steady state conditions $CD8\alpha^+$ DCs, which can be found in lymphoid organs and the spleen, are able to process antigen delivered *via* the cell surface receptor DEC 205 and induce antigen specific $Foxp3^+$ regulatory $CD4^+$ T cells in the spleen and in the lymph nodes due to their ability to produce transforming growth factor β (TGF-β) (Yamazaki *et al.*, 2008). This suggests that $CD8\alpha^+$ DCs in the steady state

may play a role in the induction and maintenance of peripheral tolerance to self-antigens.

Subsequently several elegant studies addressed the phenotype and function of cDCs in non-lymphoid organs such as lung, liver and small intestine (Bogunovic *et al.*, 2009; Plantinga *et al.*, 2010; Steinman and Cohn, 1973; Varol *et al.*, 2009). Similar to what was found in lymphoid organs, cDCs in lung, liver and the intestine can be divided into two main populations with distinct phenotypes and functions. In the lamina propria of the small intestine and the colon two subsets of cDCs can be divided by the expression of the chemokine receptor CX3CR1 and of the integrin CD103 (Cepek *et al.*, 1994; Jung *et al.*, 2000). Both populations of DCs show a similar DC like morphology and localize in the lamina propria but have distinct functional properties. $CX3CR1^+$ $CD103^-$ DCs sample luminal antigens by projecting dendrites into the gut lumen. This process is dependent on the receptor for fractalkine CX3CR1 as it is absent in CX3CR1 KO animals (Niess *et al.*, 2005). However, the exact function and mechanism of this is not yet fully understood. Additionally, $CX3CR1^+$ $CD103^-$ DCs were shown to produce high amounts of Tumour-necrosis-factor-α (TNF-α) in conditions of intestinal inflammation (Niess and Adler, 2010; Varol *et al.*, 2009). On the other hand $CX3CR1^-$ $CD103^+$ DCs where shown to be able to capture antigen in the lamina propria and transport this antigen efficiently to the mesenteric lymph node (Macpherson and Uhr, 2004; Schulz *et al.*, 2009). In the mesenteric lymph node under steady state conditions $CX3CR1^-$ $CD103^+$ DCs are able to induce $Foxp3^+$ $CD4^+$ T cells in a TGF-β and retinoic acid dependent manner (Coombes *et al.*, 2007; Sun *et al.*, 2007). This process is thought to be involved in tolerance to food antigens as well as to antigens of the commensal bacterial flora in the gut (Rescigno *et al.*, 2001). Interestingly, in the event of infection or activation of $CD103^+$ DCs, these DCs can induce T cell homing to the intestine and the mesenteric lymph node as well as an overall Th17 type immune response (Laffont *et al.*, 2010; Lewis *et al.*, 2011). Additionally a third subset of DCs can be found exclusively in the gut, which can be identified by the co-expression of CD11b and CD103. These cells do not express CX3CR1 similar to $CD103^+$ DCs. Functionally, $CD11b^+$ $CD103^+$ DCs migrate in a CCR7

dependent manner to the mesenteric lymph node and are able to prime naïve T cells as efficient as $CD103^+$ DCs.

In the lung, DCs are found as a dense network of cells distributed basolaterally to the epithelial layer as well as in the alveolar space. Two different subsets of cDCs can be found in steady state lungs of mice, $CD11b^+$ $CX3CR1^+$ DCs and $CD103^+$ $langerin^+$ DCs. $CD11b^+$ $CX3CR1^+$ DCs are located directly under the airway epithelium and do not invade the alveolar space in the steady state (Wikstrom and Stumbles, 2007). $CD11b^+$ cDCs produce a wide array of inflammatory mediators and chemokines, such as IL-6, CCL22 and CCL17, which attract and activate T cells (van Rijt *et al.*, 2005). However it is not clear what their exact contribution to T cell immunity and to the induction of allergic reactions in the lung is. $CD103^+$ DCs in the lung reside close to the alveolar epithelium and are able to sample antigens by attaching their dendrites to tight junction proteins of the airway epithelium and thereby accessing the lumen (Jahnsen *et al.*, 2006). Similar to $CD103^+$ DCs found in the gut, these cells migrate to the draining lymph nodes after antigen encounter (GeurtsvanKessel and Lambrecht, 2008; GeurtsvanKessel *et al.*, 2008). Interestingly, in contrast to $CD103^+$ DCs in the gut, lung $CD103^+$ DCs are prone to induce Th2 cells rather than regulatory T cells or Th17 cells (Lambrecht and Hammad, 2009). Furthermore careful analyses by Sung *et al.* revealed that $CD103^+$ DCs occur at a much higher frequency in the lung as compared to other non-lymphoid organs such as the intestine (Sung *et al.*, 2006).

In the skin, the body's largest organ, cDCs play a crucial role in patrolling and controlling this border to the outside world. Therefore cDCs and Langerhans cells form a dense network of cells, which are distributed in the dermis as well as in the epidermis (Merad *et al.*, 2008). In the dermis cDCs can be separated by their expression of langerin. $Langerin^+$ DC also express the marker CD103 but lack CD11b. $Langerin^-$ DCs on the other hand are $CD11b^+$ and do not express CD103 (Ginhoux *et al.*, 2009). Both cell types are negative for epithelial cell adhesion molecule (EpCAM) in contrast to Langerhans cells, which also reside in the dermis. Langerhans cells, present as migratory Langerhans cells in the dermis on their way to the lymph node, express high levels of langerin,

CD11b and EpCAM, but lack expression of CD103. This separate antigen presenting cell type can also be found in the epidermis, directly under the first layer of keratinocytes. At this location, Langerhans cells are thought to sample antigens, and are able to elicit immune responses (Helft *et al.*, 2010). Also Langerhans cells of the epidermis and the dermis are resistant to radiation in contrast to other DC subsets found throughout the body, which suggests that Langerhans cells are long-lived and are not proliferating in the steady state (Ginhoux *et al.*, 2007). Both DC subsets, $CD11b^{+}$ $CD103^{-}$ and $CD11b^{-}$ $CD103^{+}$ DCs, however are sensitive to radiation, which leads to the conclusion that these cells are actively proliferating in the steady state (Ginhoux *et al.*, 2009).

In conclusion cDCs form a diverse network of highly specialized cells, which is distributed throughout the body and is able to react to pathogens through subset specialization and utilization of differentially expressed receptors, to mount appropriate tolerogenic or immunogenic immune responses.

1.1.1.2 Plasmacytoid dendritic cells

PDCs are characterized by their unique plasma cell like morphology and by the expression of intermediate levels of the integrin CD11c, low levels of CD11b and expression of the B220 and Ly6C/G. Furthermore pDCs express the pDC specific surface molecules sialic acid binding Ig-like lectin H (Siglec H) and bone marrow stromal cell antigen 2 (BST2) (Figure 1) (Blasius *et al.*, 2006a; Blasius *et al.*, 2006b; Zhang *et al.*, 2006). PDCs can be found in all lymphoid organs. Additionally they can also be found in most non-lymphoid organs, however in substantially lower quantities compared to cDCs. The vast majority of pDCs can be found in the bone marrow (BM). Approximately 3 % of all BM hematopoietic cells can be identified as pDCs by their expression of Siglec H and BST2. Additional markers used for pDC identification albeit less specific ones are the B cell marker B220, Ly6c and Ly49q (Kamogawa-Schifter *et al.*, 2005; Toma-Hirano *et al.*, 2007). The pDC population can be further divided into $CCR9^{+}$ and $CCR9^{-}$ pDCs (Schlitzer *et al.*, 2011; Wendland *et al.*, 2007). Both populations express CD9 as long as they reside in the BM and downregulate expression of CD9 upon entry into lymphoid organs (Bjorck *et al.*, 2011).

Additionally pDCs express other surface markers in varying or low degrees such as CD8$\alpha^{(+/-)}$, CD4$^{(+/-)}$ and CD11b^{low} (Asselin-Paturel *et al.*, 2001). Interestingly, in the steady state pDCs display only low amounts of MHC class II on their surface and costimulatory molecules, like CD80 and CD86 are almost absent without appropriate stimulation.

PDCs can also be found in various lymphoid tissues such as the spleen, thymus and lymph nodes. PDCs found in these lymphoid organs are phenotypically similar to those found in the BM however lack the expression of CD9 (Bjorck *et al.*, 2011). Furthermore CCR9^{-} pDCs, which constitute up to 30 % of the pDC population in the BM, rarely exceed 10 % of the total pDC population in the peripheral lymphoid organs as shown in this dissertation (Schlitzer *et al.*, 2011). PDCs are thought to migrate to the lymphoid organs *via* the blood stream entering *via* high endothelial venules. They then migrate to the T cell areas of the lymph node in a CCR7 dependent manner (Seth *et al.*, 2011). In contrast, cDCs enter the lymphoid organs *via* afferent lymphatics.

PDCs were first characterized by their ability to produce excess quantities of type I IFN in response to viral infections (Asselin-Paturel *et al.*, 2001; Nakano *et al.*, 2001). Subsequently it was shown that pDCs are the major type I interferon producing cell type in mice and man. They utilize a specific set of innate immune receptors to sense viral and bacterial infection (Cella *et al.*, 1999). PDCs express high levels of TLR7 and 9. In response to TLR7 and 9 ligands, single stranded RNA and CpG DNA respectively, pDCs produce high levels of interferon-α (IFN-α). The ability to rapidly produce and secrete high amounts of IFN-α is due to the high expression of x-box binding protein 1 (XBP-1), a master regulatory protein of the secretory pathway and to the high constitutive expression of interferon regulatory factor (IRF) 7 in unstimulated pDCs (Iwakoshi *et al.*, 2007; Izaguirre *et al.*, 2003; Kerkmann *et al.*, 2003). Interestingly, rapid production of IFN-α is independent of the IFN-α/β receptor mediated feedback loop upon viral infection, but necessary for IFN-α production upon encounter with non-replicating virus (Blasius *et al.*, 2010; Kerkmann *et al.*, 2003; Kumagai *et al.*, 2009). Stimulation of pDCs with TLR7 or 9 ligands not

only induces IFN-α but also leads to the secretion of TNF-α, IL-12 and IL-6. Additionally upon stimulation pDCs are able to present antigens and prime T cells. They also attract other types of immune cells, such as plasma cells and NK cells (Krug *et al.*, 2003; Liu *et al.*, 2008; Sapoznikov *et al.*, 2007). PDCs, in contrast to cDCs are characterized by a low turnover and are therefore long-lived cells in contrast to cDCs (Liu *et al.*, 2007). Furthermore several studies revealed that pDCs are able to undergo a phenotypical and functional switch towards cDCs under the influence of a lymphocytic choriomeningitis virus (LCMV) infection (Zuniga *et al.*, 2008; Zuniga *et al.*, 2004). In addition to their function as sentinels and rapid producers of type I interferons pDCs are also able to instruct adaptive immunity. Le Bon *et al.* and others were able to show that type I interferon by pDCs is needed to instruct B cells to develop into antibody producing plasma cells and to do class switching from IgG to IgM (Le Bon *et al.*, 2001; Poeck *et al.*, 2004). Additionally to inducing T cell proliferation upon activation (Cella *et al.*, 2000), pDCs are also able to produce T cell attracting chemokines upon activation, such as CCL3 and CCL4, which attract $CD4^+$ as well as $CD8^+$ T cells to sites of infection (Krug *et al.*, 2002; Penna *et al.*, 2002) as well as to the T cell areas of lymph nodes.

Despite their role in the induction of immunity, pDCs of lymphoid organ origin have also been implicated in the induction of tolerance in certain experimental models. In a model of acute allogeneic graft versus host disease $CCR9^+$ pDCs were able to induce $Foxp3^+$ T cells upon adoptive transfer and suppressed disease. However, the exact mechanism of this regulatory T cell induction is not well understood (Hadeiba *et al.*, 2008). Also targeting of myelin oligodendrocyte glycoprotein (MOG) peptide to pDCs *via* Siglec H leads to belated onset of experimental autoimmune encephalomyelitis (EAE) (Loschko *et al.*, 2011). These findings further strengthen the immunoregulatory role of pDCs. Furthermore Irla *et al.* showed that selective ablation of MHC class II on pDCs leads to exacerbated course of EAE (Irla *et al.*, 2010). However pDCs have also been implicated in the break of tolerance in certain models of autoimmunity, which are associated with type I IFN, such as systemic lupus erythematodes (SLE) and psoriasis (Blomberg *et al.*, 2001; Nestle *et al.*, 2005). Several studies showed that IFN-α/β production by pDCs triggered by RNA or DNA immune-

complexes in the absence of infection can lead to a break of peripheral tolerance and to the induction of SLE or psoriasis (Gregorio *et al.*, 2010; Lande *et al.*, 2011; Lande *et al.*, 2007; Savarese *et al.*, 2006). Also in the EAE model, as shown by Isaksson *et al.*, pDCs can contribute to the pathogenesis of EAE *via* their ability to produce IFN-α, which was necessary for exacerbated disease in the early course of the disease (Isaksson *et al.*, 2009).

PDCs can also be found in lung, liver and skin as well as the small intestine and the colon. In these non-lymphoid organs the phenotype of pDCs is similar to the phenotype of pDCs, which can be found in spleen and lymph nodes. In the lung pDCs can be found in the lamina propria directly under the bronchial epithelium (Hammad and Lambrecht, 2011). De Heer *et al.* demonstrated that these pDCs are able to confer tolerance to inhaled antigen in the steady state to prevent the induction of asthma. Depletion of pDCs, before inhalation of otherwise non asthma inducing antigen, leads to the production of IgE, Th2 cytokines, airway eosinophilia and goblet cell hyperplasia and the establishment of asthma, showing that pDCs are necessary to sustain tolerance in this model (de Heer *et al.*, 2004). In addition, Goubier *et al.* were able to show that depletion of pDCs led to the abrogation of tolerance to oral antigen. This was attributed to the loss of pDCs in the liver (Goubier *et al.*, 2008).

Taken together pDCs have a diverse role in the induction and maintenance of immunity to various kinds of pathogens and in the induction of tolerance to self as well as foreign antigens.

1.1.2 Dendritic cell development

DC development is a tightly regulated multi-step process, which is able to give rise to several different populations and subsets of DC in the BM, lymphoid organs and non-lymphoid organs. This process of generating different DC types and subsets from hematopoietic precursors will be outlined in the following chapters.

1.1.2.1 Conventional dendritic cell development

Hematopoietic stem cells in the BM, which are characterized by expression of CD34 and lack of expression of lineage markers such as CD4, CD8, CD3ε, B220, CD19, CD11b, Gr-1, NK1.1 and TER-119 (termed Lin-), progressively mature to a stage were commitment to either the myeloid or lymphoid lineage occurs (Karsunky *et al.*, 2003). At this stage the common myeloid and common lymphoid precursor cells arise. Lymphoid precursors can give rise to T and B cells as well as NK cells, myeloid precursors on the other hand are restricted to differentiate into the myeloid lineage (Akashi *et al.*, 1999). Runx 1, a transcription factor involved in hematopoietic stem cell development, was shown to be important for this transition (Dominguez-Soto *et al.*, 2005). In a next step, myeloid progenitors differentiate into $FLT3^+$ Lin^- $CD117^{high}$ (c-kit), $CX3CR1^+$ $CD115^+$ (M-CSFR) monocyte/macrophage DC progenitors (MDP) in the BM, which are able to give rise to $Ly6c^{+/-}$ monocytes, certain subsets of macrophages and the common DC progenitor (CDP, Geissmann *et al.*, 2010). Transition of the MDP towards the DC lineage is mediated by FMS-like tyrosine kinase 3 ligand (FLT3L), which acts on a subset of MDPs which expresses FMS-like tyrosine kinase 3 (FLT3) but are still negative for lineage markers (Waskow *et al.*, 2008). MDPs develop into CDPs or pro-DCs under the influence of FLT3L (Onai *et al.*, 2007). CDP development is critically dependent on FLT3L as well as M-CSFR as it is severely reduced in FLT3L and macrophage colony stimulating factor (M-CSF) receptor KO animals. Furthermore it was shown by Onai *et al.* that CDPs are able to differentiate into pure $CD11c^+$ MHC class II^+ DCs upon culture with FLT3L or granulocyte macrophage colony stimulating factor (GM-CSF), further demonstrating the importance of these two growth factors (Onai *et al.*, 2007). The appropriate

receptor repertoire needed to respond to these growth factors is controlled by the master regulator of GM-CSF and FLT3L receptor expression, spleen focus forming virus proviral integration oncogene spi1 (PU.1) (Carotta *et al.*, 2010). PU.1 is able to directly regulate the expression levels of these receptors and is therefore crucial for the response to FLT3L and GM-CSF.

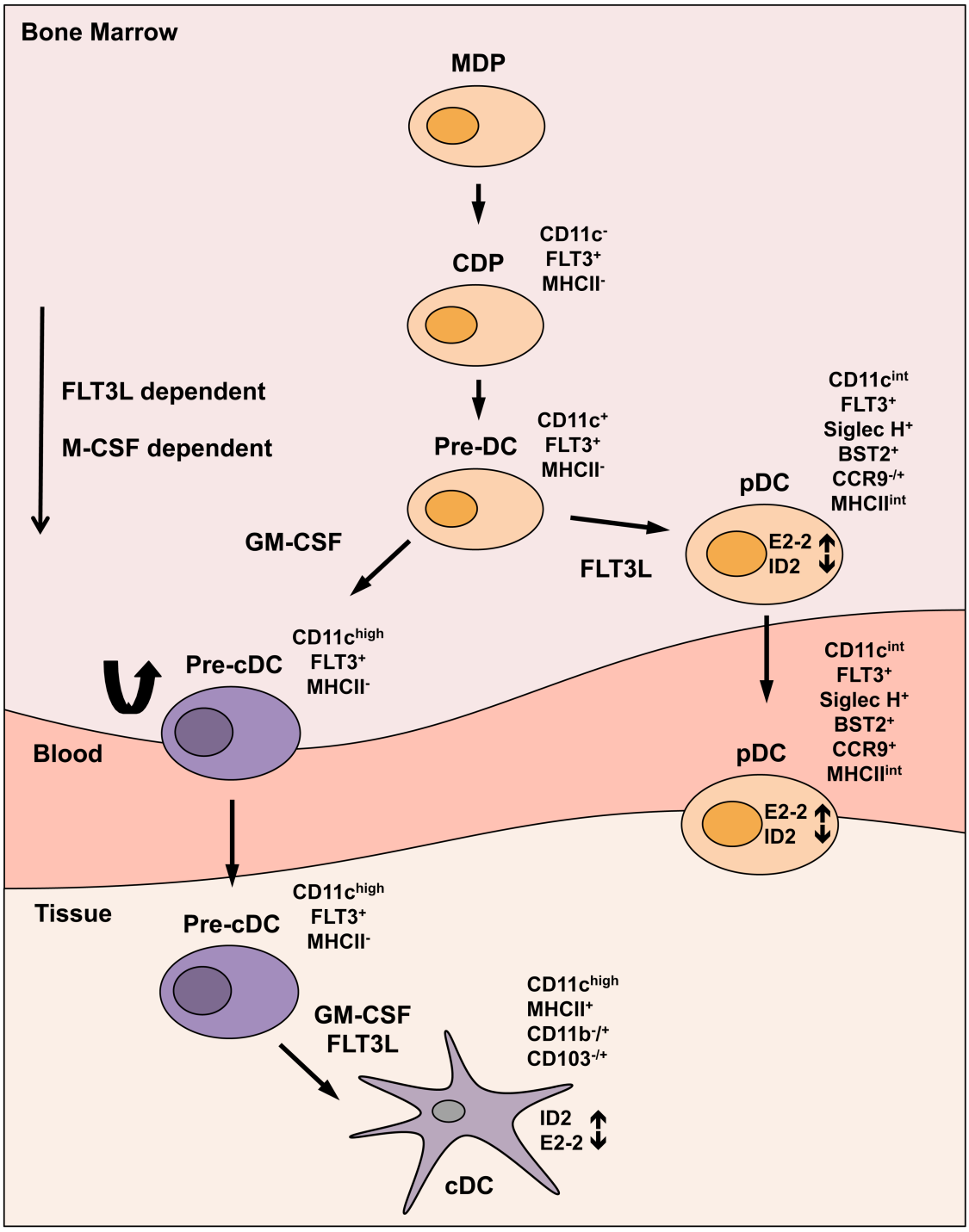

Figure 2 Schematic depiction of DC development in the BM as well as in peripheral tissues.

CDPs are committed to DC development, including the generation of cDCs and pDCs. CDPs, which are $CD11c^-$ MHC class II^- then successively develop into $CD11c^+$ MHC class II^- Pre-DCs. Pre-DCs as defined by Naik *et al.* (Naik *et al.*, 2007) are still able to differentiate into cDCs and pDCs (Naik *et al.*, 2007). Commitment to either cDC or pDC populations occurs early in the proliferation cycle of these precursors, meaning they contain single precursors which are restricted to produce pDCs or cDCs or both. Definite cDC development starts with the cDC committed DC precursor (pre-cDC) and is governed by the growth factors FLT3L, GM-CSF and M-CSF (Figure 2) (Fancke *et al.*, 2008; Kingston *et al.*, 2009; Onai *et al.*, 2007; Waskow *et al.*, 2008). Liu *et al.* set out to study the development and homeostasis of cDCs in peripheral lymphoid organs. Adoptive transfer experiments revealed that CDPs develop in the BM, into pre-cDCs which upregulate the integrin CD11c and are MHC class II^- and $FLT3^+$ (Liu *et al.*, 2009; Liu *et al.*, 2007). These Pre-cDCs are now restricted to give rise to cDCs, rather than to pDCs or monocytes. Pre-cDCs are able to leave the BM and migrate through the blood stream (0.03 % of blood lymphocytes) to peripheral lymphoid organs (Liu *et al.*, 2009). They are able to enter the lymphoid organs through high endothelial venules. Once in peripheral lymphoid organs pre-cDCs undergo multiple cell divisions, under the regulation of local FLT3L levels and differentiate into various subsets of cDCs, such as $CD8\alpha^+$ and $CD11b^+$ cDCs present in the peripheral lymphoid organs (Figure 2, (Liu *et al.*, 2009; Liu *et al.*, 2007).

However final development of cDCs subpopulations depends on different transcription factors. PU.1, signal transducers and activators of transcription protein 3 (STAT3), transducers and activators of transcription protein 5 (STAT5) and Ikaros are equally necessary for the development of both cell populations from the pre-cDC precursor (Figure 3, (Dakic *et al.*, 2005; Laouar *et al.*, 2003; Wu *et al.*, 1997). On the other hand IRF8, DNA-binding protein inhibitor 2 (ID2) and basic leucine zipper transcriptional factor ATF-like 3 (BATF3) transcription factors are critical for $CD8\alpha^+$ cDC development in lymphoid organs (Figure 3) (Aliberti *et al.*, 2003; Hacker *et al.*, 2003; Hildner *et al.*, 2008; Tsujimura *et al.*, 2003). Equally important for development of $CD11b^+$ cDCs are IRF2, IRF4 and Relb avian reticuloendotheliosis viral oncogene related B (RelB) transcription

factors (Ichikawa *et al.*, 2004; Suzuki *et al.*, 2004; Wu *et al.*, 1998). Loss of the aforementioned transcription factors leads to loss or severe reduction of one or both of the two cDC populations (Figure 3).

CDC development in non-lymphoid organs is also dependent, at least to a major part, on pre-cDC precursors and to a minor extend on monocytes (Geissmann *et al.*, 2010). Once pre-cDCs reach peripheral non-lymphoid organs like the small intestine or the lung they undergo multiple cell divisions and are able to produce several different DC subsets (Ginhoux *et al.*, 2009). Varol *et al*. and Bogunovic *et al*. were able to show that the progeny of adoptively transferred CDPs and pre-cDCs in small intestine were $CD103^+$ DCs and to a minor extend $CX3CR1^+$ $CD11b^+$ DCs (Bogunovic *et al.*, 2009; Varol *et al.*, 2009). Also adoptively transferred monocytes were not able to generate $CD103^+$ DCs, but were able to produce $CX3CR1^+$ $CD11b^+$ progeny. These two processes were both critically dependent on FLT3. However for the generation of $CD103^+$ DC GM-CSF receptor and GM-CSF were needed and according to their monocyte like ontogeny also M-CSF receptor was necessary for generation of $CX3CR1^+$ $CD11b^+$ DCs. Interestingly IRF8, ID2 and BATF3 transcription factors, which are necessary for development of $CD8\alpha^+$ DCs in lymphoid organs, are also crucial to the development of $CD103^+$ DCs in the periphery, leading to the assumption that these two cell types share a common developmental program (Figure 3) (Edelson *et al.*, 2010; Ginhoux *et al.*, 2009; Helft *et al.*, 2010).

Similar to what was found in the small intestine, $CD103^+$ cDCs in the lung also originate from pre-cDCs. $CD11b^+$ $CD103^-$ lung cDCs are in part of monocyte origin and pre-cDC origin in line with the results obtained in the small intestine (Ginhoux *et al.*, 2009; Helft *et al.*, 2010). In the skin, where $langerin^+$ and $langerin^-$ DCs can be found, only $langerin^-$ cDC originate from pre-cDCs, whereas $langerin^+$ so called Langerhans cells originate from a local precursor, which has maintained a self renewing potential within the adult skin (Chorro and Geissmann, 2010; Chorro *et al.*, 2009).

Under inflammatory conditions induced by infection or inflammation, monocytes are recruited to a greater extend than in the steady state, dependent on CCR2 and CCL2, to these sites. They also contribute substantially to the DC pool in non-lymphoid organs, by differentiating into inflammatory DCs, with the capacity to produce substantial amounts of IL-6, IL-12 and TNF-α as well as the capacity to capture antigen and present it to T cells (Auffray *et al.*, 2009; Cheong *et al.*, 2010; Shi *et al.*, 2011). Additionally it was shown that recruited monocytes can contribute to the steady state pool of $CD11b^{+}$ $CD103^{-}$ $CX3CR1^{+}$ in the intestine and that this is dependent on the presence of commensals (Niess and Adler, 2010).

1.1.2.2 Plasmacytoid dendritic cell development

PDCs can develop from the CDP, which originates from the common myeloid precursor in the BM (Figure 2) (Naik *et al.*, 2007). However, adoptive transfer experiments show that pDCs are not only able to originate from the common myeloid precursor and the CDP, but are also able to develop from the common lymphoid progenitor. This is supported by the notion that a fraction of pDCs in the BM harbours a lymphoid cell like IgH rearrangement in their DNA, hinting to a lymphoid origin (Shigematsu *et al.*, 2004). However it is not clear if pDCs arise only locally in the BM directly from the CDP or if there are intermediate more pDC committed cells, which contribute to the pDC pool, similar to the pre-cDC.

PDCs are critically dependent on the growth factor FLT3L, shown by the fact that animals, which lack FLT3L, have a strongly reduced pDC frequency in their BM (Gilliet *et al.*, 2002). However, it is not entirely clear how FLT3L is performing its pDC committing action in pDC development. A study by Sathaliyawala *et al.* showed that in contrast to other cell types, pDCs were specifically sensitive to downstream signalling triggered by FLT3L *via* the mTOR complex in DCs (Sathaliyawala *et al.*, 2010). Moreover mice that have a deletion in the genes encoding for IRF8, Ikaros, XBP-1 or growth factor independent 1 (Gfi1) show reduced or absent pDC counts in the BM and the periphery (Figure 3 (Iwakoshi *et al.*, 2007; Rathinam *et al.*, 2005; Schiavoni *et al.*, 2002; Wu *et al.*, 1997). Yet, these mutations also affect various other DC

subsets. E-protein 2-2 (E2-2, TCF4), a basic helix-loop-helix transcription factor, serves as an essential transcription factor, which is necessary and sufficient to induce pDC commitment in progenitors (Figure 3 Ghosh *et al.*, 2010). Furthermore, it is specific for the pDC lineage, as no other DC subset is deleted or reduced in E2-2 KO animals. In the BM only B cells and pDC express detectable levels of E2-2 similar to what was shown for Spi-b (Cisse *et al.*, 2008). However it is unclear how the onset of pDC commitment is regulated. One explanation for that might be that pDC express higher levels of E2-2 protein than B cells and cDCs and that in contrast to cDC, pDCs only express very low levels of ID2, which is a protein antagonist for E2-2 (Cisse *et al.*, 2008; Crozat *et al.*, 2010). In spite of this, the signals, which determine the lineage commitment of a progenitor to the pDC lineage and the ensuing upregulation of E2-2 and its target genes such as Spi-b and IRF8 is not fully understood. Additionally, E2-2 is able to repress the expression of genes involved in cDC development directly, such as ITGAX (coding for CD11c) and ID2, in contrast ID2 is able to inhibit E2-2 activity, showing that the ratio of ID2 and E2-2 is an important player in pDC commitment. This process of posttranscriptional regulation and direct transcriptional regulation is most likely very important for the commitment of precursors to the pDC lineage (Reizis, 2010; Reizis *et al.*,

DC population	Loss of population	Reduction of population	Increase of population
pDC	E2-2, IRF8, Ikaros	IRF4, Gfi1, XBP1	ID2
CD8α$^+$ CD11b$^-$ DC	IRF8, BATF3, STAT3, PU.1	Gfi1, XBP1	Runx3
CD8α$^-$ CD11b$^+$ DC	STAT3, PU.1, RelB	Gfi1, XBP1	Not known
CD103$^+$ CD11b$^-$ DC	IRF8, BATF3	Gfi1	Runx3
CD103$^-$ CD11b$^+$ DC	RelB	Gfi1	Not known
Langerhans cell	ID2, Runx3	IRF8	Gfi1

Figure 3 Table of critical DC development related transcription factors; Red colour indicates crucial transcription factors for the aforementioned population.

2011). Interestingly, a study by Ghosh *et al.* showed that E2-2 expression is not only necessary to induce commitment to the pDC lineage, but it is also crucial for maintaining a pDC phenotype, because induced ablation of E2-2 in mature pDCs led to the acquisition of a cDC phenotype in these cells (Ghosh *et al.*, 2010). Along this line Bar-On *et al.* were able to show that alternative $CD8\alpha^+$ $CX3CR1^+$ cDCs, resembling pDCs by gene expression profile, exist in the spleen, which are sensitive to E2-2 deletion and also harbour the pDC specific IgH rearrangement. It is therefore thought that these cells branch of from a not yet fully committed pDC precursor, which fails final pDC differentiation (Bar-On *et al.*, 2010).

Additionally, Zuniga *et al.* reported that under inflammatory conditions induced by LCMV infection mature pDCs were shown to acquire a cDC phenotype. This effect was critically dependent on IFN-α, elicited by the virus (Zuniga *et al.*, 2004). Interestingly, pDCs which were able to deviate from the pDC lineage lacked the expression of Siglec H, a pDC specific marker, similar to the $CD11c^+$ $B220^+$ Siglec H^- $BST2^-$ cells described by Segura *et al.* in spleen, which were cDC precursors (Segura *et al.*, 2009). In contrast to that, it was also reported that treatment with recombinant IFN-β or IFN-α, favoured the generation of pDCs instead of cDC development in FLT3L BM cultures (Watowich and Liu, 2010).

2. Aims of the study

The aim of this study was to investigate pDC lineage commitment *in vitro* and *in vivo*. It was believed that pDCs arise from the common dendritic cell progenitor (CDP) in the BM and that terminally differentiated cells leave the BM and show a stable phenotype in the periphery. As a consequence the pDC lineage would be less flexible than the cDC lineage to adapt to the requirements of specific tissues and situations, such as infection or inflammation.

CDCs can be generated from a tissue resident precursor (pre-cDC), which is able to leave the BM and to develop in the tissue into different subtypes of DCs allowing adaptation to local requirements. It had to be tested if this hypothesis was also true for pDC development. Therefore it was investigated if pDC development and its adaptation to the local tissue environment can take place in peripheral lymphoid as well as non-lymphoid tissues.

Several questions needed to be addressed in this context. First, the BM pDC population, which was believed to be a homogenous cell population had to be investigated for differences in the expression of cell surface markers to identify cell populations within the pDC population, which harbour different development potential and can therefore be precursors of locally derived pDC populations. Secondly, it had to be established that these cells were able to leave the BM and could be found in peripheral lymphoid and non-lymphoid organs; a prerequisite for further development in these tissues. Furthermore, the developmental potential of the different identified pDC populations had to be tested *in vitro* as well as *in vivo*. The aim of this was to identify cell populations within the pDC population, which were able to give rise to terminally differentiated pDCs in the tissue, thereby allowing adaptation to local microenvironments. Additionally, plasticity of different pDC subsets to deviate from the pDC lineage into the cDC lineage was assessed *in vitro* as well as *in vivo*. At last the question was asked, which cytokines are required for pDC or cDC commitment in the different tissues.

3. Material & Methods

3.1 Published results, Schlitzer *et al.*, Blood, 2011

Materials and methods used in the published results are presented in the paper (see appendix)

3.2 Material and methods (unpublished results)

3.2.3 Material

3.2.3.1 Antibodies & Cell dyes

Antibodies

Antigen	Clone	Fluorchrome	Manufacturer
BST2	120G8	FITC; APC	Prepared in our own lab
CCR4	2G12	PE	Ebioscience
CCR6	R6H1	PE-Cy5.5	Ebioscience
CCR7	4B12	PE-Cy5.5	Ebioscience
CCR9	CW1.2	PE; APC	Ebioscience
CD115	AFS98	Biotin labelled; APC	Ebioscience
CD11b	M1/70	APC; APC-efluor780	Ebioscience
CD11c	N418	PE-Cy7	Ebioscience
CD135	A2F10	Biotin labelled; PE	Ebioscience
CD4	GK1.45	APC	Ebioscience

CD8	53-6.7	APC	Ebioscience
CXCR3	173	PE-Cy5.5	Ebioscience
CXCR4	2B11	PE-Cy5.5	Ebioscience
MHC class II	M5 / 114.15.2	Efluor 450; PE; APC-Efluor780	Ebioscience
Sca-1	D7	PE-Cy5.5	Ebioscience
Siglec H	440c	FITC; APC	Prepared in our own lab

Cell Dyes

Dye	Final concentration used	Manufacturer
Violet Trace; Efluor 450	5µM	Invitrogen

3.2.3.2 Cell culture solutions, media, buffers

Media

Name	Formulation	Manufacturer
RPMI	RPMI	Gibco
RPMI complete	RPMI 10 % FCS 1 % non essential AA 1 % Glutamax 1 % Penicillin/Streptomycin	Gibco
DC Medium	RPMI 10 % FCS	Promocell

1 % non essential AA
1 % Glutamax
1 % Penicillin/Streptomycin
500 mM β-mercaptoethanol

Cell culture solutions

Name	Manufacturer
β-mercatopoethanol solution 14.2 M	Sigma-aldrich
EDTA solution 0.5 M	Gibco
Glutamax 100x	PAA
Non essential amino acids 100x	PAA
Penicillin / Streptomycin solution 100x	PAA
Sodium pyruvate solution 100 mM	PAA
Trypsin/EDTA solution	PAA

Buffers

Name	Formulation	Manufacturer
In vivo injection PBS	PBS without Ca^{2+}/Mg^{2+}	PAA
FACS buffer	PBS without Ca^{2+}/Mg^{2+} 2 % FCS	PAA
MACS buffer / Sort buffer	PBS without Ca^{2+}/Mg^{2+} 2 % FCS 2 mM EDTA	PAA
Red blood cell lysis buffer	4.1 g ammoniumchlorid 500 µl 1M Tris HCL Add sterile water up to 500 ml Adjust pH to 7.5	Prepared in our own lab

3.2.3.3 Cell lines

All cell lines and primary tissue digestions were performed at 37 °C and 5 % CO_2 in a humidified incubator

B16 FLT3L melanoma cell line

As described in Dranoff *et al.* (Dranoff *et al.*, 1993) B16 melanoma cell line expressing FLT3L was maintained in RPMI complete at 5 % CO_2, 37 °C in a humidified incubator and cultured for 3 days prior to s.c. injection into C57BL/6 mice.

3.3.3.4 Mice

Strain	Background	Source
C57BL/6	C57BL/6	Harlan, Paderborn
CX3CR1-GFP	C57BL/6	Jan Hendrik Niess / University of Ulm (Niess *et al.*, 2005)
CSF2rβ KO	C57BL/6	Tobias Suter / ETH Zurich (Robb *et al.*, 1995)

3.3.3.5 Equipment

Device	Manufacturer
MoFlow II cell sorter	Beckman Coulter
Gallios Flow cytometer	Beckman Coulter
FACS Aria II cell sorter	Becton Dickinson
FACS Calibur Flow cytometer	Becton Dickinson

3.2.4 Methods

3.2.4.1 Cell culture of B16 FLT3L secreting melanoma

B16 FLT3L secreting melanoma cells were seeded in a cell culture flask (75 cm^2) directly after thawing in RPMI complete medium. After 2 days of culture at 37 °C with 5 % CO_2 cells were harvested using 5 ml Trypsin/EDTA for 10 min at 37 °C and cells were removed subsequently from the tissue culture flask and centrifuged for 5 min at 1500 rpm at 4 °C. Cells were resuspended in RPMI complete and split at a ratio of 1:15. After 3 days of additional growth cells were harvested using Trypsin/EDTA.

3.2.4.2 Expansion of DCs by treatment with FLT3L secreting B16 melanoma

B16 FLT3L secreting melanoma cells were cultured and harvested as described in 3.2.4.1. After harvesting, cells were centrifuged for 5 min at 1500 rpm at 4 °C. After centrifugation cells were resuspended in PBS in 200 µl injection volume per animal using a 1 ml insulin syringe. For injection animals were anesthetized with isoflurane and the injection was done subcutaneously (s.c.) in the neck. Mice were sacrificed 7 days after injection.

3.2.4.3 Isolation of murine BM

6-8 weeks old mice were sacrificed by CO_2 asphyxia. Hind legs were removed from the corpus and bones were dissected in a petri dish. Subsequently the single femurs and tibias were opened on both sides and the BM was flushed out with RPMI using a 10 ml syringe with a 20 G needle. By pipetting up and down remaining parts of the BM were resuspended and the BM single cell solution was transferred into a 50 ml Falcon tube and centrifuged for 5 min at 1500 rpm at 4 °C. After centrifugation the supernatant was removed and the pellet was resuspended in 600 µl red blood cell lysis buffer and incubated for 5 min

at RT. The reaction was quenched with 20 ml of RPMI complete medium and centrifuged for 5 min at 1500 rpm at 4 °C. BM cells were resuspended in FACS or Sort buffer.

3.2.4.4 FACS sorting of PDC subsets for adoptive transfer

BM cells of B16 FLT3L melanoma injected mice were isolated as described in 3.2.4.3. Cells were resuspended in Sort buffer and centrifuged for 5 min at 1500 rpm at 4 °C. For antibody staining, 800 µl Fc receptor blocking antibody containing hybridoma supernatant was mixed with antibodies (antibody dilution 1:200). Cells were incubated with the staining solution in the dark for 15 min at 4 °C. Subsequently, cells were washed once with 10 ml of Sort buffer and centrifuged for 5 min at 1500 rpm at 4 °C. Cells were then sorted using a Beckman coulter MoFlow cell sorter or a Becton Dickinson FACS Aria, to a purity higher then 95 % (For sorting strategy see Figure 4). Cells were sorted into 3 ml of 100 % FCS into 15 ml polystyrene tubes (Nunc, Rochester, USA). Quality control was done using a Beckman coulter Gallios flow cytometer after sorting. Data was analysed using Flow Jo flow cytometry analysis software (Tree star, Ashland, USA).

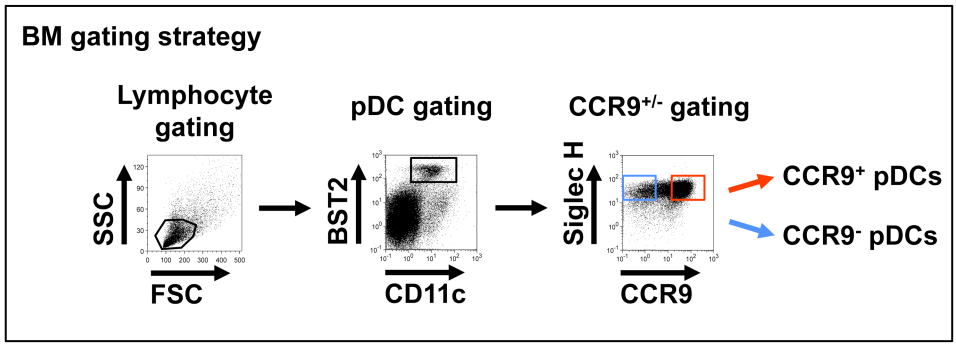

Figure 4 Sorting strategy for CCR9$^+$ and CCR9$^-$ pDCs from BM.

3.2.4.5 Labelling of cells with Violet trace

After FACS sorting, pDCs were centrifuged and resuspended in PBS at a concentration of 1 x 10^6 per ml and Violet trace was added at a concentration of 5 µM and incubated for 20 min at 37 °C in a water bath. The staining reaction was quenched using 10 ml of RPMI complete

medium and cells were incubated for 5 min at RT. Subsequently cells were centrifuged for 5 min at 1500 rpm at 4 °C and resuspended in 150 µl of PBS per injection.

3.2.4.6 Adoptive transfer of pDC subsets

After FACS sorting and labelling of pDC subsets cells were injected intravenously (i.v.) in the tail veins of mice using a 1 ml insulin syringe (150 µl PBS injection volume)

3.2.4.7 Isolation of cells from lymphoid tissues of mice

Mice were sacrificed as described in 3.2.4.3 and lymphoid tissues, such as the mesenteric and inguinal lymph nodes as well as the spleen were excised. After excision, organs were minced and digested in RPMI containing collagenase and DNAse (concentration: collagenase 500 µg/ml; DNAse 100 µg/ml) for 45 min at 37 °C. After incubation cells were passed through a 100 µm cell strainer (BD Falcon, Bedford, USA) and the strainer was washed twice with 10 ml of RPMI medium. Cells were then centrifuged for 5 min at 1500 rpm at 4 °C. After centrifugation red blood cell lysis was performed for 5 min as described. Lysis was stopped by adding 2 ml of FACS buffer and cells were then centrifuged for 5 min at 1500 rpm at 4 °C and resuspended in FACS buffer.

3.2.4.8 Isolation of murine leucocytes from the lung

Mice were sacrificed as described in 3.2.4.2. Lungs were perfused with ice-cold PBS, *via* the heart. Lungs were carefully excised and cut in 5 mm x 5 mm size pieces and digested in RPMI containing collagenase (500 µg/ml) and DNAse (100 µg/ml) for 1 h at 37 °C. After digestion the solution was passed through a 100 µm cell strainer and the strainer was washed twice with 10 ml RPMI. After washing cells were centrifuged for 5 min at 1500 rpm 4 °C. Cells were subsequently stained for FACS analysis as described in 3.2.4.12.

3.2.4.9 Isolation of murine leucocytes from the liver

Liver was perfused with ice-cold PBS *via* the portal vein and cut into 5 mm x 5 mm size pieces. Subsequently, pieces were digested for 1 h in RPMI containing collagenase IV (500 µg/ml) and DNAse (100 µg/ml). After digestion, liver pieces were passed through a 100 µm cell strainer and the strainer was washed two times with 10 ml of RPMI. Cell solution was then spun down at 1500 rpm at 4 °C for 5 min. After centrifugation, the pellet was resuspended in 5 ml of RPMI in a 15 ml Falcon tube and 2.1 ml of Percoll (Sigma-aldrich, Munich, Germany) was added on top of the cell solution and vortexed. After vortexing the cell-Percoll solution was centrifuged for 15 min at 2100 rpm at room temperature without acceleration and deceleration. After centrifugation, the pellet contains the lymphocytes. The hepatocytes form a sticky layer on top of the solution, which is discarded. The pellet was transferred into a new tube and red blood cell lysis was performed. Cells were resuspended in FACS buffer.

3.2.4.10 Isolation of murine leucocytes from blood

Mice were sacrificed by inhalation of excess isoflurane. Directly after death, the chest was opened and the heart was exposed. Blood was drawn directly from the heart using a 1 ml 26 G Sub-Q syringe (BD, Franklin Lakes, USA). 50 µl of heparin solution was provided in a 15 ml Falcon tube in which the blood was collected. Subsequently, 10 ml of red blood cell lysis was added to the falcon tube and incubated for 15 min at RT. After incubation the solution was spun down at 1500 rpm at 4 °C for 5 min. After centrifugation, supernatant was removed and lymphocytes were resuspended in FACS buffer.

3.2.4.11 Isolation of murine leucocytes from the colon and the small intestine

Mice were sacrificed as described in 3.2.4.3 and colon and / or small intestine were excised carefully and attached fat was removed. Subsequently faecal content was removed and colon and / or small intestine were flushed with PBS to remove additional faecal content.

Colon and small intestine were opened longitudinally and were cut into 3 mm long pieces and put into ice-cold PBS and shaken vigorously. After shaking and settling of the tissue parts to the bottom of the Falcon tube, supernatant was removed and washing was performed 3 additional times. Tissue parts were then transferred into a 50 ml beaker tube containing 50 ml PBS, 2 mM DTT, 5 mM EDTA and incubated for 30 min at 37 °C stirring. After incubation, tissue pieces were passed through a 100 µm cell strainer. To isolate intra-epithelial lymphocytes (IEL fraction) of the colon and the small intestine filtrate of the last cell straining process is collected in a 50 ml Falcon tube and passed through a 10 ml syringe filled with glass wool, up to the 3 ml mark. After glass wool filtration, filtrate is centrifuged for 5 min at 1500 rpm at 4 °C. After centrifugation the IEL fraction is resuspended in FACS buffer and can be analysed further. For isolation of lamina propria lymphocytes (LPL fraction) tissue parts remaining in the cell strainer were digested for 15 min in RPMI containing DNAse (100 µg/ml) and collagenase (500 µg/ml) and shaken vigorously prior to incubation at 37 °C. After incubation tissue parts were passed through a 100 µm cell strainer and flow through was collected and centrifuged at 1500 rpm for 5 min at 4 °C. After centrifugation the pellet was resuspended in FACS buffer and analysed further.

3.2.4.12 FACS analysis of adoptively transferred pDC subsets

FACS staining of cells isolated as described in 3.2.4.7 - 11 was done, by seeding cells in 96 well u-shaped plates, followed by a centrifugation for 5 min at 1500 rpm at 4 °C. Cells were subsequently stained using 70 µl Fc receptor antibody containing hybridoma supernatant mixed with various combinations of antibodies to mouse cell surface antigens per well. Cells were resuspended in the antibody FC receptor supernatant mixture (dilution 1:200) and incubated for 15 min at 4 °C in the dark. After incubation cells were washed twice using 200 µl of FACS buffer for each staining reaction and centrifuged for 5 min at 1500 rpm at 4 °C. After washing cells were resuspended in 200 µl FACS buffer and analysed

using a Gallios flow cytometer. Directly before analysis, 5 µl of PI (concentration: 50 µg/ml) was added to the cell solution for exclusion of dead cells. Data was analysed using Flow Jo flow cytometry analysis software. Gating for Violet trace^{+} CD11c^{+} cells in the PI^{-} cell fraction identified transferred cells.

3.2.3.13 Assessment of proliferation using dilution of Violet trace cell dye

Proliferation of transferred cells was assessed by dilution of Violet trace cell dye. Cells, which proliferated showed reduction of fluorescence of Violet trace in comparison to unproliferated cells directly after staining with Violet trace. Level of Violet trace fluorescence was assessed using a Gallios Flow cytometer.

3.2.3.14 Statistical analysis of results

For statistical analysis of results shown in this thesis Student's t-test (unpaired, two-tailed) was used to test for statistical significance * indicates $p < 0.05$; ** indicates $p < 0.01$.

4. Results

4.1 Summary of results published in Blood by Schlitzer *et al.*, 2011

4.1.1 CCR9$^-$ MHC class II^{low} pDCs are immediate precursors of CCR9$^+$ MHC class II^{int} pDCs

While other lymphoid organs such as the spleen and lymph nodes only have very low numbers of pDCs, the BM has about 3 % pDCs in its leucocyte population. Accounting for 39 % ± 6.2 % of CD11c$^+$ cells (mean ± SD, n=3). Within this population of BM pDCs a substantial fraction of 24.1 % ± 5.3 % (n=4) lack or express at a very low level the chemokine receptor CCR9 (Figure 1 A in Schlitzer *et al.*, Blood, 2011). Both populations whether being CCR9$^+$ or CCR9$^{-/low}$ express critical pDC markers such as BST2, B220 and Siglec H, however CCR9$^-$ pDC express these at slightly lower levels. Furthermore, expression of MHC class II, CD80 and CD86 was lower on CCR9$^-$ pDCs compared to CCR9$^+$ pDCs in the BM, showing the lower activation and less differentiated status of CCR9$^-$ pDCs (Figure 1 A and B, Schlitzer *et al.*, Blood, 2011). Additionally, expression of CD135 (FLT3) and CD115 (MCSF-R) was similar between CCR9$^+$ and CCR9$^-$ pDCs. Interestingly a small subpopulation of CCR9$^-$ pDCs showed a higher expression of CD115. Also mRNA expression of CSF2αr, the specific GM-CSF receptor chain, was similar between the two pDC subsets (Figure 1 B in Schlitzer *et al.*, Blood, 2011). However CX3CR1, a chemokine receptor expressed on all splenic DC subsets, was also expressed on both pDC subsets albeit at slightly higher levels on CCR9$^-$ pDCs, as determined by mRNA expression as well as FACS analysis. Thus these results show that except for the lower expression of Sca-1 and activation associated markers such as MHC class II, CD80 and CD86, both populations have a characteristic pDC surface phenotype, concluding that this marks a close developmental relationship.

E2-2 was described as the critical transcription factor driving pDC development. We therefore determined expression of E2-2 and other DC development related transcription factors in $CCR9^-$ and $CCR9^+$ pDCs sorted from the BM. E2-2 and IRF8 where both expressed in $CCR9^-$ pDCs albeit at slightly lower levels compared to $CCR9^+$ pDCs. Spi-b, ID2 and BATF3, were also expressed in both populations, however no significant differences could be detected (Figure 1 B in Schlitzer *et al.*, Blood, 2011). Interestingly PU.1 was significantly higher expressed in $CCR9^-$ pDCs than in $CCR9^+$ pDCs. Functionally, stimulation with the TLR9 ligand CpG 2216 revealed differences between the $CCR9^-$ and $CCR9^+$ pDC subset. $CCR9^-$ pDCs responded with higher levels of IFN-α, a pDC signature cytokine, to overnight stimulation with CpG 2216 compared to $CCR9^+$ pDCs. Furthermore IL-6 and IL-12 was induced by CpG 2216 stimulation in $CCR9^-$ pDCs, whereas $CCR9^+$ pDCs only produced very little amounts of these cytokines (Figure 1 D in Schlitzer *et al.*, Blood, 2011).

The rearrangement of the immunoglobulin gene locus in $CCR9^-$ and $CCR9^+$ pDCs was analysed by genomic DNA PCR. As shown in Figure 1 C (Schlitzer *et al.*, Blood, 2011) no rearrangement could be detected in $CCR9^-$ pDCs sorted from the BM, whereas $CCR9^+$ pDCs showed D_H-J_H immunoglobulin gene rearrangement, which has been described before for a subpopulation of pDCs.
This analysis shows that except the lack of D_H-J_H immunoglobulin rearrangement $CCR9^-$ and $CCR9^+$ pDC share most of the characteristic pDC features, including the ability to produce IFN-α upon TLR stimulation. We therefore investigated if $CCR9^-$ pDCs can give rise to fully differentiated $CCR9^+$ pDCs and incubated $CCR9^-$ and $CCR9^+$ pDCs for 48h with or without FLT3L. $CCR9^-$ pDCs gave rise to fully differentiated $CCR9^+$ MHC class II^+ pDCs 48h after incubation with or without FLT3L, showing that $CCR9^-$ pDCs can be precursors of fully differentiated $CCR9^+$ pDCs (Figure 2 in Schlitzer *et al.*, Blood, 2011).

Additionally, $CCR9^-$ pDCs were not only found in the BM, but also in spleen, lymph nodes and the colon, albeit at lower numbers (supplemental figure 1 B, Schlitzer *et al.*, Blood, 2011).

4.1.2 CCR9$^-$ MHC class II^{low} pDC retain plasticity to acquire phenotype and function of CD11b$^+$ MHC class II^{high} cDC-like cells

It was shown recently that subsets of intestinal DCs derive from local precursors and that this process is shaped by the intestinal microenvironment. We therefore tested if the development of CCR9$^-$ pDCs can be influenced by a supernatant derived from intestinal epithelial cells (IEC-SN). We incubated CCR9$^-$ or CCR9$^+$ pDCs sorted from the BM for 48h with 50 % of IEC-SN and assessed the phenotype and function of CCR9$^-$ and CCR9$^+$ pDCs 48h after incubation. Exposure of CCR9$^-$ pDCs to IEC-SN leads to the downregulation of pDC markers such as BST2, Siglec H and B220 in a substantial fraction of CCR9$^-$ pDCs (35 % ± 7.4 %, mean ± SD, n=5, Figure 3 A, Schlitzer *et al.*, Blood, 2011). Also lower expression of CCR9, CD8α and CD4 could be detected in CCR9$^-$ cultures 48h after incubation with IEC-SN compared to CCR9$^+$ cultures. As shown in Fig 3 A (Schlitzer *et al.*, Blood, 2011) exposure to IEC-SN for 48h lead to the generation of a CD11b^{high} MHC II^{high} BST2^{low} population only in cultures of CCR9$^-$ pDC but not in CCR9$^+$ pDC cultures. This newly developed subpopulation of IEC-SN DCs showed high expression of CD11b, MHC class II as well as CD80 and CD86, thus resembling cDCs. Remaining BST2$^+$ cells in these cultures retained a normal pDC phenotype and also upregulated CCR9, to become fully differentiated pDCs (Figure 3 A, Schlitzer *et al.*, Blood, 2011). We furthermore assessed the functional properties of IEC-SN treated CCR9$^-$ pDC cultures. Stimulation of CCR9$^-$ pDCs cultured with IEC-SN and CpG 2216 results in lower secretion of IFN-α, but higher secretion of IL-6 and IL-12 compared to CCR9$^-$ pDCs cultured with medium (Figure 3 B, Schlitzer *et al.*, Blood, 2011). We therefore hypothesized that BST2^{low} cells from IEC-SN conditioned CCR9$^-$ cultures could not only have acquired the phenotype of cDCs but also functions of cDCs, such as efficient T cell activation. To test this we sorted IEC-SN DCs and IEC-SN pDCs from 48h conditioned CCR9$^-$ pDCs cultures and cultured both subsets, after pulsing with OVA protein or OVA peptide, for 4 days with OT I (CD8$^+$) or OT II (CD4$^+$) T cells respectively. As shown in figure 4 A (Schlitzer *et al.*, Blood, 2011) IEC-SN DCs were significantly more efficient in inducing T cell proliferation as well as IFN-γ

secretion by $CD4^+$ (OT II) T cells compared to IEC-SN pDCs. Also IEC-SN DCs were more efficient than splenic pDCs and $CD8\alpha^-$ DCs in inducing OT I $CD8^+$ T cell proliferation and IFN-γ production but less potent then $CD8\alpha^+$ DCs from the spleen, which are the most efficient cross-presenting DCs. (Figure 4 B, Schlitzer *et al.*, Blood, 2011). Furthermore, we carefully assessed the DC compartment in Peyer's patches of steady state mice and found a population of DCs which expresses lower levels of BST2, Siglec H and Sca-1 then $BST2^{high}$ pDCs but higher levels of MHC class II and CD11b, thereby closely resembling IEC-SN induced cDC-like cells derived from $CCR9^-$ pDCs (Figure 3 C, Schlitzer *et al.*, Blood, 2011).

These results show that $CCR9^-$ pDCs retain plasticity to divert from the pDC lineage under the influence of IEC-SN and are able to give rise, in addition to fully differentiated pDCs, to a $CD11b^+$ MHC class II^{high} cDC-like cell subset, with the capacity to produce high amounts of pro-inflammatory cytokines and to induce robust $CD4^+$ as well as $CD8^+$ T cell responses. A similar cell type exists in the Peyer's patches. Therefore this differentiation may also occur *in vivo*.

4.1.3 Diversion of $CCR9^-$ MHC class II^{low} pDCs from the pDC lineage is induced by GM-CSF

Intestinal epithelial cells such as the PTK6 cell line used in this study are able to produce various factors, which are able to interfere with DC development. We were not able to detect TNF-α, IL-10, TGF-β, IL-6 or type I IFN (Fig.S4 C) in the IEC-SN, which have all been described to influence pDC development. GM-CSF however, which is produced constitutively by intestinal epithelial cells and further upregulated upon inflammation, could be detected at low but reproducible levels in supernatants obtained from PTK6 monolayers (56 ± 7.5 pg/ml, n=3). We therefore tested if pre-treatment of IEC-SN with neutralizing anti-GM-CSF antibody inhibited the generation of cDC-like cells ($BST2^{low}$, $CD11c^{high}$, $CD11b^{high}$, MHC class II^{high} cells) in $CCR9^-$ pDC cultures. After neutralization of GM-CSF in the IEC-SN, the generation of $CD11b^+$ MHC class II^{high} cells in $CCR9^-$ pDC cultures was abrogated (Figure 5 A, Schlitzer *et al.*, Blood, 2011). Also the observed enhanced production of IL-6 and IL-12 was

blocked by treatment of the IEC-SN with anti-GM-CSF antibody. Decreased production of IFN-α in IEC-SN treated CCR9$^-$ pDC cultures however was unaffected by neutralizing GM-CSF in the IEC-SN (Figure 5 B, Schlitzer *et al.*, Blood, 2011). Vice versa addition of recombinant GM-CSF to CCR9$^-$ pDC cultures for 48h induced the formation of CD11b$^+$ MHC class II^{high} cells from CCR9$^-$ pDC precursors (Figure 5 C, Schlitzer *et al.*, Blood, 2011). Furthermore secretion of IL-6 and IL-12 was enhanced upon stimulation with CpG 2216 24h after conditioning with GM-CSF, however secretion of IFN-α was not inhibited by culture with recombinant GM-CSF suggesting that there are additional factors in the IEC-SN, which contribute to this effect (supplemental Figure 4 B, Schlitzer *et al.*, Blood, 2011). In contrast to GM-CSF FLT3L or M-CSF added to CCR9$^-$ pDC cultures did not interfere with the generation CCR9$^+$ fully differentiated pDCs in CCR9$^-$ pDC cultures and did not induce the generation of CD11b$^+$ MHC class II^{high} cDC-like cells (Figure 5 C, Schlitzer *et al.*, Blood, 2011). Thus, we can conclude that GM-CSF secreted by intestinal epithelial cells is able to mediate deviation of CCR9$^-$ pDCs from the pDC lineage towards the cDC lineage and leads to the acquisition of cDC markers and enhanced production of proinflammatory cytokines.

4.1.4 Diversion of CCR9$^-$ MHC class II^{low} pDCs from the pDC lineage is marked by a profound change in the expression of transcription factors

We hypothesized that the observed phenotypic and functional shift of CCR9$^-$ pDCs towards a cDC-like phenotype is not only transient but resembles a profound deviation from the pDC lineage commitment and should therefore be regulated on the level of transcription factors. We therefore sorted IEC-SN pDCs as well as IEC-SN DCs derived from CCR9$^-$ pDCs cultured with IEC-SN and compared expression of DC lineage specific transcription factors with expression in CD8α^+ as well as CD8α^- cDCs from the spleen by qRT-PCR analysis.

This analysis showed that expression of E2-2 was significantly downregulated in IEC-SN DC as compared to IEC-SN pDCs. This was also true for E2-2

regulated transcripts of IRF8 and Spi-b. IRF8 levels expressed by IEC-SN DC were as low as levels found in CD8α^- DCs but not CD8α^+ DCs. Additionally, cDC development associated transcription factors such as ID2, BATF3 and PU.1 were significantly upregulated in IEC-SN DC compared to IEC-SN pDCs, thereby showing a cDC associated expression pattern (Figure 6, Schlitzer *et al.*, Blood, 2011).

These results show that upon exposure to GM-CSF containing IEC-SN CCR9$^-$ pDC precursors are able to deviate from the pDC lineage to the cDC lineage and that this developmental shift is reflected by a change in the transcription factor profile of the newly developed cDC-like cell.

4.1.5 Author contributions

I am the first author of the study presented in this thesis. I generated all experimental data on my own with some help by Jakob Loschko. Also Katrin Mair maintained PTK6 cells for generation of IEC-SN in the laboratory of Roger Vogelmann. Cell sorting was done with help from Lynette Henkel in the Cell sorting facility of the Institute for Medical Microbiology, Hygiene and Immunology (TU München), the sorting facility is headed by Matthias Schiemann. Jan Hendrik Niess and Henrik Einwächter contributed reagents and mice towards this study. All data was analysed by myself. Wolfgang Reindl contributed to interpreting the data. Anne Krug and myself interpreted the data. Anne Krug supervised the study.

4.2 Unpublished results

4.2.1 CCR9$^-$ and CCR9$^+$ pDCs from the BM maintain pDC phenotype characteristics after *in vivo* expansion by FLT3L

After identifying CCR9$^-$ pDCs in the BM as a potential precursor for CCR9$^+$ pDCs and furthermore demonstrating that CCR9$^-$ pDCs are able to undergo lineage diversion from the pDC lineage to the cDC lineage *in vitro* (Schlitzer *et al.*, 2011), we set out to investigate if this phenomenon can also be observed *in vivo* and if CCR9$^-$ pDCs can act as precursors to CCR9$^+$ pDCs *in vivo* in the steady state. We therefore used an adoptive transfer model to assess the fate of CCR9$^-$ and CCR9$^+$ pDCs *in vivo*.

To obtain sufficient numbers of CCR9$^-$ and CCR9$^+$ pDCs for transfer experiments DCs were expanded *in vivo* by subcutaneously injecting B16 melanoma cells, which secrete high amounts of FLT3L. This syngeneic tumour is implanted s.c. in the neck of C57BL/6 mice and allowed to grow for 7 days.
After expansion of DC populations we sorted CCR9$^-$ and CCR9$^+$ pDC populations by the use of the pDC markers BST2 and Siglec H and the integrin CD11c. However to test if the expansion of pDCs by FLT3L secreting tumour cells changed the phenotype of pDCs in the BM or the ratio of subpopulations, we monitored expression of pDC signature molecules on CCR9$^-$ and CCR9$^+$ pDC directly after sorting. As shown in Figure 5 CCR9$^+$ pDCs expressed all pDC related markers at a high level, such as Siglec H and BST2. Furthermore CCR9$^+$ pDCs show intermediate expression for CD11c and CX3CR1 and only low levels of CD11b, which is in accordance to the published literature and our previous findings (Schlitzer *et al.*, 2011). CCR9$^-$ pDCs, depicted in Figure 5, expressed similar levels of the pDC specific markers Siglec H and BST2 compared to CCR9$^+$ pDCs. Furthermore the expression levels of CD11c, CD11b are very similar to those of CCR9$^+$ pDCs. However CX3CR1 expression was elevated in CCR9$^-$ pDCs, similar to levels which are found in cDC populations in the spleen. Higher expression of CX3CR1 was also seen in the BM of unmanipulated mice (shown in Schlitzer *et al.*).

MHC class II can be used as a marker of pDC differentiation and maturation, therefore we also assessed MHC class II expression on both $CCR9^+$ and $CCR9^-$ pDCs and found that $CCR9^+$ pDCs expressed higher levels of MHC class II on their surface than $CCR9^-$ pDCs, hinting at a more differentiated and mature status of the $CCR9^+$ pDCs. Also the level of MHC class II expression was slightly higher on $CCR9^-$ pDC from BM of mice bearing the FLT3L producing tumour then for untreated mice.

Taken together this data shows that $CCR9^+$ and $CCR9^-$ pDCs both express pDC specific markers, therefore are both part of the pDC lineage, but differ in differentiation and maturation status and the expression of CX3CR1. $CCR9^-$ $MHCII^{low}$ $CX3CR1^{high}$ pDCs are phenotypically less mature and differentiated in comparison to $CCR9^+$ pDCs. Additionally FLT3L-mediated expansion does not greatly alter expression of pDC surface molecules in the BM of WT mice.

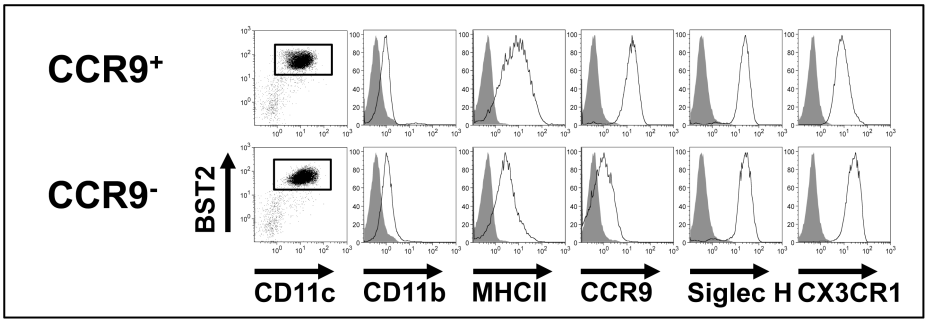

Figure 5 $CCR9^-$ pDC show pDC surface marker expression, but immature differentiation and maturation status compared to $CCR9^+$ pDCs; FACS sorted 7 day FLT3L expanded BM $CCR9^-$ and $CCR9^+$ pDCs were assessed for the expression of depicted markers in steady state C57BL/6 mice by flow cytometry. One representative experiment shown (n=3).

4.2.2 CCR9$^-$ and CCR9$^+$ pDCs are equally expanded by treatment with FLT3L

To assess if both pDC subsets in the BM are equally responsive to FLT3L, a critical growth factor for DCs, we transplanted FLT3L secreting melanoma cells into steady state C57BL/6 mice and analysed transplanted and non-transplanted mice 7 days later. As shown in Figure 6 A 7 day FLT3L exposure induced a robust 3-fold expansion of the pDC subset in the BM (FLT3L treated 9.75 % ± 1.75 %; untreated 3.18 % ± 0.41 %; mean ± SD). Both the CCR9$^+$ and CCR9$^-$ pDC subsets in the BM were expanded upon exposure to FLT3L (Figure 5 B). CCR9$^+$ pDCs accounted for 83.6 % ± 1.6 % (mean ± SD) in FLT3L exposed mice versus 85.3 % ± 3.1 % (mean ± SD) in untreated mice of the total pDC population in the BM. The proportion of the CCR9$^-$ pDC subset remained unchanged (12 % ± 3.2 % in FLT3L exposed mice; 14.7 % ± 3.2 % in untreated mice; mean ± SD), showing that both populations respond similarly to FLT3L.

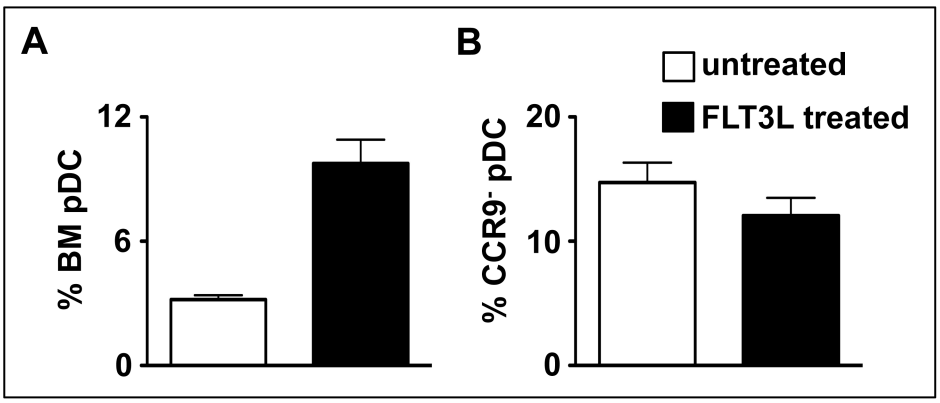

Figure 6 Exposure to FLT3L expands both the CCR9$^-$ and CCR9$^+$ pDC populations equally; (A) Mice were exposed or not for 7 days to B16 melanoma secreting FLT3L. Proportion of pDCs in total PI$^-$ BM leucocytes was monitored by flow cytometry (n=5, mean ± SD); **(B)** Percentage of CCR9$^-$ pDC within the total pDC population in the BM of day 7 FLT3L exposed and not exposed mice (n=5, mean ± SD).

4.2.3 CCR9$^-$ pDCs are present in all major lymphoid and non-lymphoid organs

After having found that CCR9$^-$ pDCs isolated from the BM are able to serve as CCR9$^+$ pDC precursors and can also differentiate into cDC-like cells in response to tissue-derived factors *in vitro*, we determined the abundance of CCR9$^-$ pDC precursors in blood, lymphoid organs and non-lymphoid organs of steady state mice.

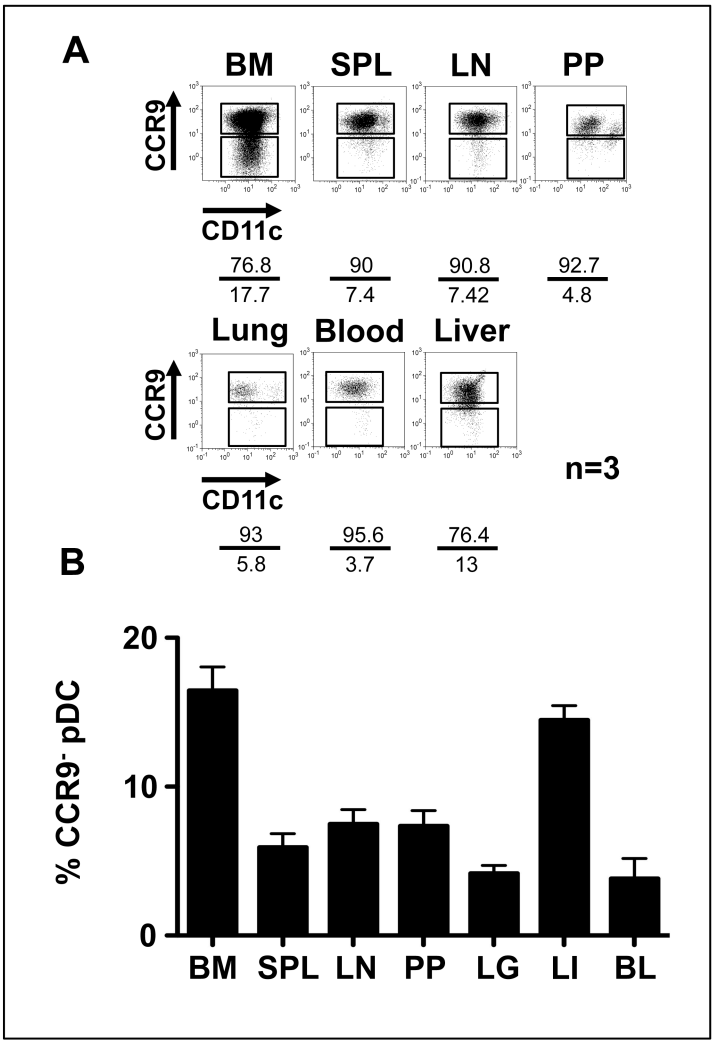

Figure 7 CCR9$^-$ pDCs are present in all major lymphoid and non-lymphoid organs; (A) BM, SPL, LN, PP, LG, BL and LI were analysed by flow cytometry for the expression of CCR9 and CD11c in the BST2$^+$ Siglec H$^+$ CD11c^{int} PI$^-$ population. Numbers indicate percentages within the total pDC population. One representative experiment out of 3 is shown **(B)** Statistical analysis of CCR9$^-$ pDC abundance in the pDC population in BM, SPL, LN, PP, LG, LI, BL is shown (n=3, mean ± SD).

Analysis of BM, spleen (SPL), lymph nodes (pooled mesenteric and inguinal lymph nodes, LN), Peyer's patches (PP), lung (LG), blood (BL) and liver (LI) revealed that within the Siglec H^+ $BST2^+$ $CD11c^{int}$ PI^- population of pDCs a significant fraction of $CCR9^-$ pDCs was present in all major organs and the blood (Figure 7 A and B).

In the BM, which has approximately 3 % pDCs among its lymphocyte population, 16.5 % ± 3.1 % (mean ± SD) $CCR9^-$ pDCs were found in the steady state. In spleen, lymph node and Peyer's patches, which harbour significantly smaller pDCs populations than the BM (between 0,5 % and 1 % of the total lymphocyte population) still between 5 % and 7 % of the pDC population were negative or low for CCR9. In the blood $CCR9^-$ pDCs were also observed (3.8 % ± 2.9 %; mean ± SD) showing that $CCR9^-$ pDCs circulate within the blood stream and are thus able to seed lymphoid as well as non-lymphoid organs. $CCR9^-$ pDCs can also be identified in non-lymphoid organs such as the lung and liver. Interestingly the frequency of $CCR9^-$ pDCs was highest in hematopoietic organs – BM and liver. Showing that $CCR9^-$ pDCs are not only present in lymphoid organs and the blood, but are also able to enter non-lymphoid organs and persist in these organs.

4.2.4 $CCR9^-$ and $CCR9^+$ pDCs display a similar chemokine receptor expression pattern

To address the question if these two cell populations differ in their abilities to home to different sites in the body, expression of several chemokine receptors was measured on the surfaces of $CCR9^-$ and $CCR9^+$ pDCs. In pDCs CCR7, CXCR3 and CXCR4 are indicated in the recruitment to peripheral organs and especially to the lymph node (Seth *et al.*, 2011). Furthermore we investigated the expression of CCR6 and CCR4, which are chemokine receptors associated with homing of leucocytes to the gut and the lung under steady state and inflammatory conditions (Heiseke *et al.*, 2011; Ito *et al.*, 2011). As shown in Figure 8 $CCR9^-$ pDCs showed low but significant expression of the chemokine receptor CCR7. Interestingly the more mature population of $CCR9^+$ pDCs displayed higher levels of CCR7 in the steady state in BM and spleen.

Furthermore CCR9$^+$ pDCs in the BM and the spleen also display elevated levels of CCR6, a chemokine receptor associated with homing to mucosal interfaces, especially important in the small intestine. Additionally CCR9$^+$ pDCs have a higher expression of the inflammation associated chemokine receptors CXCR3, CXCR4 and CCR4 in BM and spleen compared to only low levels of these chemokine receptors on the surface of CCR9$^-$ pDCs, as determined by flow cytometric analysis. Higher expression of chemokine receptors in CCR9$^+$ pDC in the spleen as well as in the BM correlate with the more mature phenotype of the CCR9$^+$ pDCs.

As shown in Figure 8 C, CCR9$^-$ and CCR9$^+$ pDCs in the blood of steady state WT mice do not differ substantially in the expression of CCR4 on their surfaces.

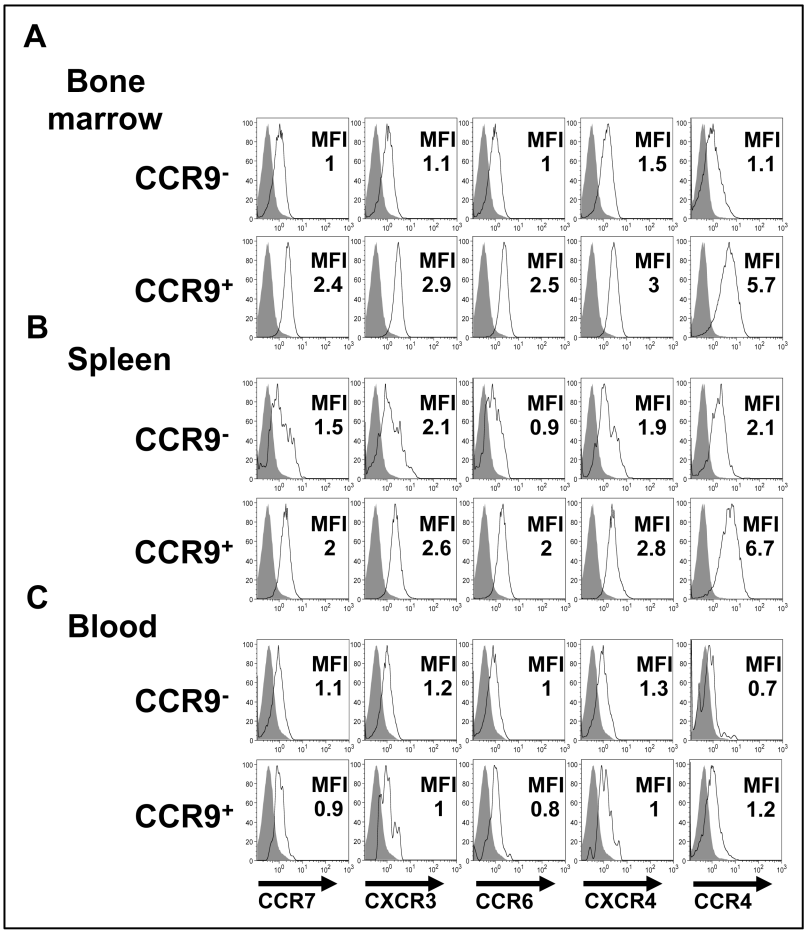

Figure 8 Analysis of chemokine receptor expression on CCR9$^-$ and CCR9$^+$ pDCs in BM, spleen and blood of WT mice; CCR9$^-$ and CCR9$^+$ Siglec H$^+$ BST2$^+$ CD11c^{int} PI$^-$ pDCs in BM, spleen and blood were analysed by flow cytometry for the expression of CCR7, CXCR3, CCR6, CXCR4 and CCR4 directly after isolation. One representative experiment shown out of 3. Histograms show antibody staining (open histograms) against unstained control (filled histograms).

However CCR4 expression on CCR9$^+$ pDCs is higher then on CCR9$^-$ pDCs in the blood similar to what was found in BM and spleen. Additionally comparing BM, spleen and blood CCR9$^+$ pDCs and their expression of the tested chemokine receptors, CCR9$^+$ pDCs downregulate all five tested receptors once they enter the blood stream, whereas CCR9$^-$ pDCs upregulate CCR4 upon entry to the blood stream in steady state WT mice.

4.2.5 Rapid accumulation of CCR9$^+$ and CCR9$^-$ pDCs in lymphoid organs after adoptive transfer in steady state mice

The distribution of CCR9$^-$ and CCR9$^+$ pDCs in the organs *in vivo* was assessed by the use of an adoptive transfer system. CCR9$^+$ or CCR9$^-$ pDCs were isolated

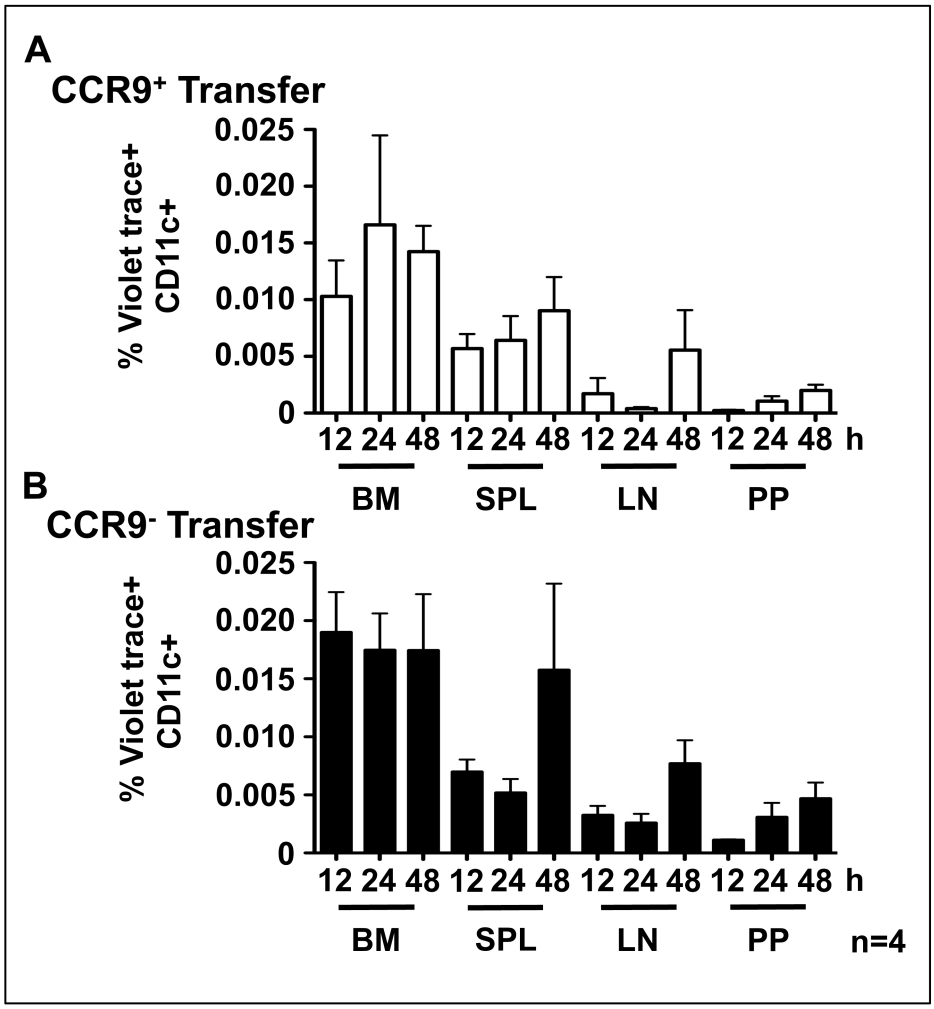

Figure 9 Recovery of adoptively transferred CCR9$^+$ and CCR9$^-$ pDCs 12h, 24h and 48h after transfer in BM, SPL, LN and PP; (A) 12 – 48h kinetic of WT CCR9$^+$ pDC recovery (Violet trace$^+$, CD11c$^+$, PI$^-$) transferred into WT steady state recipients; **(B)** 12 – 48h kinetic of WT CCR9$^-$ pDC recovery (Violet trace$^+$, CD11c$^+$, PI$^-$) transferred into WT steady state recipients; Recovery was assessed by flow cytometry, at the time points indicated. Pooled results of 4 independent experiments are shown (n=4, mean ± SD).

from FLT3L expanded C57BL/6 WT mice and labelled with Violet trace. After labelling, 5 x 10^5 $CCR9^-$ or $CCR9^+$ pDC were injected i.v. into WT steady state recipients and detected in the BM and lymphoid organs at 12h, 24h and 48h post transfer. As shown in Figure 9 A $CCR9^+$ pDCs enrich in the BM of recipients and reach a plateau at the 24h time point whereas in spleen, lymph node and Peyer's patches appearance of $CCR9^+$ pDC gradually increased until 48h after injection. In contrast to that $CCR9^-$ pDCs seemed to be recruited more rapidly to the BM, because recovery of $CCR9^-$ pDCs after transfer was already maximal 12h post injection and did not increase substantially after this time point. In the periphery however, $CCR9^-$ pDCs increase in spleen, lymph nodes and Peyer's patches until 48h after injection similar to what is seen after transfer of $CCR9^+$ pDCs (Figure 9 B). These kinetics show that $CCR9^+$ as well as $CCR9^-$ pDCs are rapidly recruited to the BM as well as to peripheral lymphoid tissues such as the spleen, lymph nodes and Peyer's patches. However it is not clear if there is further increase in spleen, lymph nodes and Peyer's patches, which is due to proliferation or secondary recruitment, as our analysis did not expand to more then 48h. Despite this, the 48h time point was chosen as a suitable time point for phenotypic analysis, because sufficient numbers of transferred cells could be recovered from all assessed organs.

4.2.6 $CCR9^-$ and $CCR9^+$ pDCs accumulate in all major lymphoid and non-lymphoid organs upon adoptive transfer

After identifying 48h as a time point, which is suitable for analysis of transferred $CCR9^-$ and $CCR9^+$ pDCs after adoptive transfer, we monitored recovery of Violet $trace^+$ $CD11c^+$ PI^- pDC populations at 48h after transfer by flow cytometry. As shown in Figure 10 $CCR9^-$ and $CCR9^+$ pDCs were able to home to the BM to the same extent 48h after transfer. Similarly also in the spleen and lymph node no difference in recovery of $CCR9^-$ and $CCR9^+$ pDCs could be detected indicating that homing to these secondary lymphoid organs is not different between $CCR9^-$ and $CCR9^+$ pDCs, which is in accordance with the only minimal difference in expression of CCR7 (slightly lower expression of CCR7 on $CCR9^-$ pDCs). However $CCR9^-$ pDCs homed significantly better to Peyer's patches than $CCR9^+$ pDCs upon adoptive transfer (Figure 10 A). This

data shows that CCR9$^-$ pDCs 48h after adoptive transfer home efficiently to the BM and secondary lymphoid organs such as the spleen and lymph nodes and furthermore have an advantage over CCR9$^+$ pDCs to enter the Peyer's patches

We also assessed recovery of transferred cell populations in the blood and in non-lymphoid organs such as the lung, liver, small intestine and the colon (Figure 10 B and C). 48h after adoptive transfer donor cells could be recovered in all analysed organs and the blood. However, several interesting differences in the homing properties of CCR9$^-$ and CCR9$^+$ pDCs to non-lymphoid organs could be observed. Transferred CCR9$^-$ pDCs homed significantly better to the lung and the colon of recipient mice, although only few cells were recovered

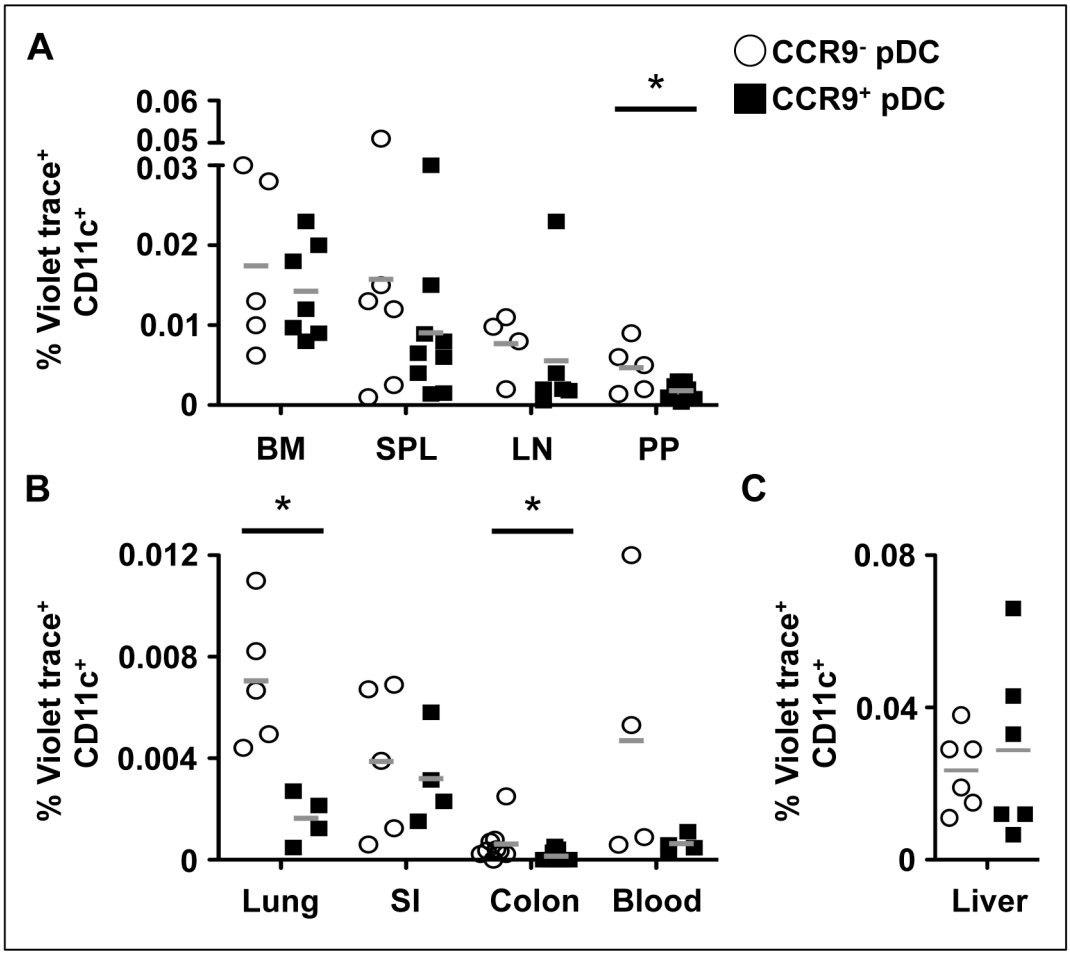

Figure 10 Recovery of adoptively transferred CCR9$^-$ and CCR9$^+$ pDCs 48h after transfer in lymphoid and non-lymphoid organs; (A) Recovery of transferred Violet trace$^+$ CD11c$^+$ PI$^-$ CCR9$^-$ or CCR9$^+$ pDCs 48h after i.v. transfer in BM, SPL, LN and PP. **(B)** Recovery of transferred Violet trace$^+$ CD11c$^+$ PI$^-$ CCR9$^-$ or CCR9$^+$ pDCs 48h after i.v. transfer in lung, SI, colon and blood. **(C)** Recovery of transferred Violet trace+ CD11c$^+$ PI$^-$ CCR9$^-$ or CCR9$^+$ pDCs 48h after i.v. transfer in liver. Recovery was assessed by flow cytometry in the indicated organs. Pooled results of 3 independent experiments are shown. Asterisks indicate statistical significance; $p<0.05$, Student's t-test.

from the colon. However, there was no difference in homing to the liver and the small intestine (Figure 10 B and C). Also no significant difference could be observed in the ability of $CCR9^-$ or $CCR9^+$ pDCs to recirculate or to persist in the blood 48h after transfer, although a trend towards a higher $CCR9^-$ pDC recovery in the blood was detected.

In conclusion $CCR9^-$ and $CCR9^+$ pDCs can be recovered to a similar extend in BM, spleen, lymph nodes (mesenteric and inguinal lymph nodes, pooled), small intestine, liver and the blood, but differ in their ability to enter or to persist in the lung, in the colon and in the Peyer's patches.

4.2.7 CCR9$^-$ but not CCR9$^+$ pDCs downregulate pDC-specific surface molecules in lymphoid and non-lymphoid organs

Since CCR9$^-$ pDCs were able to generate mature CCR9$^+$ pDCs *in vitro* but could still deviate from the pDC lineage to the cDC lineage, we hypothesized that this could also occur *in vivo*. We therefore monitored expression of BST2 and CD11c on Violet trace-labelled CCR9$^-$ and CCR9$^+$ pDCs 48h after transfer in lymphoid and non-lymphoid organs. As shown in Figure 11 A, transferred CCR9$^+$ pDCs largely maintained expression of BST2 48h after transfer, whether in the BM or in the peripheral lymphoid organs, such as the spleen, lymph nodes and Peyer's patches. This was also the case in liver, small intestine and in the lung, although the pDC phenotype of transferred CCR9$^+$ pDCs in the lung

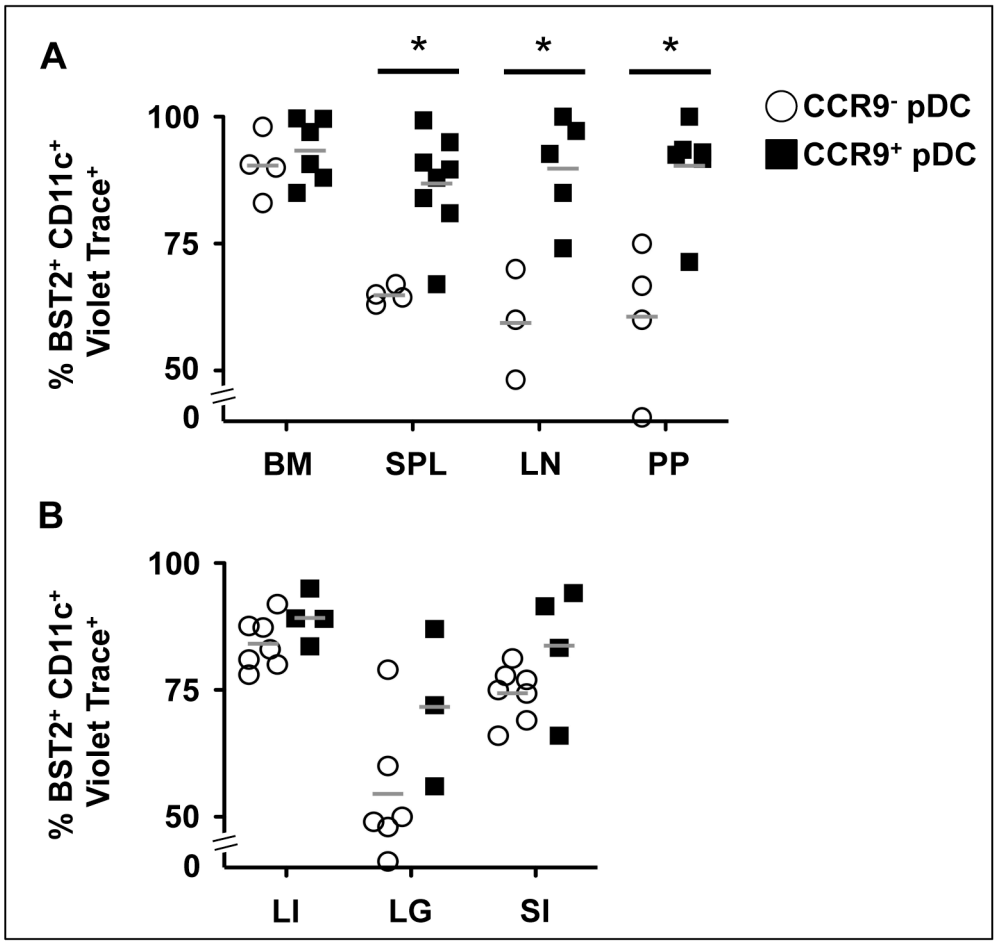

Figure 11 Percentage of BST2$^+$ CD11c$^+$ Violet trace$^+$ PI$^-$ cells in lymphoid and non-lymphoid organs 48h after adoptive transfer of CCR9$^-$ or CCR9$^+$ pDCs; (A) Percentage of BST2$^+$ CD11c$^+$ Violet trace$^+$ CCR9$^-$ or CCR9$^+$ cells 48h after i.v. transfer in BM, SPL, LN, PP. **(B)** Percentage of BST2$^+$ CD11c$^+$ Violet trace$^+$ CCR9$^-$ or CCR9$^+$ cells 48h after i.v. transfer in LI, LG and SI. Expression of BST2, CD11c and Violet trace was monitored by flow cytometry. Pooled results of 3 independent experiments are shown. Asterisks indicate statistical significance; $p<0.05$, Student's t-test.

was less stable. In contrast, a fraction of transferred CCR9$^-$ pDCs had already downregulated BST2 expression 48h after transfer in spleen (64.8 % ± 1.6 % BST2$^+$ cells; mean ± SD), lymph nodes (59 % ± 10.9 % BST2$^+$ cells; mean ± SD) and Peyer's patches (60.6 % ± 14.5 % BST2$^+$ cells; mean ± SD) (Figure 11 A) and also in non-lymphoid organs such small intestine (74.1 % ± 5.2 % BST2$^+$ cells; mean ± SD) and the lung (54.5 % ± 13.4 % BST2$^+$ cells; mean ± SD) (Figure 11 B). Downregulation of BST2, here used as a surrogate marker for a pDC specific phenotype, was significantly downregulated in spleen, lymph nodes and Peyer's patches, however was maintained in the BM (90.4 % ± 6.1 % BST2$^+$ cells; mean ± SD) (Figure 11 A). Furthermore downregulation of BST2 was also found in the lung and small intestine, but was maintained in the liver (84.1 % ± 5 % BST2$^+$ cells; mean ± SD) (Figure 11 B). These results show that a substantial fraction of CCR9$^-$ pDCs downregulated BST2 48h after adoptive transfer deviating from the pDC lineage. This loss of pDC phenotype was found in peripheral lymphoid organs and non-lymphoid organs but not in BM and liver. At these hematopoietic and former hematopoietic sites pDC specific marker expression is maintained.

In conclusion CCR9$^-$ pDCs retain the potential to divert from the pDC lineage. This event occurs in the steady state *in vivo* and depends on tissue specific factors.

4.2.8 CCR9$^-$ pDCs give rise to CCR9$^+$ pDCs and to CD11b$^+$ MHC class II^{high} cDCs locally in the tissues

To further elucidate the phenotypical changes of transferred CCR9$^-$ pDCs and to prove the hypothesis that CCR9$^-$ pDCs can give rise to CCR9$^+$ fully

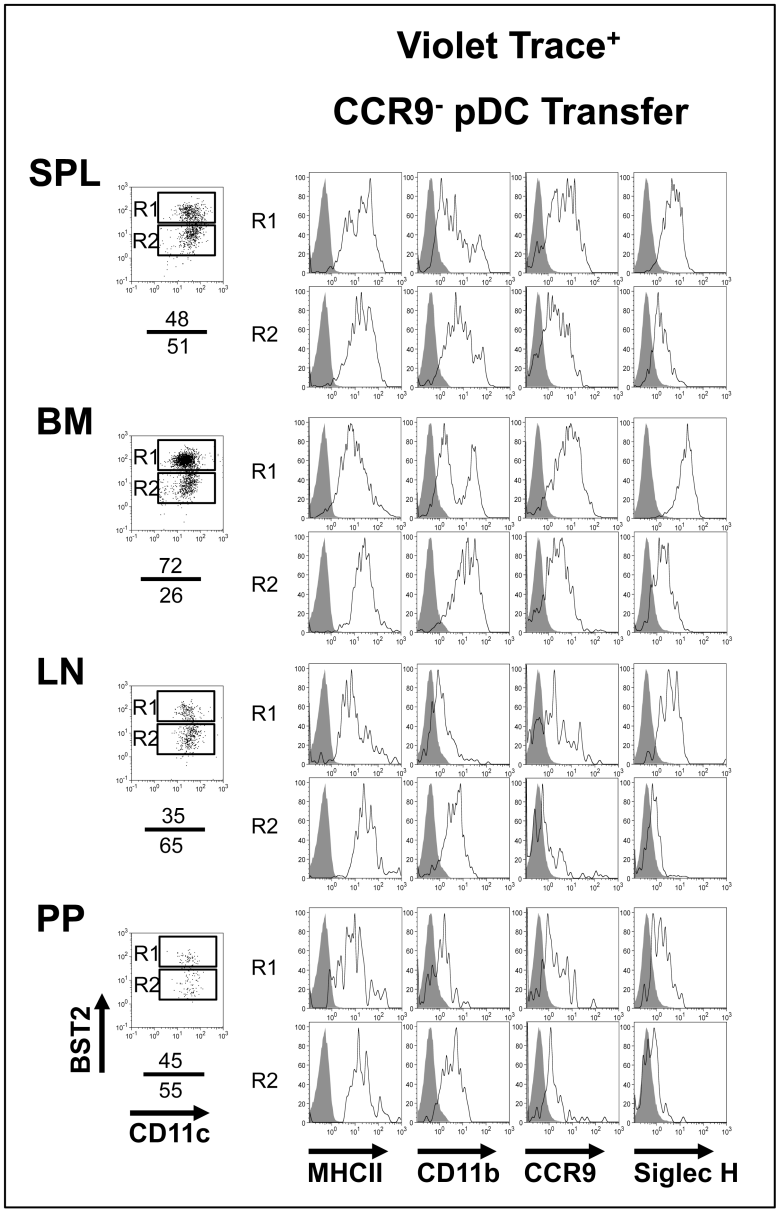

Figure 12 Phenotype of adoptively transferred CCR9$^-$ pDCs 48h after transfer; FACS sorted BM Violet trace$^+$ CCR9$^-$ pDCs from FLT3L expanded donors, were transferred i.v. into steady state hosts and surface expression of MHC class II, CD11b, CCR9 and Siglec H was assessed by flow cytometry in SPL, BM, LN and PP 48h after transfer. Cells shown in dot blots and histograms were gated PI$^-$. One representative experiment shown out of 3. Histograms show antibody staining (open histograms) against unstained control (filled histograms).

differentiated pDCs like a *bona fide* pDC precursor *in vivo*, we transferred sorted Violet trace-labelled CCR9$^-$ pDCs into steady state hosts and analysed their phenotype 48h after i.v. transfer. We analysed the surface expression of BST2, CD11c, MHC class II, CD11b, CCR9 and Siglec H on Violet trace$^+$ PI$^-$ cells in spleen, BM, lymph nodes and Peyer's patches by flow cytometric analysis. As shown in Figure 12, a fraction of Violet trace$^+$ cells in the spleen showed downregulation of BST2 as well as Siglec H 48h after transfer. Additionally, cells which downregulated BST2 and Siglec H (SPL, R2 upper panel) upregulated CD11b and strongly upregulated MHC class II, in contrast to pre transfer levels (see Figure 5) resembling the phenotype of recipient cDCs in the spleen (Figure 13). A fraction of cells with a cDC-like phenotype can be found in all analysed tissues (R2 gate in BM, lymph nodes and Peyer's patches). Cells, derived from transferred CCR9$^-$ pDCs, which maintain

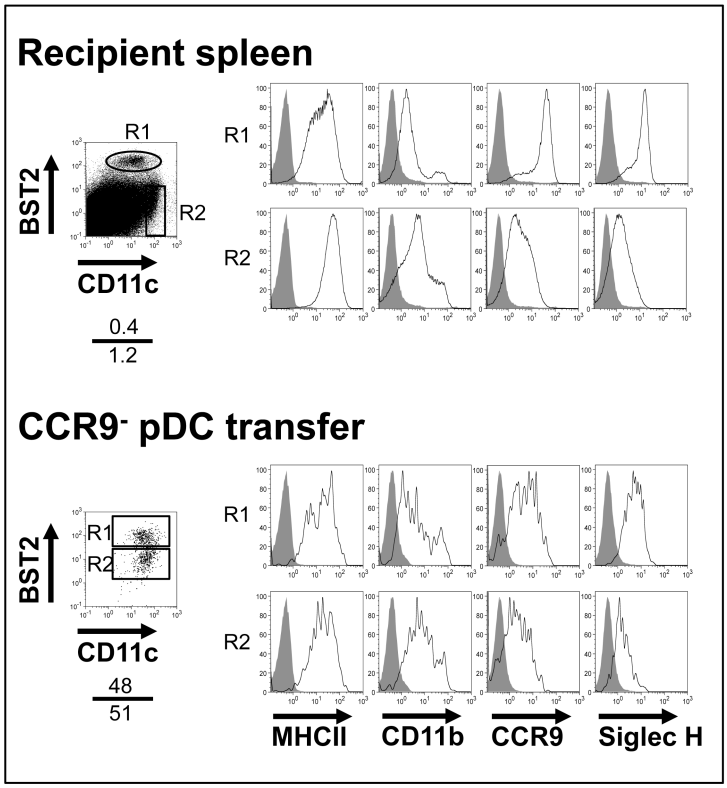

Figure 13 CCR9$^-$ pDC originated pDCs and cDCs share surface characteristics with the endogenous pDC and cDC pool; Comparison between splenic pDC and cDC to adoptively transferred CCR9$^-$ pDC progeny 48h after transfer in the spleen of steady state mice. All blots gated PI$^-$. Histograms show antibody staining (open histograms) against unstained control (filled histograms).

expression of the pDC specific surface markers BST2 and Siglec H (depicted in the R1 gates in Figure 12) develop into mature $CCR9^+$ pDCs characterized by a high expression of CCR9, intermediate MHC class II expression (higher than pre transfer levels) and low expression of the integrin CD11b. These results show that $CCR9^-$ pDCs can develop locally into phenotypically mature $CCR9^+$ pDCs. $CCR9^-$ pDCs are therefore true precursors of $CCR9^+$ pDCs. Also in comparison to the recipient's pool of pDCs and $CCR9^+$ pDCs in the spleen, $CCR9^+$ pDCs originating from $CCR9^-$ pDC precursors share similar expression levels of Siglec H, CD11b and CCR9 with their endogenous counterparts (Figure 13). However, the percentages of cells which deviate from the pDC lineage by downregulation of pDC markers (BST2, Siglec H) and upregulation of CD11b and MHC class II are much higher in secondary lymphoid organs than in BM. Where this effect seems to take place only to a very limited extend. These data indicates that $CCR9^-$ pDCs retain plasticity to divert towards the cDC lineage *in vivo* in the absence of immune stimulation.

4.2.9 CX3CR1 is not required for CCR9$^+$ or CCR9$^-$ pDC recruitment to the BM or peripheral lymphoid organs upon adoptive transfer

As CX3CR1 was indicated as a critical factor for monocyte homing and is also critical for localization and function of DCs in the gut and the lung, we tested if deficiency in CX3CR1 affects the distribution of transferred CCR9$^+$ or CCR9$^-$ pDCs in BM, spleen, lymph nodes or Peyer's patches (Auffray *et al.*, 2007; Jakubzick *et al.*, 2008; Niess *et al.*, 2005). We therefore transferred 5 x 10^5 CX3CR1 deficient CCR9$^+$ or CCR9$^-$ pDCs into steady state WT recipients and compared the recovery in the aforementioned organs with that of WT cells 48h after transfer. As shown in Figure 14 A, adoptively transferred CX3CR1-deficient CCR9$^+$ pDCs could be recovered to a comparable extend as WT CCR9$^+$ pDCs (Same WT data shown as in Figure 10). Furthermore only small reductions in recovery of CCR9$^+$ pDCs lacking CX3CR1 could be observed in spleen and lymph nodes, which did not reach statistical significance. Recovery in Peyer's patches was not changed by the lack of CX3CR1 in CCR9$^+$ pDCs. Similar to what was seen in CX3CR1 deficient CCR9$^+$ pDCs no differences in BM recovery could be observed for CX3CR1$^{-/-}$ compared to WT CCR9$^-$ pDCs 48h after transfer. Also no statistically significant differences could be discovered in the appearance of CX3CR1$^{-/-}$ CCR9$^-$ pDCs in spleen, lymph node and Peyer's patches. Additionally we monitored expression of BST2 on transferred CCR9$^-$ and CCR9$^+$ pDCs 48h after transfer. However BST2 downregulation was not affected by CX3CR1 deficiency (data not shown). We therefore conclude that CX3CR1 is not required for the recruitment and maintenance of CCR9$^+$ and CCR9$^-$ pDCs in BM, spleen, lymph nodes or Peyer's patches.

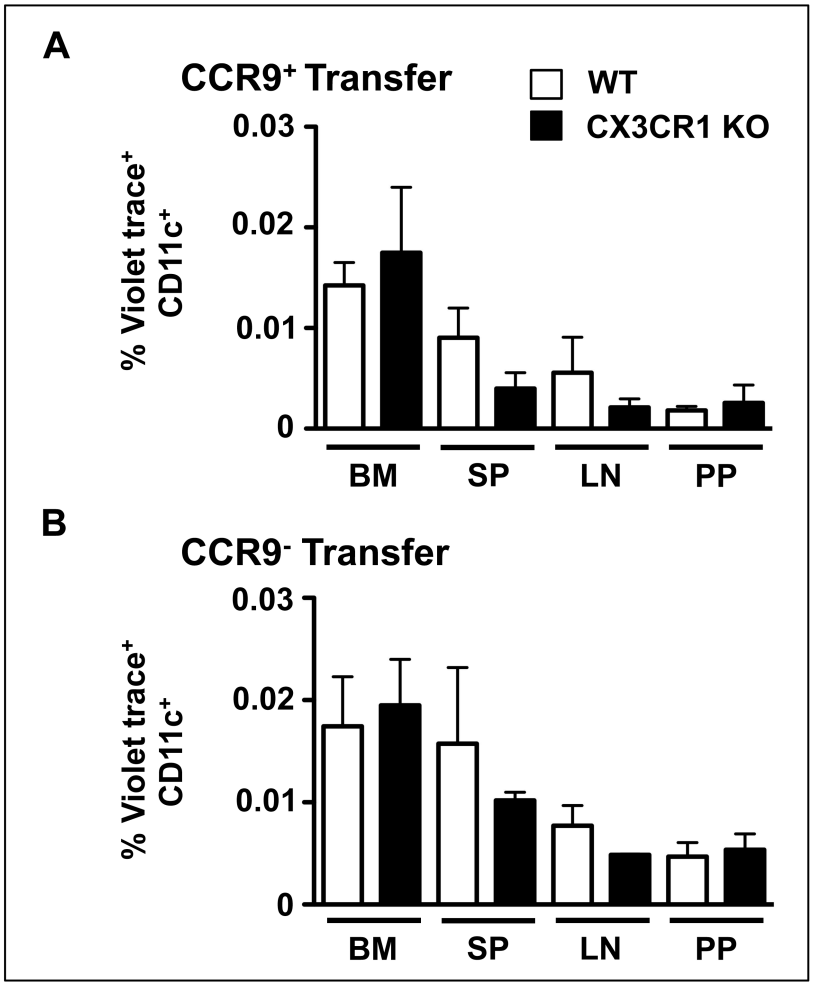

Figure 14 Recovery of transferred CCR9$^+$ and CCR9$^-$ WT or CX3CR1 KO pDCs 48h after adoptive transfer to WT recipients in BM, SPL, LN and PP; WT or CX3CR1 KO CCR9$^+$ or CCR9$^-$ pDCs were FACS sorted from the BM of day 7 FLT3L treated animals and transferred i.v. into steady state hosts. **(A)** FACS analysis of adoptively transferred CCR9$^+$ pDCs 48h after transfer. Recovery of transferred cells gated PI$^-$. **(B)** FACS analysis of adoptively transferred CCR9$^-$ pDCs 48h after transfer. Recovery of transferred cells gated PI$^-$. Pooled results of 2 independent experiments are shown (n=4, mean ± SD).

4.2.10 A role for GM-CSF in the tissue specific migration of CCR9$^-$ and CCR9$^+$ pDCs

Blocking GM-CSF prevented the differentiation of CCR9$^-$ pDCs upon conditioning with IEC-SN *in vitro* (Schlitzer *et al.*, 2011). We therefore set out to study the role of GM-CSF receptor signalling on the fate of transferred CCR9$^-$ or CCR9$^+$ pDCs *in vivo*. We therefore isolated pDCs from the BM of mice deficient in the common β chain of the GM-CSF receptor (CSF2rβ), which is required for GM-CSF, IL-3 and IL-5 signalling. CCR9$^-$ and CCR9$^+$ pDCs were sorted from these mice 7 days after implantation of FLT3L-secreting melanoma. We then transferred 5 x 10^5 CSF2rβ KO CCR9$^-$ or CCR9$^+$ pDCs into WT recipients and analysed the recovery in BM, spleen, lymph nodes, Peyer's patches, lung, small

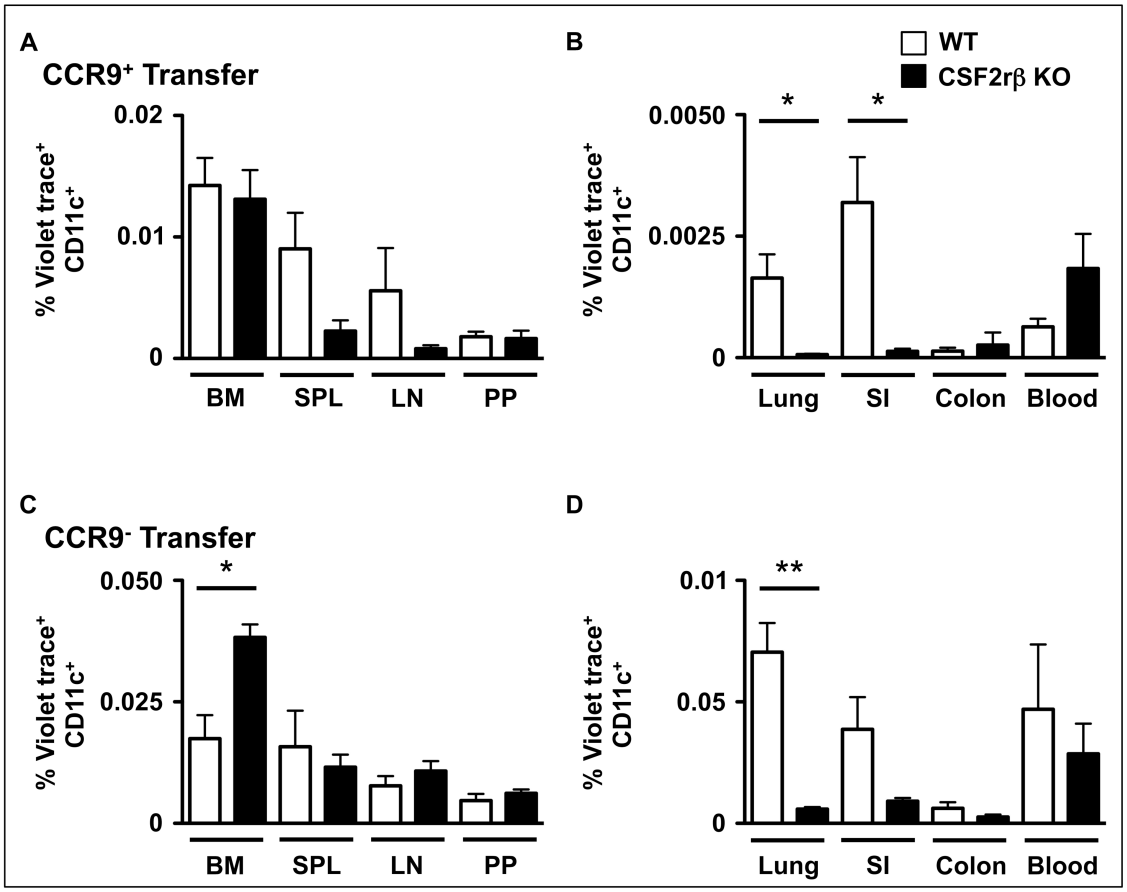

Figure 15 Recovery of WT and CSF2rβ deficient CCR9$^+$ and CCR9$^-$ pDCs into WT steady state recipients; (A) Recovery of Violet trace$^+$ CD11c$^+$ PI$^-$ cells in BM, SPL, LN and PP 48h after transfer of WT or CSF2rβ deficient CCR9$^+$ pDCs; **(B)** Recovery of Violet trace$^+$ CD11c$^+$ PI$^-$ cells in lung, SI, colon and blood 48h after transfer of WT or CSF2rβ deficient CCR9$^+$ pDCs; **(C)** Recovery of Violet trace$^+$ CD11c$^+$ PI$^-$ cells in BM, SPL, LN and PP 48h after transfer of WT or CSF2rβ-deficient CCR9$^-$ pDCs; **(D)** Recovery of Violet trace$^+$ CD11c$^+$ PI$^-$ cells in lung, SI, colon and blood 48h after transfer of WT or CSF2rβ-deficient CCR9$^-$ pDCs; pooled results of 2 independent experiments are shown; Asterisks indicate statistical significance; *p<0.05, **p<0.01, Student's t-test; (n=4, mean ± SD).

intestine, colon and blood 48h later in comparison to WT cells (WT data taken from experiments shown in Figure 10) CSF2rβ deficient $CCR9^+$ pDCs could be recovered from the BM to a similar degree as their WT counterparts. However in spleen and lymph node fewer but not significantly less CSF2rβ deficient $CCR9^+$ pDC were found (Figure 15 A), showing a trend towards a reduced appearance of $CSF2r\beta^{-/-}$ $CCR9^+$ pDCs in peripheral lymphoid organs. Furthermore significantly reduced numbers of $CSF2r\beta^{-/-}$ $CCR9^+$ pDCs were observed in the lung and small intestine in comparison to WT $CCR9^+$ pDCs. Appearance of $CCR9^+$ pDCs in the colon seemed to be independent of CSF2rβ. Additionally CSF2rβ deficient $CCR9^+$ pDCs showed a trend to enrich in the blood compared to WT $CCR9^+$ pDCs, however this effect did not reach statistical significance (Figure 15 B). In contrast to CSF2rβ deficient $CCR9^+$ pDCs which appeared in similar numbers in the BM 48h after transfer, CSF2rβ deficient $CCR9^-$ pDCs enriched significantly in the BM of WT steady state recipients 48h after transfer. Also CSF2rβ deficient $CCR9^-$ pDCs showed no reduction in recovery in peripheral lymphoid organs (Figure 15 C). However in lung and small intestine a reduction in recovery of $CSF2r\beta^{-/-}$ $CCR9^-$ pDCs could be observed similar to what was found in $CSF2r\beta^{-/-}$ $CCR9^+$ pDCs (Figure 15 D). These results suggest that CSF2rβ plays a role for the accumulation of $CCR9^+$ pDCs in peripheral lymphoid organs and is required for $CCR9^+$ pDCs to accumulate in the lung and the small intestine after transfer. $CCR9^-$ pDCs however seem to maintain their ability to accumulate in lymphoid organs in the absence of signalling through the CSF2rβ but also seem to be critically dependent on CSF2rβ for accumulating in mucosal tissues, such as the lung and the small intestine.

4.2.11 CSF2rβ signalling controls proliferation of CCR9$^-$ and CCR9$^+$ pDCs

Reduced recovery of transferred cells from the organs could be due to homing defects, reduced survival or reduced proliferation of the cells in the respective organs. It is know that GM-CSF is able to actively inhibit FLT3L induced gene programs *in vivo* and *in vitro* (Esashi *et al.*, 2008; Gilliet *et al.*, 2002). We

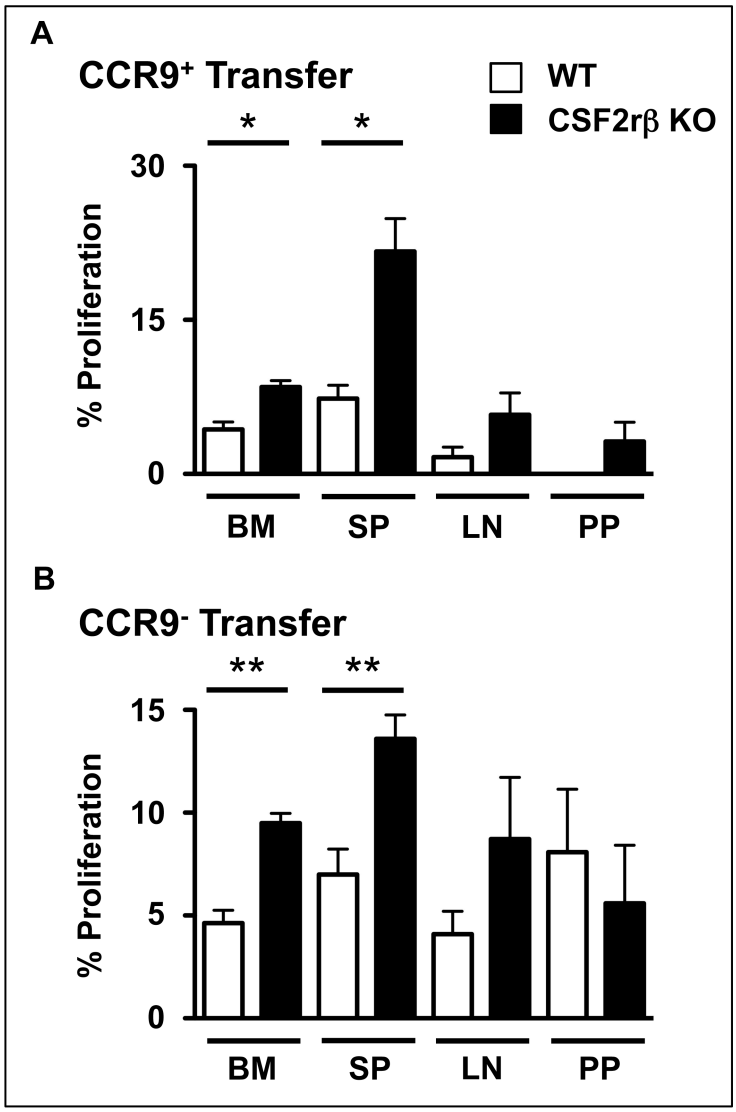

Figure 16 Proliferation of WT or CSF2rβ deficient CCR9$^-$ or CCR9$^+$ pDCs in BM, SP, LN and PP 48h after adoptive transfer; (A) Proliferation of CD11c$^+$ Violet trace$^+$ cells in WT or CSF2rβ deficient CCR9$^+$ pDC transferred WT recipients. **(B)** Proliferation of CD11c$^+$ Violet trace$^+$ cells in WT or CSF2rβ deficient CCR9$^-$ pDC transferred WT recipients; Proliferation was determined by dilution of Violet trace. Expression of BST2, CD11c and Violet trace was monitored by flow cytometry 48h after adoptive transfer in the indicated organs. Pooled results of 2 independent experiments are shown. Asterisks indicate statistical significance; *p<0.05, **p<0.01, Student's t-test (n=4, mean ± SD).

therefore investigated the effect of GM-CSF on pDC expansion and proliferation. Proliferation was measured by Violet trace dye dilution 48h after transfer of $CCR9^-$ and $CCR9^+$ pDCs from WT or $CSF2r\beta^{-/-}$ animals into WT steady state recipients. As shown in Figure 16 A $CSF2r\beta^{-/-}$ $CCR9^+$ pDCs showed significantly enhanced proliferation in BM and spleen 48h after transfer (BM: CSF2rβ KO: 8.5 % ± 1.2 % compared to WT: 4.3 % ± 1.7 % $p< 0.05$; spleen: CSF2rβ KO: 21.7 % ± 6.4 % compared to WT: 7.3 % ± 3.1 % $p< 0.05$; mean ± SD) and also showed a trend towards more proliferation in LN and PP of the same animals. Additionally, $CSF2r\beta^{-/-}$ deficient $CCR9^-$ pDCs also proliferate more than WT $CCR9^-$ pDCs (Figure 16 B). In the BM up to 9.5 % ± 0.95 % of CSF2rβ deficient $CCR9^-$ pDCs showed dilution of Violet trace compared to 4.6 % ± 1.4 % WT cells ($p< 0.01$; mean ± SD). Also in the spleen of these mice 13.6 % ± 2.7 % of CSF2rβ deficient $CCR9^-$ pDCs proliferated 48h after transfer compared to 7 % ± 2.8 % of WT cells ($p< 0.01$; mean ± SD). In conclusion these results show that CSF2rβ controls proliferation of $CCR9^+$ and $CCR9^-$ pDCs in the BM and in the spleen. Enhanced proliferation of CSF2rβ-deficient $CCR9^-$ pDCs may therefore contribute to their accumulation in the BM (Figure 15 C). In contrast, in the spleen increased proliferation of CSF2rβ-deficient $CCR9^-$ and $CCR9^+$ pDCs did not compensate for defects in migration and/or survival of the cells leading to similar or even reduced recovery of transferred cells in the spleen.

4.2.12 Lack of CSF2rβ on CCR9$^-$ or CCR9$^+$ pDCs does not impair the plasticity of CCR9$^-$ pDCs

We hypothesized that GM-CSF would be required for CCR9$^-$ pDC to deviate from the pDC lineage, as our *in vitro* experiments showed that antibody mediated blocking of GM-CSF is sufficient to inhibit differentiation of CCR9$^-$ pDCs towards a cDC-like cell type after exposure to epithelial cells derived factors. To prove this concept *in vivo* we measured expression of CD11c and BST2 on cells derived from CCR9$^-$ and CCR9$^+$ pDCs (WT vs. CSF2rβ KO) 48h after transfer in BM, spleen, lymph node and Peyer's patches (Same experiments as shown in Figure 15, Figure 16 and Figure 10). As shown in Figure 17 A WT or CSF2rβ CCR9$^+$ pDCs showed no or only minor downregulation of pDC markers such as BST2 in the BM (95 % ± 2.24 % BST2$^+$ cells; mean ± SD), spleen (73.5 % ± 11.3 % BST2$^+$ cells; mean ± SD), lymph nodes (83.3 % ± 12.2 % BST2$^+$ cells; mean ± SD) and Peyer's patches (93 % ± 12 % BST2$^+$ cells; mean ± SD) (Figure 17 B). Interestingly CSF2rβ deficient CCR9$^-$ pDCs, did not loose their ability to deviate from the pDC lineage, despite lack of GM-CSF signalling (Figure 17 A and C). 48h after transfer of CSF2rβ deficient CCR9$^-$ pDCs downregulation of pDC specific markers, such as BST2 was comparable to WT CCR9$^-$ pDCs in spleen (49.1 % ± 4.8 % BST2$^+$ cells; mean ± SD), lymph nodes (54.7 % ± 7.3 % BST2$^+$ cells; mean ± SD) and Peyer's patches (51.9 % ± 12.6 % BST2$^+$ cells; mean ± SD). Tissue specific downregulation of BST2 was maintained in CSF2rβ deficient CCR9$^-$ pDC, as in the BM only a minor downregulation of BST2 could be observed (88 % ± 1.4 % BST2$^+$ cells; mean ± SD, Figure 17 A). Downregulation of BST2 was paralleled by downregulation of Siglec H and upregulation of CD11b and MHC class II, indicating a cDC-like phenotype as described before. These results indicate that in contrast to the results obtained *in vitro*, activity of constitutively produced GM-CSF (*via* the CSF2rβ) is not critical for mediating pDC lineage diversion of CCR9$^-$ pDCs to cDC-like cells *in vivo*. Therefore it is likely that other factors are involved and can substitute for GM-CSF *in vivo*.

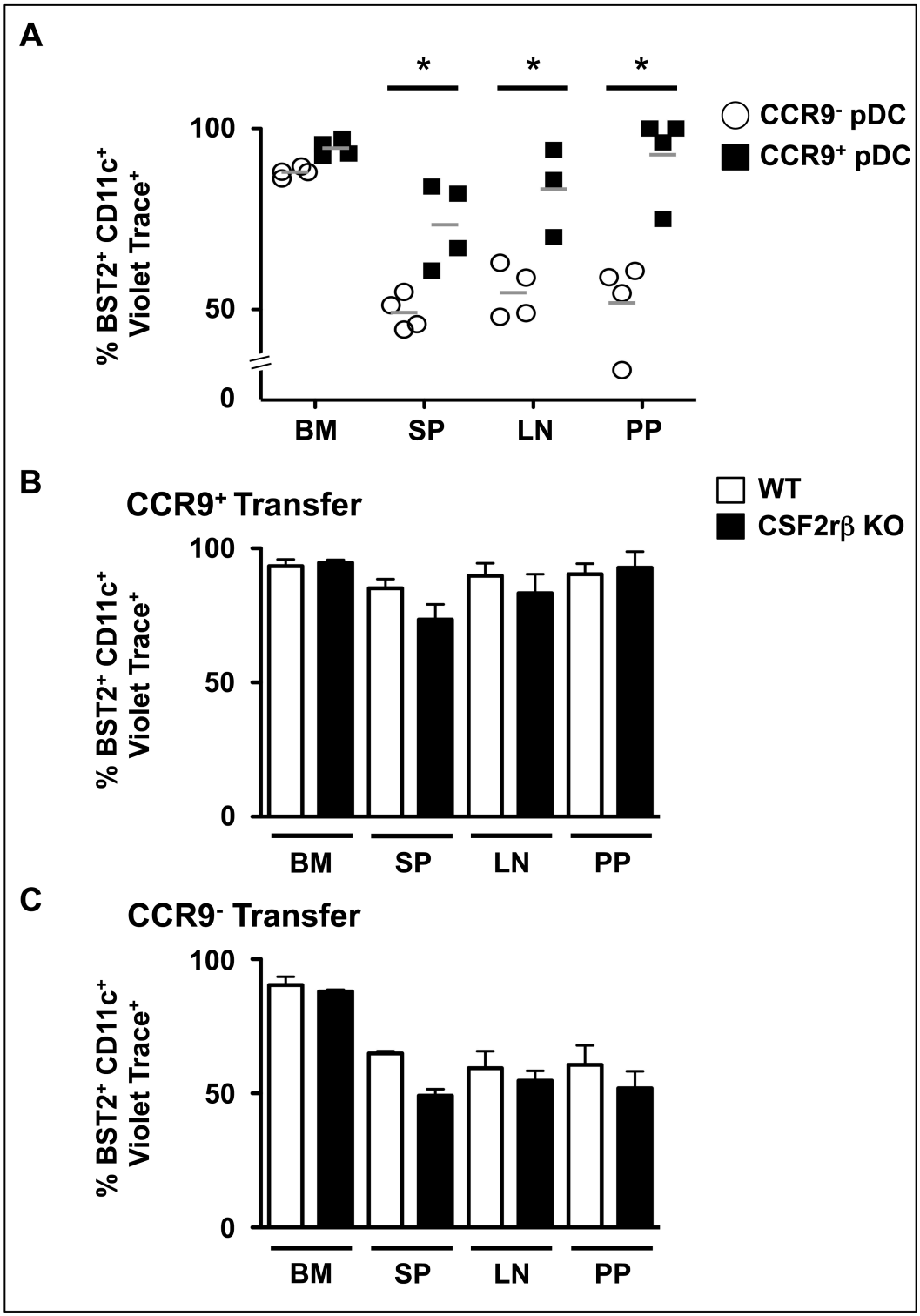

Figure 17 Percentage of BST2$^+$ CD11c$^+$ Violet trace$^+$ PI$^-$ cells in BM, SPL, LN and PP 48h after adoptive transfer of CSF2rβ KO CCR9$^-$ or CCR9$^+$ pDCs; (A) Transfer of CSF2rβ deficient CCR9$^-$ or CCR9$^+$ pDCs into steady state WT recipients; Expression of BST2, CD11c and Violet trace was monitored by flow cytometry 48h after adoptive transfer in the indicated organs. Pooled results of 2 independent experiments are shown (n=4) **(B)** Transfer of CCR9$^+$ WT and CSF2rβ deficient pDCs into steady state WT recipients; Expression of BST2, CD11c and Violet trace was monitored by flow cytometry 48h after adoptive transfer in the indicated organs. Pooled results of 2 independent experiments are shown (n=4, mean ± SD) **(C)** Transfer of CCR9$^-$ WT and CSF2rβ deficient pDCs into steady state WT recipients; Expression of BST2, CD11c and Violet trace was monitored by flow cytometry 48h after adoptive transfer in the indicated organs. Pooled results of 2 independent experiments are shown (n=4, mean ± SD). Asterisks indicate statistical significance; *p<0.05, **p<0.01, Student's t-test.

5. Discussion

In this study we identify two distinct subpopulations in murine BM pDCs. These two populations, which are both highly positive for the pDC-specific surface molecules BST2 and Siglec H, differ in their expression of CCR9 and MHC class II but also have different developmental and functional properties *in vitro* and *in vivo*.

$CCR9^+$ pDC, which are terminally differentiated pDCs, similar to the majority of pDCs found in secondary lymphoid organs secrete IFN-α and low amounts of proinflammatory cytokines after stimulation with a TLR9 ligand. $CCR9^-$ pDCs secrete even higher amounts of IFN-α and higher amounts of IL-6 and IL-12. $CCR9^-$ pDCs express lower levels of costimulatory molecules and MHC class II compared to $CCR9^+$ pDCs. On the level of transcription factors both, $CCR9^-$ and $CCR9^+$ pDCs from the BM, express critical pDC related genes such as E2-2 and Spi-b, although $CCR9^-$ pDCs express these at lower levels. $CCR9^+$ pDCs are stable in their surface phenotype *in vitro* or after adoptive transfer *in vivo*. In contrast to that a subpopulation of $CCR9^-$ pDCs downregulated pDC markers and upregulated CD11b and MHC class II thus acquiring a cDC-like phenotype. The remaining $CCR9^-$ pDCs developed, spontaneously *in vitro* or after adoptive transfer, into fully differentiated $CCR9^+$ pDCs, showing a developmental relationship between these two subsets. Additionally $CCR9^-$ pDCs are present in every lymphoid and non lymphoid organ and can develop independently of the BM environment into mature $CCR9^+$ pDCs at these sites. Generation of terminally differentiated $CCR9^+$ pDCs was regulated in a tissue specific manner, being more pronounced in the BM and liver than in spleen, lymph nodes, lung or small intestine.

As a critical factor for the appearance of $CCR9^-$ pDCs and $CCR9^+$ pDCs in lung and small intestine, CSF2rβ was identified. Furthermore CSF2rβ controls local proliferation of $CCR9^+$ and $CCR9^-$ pDCs in BM and spleen, showing two unexpected roles for CSF2rβ in the control of pDC distribution and development.

5.1 CCR9$^-$ pDCs are part of the pDC lineage

In this study we show that CCR9$^-$ pDCs and CCR9$^+$ pDCs have a broadly overlapping phenotype despite the difference in CCR9 expression and the lower expression of MHC II, CD80 and CD86 on CCR9$^-$ pDCs. Furthermore higher expression of CX3CR1 and lower expression of CD4 and CD8 was detected on CCR9$^-$ pDCs. This shows that phenotypically CCR9$^-$ pDCs and CCR9$^+$ pDCs both appear to belong to the pDC lineage. Also functionally both subsets fulfill the characteristics of pDCs. On the level of transcription factors both subsets harbour a pDC development program shown by the high expression of the essential pDC transcription factor E2-2 as well as IRF8 and Spi-b. However one major difference between CCR9$^-$ pDCs and CCR9$^+$ pDCs remains. CCR9$^-$ pDCs do not harbour a rearrangement in their *Ig* genes as CCR9$^+$ pDCs do. This corresponds to a GFP$^-$ population of pDCs identified in RAG/GFP reporter animals, which have a higher capacity to produce IFN-α (Pelayo *et al.*, 2005).

Furthermore CCR9 expression on pDCs isolated from the BM seemed not to be a requirement to enter the intestine and other mucosal associated tissue, such as the lung or Peyer's patches upon adoptive transfer. Additionally in contrast to the published literature (Wendland *et al.*, 2007), CCR9$^-$ pDCs were more efficient in homing to the Peyer's patches and the lung and not significantly impaired in homing to the small intestine, hinting on the involvement of a different chemokine receptor for pDC homing to these peripheral tissue sites.

Furthermore CCR9$^-$ pDCs can be found in all organs of the body, irrespective if lymphoid or non-lymphoid. This suggests that pDC development can also occur locally in the organs and is therefore able to adapt to the microenvironment. In this study treatment with FLT3L was used to expand the DC compartment in steady state mice, this model is widely used and has the advantage of expanding all DC and DC progenitor subsets allowing isolation of sufficient quantities of rare cell populations such as pDCs (Mach *et al.*, 2000; Miller *et al.*, 2003). We compared the surface phenotypes of pDCs in untreated and FLT3L treated mice and did not observe differences in activation or maturation associated markers on pDCs. Also CCR9$^-$ and CCR9$^+$ pDCs are expanded

equally, showing that this model is suitable to expand pDCs for the use in adoptive transfer experiments.

5.2 Plasticity of CCR9$^-$ pDCs

CCR9$^+$ pDCs can be found in lymphoid organs as well as the lung, small intestine and liver 48h after adoptive transfer, however the phenotype of transferred cells is stable. CCR9$^-$ pDCs however, which can be found in lymphoid organs, lung, small intestine, colon and the liver show a less stable more plastic phenotype. Upon adoptive transfer a subpopulation of the CCR9$^-$ pDC-like cells have the potential to generate both pDCs - upregulating CCR9, MHCII, CD80 and CD86 – and cDCs downregulating pDC markers and upregulating CD11b and MHCII. They therefore resemble other progenitor and precursor populations functionally but not phenotypically.

Pro-DCs or common DC progenitors (CDP, Lin$^-$ CD117^{int} CD115$^+$ MHC II$^-$) were previously described to be able to generate pDCs and cDCs under steady state conditions *in vitro* (FLT3L BM cultures) (Naik *et al.*, 2007; Onai *et al.*, 2007). Additionally the macrophage dendritic cell progenitor (MDP, CD135$^+$ CD115$^+$ CX3CR1$^+$) is thought to contribute towards the DC and the macrophage lineage (Auffray *et al.*, 2009). Neither the MDP nor CDP express lineage markers such as B220 and lack CD11c expression, thereby being excluded from the CCR9$^-$ pDC population (BST2$^+$ Siglec H$^+$ B220$^+$ CD11c$^+$). Furthermore Pro-DCs/CDPs and MDPs retain the potential to proliferate extensively *in vivo*. Proliferation was only observed for ~ 5 % of the CCR9$^-$ pDC population under steady state conditions, making it unlikely that one of the aforementioned progenitor populations contributed to the cDC lineage differentiation of 30 - 40 % of CCR9$^-$ pDCs. Careful phenotypic analysis also excluded the possibility that precursors of cDCs identified by Segura *et al.* contaminated the CCR9$^-$ pDC population causing this shift in phenotype. B220$^+$ CD11c$^+$ BST2$^-$ CCR9$^-$ cells from the spleen were indicated in a recent report to give rise to a population of cDCs in the spleen under steady state conditions (Segura *et al.*, 2009). However we were not able to detect such a population within the CCR9$^-$ pDCs, because CCR9$^-$ pDCs show high expression of BST2. Additionally, contamination with

pre-cDCs was a possibility. Pre-cDC (Lin^- $CD11c^+$ $BST2^{low}$ $MHCII^-$ $CD135^+$), described by Liu *et al.*, are local precursors of cDC subsets in their various target organs, but are generated in the BM. A contamination by pre-cDCs could therefore contribute to the cDC potential of our $CCR9^-$ pDC population (Liu *et al.*, 2007). However pre-cDCs are devoid of Siglec H expression and show only very low BST2 and B220 expression. Thus pre-cDCs are excluded by the sorting strategy (Figure 4, $BST2^+$ Siglec H^+ $CD11c^{int}$) used in the present study. Additionally we also obtained gene expression profiles from $CCR9^-$ and $CCR9^+$ pDCs sorted from the BM by microarray and compared these to CDP gene expression data from the Immgen database. This analysis showed that genes, which are upregulated in CDPs, are also upregulated in $CCR9^-$ pDCs but not in $CCR9^+$ pDCs, providing further evidence for a progenitor cell gene expression signature (data not shown).

Therefore $CCR9^-$ pDCs are not only immediate tissue resident precursors of fully differentiated $CCR9^+$ pDCs but are also able to deviate from the pDC lineage under tissue specific environmental conditions. This study identifies a novel DC precursor that can contribute to both the pDC as well as the cDC pool.

The identity of the cDC-like cells generated by $CCR9^-$ pDCs is still unclear. These cells resemble cDCs in phenotype and function but still maintain some pDC-like features, such as expression of BST2 and Siglec H at low levels, at least 48h after transfer. Recently, Jung *et al.* discovered a similar cDC-like population in the spleen of mice (Bar-On *et al.*, 2010). They identified a population similar to our cDC-like cells. pDC related cDCs identified by Bar-On *et al.* are critically dependent on E2-2, which relates them closely to the pDC lineage, similar to the cDC-like cells which we observed. However certain differences between the two populations remain. pDC related cDCs from the paper by Bar-On *et al.* express high levels of $CD8\alpha$ and CX3CR1 but fail to cross prime T cells and lack production of IL-12p70. In contrast to that cDC-like cells originating from $CCR9^-$ pDC precursors produce high amounts of IL-12 and are able to cross prime T cells upon *in vitro* co-cultivation, although they are less efficient than $CD8\alpha^+$ splenic cDCs (Bar-On *et al.*, 2010). However, expression levels of CX3CR1 were very similar between the two cell

populations. Also CCR9$^-$ pDCs, which are the precursors of cDC-like cells, show no rearrangement in the *Ig* gene locus, in contrast to pDC related cDCs from the paper by Bar-On *et al.*, making it unlikely that these cells arise from the same precursor.

cDC-like cells, which are generated from CCR9$^-$ pDCs and deviate from the pDC lineage, not only change their phenotype, but also activate a different gene expression program *in vitro* similar to what is found in splenic cDCs. This cDC-like gene expression program was marked by a very low expression of the transcription factors IRF8 and E2-2 and upregulation of BATF3 and notably ID2. ID2 counteracts E2-2 - the essential pDC transcription factor - by forming E2-2/ID2 heterodimers and thereby inhibiting E2-2 functionality (Ghosh *et al.*, 2010; Hacker *et al.*, 2003). This might be one explanation for the acquisition of their cDC-like gene expression profile and surface phenotype. However, it is not entirely clear which cDC subtype cDC-like cells generated from CCR9$^-$ pDCs *in vitro* as well as *in vivo* resemble exactly. Low IRF8 expression is a hallmark of CD11b$^+$ CD8α^- DCs in the spleen and peripheral tissues (Ginhoux *et al.*, 2009); therefore gene expression observed in cDC-like cells would fit this cDC subtype's gene expression profile. cDC-like cells however cross-presented soluble antigen more efficiently than CD11b$^+$ CD8α^- splenic DCs but less efficiently than CD11b$^-$ CD8α^+ splenic DCs *in vitro*. One explanation for this discrepancy could be that cDC-like cells generated, after 48h of incubation with IEC-SN or GM-CSF or post transfer from CCR9$^-$ pDC precursors are not yet fully developed and therefore assume an intermediate state between CD8α^- and CD8α^+ cDCs.

Additionally it is not understood whether CCR9$^-$ pDCs directly develop into cDC-like cells or if there is a pre-cDC intermediate, which then develops into the various types of cDCs in peripheral tissues. This would make the CCR9$^-$ pDC an additional source for pre-cDCs in peripheral tissues.

In vitro lineage diversion of CCR9$^-$ pDCs to cDC-like cells was critically dependent on the availability of GM-CSF, either recombinant or its presence in

the IEC-SN. It was recently observed that development of certain subpopulations of cDCs ($CD11b^+$ $CD103^+$ cDCs) especially in the lamina propria of the small intestine is critically dependent on GM-CSF (Bogunovic *et al.*, 2009). Also GM-CSF is produced under steady state conditions at mucosal surfaces such as the lung and the intestine but also in the skin (Reed and Whitsett, 1998; Yokota *et al.*, 2009). Furthermore GM-CSF can be induced at higher levels during inflammation such as colitis and *citrobacter rodentium* infection (Heiseke *et al.*, 2011; Hirata *et al.*, 2010). Therefore exposure of $CCR9^-$ pDCs to GM-CSF in peripheral non-lymphoid tissues is likely to occur in the resting state and even more so during inflammation. Additionally increased expression of PU.1 in cDC-like cells originating from $CCR9^-$ pDCs was detected. PU.1 is the major regulator of the expression of FLT3 and GM-CSF receptor further demonstrating a role for GM-CSF signalling in DC lineage commitment (Carotta *et al.*, 2010). The role of GM-CSF was therefore tested *in vivo*. A knockout mouse model was used, which is deficient in the common IL-3, IL-5, and GM-CSF receptor chain (CSF2rβ). This model is deficient in the signalling of IL-5 and GM-CSF receptor but still able to signal through the IL-3 receptor *via* an alternative β chain (Nishinakamura *et al.*, 1995). After transfer of CSF2rβ deficient $CCR9^-$ pDCs or $CCR9^+$ pDCs into WT recipients unexpected findings were obtained. Deficiency in CSF2rβ did not impair the ability of $CCR9^-$ pDCs to differentiate to $CCR9^+$ fully differentiated pDCs. Also formation of the cDC-like cells was not inhibited, as it would have been expected according to the *in vitro* data. One explanation for this might be that IL-3 substitutes for GM-CSF *in vivo* as the signalling of this closely related receptor is still intact. Furthermore in human DC development IL-3 and GM-CSF have similar roles in the establishment of lineage identity in *in vitro* culturing systems (Caux *et al.*, 1996).

Also tissue specific downregulation of pDC markers and establishment of a cDC-like cell population from $CCR9^-$ pDCs was observed. $CCR9^-$ pDCs show both pDC and cDC potential but it depends on the tissue whether pDCs or cDCs are generated during these final differentiation steps. Cells generated from $CCR9^-$ pDCs in BM and liver showed only minor downregulation of BST2,

whereas strong downregulation was observed in secondary lymphoid organs, lung and small intestine. It is not clear which factors contribute to this effect, however M-CSF was indicated to play a major role in the final differentiation of pDCs in the BM, therefore this might play a role in favouring generation of $CCR9^+$ pDCs from $CCR9^-$ pDCs (Fancke *et al.*, 2008). Availability of FLT3L may also play a major role in these final differentiation steps (Waskow *et al.*, 2008).

In the BM, stromal cells exist which are able to build specific cell niches to support the differentiation process of certain cell types, such as plasma cells or hematopoietic stem cells (Despars *et al.*, 2007; Tokoyoda *et al.*, 2009). Therefore it is plausible that in BM and liver, hematopoietic and former hematopoietic organs, similar specific niches may exist which support pDC lineage commitment. Another interesting observation is that the lung and the small intestine are sites of microbial colonization and that this influences composition and function of the DC pool (Ng *et al.*, 2010; Niess and Adler, 2010), by altering the tissue microenvironment. Therefore these factors may have an impact on the lineage commitment of $CCR9^-$ pDCs in lung and small intestine but less in BM and liver.

5.3 GM-CSF, a novel factor involved in pDC homing and proliferation

$CCR9^-$ pDCs as well as $CCR9^+$ pDCs express the specific CSF2rα chain of the GM-CSF receptor at a similar level and GM-CSF signalling was crucial for deviation of $CCR9^-$ pDCs from the pDC lineage. Despite seeing no effect on the generation of cDC-like cells from CSF2rβ receptor deficient $CCR9^-$ pDCs we could see a dramatic effect on the homing capacities of $CCR9^-$ pDCs as well as $CCR9^+$ pDCs. $CCR9^-$ pDCs as well as $CCR9^+$ pDC were not able to accumulate in the lung as well as in the small intestine. Furthermore $CCR9^-$ pDCs accumulated significantly in the BM. This can be due to maintenance, migration/recruitment or proliferation of pDC subsets. Recent reports showed that GM-CSF is necessary for the development and maintenance of several DC populations in the intestine as well as in the lung, including the $CD103^+$ DCs and the $CD11b^+$ $CD103^+$ DCs (Ginhoux *et al.*, 2009). Therefore, it is possible

that both pDC subsets need the GM-CSF signal to be viable in small intestine and lung.

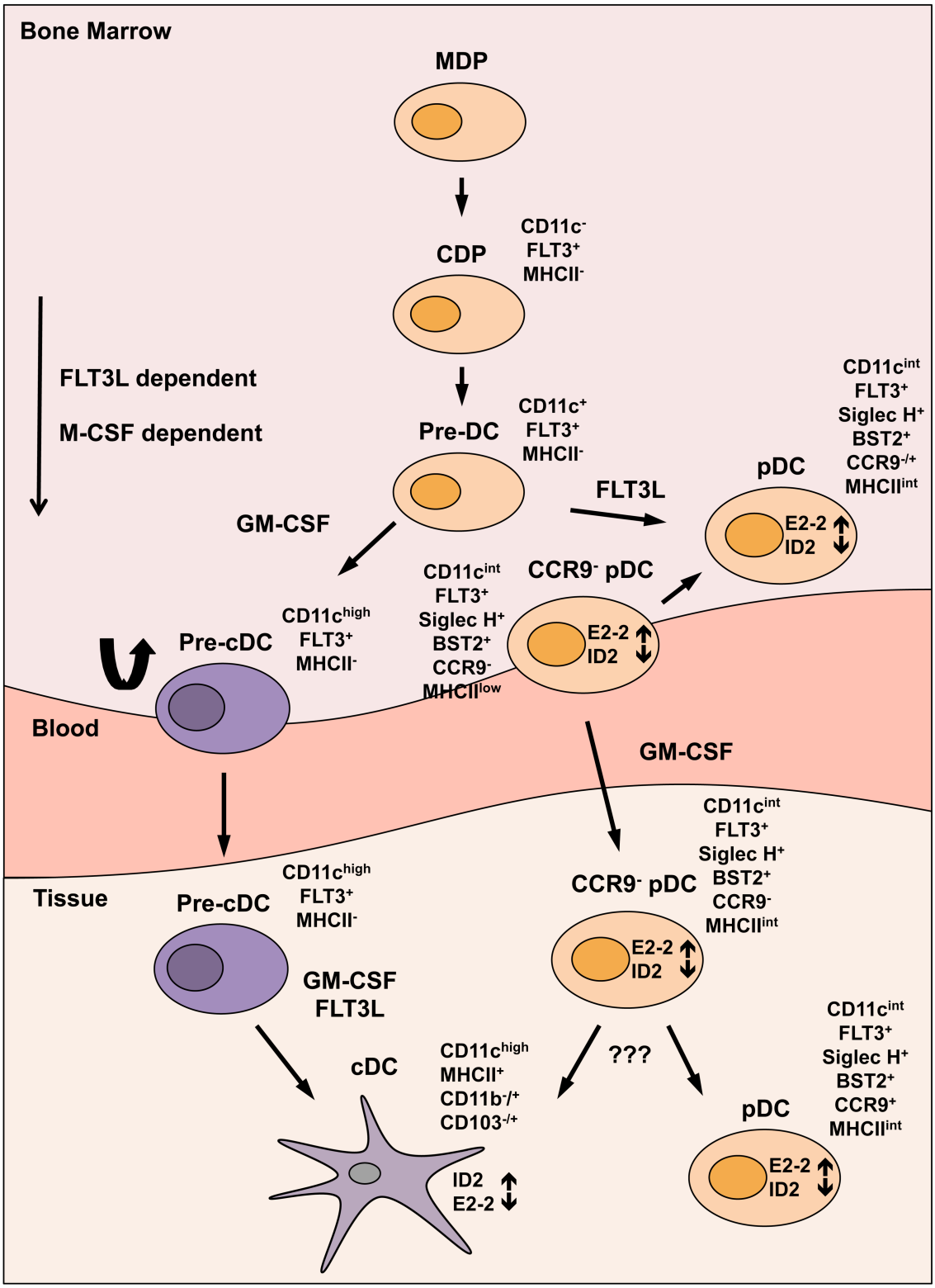

Figure 18 New model for DC development in the BM as well as in peripheral lymphoid and non-lymphoid organs.

Also it has been shown that GM-CSF is able to act like a chemokine. It was reported that GM-CSF is able to mobilize neutrophils from the microvasculature in a GM-CSF receptor β chain and PI3 Kinase dependent manner (Khajah *et al.*, 2011). Furthermore there is evidence from a rat experimental model that GM-CSF also controls myocardial invasion of macrophages upon cardiac infarction (Kellar *et al.*, 2011). Taken together this evidence also points to the role of GM-CSF as a critical factor for pDC migration and entrance to mucosal surfaces.

We also observed an additional effect of the loss of GM-CSF receptor signalling in $CCR9^-$ pDCs as well as $CCR9^+$ pDCs. Transferred $CCR9^-$ pDCs as well as $CCR9^+$ pDCs lacking the GM-CSF receptor show a substantial increase in proliferation in BM and spleen compared to WT cells. Several different reports have shown that GM-CSF is able to actively suppress pDC development and expansion in the BM as well as in *in vitro* cultures (Carotta *et al.*, 2010; Cisse *et al.*, 2008; Esashi *et al.*, 2008; Ghosh *et al.*, 2010). Thereby it is possible that in the absence of GM-CSF receptor signals, FLT3L exposure in the BM and in the spleen leads to a re-induction of the FLT3L mediated pDC expansion.

5.4 The origin of $CCR9^-$ pDCs

An interesting question, which arises from these data is the origin of the $CCR9^-$ pDC. It is thought that mature $CCR9^+$ pDCs arise from the CDP and from a yet unknown cell downstream of the lymphoid precursor (Shigematsu *et al.*, 2004). Yet $CCR9^-$ pDCs do not harbour a rearrangement in their *Ig* genes and therefore most likely originate from a myeloid progenitor e.g. the CDP. However, it is unclear at which step of development *Ig* rearrangement in pDCs occurs and if only cells, which originate from the lymphoid precursor undergo the rearrangement. Therefore this is not a proof for this concept. Furthermore it is not clear whether CDPs can develop into $CCR9^+$ pDCs directly without having to go through a pre-pDC stage such as the $CCR9^-$ pDC. Furthermore the question remains if only pDCs, which develop in the BM originate directly from the CDP or the lymphoid precursor, thereby skipping the pre-pDC stage. pDCs

which develop in the periphery however might originate from a local precursor, such as the CCR9$^-$ pDC to adapt to local tissue conditions.

5.5 A new model for DC development

It was thought that pDC development is exclusively happening in the BM and that pDCs originate directly from the CDP and a yet unknown lymphoid precursor cell, which harbours a *Ig* rearrangement, thereby limiting the capacity of pDCs to adapt to local tissue conditions and microenvironments. Concluding from the data presented here a new model for DC development is proposed (Figure 18): This study shows that terminally differentiated pDCs can arise from tissue resident precursors, such as CCR9$^-$ pDCs, directly in the peripheral organs thus allowing pDCs to adapt to the local microenvironment. Furthermore CCR9$^-$ pDC still retain plasticity to give rise to cDC-like cells, regulated by local tissue specific factors. This demonstrates that CCR9$^-$ pDCs are precursor cells which contribute to the pDC and the cDC pool and can thereby be seen as novel players in DC development, which are able to undergo extensive tissue adaptation due to their presence and development in peripheral lymphoid as well as non lymphoid organs.

6. Summary

The data presented in this thesis identifies a subpopulation of CCR9$^-$ MHC class II^{low} BST2$^+$ Siglec H$^+$ plasmacytoid dendritic cells (pDCs) within the bone marrow (BM) pDC population. CCR9$^-$ MHC class II^{low} BST2$^+$ Siglec H$^+$ pDCs express the essential pDC transcription factor E2-2 and produce high levels of interferon-α (IFN-α) and proinflammatory cytokines upon toll-like receptor 9 (TLR9) stimulation. This phenotypically immature pDC population is an immediate precursor for fully differentiated CCR9$^+$ pDCs *in vitro* as well as *in vivo*, but does not harbour a pDC-specific gene rearrangement in the *Ig* gene locus.

CCR9$^-$ pDCs retain plasticity to downregulate pDC specific surface molecules and upregulate CD11b and MHC class II, acquiring phenotype and function of CD8α^- CD11b$^+$ conventional dendritic cell (cDC) - like cells after conditioning with supernatant derived from colonic epithelial cells or exposure to recombinant granulocyte macrophage colony stimulating factor (GM-CSF) *in vitro*. Functionally cDC-like cells generated from CCR9$^-$ pDCs acquire properties of cDCs such as efficient T cell activation and the production of high levels of proinflammatory cytokines comparable to those of splenic CD8α^- DCs. This phenotypic and functional change is also reflected on the level of transcription factor expression by downregulation of E2-2, Spi-b and IRF8 but upregulation of ID2, PU.1 and BATF3.

CCR9$^-$ pDCs can give rise to fully differentiated CCR9$^+$ pDCs and CD11b$^+$ MHC class II^{high} cDC-like cells locally in the tissue *in vivo* in the steady state. However the plasticity and lineage commitment of CCR9$^-$ pDCs is regulated in a tissue specific manner. In BM and liver CCR9$^-$ pDCs primarily give rise to CCR9$^+$ pDCs, whereas in spleen, lymph nodes, lung and small intestine a substantial fraction deviates from the pDC lineage to the cDC lineage.

Furthermore, this study shows that GM-CSF is necessary for the appearance of CCR9$^-$ pDCs and CCR9$^+$ pDCs in lung and small intestine but is dispensable for the generation of CD11b$^+$ MHC class II^{high} cDCs from CCR9$^-$ pDCs *in vivo*.

Moreover, GM-CSF controls the proliferation of $CCR9^-$ and $CCR9^+$ pDCs in BM and spleen upon adoptive transfer.

In conclusion these results show that $CCR9^-$ pDCs are tissue resident precursors of pDCs and cDCs and that the generation of DC subsets is regulated by tissue derived factors, thereby allowing adaptation to local microenvironments. This increases the flexibility of the DC compartment under circumstances of infection or inflammation.

7. Zusammenfassung

Die vorliegende Dissertation identifiziert eine Subpopulation der plasmazytoiden dendritischen Zellen (pDZ) im Knochenmark (KM). Diese Subpopulation exprimiert die pDZ spezifischen Oberflächenmoleküle BST2 und Siglec H, jedoch nur wenig MHC Klasse II, sowie kein oder nur wenig CCR9. Weiterhin exprimieren $CCR9^-$ $BST2^+$ Siglec H^+ pDZ den essentiellen pDZ Transkriptionsfaktor E2-2 und können außerdem nach Stimulation von TLR9 grosse Mengen an IFN-α und geringere Mengen an inflammatorischen Zytokinen (IL-6 und IL-12) produzieren. $CCR9^-$ pDZ sind phänotypisch unreife pDZ und unmittelbare Vorläufer von voll ausdifferenzierten pDZ sowohl *in vitro* als auch *in vivo*, tragen allerdings keine pDZ spezifischen rearrangierten Immunglobulingene.

Nach Inkubation mit Überstand von intestinalen Epithelzellen oder mit rekombinantem GM-CSF *in vitro* sind $CCR9^-$ pDZ in der Lage, die Expression der pDZ spezifischen Oberflächenmoleküle zu verringern, als auch die Expression von CD11b und MHC Klasse II zu erhöhen, und werden damit konventionellen DZ (kDZ) ähnlich. Auch funktionell zeigen diese Zellen nun Merkmale von kDZ. kDZ-ähnliche Zellen sind in der Lage, T-Zellen effizient zu aktivieren und können ebenso nach TLR9-Stimulation grosse Mengen an inflammatorischen Zytokinen (IL-6 und IL-12) produzieren. Des Weiteren spiegelt sich die Veränderung des Phänotyps und der Funktionalität auch auf Ebene der Genexpression wieder, da E2-2, Spi-b und IRF8 herunterreguliert und ID2, PU.1 und BATF3 hochreguliert werden.

Nach adaptivem Zelltransfer sind $CCR9^-$ pDZ dazu in der Lage, sich sowohl zu voll ausdifferenzierten $CCR9^+$ pDZ als auch zu kDZ zu entwickeln. Diese Plastizität ist gewebsspezifisch. Im KM und in der Leber entwickeln sich $CCR9^-$ pDZ hauptsächlich zu voll ausdifferenzierten pDZ, wohingegen in der Milz, den Lymphknoten sowie der Lunge und dem Dünndarm hauptsächlich eine Entwicklung zu kDZ zu beobachten ist.

Weiterhin zeigt diese Arbeit, dass GM-CSF nach adaptivem Zelltransfer ausschlaggebend dafür ist, dass $CCR9^-$ und $CCR9^+$ pDZ sich in der Lunge und im Dünndarm ansammeln. Darüber hinaus reguliert GM-CSF nach adaptivem Zelltransfer die Proliferation von $CCR9^+$ und $CCR9^-$ pDZ sowohl in der Milz als auch im KM. *In vivo* in Abwesenheit von Immunstimulation ist GM-CSF jedoch nicht der kritische Faktor, der die Generation von kDZ-ähnlichen Zellen aus $CCR9^-$ pDZ bewirkt.

Zusammenfassend zeigen diese Ergebnisse, dass $CCR9^-$ pDZ sowohl in lymphoiden als auch in nicht-lymphoiden Geweben zu finden sind. $CCR9^-$ pDZ können dort in Abhängigkeit von lokalem Milieu entweder vermehrt in pDZ oder kDZ differenzieren und sich so den jeweiligen Bedingungen in den einzelnen Organen anpassen. Dieser Mechanismus erhöht die Flexibilität des DZ-Kompartiments und damit die Adaption der Immunantwort bei Infektion und Entzündung.

8. References

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9. Acknowledgements

First of all I would like to thank PD Dr. med Anne Krug, Dr. med. Wolfgang Reindl and Prof. Dr. Stefan Bauer for supervision, guidance and help during my PhD thesis. In particular I would like to thank Anne Krug for mentoring and for giving me the opportunity to work in a thriving and motivating environment, for supporting the study scientifically and financially and for giving me the possibility to attend international meetings to present my work

Also I am deeply thankful to all members of the lab.
Katharina, Alexander, Jakob and Daniela, thank you for all the good times we shared I will always remember that!

Lynette thank you for spending so much time with me at the sorter. This was a crucial part of my project. Thank you!

I would also like to thank my collaborators, Tobias Suter and Jan Hendrik Niess for providing knockout mice for this project.

Ksenija, Stefan, Martin, Marian and Stefanie it was always a pleasure to talk science with you guys! Thank you very much for that and everything else!

At last I want to thank Ana, you are my one and only. Everything would not have been possible without your help, strength and all the trust you put into me! I am very very thankful for that and
I love you!

10. Verzeichnis der akademischen Lehrer

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Agrawal	Universität Marburg
Bauer	Universität Marburg
Bölker	Universität Marburg
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Brandl	Universität Marburg
Brändle	Universität Marburg
Bremer	Universität Marburg
Buckel	Universität Marburg
Cruickshank	University of Manchester
Else	University of Manchester
Feuser	Universität Marburg
Galland	Universität Marburg
Greiner	Universität Marburg
Grencis	University of Manchester
Hassel	Universität Marburg
Homberg	Universität Marburg
Imhof	Universität Marburg
Kahmann	Universität Marburg
Lingelbach	Universität Marburg
Maier	Universität Marburg
Moore	University of Manchester
Renkawitz-Pohl	Universität Marburg
Zauner	Universität Marburg
Ziegenhagen	Universität Marburg

11. Appendix

11.1 Schlitzer *et al.*, Blood, 2011

Identification of CCR9$^-$ murine plasmacytoid DC precursors with plasticity to differentiate into conventional DCs

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Whereas the final differentiation of conventional dendritic cells (CDCs) from committed precursors occurs locally in secondary lymphoid or peripheral tissues, plasmacytoid dendritic cells (PDCs) are thought to fully develop in the bone marrow from common DC progenitors before migrating to the periphery. In our study, we define, for the first time, a subpopulation of CCR9$^-$ major histocompatibility complex class II^{low} PDCs in murine bone marrow, which express E2-2 and are immediate precursors of CCR9$^+$ fully differentiated PDCs. However, CCR9$^-$ PDCs have the plasticity to acquire the phenotype and function of CD11b$^+$ CD8α^- major histocompatibility complex class II^{high} CDC-like cells under the influence of soluble factors produced by intestinal epithelial cells or recombinant GM-CSF. This deviation from the PDC lineage commitment is regulated on the level of transcription factors reflected by down-regulation of E2-2 and up-regulation of ID2, PU.1, and BATF3. Thus, CCR9$^-$ PDCs are immediate PDC precursors that can be reprogrammed to differentiate into CDC-like cells with higher antigen-presenting and cytokine-producing capacity under the influence of the local tissue microenvironment. (*Blood*. 2011; 117(24):6562-6570)

Introduction

Although plasmacytoid dendritic cells (PDCs) express markers of the "lymphoid" lineage, they can be derived from both common myeloid and common lymphoid progenitors.[1] Several elegant studies have convincingly shown that PDCs arise from common dendritic cell (DC) progenitors (or pro-DCs) in the bone marrow (BM),[2,3] which have the additional potential to generate precursor cells committed to conventional dendritic cell (CDC) development (pre-CDCs).[4,5] In contrast, common DC progenitors derived precursor cells committed exclusively to PDC development have not been described. Generation of PDCs is dependent on transcription factor E2-2, which drives the expression of other key transcription factors involved in PDC development and function (IRF8, Spi-B, IRF7) as well as PDC-specific markers (BST2 and Siglec H in murine PDCs, BDCA2 in human PDCs).[6]

It has been reported that a population of Siglec-H-negative PDC-like cells in murine BM, which is only present in lymphocytic choriomeningitis virus-infected mice is capable of differentiating into CDCs during viral infection in a type I interferon (IFN)-dependent manner.[7,8] The potential of PDCs or committed PDC precursors to differentiate into other DC subpopulations in the absence of infection is unknown.

It has been assumed from the available data that PDCs fully differentiate in the BM, circulate in the blood, and then enter lymphoid organs and peripheral tissues. CDCs, however, are generated from circulating committed precursors (pre-CDCs), which differentiate locally in lymphoid and peripheral tissues under the control of growth factors, such as Fms-like tyrosine kinase 3 ligand (Flt3L).[4,9] Thus, the development of CDC subpopulations is shaped by the local microenvironment allowing adaptation to tissue-specific functions. This has been shown recently for DCs in the intestinal lamina propria whose development from precursors is driven by local growth factors and the enteric microbial flora.[10-12] It is so far not clear whether PDCs in the intestine or in other peripheral tissues exclusively derive from fully differentiated circulating PDCs[13] or whether they can also differentiate locally from committed precursor cells under the influence of the specific tissue microenvironment.

In this study, we identify an immediate PDC precursor in murine BM, which is characterized by expression of the transcription factor E2-2, PDC-specific markers (BST2, Siglec H), and production of type I IFN but lack of CCR9 and low major histocompatibility complex (MHC) class II expression. We show that CCR9$^-$ MHCII^{low} PDCs spontaneously give rise to fully differentiated CCR9$^+$ MHCII$^+$ PDCs. However, in contrast to CCR9$^+$ PDCs, these cells retain the ability to divert from the PDC lineage and differentiate into MHCII^{high} CD11b$^+$ CD8α^- antigen-presenting CDCs under the influence of GM-CSF, which is produced constitutively by intestinal epithelial cells. Down-regulation of the PDC-specific transcription factor E2–2 and concomitant up-regulation of transcription factors involved in CDC development show that this developmental shift is transcriptionally regulated. Our results demonstrate the existence of an immediate PDC precursor whose final differentiation can be shaped by the local tissue microenvironment.

Submitted December 21, 2010; accepted April 8, 2011. Prepublished online as *Blood* First Edition paper, April 20, 2011; DOI 10.1182/blood-2010-12-326678.

The online version of this article contains a data supplement.

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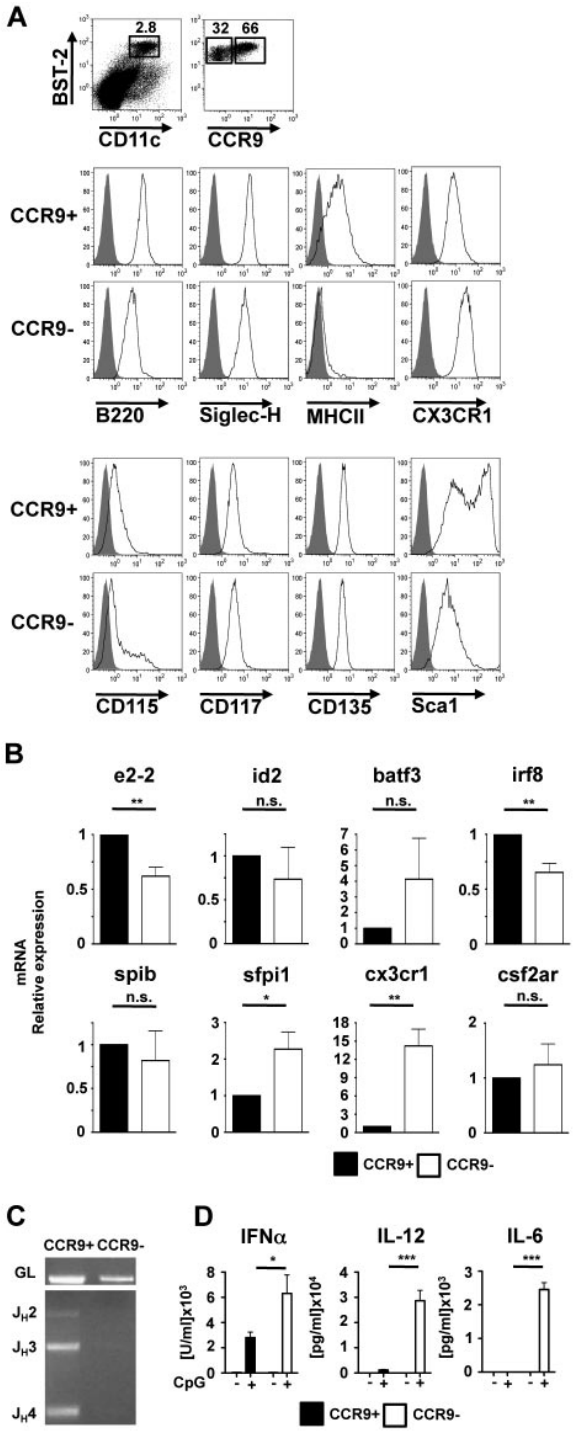

Figure 1. Characterization of CCR9⁻ and CCR9⁺ PDCs in murine bone marrow. Expression of the indicated markers was analyzed by FACS in primary BM cells. Dot plots show expression of CD11c versus BST2 in propidium iodide-negative cells and expression of BST2 versus CCR9 in BST2⁺ CD11c⁺ cells. Expression of the indicated markers in CCR9⁺ and CCR9⁻ BST2⁺ CD11c⁺ cells is shown below (overlay with fluorescence minus one [FMO] control, filled histograms) (A). Relative mRNA expression was quantified in CCR9⁺/BST2⁺/CD11c⁺ and CCR9⁻/BST2⁺/CD11c⁺ PDC populations sorted from primary BM cells by quantitative RT-PCR (B, mean ± SD, n = 3). Immunoglobulin D_H-J_H rearrangement was detected by PCR in genomic DNA isolated from CCR9⁺ and CCR9⁻ PDCs (C). CCR9⁺ and CCR9⁻ PDCs were incubated with medium or CpG 2216 for 24 hours. IFN-α, IL-12p40, and IL-6 were measured in the supernatants by ELISA (D, mean ± SD, n = 3). *$P < .05$. **$P < .01$. ***$P < .001$. n.s. indicates not significant.

Methods

Mice

Specific pathogen-free, female 6- to 8-week-old C57BL/6 mice were purchased from Harlan Winkelmann. OT II and OT I mice and *Cx3cr1-egfp* reporter mice (C57BL/6 background)[11] were bred under specific pathogen-free conditions. Experiments were performed in accordance with German animal care and ethics legislation and have been approved by the local government authorities.

Generation of Flt3-ligand generated BM-DCs

BM cells were cultured in DC medium containing 20 ng/mL recombinant human (rh) Flt3-L for 7 days as described (FL-DCs).[14]

Primary cell isolation and sorting

Spleen, Peyer patches, and mesenteric and inguinal lymph nodes were digested with collagenase D and DNase I. Single-cell suspensions from these organs and from BM were stained with the indicated markers and sorted using a FACSAria (BD Biosciences) or MoFlow cell sorter (Beckman Coulter). CD4⁺ and CD8⁺ T cells were isolated from splenocytes by negative selection using MACS technology (Milteny Biotec).

Preparation of intestinal epithelial supernatant

The murine colonic epithelial cell line PTK6 was maintained in RPMI 1640/5% fetal bovine serum/1 μg/mL insulin-transferrin-selenium/10 U/mL rmIFN-γ at 33°C as described.[15] PTK6 cells were seeded on 0.5 μm pore size transwell filters and cultured at 37°C until formation of polarized monolayers. Monolayers were washed thoroughly with PBS and inserted into fresh DC medium. Supernatants of intestinal epithelial cells (IEC-SN) were harvested from the basolateral side after 24 hours, centrifuged, and sterile-filtered before use.

DC culture and stimulation

FL-DCs and PDC subpopulations sorted from FL-DCs or primary BM cells were cultured with 50% IEC-SN or recombinant GM-CSF or M-CSF (concentrations indicated in figure legends) at a density of 2.5×10^6/mL for 48 hours. Cells were subsequently harvested for RNA isolation, FACS analysis, and cell sorting. Where indicated, CpG 2216 (0.5μM, MWG Biotech) was added after 24 hours for further 24 hours.

Antigen presentation assays

Sorted DC populations were loaded with OVA peptide (MHC class II–specific peptide: ISQAVHAAHAEINEAGR; aa 323-339) or OVA protein (5μM) for 1 hour and cocultured with carboxyfluorescein succinimidyl ester (CFSE)–labeled CD4⁺ OT II or CD8⁺ OT I T-cells at a ratio of 1:10 for 4 days. T cells were analyzed by flow cytometry for proliferation (CFSE dilution) and intracellular IFN-γ and IL-2 after restimulation with phorbol myristate acetate (PMA)/ionomycin.

Flow cytometry

For FACS analysis, cells were stained using fluorescently labeled antibodies directed against the indicated cell surface antigens (eBioscience) as described.[14] Anti-BST2 (120G8, rat IgG1)[16] and anti-Siglec H antibodies (440c, rat IgG2b)[17] were conjugated with fluorescein isothiocyanate or biotin. XCR1 staining reagent was kindly provided by R. Kroczek. Propidium iodide was added to exclude dead cells from analysis. T cells were stimulated with PMA/ionomycin (20 ng/mL/1 μg/mL) for 6 hours adding GolgiPlug and GolgiStop (BD Biosciences). Cells were then fixed in 2% paraformaldehyde, permeabilized with 0.5% saponin, and stained with anti–IFN-γ–phycoerythrin and anti–IL-2–allophycocyanin (BD Biosciences). Cells were acquired using a FACSCalibur flow cytometer (BD Biosciences) or a Gallios flow cytometer (Beckman Coulter).

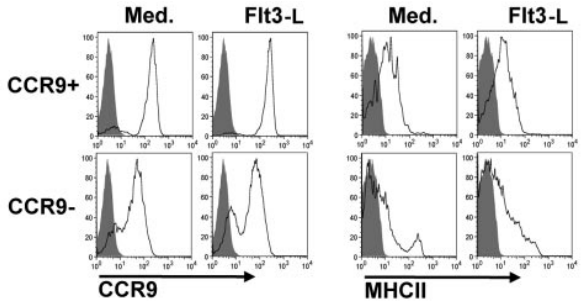

Figure 2. Differentiation of CCR9$^-$ MHCII^{low} PDCs into CCR9$^+$ MHCII$^+$ PDCs. CCR9$^+$ and CCR9$^-$ PDCs were incubated with or without Flt3-L for 48 hours. Expression of CCR9 and MHC class II was measured by FACS. Filled histograms represent FMO control. Results of one representative experiment are shown.

RNA isolation, cDNA preparation, and quantitative real-time PCR

RNA was isolated using the RNeasy mini kit (QIAGEN) and transcribed to cDNA using SuperScript III reverse transcriptase (Invitrogen). Quantitative RT-PCR was performed using TaqMan Gene expression Master Mix together with a TaqMan Step One Plus instrument. TaqMan probes are: HPRT Mm00446968_m1, Tcf4 Mm01262526_g1, Sfpi1 Mm00488142_m1, Batf3 Mm01318274, Irf8 Mm00492567_m1, Csf2ar Mm00438331_g1, Id2 Mm00711781_m1, Cx3cr1 Mm00438354, and Spib Mm01719550_s1 (Invitrogen).

Diagnostic PCR for Ig rearrangement

Genomic DNA was isolated from 5×10^5 FACS-sorted cells using the DNAeasy Blood & Tissue Kit (QIAGEN). PCR primers specific for rearranged and nonrearranged Ig loci were used to diagnose IgH D-J rearrangement as previously described.[18]

Cytokine measurement by ELISA

GM-CSF was measured by ELISA kit from eBioscience. IFN-α was measured in supernatants by ELISA as described.[14] Matched antibody pairs and streptavidin-horseradish peroxidase (GE Healthcare) were used for IL-6 ELISA (BD Biosciences) and IL12p40 ELISA (BD Biosciences) as described.[14]

Statistical analysis

Paired, 2-tailed Student *t* test was used to determine statistically significant differences.

Results

CCR9$^-$ MHCII^{low} PDCs are immediate precursors of CCR9$^+$ MHCII$^+$ PDCs

Compared with spleen and lymph nodes where the majority of CD11c$^+$ DCs are CDCs, murine BM contains a higher percentage of PDCs (39% ± 6.2% of CD11c$^+$ cells, n = 3) accounting for approximately 3% of total BM leukocytes. Among these, we identified a fraction of 24.1% ± 5.3% (n = 4) PDCs lacking expression or expressing very low levels of the chemokine receptor CCR9 (Figure 1A dot plots). This population is designated as CCR9$^-$ PDCs in this manuscript. Further characterization of CCR9$^+$ and CCR9$^-$ PDCs in the BM revealed that both populations show a phenotype characteristic for PDCs (Figure 1A; supplemental Figure 1A, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). In contrast to CCR9$^+$ PDCs, however, CCR9$^-$ PDCs expressed very low levels of MHC class II and lower levels of costimulatory molecules CD80 and CD86, demonstrating that CCR9$^-$ PDCs are less activated than CCR9$^+$ PDCs. In addition, we observed that CD8α, CD4, and Sca-1 were also expressed at lower levels in CCR9$^-$ than CCR9$^+$ PDCs. CD115 (macrophage colony-stimulating factor receptor [M-CSFR]) was expressed at similar levels in both populations with a small subpopulation of CD115^{high} CCR9$^-$ PDCs. Expression of GM-CSF receptor (csf2ar) on mRNA level was also comparable in both populations (Figure 1B bottom right panel). CX3CR1, which is expressed on common DC progenitors and at variable levels on all splenic DC subpopulations, was expressed on CCR9$^-$ as well as CCR9$^+$ BM PDCs with higher expression levels in CCR9$^-$ PDCs (Figure 1A-B). Thus, with the exception of very low MHC class II expression and lack of CCR9, CCR9$^-$ PDCs phenotypically resemble CCR9$^+$ PDCs, suggesting a close developmental relationship between these subpopulations.

The transcription factor E2-2 is critical for development of the PDC lineage.[6] We therefore determined relative mRNA expression of E2-2 and other transcription factors involved in DC development in sorted CCR9$^+$ and CCR9$^-$ BM PDCs (Figure 1B). E2-2 and IRF8 were expressed in both populations, albeit at slightly lower levels in CCR9$^-$ PDCs. No significant difference was observed in the expression of Spi-B, ID2, and BATF3, whereas PU.1 (sfpi1) showed a higher expression in CCR9$^-$ PDCs. PCR analysis of genomic DNA obtained from sorted CCR9$^-$ and CCR9$^+$ PDCs revealed that only CCR9$^+$ PDCs have undergone D_H-J_H immunoglobulin gene rearrangement (Figure 1C). High level type I IFN production is a hallmark feature of PDCs, so we stimulated BM PDCs with TLR9 ligand CpG 2216 and were able to show that CCR9$^-$ PDCs secreted even higher amounts of IFN-α than the CCR9$^+$ PDCs. In addition, CpG-stimulated CCR9$^-$ PDCs produced significant amounts of IL-12 and IL-6, whereas CCR9$^+$ PDCs produced only low levels of IL-12 (1.2 ± 0.3 ng/mL; Figure 1D).

Our analysis shows that CCR9$^-$ BM PDCs possess most of the characteristics of CCR9$^+$ differentiated PDCs, with the exception of CCR9 expression and D_H-J_H rearrangement. We therefore investigated whether CCR9$^-$ PDCs can give rise to CCR9$^+$ PDCs by exposing them to culture medium with or without Flt3L for 48 hours. In both conditions, the majority of CCR9$^-$ MHCII^{low} PDCs differentiated into CCR9$^+$ MHCII$^+$ PDCs (Figure 2). We therefore conclude that CCR9$^-$ PDCs are precursors of CCR9$^+$ PDCs.

CCR9$^-$ PDCs are also found in spleen and lymph nodes, although the size of this subpopulation is smaller and more variable in these organs than in the BM (11.8% ± 6.4% of splenic PDCs, n = 3, supplemental Figure 1C). CCR9$^-$ PDCs were also found in mesenteric lymph nodes and in the colon. In Peyer patches, however, CCR9 expression in PDCs was uniformly lower than in other lymphoid organs, and a distinct population of CCR9$^-$ PDCs could not be detected (supplemental Figure 1D). The CCR9$^-$ PDC subpopulation in the spleen showed an almost identical phenotype to its counterpart in the BM except for higher expression of MHC class II (supplemental Figure 1B). Thus, CCR9$^-$ PDCs exit the BM and could give rise to CCR9$^+$ PDCs in the periphery.

CCR9$^-$ MHCII^{low} PDC precursors can differentiate to CD11b$^+$ MHCII^{high} CDC-like cells upon exposure to intestinal epithelial cell produced factors

It has been reported that CD11c^{high} DC subpopulations within the intestinal lamina propria are generated locally from precursors

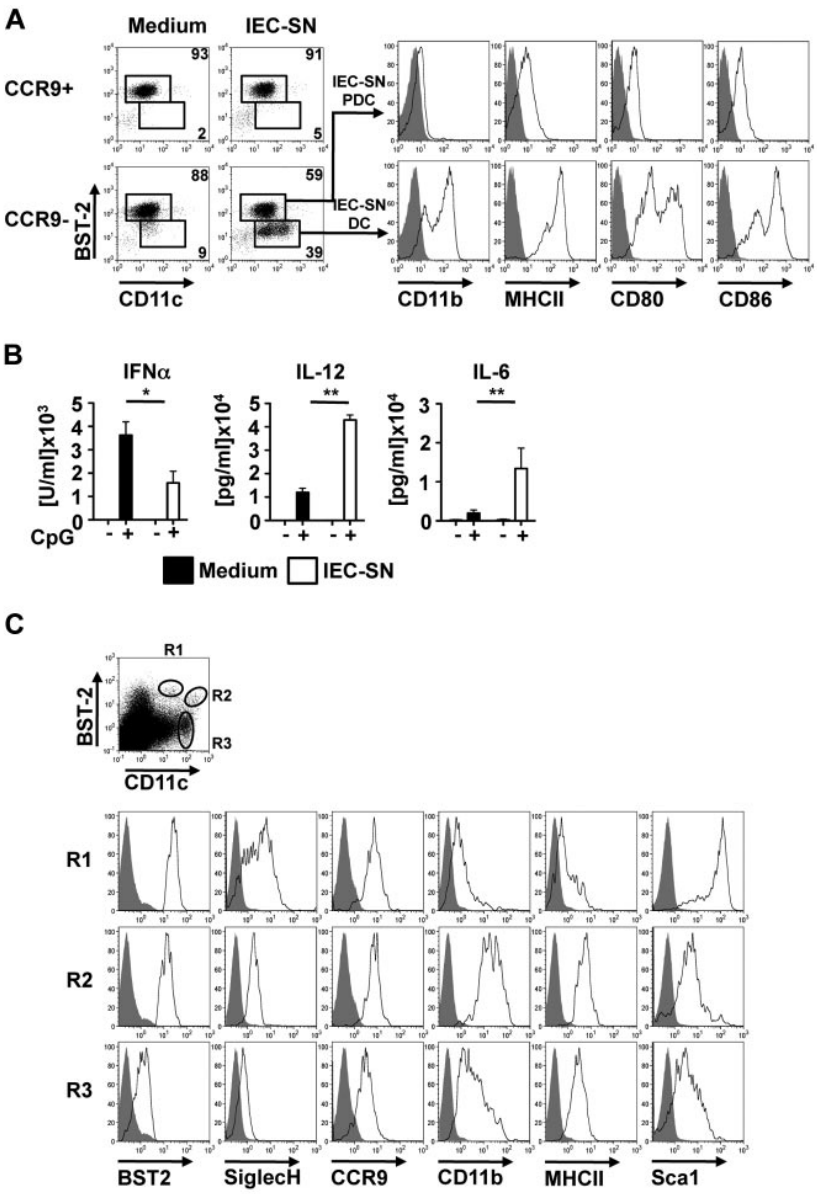

Figure 3. Generation of CD11b$^+$ MHCII^{high} CDC-like cells from CCR9$^-$ MHCII^{low} PDCs. CCR9$^+$ and CCR9$^-$ PDC populations were sorted from primary BM cells, incubated with medium or IEC-SN for 48 hours, and stained for FACS analysis. Surface expression of BST2 versus CD11c is shown in the dot plots. Expression levels of CD11b, MHC class II, CD80, and CD86 in BST2^{high} IEC-SN PDCs and BST2^{low} IEC-SN DCs generated from CCR9$^-$ BM PDCs after treatment with IEC-SN are shown in the histograms (A, filled histograms: FMO control, results of one representative of 5 experiments). CCR9$^+$ and CCR9$^-$ PDCs were incubated with medium or 50% IEC-SN. After 24 hours, CpG 2216 (0.5μM) was added and cytokine concentrations were measured in the supernatants by ELISA after further 24 hours (B, mean ± SD, n = 3). Cells were isolated from Peyer patches digested with DNase/collagenase and subsequently analyzed by FACS for surface expression of the depicted markers. Dot blots show expression of BST2 and CD11c in propidium iodide-negative cells. Expression of the indicated markers in BST2^{high} CD11c$^+$ (R1), BST2^{low} CD11c^{high} (R2) and CD11c^{high} (R3) is shown below (C, filled histograms: FMO control). *$P < .05$. **$P < .01$.

under the influence of the tissue microenvironment. To investigate whether differentiation of PDCs from CCR9$^-$ immediate PDC precursors is influenced by the gut microenvironment, sorted CCR9$^+$ and CCR9$^-$ BM PDC subpopulations were exposed to 50% IEC-SN or medium alone for 48 hours. As shown in Figure 3A and supplemental Figure 2A, exposure of CCR9$^-$, but not CCR9$^+$, BM PDCs to IEC-SN led to the generation of a substantial population of BST2^{low}/CD11c$^+$ cells (35% ± 7.4%, mean ± SD, n = 5), which were CD11b$^+$ and expressed high levels of MHC class II and costimulatory molecules CD80 and CD86 (IEC-SN DC, Figure 3A histograms). The remaining BST2^{high} PDCs in these cultures (IEC-SN PDCs) maintained their PDC phenotype and further up-regulated CCR9 expression (Figure 3A; supplemental Figure 2B). Extensive phenotypic analysis of BST2^{low} CD11c$^+$ cells newly generated from CCR9$^-$ BM PDCs in culture with IEC-SN (IEC-SN-DCs) showed that these cells expressed lower levels of B220, Siglec H, CCR9, CD8α, CD4, and Sca-1, but higher levels of MHC class II, CD80, CD86, CD11b, CD24, CD115, CD117, and CD135 after exposure to IEC-SN than the PDCs present in the same cultures (IEC-SN PDCs), thus resembling CDCs. In contrast, CCR9$^+$ BM PDCs showed a stable PDC phenotype even after exposure to IEC-SN (PDC + IEC-SN, supplemental Figure 2B). Although IEC-SN DCs expressed CD8α at low to intermediate level, they lacked expression of XCR1, a conserved marker of murine CD8α$^+$ splenic CDC equivalents (supplemental Figure 2B).[19] In parallel to the observed change in phenotype, CCR9$^-$ BM PDCs cultured with IEC-SN produced significantly less IFN-α but more IL-12 and IL-6 in response to CpG 2216 compared with cells cultured with medium alone (Figure 3B).

Phenotypic analysis of BST2$^+$ SiglecH$^+$ CD11c$^+$ cells in Peyer patches underlying the intestinal epithelium revealed a subpopulation of BST2^{low} CD11c^{high} cells expressing lower levels of Siglec H and Sca-1 as well as higher levels of MHC class II and CD11b than BST2^{high} CD11c^{low} PDCs (Figure 3C). This DC subpopulation,

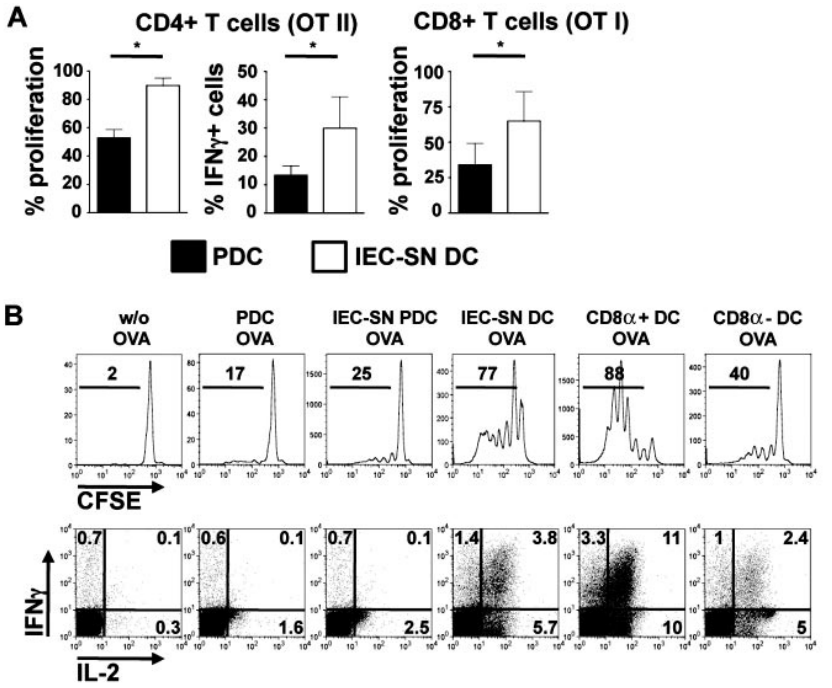

Figure 4. CD4$^+$ and CD8$^+$ T-cell proliferation and cytokine secretion in response to antigen presentation by CD11b$^+$ MHCII^{high} CDC-like cells. BST2^{high} PDCs and BST2^{low} IEC-SN DCs were pulsed with OVA peptide and cocultured with CFSE-labeled CD4$^+$ OT II T cells or were pulsed with OVA protein and cocultured with CD8$^+$ OT I T cells for 4 days. Proliferation was determined by CFSE dilution, and intracellular IFN-γ was measured by FACS after stimulation with PMA/ionomycin (A, mean ± SD, n = 3). *$P < .05$. PDCs, BST2^{high} IEC-SN PDCs, BST^{low} IEC-SN DCs, splenic CD8α$^+$ DCs, and splenic CD8α$^-$ DCs were cocultured with OT I T cells in the presence of OVA protein. As control, PDCs were cocultured with OT I T cells in the absence of antigen. After 4 days, proliferation was determined by CFSE dilution, and intracellular IFN-γ and IL-2 were detected by FACS after restimulation with PMA/ionomycin (B, results of 1 representative of 2 experiments are shown).

which was found in Peyer patches but not in spleen or lymph nodes, closely resembles the CDC-like cells generated from CCR9$^-$ PDCs in vitro under the influence of IEC-SN, with the exception of CCR9 expression, which may be influenced by local factors. Thus, the observed deviation from the PDC lineage to a CDC-like cell type may also occur in vivo in the intestinal microenvironment of the Peyer patches.

CD11b$^+$ MHCII^{high} CDC-like cells generated from CCR9$^-$ PDCs are efficient in presenting antigens to CD4$^+$ and CD8$^+$ T cells

To obtain a larger amount of CCR9$^-$ PDCs for functional studies, we turned to CD11c$^+$ BST2$^+$ PDCs from Flt3L-cultured BM cells (FL-DCs), which phenotypically correspond to primary CCR9$^-$ BM PDCs and down-regulated BST2 as well as Siglec H while up-regulating expression of CD11b, MHC class II, and CX3CR1 after exposure to IEC-SN (supplemental Figure 3A-B). The differentiation process did not involve proliferation as shown by lack of CFSE dilution during the 48-hour culture period (supplemental Figure 3B histograms). Therefore, with regard to both phenotype and function, CCR9$^-$ PDCs from FL-DC cultures are a valid model to study the developmental fate of CCR9$^-$ BM PDCs and the functional properties of the resultant IEC-SN DCs.

IEC-SN DCs generated from CCR9$^-$ PDCs resembled CDCs in that they expressed high levels of MHC class II and costimulatory molecules and produced high amounts of proinflammatory cytokines. Based on these observations, we hypothesized that these cells would be efficient T-cell activators. To compare their antigen presentation capacity, we sorted BST2^{low} DCs generated by treatment of FL-DCs with IEC-SN (IEC-SN DCs) as well as BST2^{high} PDCs from medium-treated FL-DCs and cocultured them for 4 days with CD4$^+$ OT II T cells or CD8$^+$ OT I T cells after pulsing with OVA peptide or OVA protein, respectively. IEC-SN DCs were significantly more efficient in inducing CD4$^+$ T-cell proliferation and IFN-γ production as well as cross-presenting soluble antigen to CD8$^+$ T cells than PDCs (Figure 4A). IEC-SN DCs were more efficient in inducing proliferation and cytokine production of CD8$^+$ OT I T cells than CD8α$^-$ splenic CDCs but less efficient than CD8α$^+$ splenic CDCs (Figure 4B). Thus, CCR9$^-$, but not CCR9$^+$, PDCs maintain the plasticity to divert from the PDC lineage and differentiate into CD11b$^+$ MHCII^{high} CDC-like cells with higher capacity to produce inflammatory cytokines and present as well as cross-present exogenous antigens upon exposure to IEC-derived factors.

Generation of CD11b$^+$ MHCII^{high} CDC-like cells from CCR9$^-$ PDC precursors is mediated by GM-CSF

Several soluble factors, which can be produced by IEC and could be involved in inducing differentiation of CCR9$^-$ PDCs to CD11b$^+$ MHCII^{high} CDC-like cells were not detectable in IEC-SN (TNF-α, IL-10, TGF-β, IL-6, type I IFN; supplemental Figure 4C). GM-CSF is produced at low levels in various tissues, including the intestine in the steady state, and is further induced during inflammation and infection.[20,21] Indeed, IEC-SN obtained from PTK6 cell monolayers contained low concentrations of GM-CSF (56 ± 7.5 pg/mL, n = 3). Pretreatment of IEC-SN with neutralizing antibody against GM-CSF largely abrogated down-regulation of BST2 (Figure 5A) and the enhanced inflammatory cytokine production in FL-DCs exposed to IEC-SN (Figure 5B). The reduction in IFN-α production was unaffected by blocking GM-CSF despite maintenance of the PDC population (Figure 5B). Conversely, addition of recombinant GM-CSF induced down-regulation of BST2, up-regulation of MHC class II, and increased IL-12 and IL-6 production similar to IEC-SN (supplemental Figure 4A-B). In contrast to IEC-SN, however, recombinant GM-CSF did not inhibit but rather enhanced the IFN-α response of FL-DCs to CpG 2216, suggesting that additional IEC-derived factors are involved in down-modulation of the IFN-α response by IEC-SN (supplemental Figure 4B). These results demonstrate that the differentiation of CCR9$^-$ MHCII^{low} PDCs to CD11b$^+$ MHCII^{high} CDC-like cells

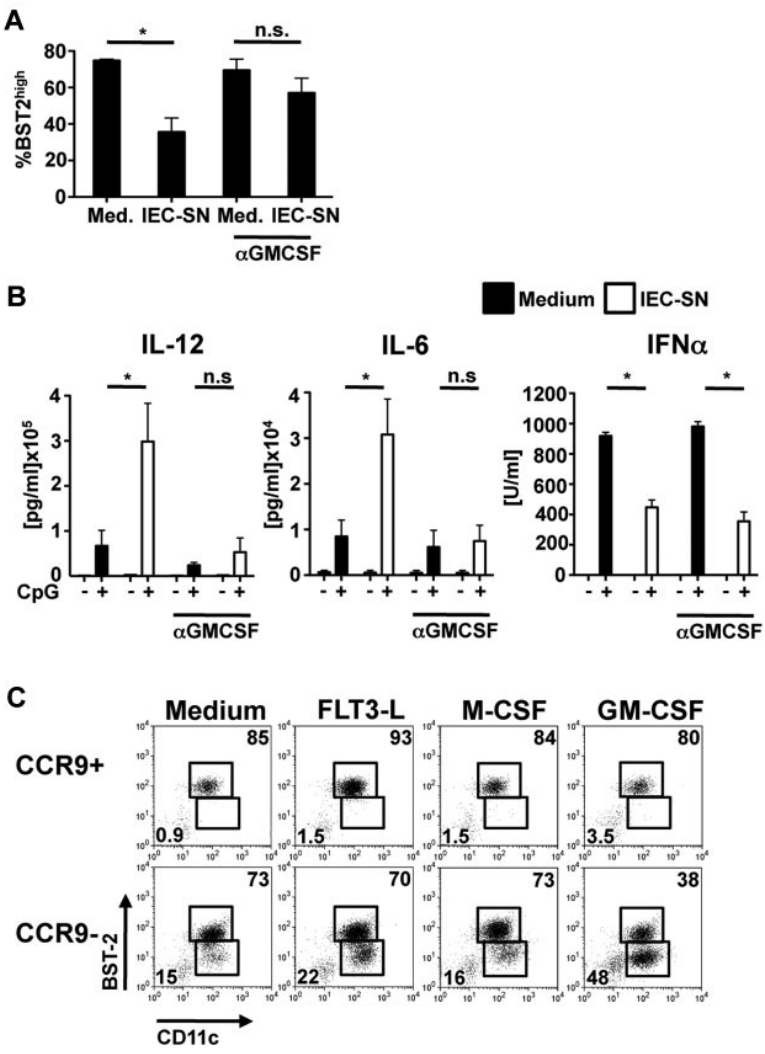

Figure 5. Role of GM-CSF for differentiation of CCR9⁻ PDCs to CD11b⁺ MHCIIhigh CDC-like cells. Neutralizing antibody against GM-CSF (1 μg/mL) was added to IEC-SN or medium 2 hours before addition to FL-DCs for 48 hours (A-B). The percentage of BST2high PDCs of all CD11c⁺ cells was determined by FACS (A, mean ± SD, n = 3). CpG 2216 (0.5μM) was added after 24 hours, and cytokines were measured in the supernatants after further 24 hours (B, mean ± SD, n = 3). *$P < .05$. n.s. indicates not significant. CCR9⁺ and CCR9⁻ PDCs sorted from primary BM cells were cultured with medium alone or medium supplemented with Flt3L (20 ng/mL), M-CSF (1 ng/mL) or GM-CSF (1 ng/mL) for 48 hours. Expression of CD11c and BST2 was measured by FACS. Results of one representative experiment are shown (C).

with higher inflammatory cytokine-producing ability is mediated by GM-CSF produced by IEC.

The observed effect of GM-CSF on the differentiation of CCR9⁻ PDCs was confirmed in CCR9⁻ PDCs freshly isolated from primary BM cells. After exposure to GM-CSF (even at very low doses) but not Flt3L or M-CSF CCR9⁻, but not CCR9⁺, PDCs down-regulated BST2 expression (Figure 5C; supplemental Figure 4E) and up-regulated CD11b and MHC class II expression (supplemental Figure 4D). Whereas the majority of CCR9⁻ PDCs further differentiated to CCR9⁺ PDCs in the absence of growth factors or in the presence of Flt3L or M-CSF, a substantial fraction of CCR9⁻ PDCs differentiated into CD11b⁺ MHCIIhigh CDC-like cells on exposure to IEC-SN containing GM-CSF or low concentrations of recombinant GM-CSF. Thus, CCR9⁻ immediate PDC precursors constitute an alternative source for the generation of CD11b⁺ MHCIIhigh CDC-like cells under conditions where GM-CSF is produced.

Diversion of CCR9⁻ PDC precursors to CD11b⁺ MHCIIhigh CDC-like cells is regulated by transcription factors

We hypothesized that the observed differentiation of CCR9⁻ PDCs to CD11b⁺ MHCIIhigh CDC-like cells is not merely a transient shift in phenotype but rather a true diversion of lineage commitment that should be reflected in altered expression of lineage-specific transcription factors. We therefore sorted BST2high PDCs (IEC-SN PDCs) and BST2low CDCs (IEC-SN DCs) generated in cultures of primary CCR9⁻ BM PDCs with IEC-SN as well as BST2high PDCs, which had been cultured with medium alone. For comparison, CD8α⁺ and CD8α⁻ CDCs were sorted from splenocytes. Expression levels of transcription factors involved in PDC and CDC development were quantified by quantitative RT-PCR.

Expression of E2-2 was greatly reduced in IEC-SN DCs compared with the remaining IEC-SN PDCs or PDCs treated with medium alone (Figure 6). Expression of E2-2 regulated transcription factors IRF8 and SpiB was also reduced in IEC-SN DCs compared with PDCs. IEC-SN DCs expressed similarly low levels of IRF8 as CD8α⁻ splenic CDCs distinct from CD8α⁺ splenic CDCs. Expression of transcription factors regulating CDC development (ID2, BATF3, and PU.1),[22-24] was greatly increased in IEC-SN DCs compared with PDCs, thus resembling the expression pattern found in splenic CDCs. Thus, loss of PDC-specific phenotype and function directly correlates with down-regulation of E2–2 and concomitant up-regulation of transcription factors involved in CDC development. Upon exposure to GM-CSF–

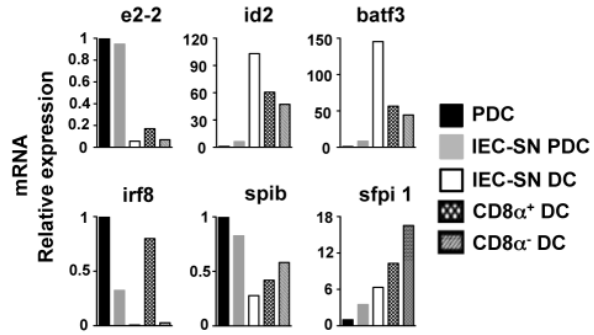

Figure 6. Transcription factor expression pattern in CD11b$^+$ MHCII^{high} CDC-like cells generated from CCR9$^-$ PDCs. CCR9$^+$ and CCR9$^-$ PDCs sorted from primary BM cells were incubated with medium alone or 50% IEC-SN for 48 hours. Relative mRNA expression of transcription factors was measured by quantitative RT-PCR in sorted BST2^{low} IEC-SN DCs and BST2^{high} IEC-SN PDCs generated from CCR9$^-$ PDCs cultured with IEC-SN as well as in sorted BST2^{high} PDCs generated from CCR9$^+$ PDCs cultured with medium alone. The expression patterns of transcription factors in splenic CD8α$^+$ and CD8α$^-$ DCs are shown for comparison. Results of one representative of 2 experiments are shown.

containing IEC-SN CCR9$^-$ immediate PDC precursors undergo a developmental shift from the PDC lineage to the CDC lineage, which is regulated on the level of transcription factors.

Discussion

In this study, we identify an immediate CCR9$^-$ MHCII^{low} precursor of PDCs imprinted with a PDC developmental program, which leads to spontaneous differentiation into CCR9$^+$ MHCII$^+$ PDCs. This population can be reprogrammed to differentiate into a CD11b$^+$ MHCII^{high} CDC-like population upon exposure to supernatants of IECs with low concentrations of GM-CSF as an essential factor. This developmental shift correlates with down-regulation of E2-2 and up-regulation of transcription factors promoting CDC development. Thus, immediately before the final differentiation step, exposure to GM-CSF forces CCR9$^-$ PDCs to branch off their developmental course and contribute to the generation of CDCs. This alternative differentiation is likely to be relevant in peripheral tissues, such as the intestine or at sites of inflammation, where GM-CSF is produced.

We show in our study that CCR9$^-$ MHCII^{low} BM PDCs are closely related to CCR9$^+$ MHCII$^+$ PDCs: Their phenotype largely overlaps with the characteristic phenotype of CCR9$^+$ PDCs in BM as well as secondary lymphoid organs with few notable exceptions: Lack of CCR9 expression, very low expression of MHC class II, and lower expression of CD4, CD8α, CD80, and CD86 demonstrating the lower differentiation and activation status of CCR9$^-$ versus CCR9$^+$ PDCs. Higher levels of Sca-1 expression may reflect that CCR9$^+$ BM PDCs are developmentally closer to splenic CCR9$^+$ PDCs, which are Sca-1^{high} (supplemental Figure 1). The CCR9$^-$ BM PDC population secreted high amounts of IFN-α upon stimulation and expressed E2-2, the essential transcription factor driving PDC development. Thus, CCR9$^-$ BM PDCs already possess most of the characteristic features of differentiated PDCs. Interestingly, D_H-J_H immunoglobulin rearrangement was not detected in CCR9$^-$ PDCs in contrast to CCR9$^+$ PDCs, yet it is already known that expression of recombination activation gene 1 (RAG1) and subsequent D^H-J^H immunoglobulin rearrangement occurs only in a fraction of PDCs (~ 50%) during later steps of development after the common DC progenitor stage.[25,26] The *Rag1*$^{-/-}$ BM PDC population characterized by Pelayo et al[25] responded to CpG stimulation with higher IFN-α and inflammatory cytokine production, similar to our results obtained with CCR9$^-$ BM PDCs.

CCR9$^-$ MHCII^{low} PDCs are immediate PDC precursors that retain the ability to divert from commitment to the PDC lineage and differentiate into CD11b$^+$ MHCII^{high} CDC-like cells in the presence of IEC-SN or GM-CSF. This population, however, is distinct from previously described common DC progenitors or pro-DCs (Lin$^-$ c-kit^{int} Flt3$^+$ M-CSFR$^+$) because of expression of lineage markers B220, CD11c, CD4, CD8α, and CD86, heterogeneous expression of M-CSFR (CD115), and lack of proliferation and CDC differentiation in response to Flt3L.[2,3] CCR9$^-$ MHCII^{low} PDC precursors may be contained within CD11c$^+$ Ly6C$^+$ CD31$^+$ B220$^+$ "preimmunocytes" or CD11c$^+$ MHCII$^-$ B220$^+$ precursors in the BM.[27,28] In contrast to these studies, which were performed before the identification of PDC-specific markers, we clearly show that CCR9$^-$ MHC^{low} PDC precursors are a well-defined mostly homogeneous population, which is far advanced in their differentiation to CCR9$^+$ MHCII$^+$ PDCs and committed to PDCs under steady-state conditions (culture with medium or Flt3L). Because of the expression of the PDC-specific markers BST2 and Siglec H, this population does not overlap with the CD11c$^+$ B220$^+$ BST2$^-$ CCR9$^-$ DCs in the spleen, which give rise to splenic CDC subsets.[29]

Despite their close relationship to finally differentiated CCR9$^+$ PDCs, the CCR9$^-$ MHCII^{low} PDC precursors identified in our study retain the potential to significantly contribute to the generation of CD11b$^+$ MHCII^{high} CDC-like cells under conditions where GM-CSF is produced. Outside of the BM, CCR9$^-$ PDC precursors, which have the same phenotype as their BM counterparts but express MHC class II at higher levels, were also found in spleen and lymph nodes, suggesting that these immediate PDC precursors leave the BM. In CCR9-deficient mice, the PDC population is greatly diminished in the small intestine but only partially in Peyer patches[13] and not reduced in the colon.[30] Therefore, PDC precursors may reach Peyer patches and colonic lamina propria despite the lack of or low expression of CCR9. We found a subpopulation of BST2^{low} CD11c^{high} cells in Peyer patches, which phenotypically closely resembles the CDC-like cells generated from CCR9$^-$ PDCs in vitro. The PDC-specific marker Siglec H was still expressed on this subpopulation, although at lower levels than in PDCs, suggesting a close developmental relationship to PDCs. Thus, differentiation of immediate PDC precursors into CDC-like cells may also occur in vivo at this site. The final differentiation of PDC precursors to either CCR9$^+$ PDCs or CD11b$^+$ MHCII^{high} CDC-like cells is therefore subjected to conditions of the local tissue microenvironment, such as the intestine, allowing adaptation to local requirements in the steady state as well as during inflammation or infection.

Careful phenotypic analysis of the MHCII^{high} CDC-like cells, which were generated from CCR9$^-$ PDC precursors under the influence of GM-CSF containing IEC-SN (supplemental Figures 2, 3), revealed that these cells expressed CD11b and CX3CR1, low levels of CD8α and CD4, but lacked significant XCR1 and CD103 expression, markers of spleen CD8α$^+$ DC equivalents.[19] This phenotype therefore resembles that of CD8α$^-$ CD11b$^+$ splenic CDCs or related DCs in peripheral tissues.

Very low IRF8 expression combined with increased expression of BATF3, ID2, and PU.1 is also congruent with this phenotype. The CD11b$^+$ MHCII^{high} CDC-like cells, which were generated from CCR9$^-$ PDC precursors might also be related to the

CX3CR1$^+$ CD8α$^+$ DC population recently identified in the spleen whose gene expression profile partially overlaps with that of PDCs.[31] Functional analysis showed that the CD11b$^+$ MHCII^{high} CDC-like cells generated from CCR9$^-$ PDC precursors produce higher levels of inflammatory cytokines and present exogenous antigens to CD4$^+$ T cells and CD8$^+$ T cells more efficiently than PDCs. The cross-presenting capacity of this CDC-like population generated from CCR9$^-$ PDCs in vitro was higher than that of splenic CD8α$^-$ CDCs, but lower than that of splenic CD8α$^+$ CDCs. We conclude from these functional data that diversion from the PDC commitment at this late stage in development allows the generation of CDC-like cells with higher potential for efficient induction of adaptive immune responses, which may be beneficial in specific organs or during infections.

We found that GM-CSF is the major factor produced by IEC, which was responsible for the observed differentiation of CCR9$^-$ PDC precursors to CD11b$^+$ MHCII^{high} CDC-like cells in response to IEC-SN. Type I IFN, which has been shown to drive the generation of BST2$^+$ Siglec H$^-$ PDC-like cells in the BM, which convert to CDCs during lymphocytic choriomeningitis virus infection, was not produced by IEC and could thus be excluded as a relevant factor.[7,8] GM-CSF is not detectable in the serum in the steady state but increases during inflammation.[20] It has been assumed that GM-CSF is dispensable for DC development in the steady state but influences DC differentiation during inflammation by inducing the generation of "inflammatory" DCs from precursors. However, it has been shown recently that GM-CSF controls the development of subpopulations of lamina propria CDCs and dermal DCs from precursor cells.[10,12,32] Thus, the observed differentiation of CCR9$^-$ PDC precursors to CDCs could contribute to the generation of CDCs in peripheral tissues in the steady state. However, GM-CSF is strongly induced and critical for host defense during intestinal infection with the mouse enteric pathogen *Citrobacter* rodentium.[21] GM-CSF is also induced and plays an essential role in autoimmune inflammatory diseases, such as experimental autoimmune encephalomyelitis, collagen-induced arthritis, and myocarditis.[20,33] Thus, differentiation of CCR9$^-$ PDC precursors to CDC-like cells is probably relevant during infection and inflammation, when GM-CSF expression is induced.

E2-2 is the critical transcription factor for PDC development and lineage stability.[6] CCR9$^-$ PDCs differentiating to CD11b$^+$ MHCII^{high} CDC-like cells in the presence of IEC-SN down-regulate expression of E2-2 and E2-2–regulated Spi-B and IRF8, whereas CCR9$^+$ PDCs maintain their high level expression of E2-2. DC development is regulated by transcription factors in a dose-dependent manner,[6,22] and differentiation to DC subpopulations appears to depend on the ratio of the different transcription factors. ID2-mediated inhibition of E protein activity by formation of inactive ID2/E2-2 heterodimers prevents PDC development[34] and allows the generation of CDCs.[35] Thus, down-regulation of E2-2 and up-regulation of ID2 in CCR9$^-$ PDCs exposed to IEC-SN is expected to prevent the activation of E2-2 target genes required for full differentiation and stability of PDCs. The decrease in the E2-2/ID2 ratio found in CCR9$^-$ PDCs on treatment with IEC-SN therefore provides a good explanation for the observed diversion from the PDC lineage commitment and differentiation into CDC-like cells. PU.1 dose-dependently regulates DC development by inducing and maintaining Flt3 and GM-CSF–receptor expression, which is more critical for CDC than for PDC development. Divergence of the CDC and PDC lineages was shown to be paralleled by high versus low PU.1 expression.[22] This is confirmed in our study also for the reprogramming of PDC precursors to CDC-like cells.

In conclusion, we identify CCR9$^-$ PDCs, which are found in BM and secondary lymphoid organs as immediate PDC precursors whose final differentiation can be shaped by the local tissue microenvironment. In the presence of GM-CSF, which is produced constitutively in peripheral tissues or is induced at sites of inflammation, these immediate PDC precursors undergo profound changes in transcription factor expression pattern leading to a developmental shift toward CD11b$^+$ MHCII^{high} CDC-like cells with higher cytokine-producing and antigen-presenting capacity. This plasticity at a late stage in the PDC developmental pathway may promote inflammation but may also be beneficial for immune defense against pathogens.

Acknowledgments

The authors thank Giorgio Trinchieri, Marco Colonna, and Richard Kroczek for providing reagents, Viktoria Doll for cooperation, Annika Bosch and Julia Geitner for excellent technical assistance, and the Faculty Graduate Center Weihenstephan of TUM Graduate School at Technical University Munich, Germany for support.

A. K., A. S., J. L., and W. R. were supported by the German Research Foundation (grants KR2199/1-4, KR2199/3-1, SFB 455, SFB 571, and GRK 1482). J.-H.N. is supported by the German Research Foundation (grants Ni575/6-2 and Ni575/7-1).

This work is submitted in partial fulfillment of the PhD thesis of A.S.

Authorship

Contribution: A.S. designed research, performed experiments, analyzed data, and wrote the paper; J.L., K.M., and L.H. performed experiments; R.V., M.S., and J.-H.N. contributed reagents and mice; H.E. analyzed data; and W.R. and A.K. designed the study, interpreted data, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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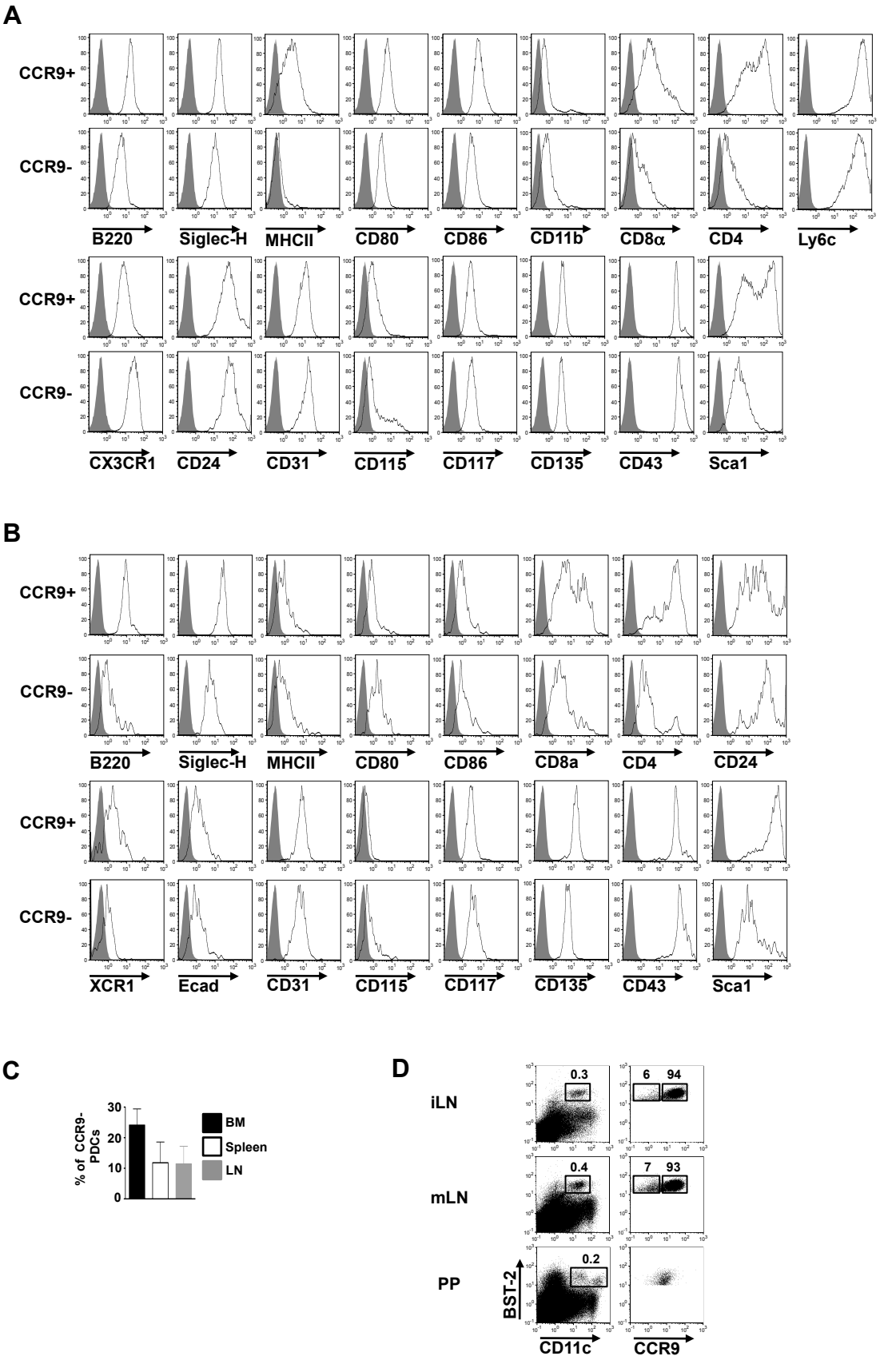
Fig. S1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CX3CR1
CD24
CD31
CD115
CD117
CD135
CD43
Sca1
B
CCR9+
CCR9-
B220
Siglec-H
MHCII
CD80
CD86
CD8a
CD4
CD24
CCR9+
CCR9-
XCR1
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CD115
CD117
CD135
CD43
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C
% of CCR9- PDCs
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20
10
0
BM
Spleen
LN
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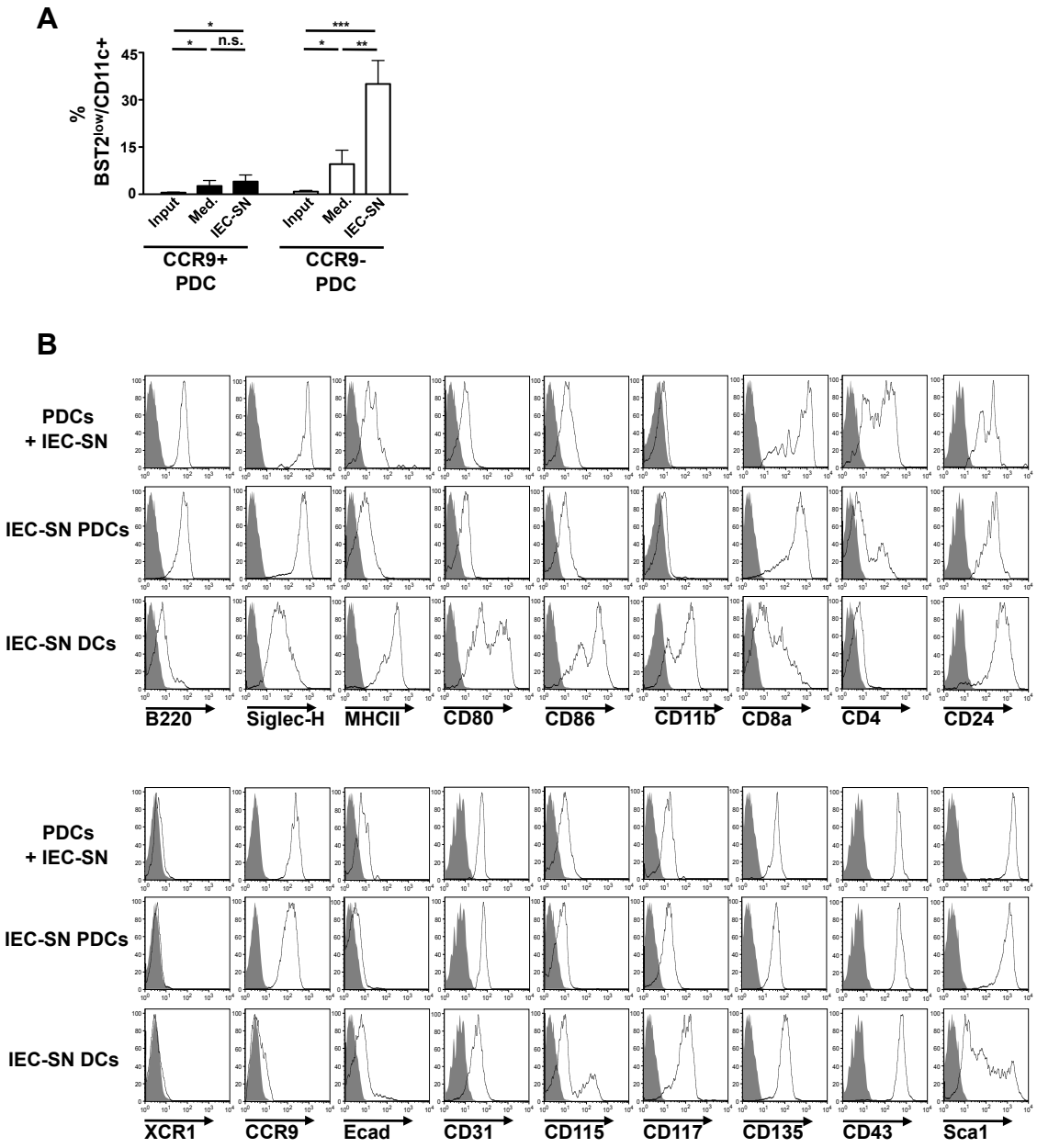
Fig. S2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Input
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+ IEC-SN
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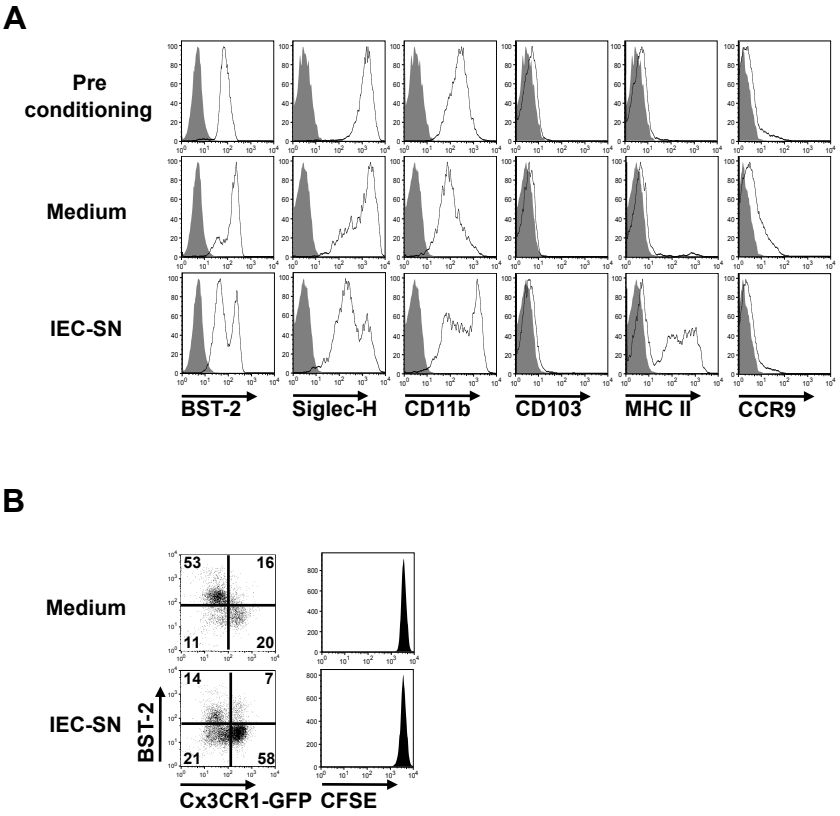
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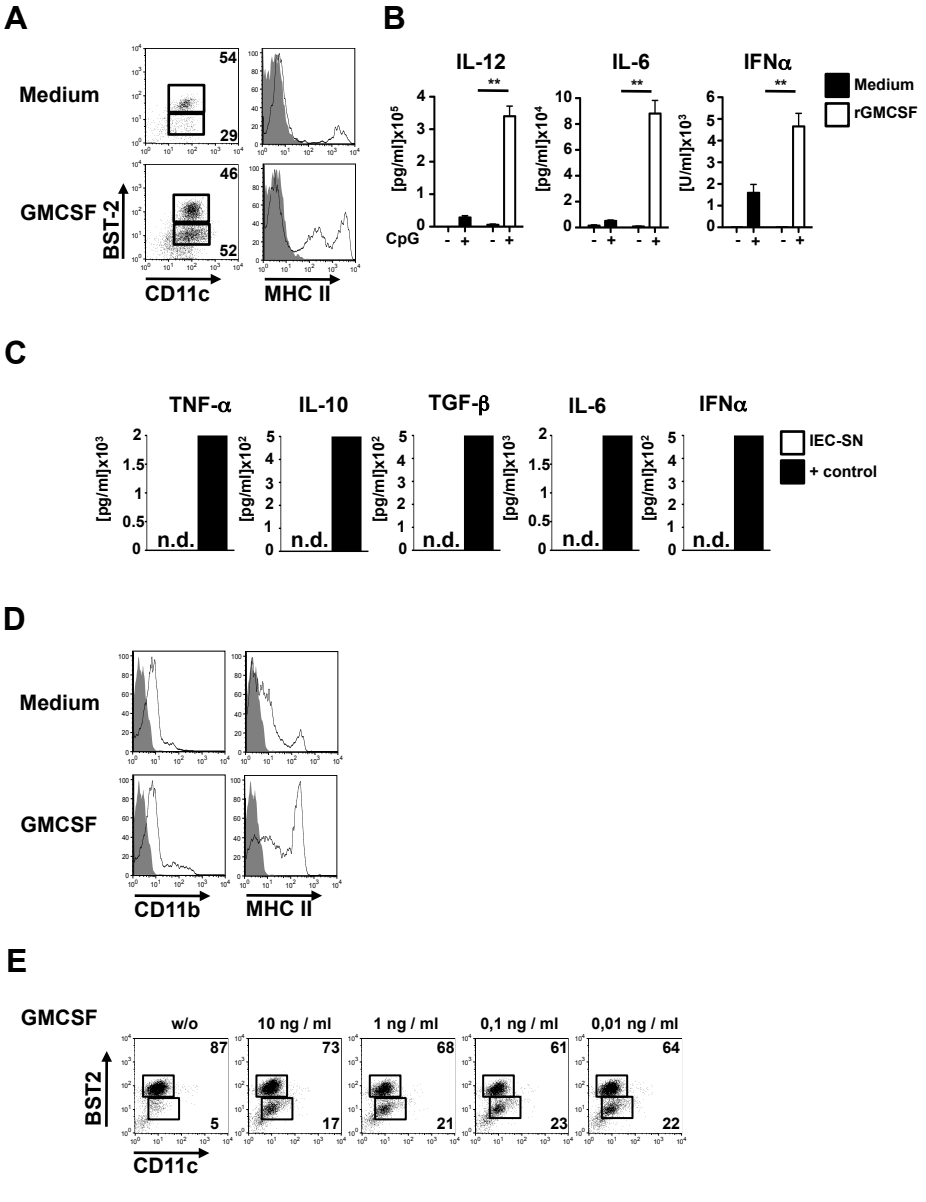
Fig. S4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IFNα
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n.d.
IEC-SN
+ control
D
Medium
GMCSF
CD11b
MHC II
E
GMCSF
w/o
10 ng / ml
1 ng / ml
0,1 ng / ml
0,01 ng / ml
87
5
73
17
68
21
61
23
64
22
BST2
CD11c

Fig. S1 Phenotypic characterization of CCR9$^+$ and CCR9$^-$ PDCs in BM and spleen

Expression of the indicated cell surface molecules was analysed by FACS in CCR9$^+$ and CCR9$^-$ BST2$^+$ CD11c$^+$ BM PDCs (A) and splenic PDCs (B). Percentage of CCR9$^-$ BST2$^+$ CD11c$^+$ PDCs in BM, Spleen and mesenteric lymph node (C, mean ± SD, n=3). FACS analysis of CCR9 expression in BST2$^+$CD11c$^+$ PI-negative cells in inguinal and mesenteric lymph nodes as well as Peyer's patches (D).

Fig. S2 Characterization of CD11b$^+$ MHCII^{high} CDCs generated from CCR9$^-$ BM PDCs upon exposure to IEC-SN

The percentage of BST2^{low} CD11c$^+$ cells generated from CCR9$^+$ and CCR9$^-$ BM PDC after 48 hrs culture with medium or IEC-SN versus before culture is shown (A, mean ± SD, n=5). CCR9$^+$ and CCR9$^-$ PDCs were sorted from primary BM cells and cultured with 50 % IEC-SN for 48 hrs. Expression of the indicated cell surface molecules was analysed by FACS in BST2^{high} PDCs present in the cultures of CCR9$^+$ PDCs with IEC-SN (PDCs + IEC-SN, upper row) as well as in BST2^{high} PDCs (IEC-SN PDCs, middle row) and BST2^{low} DCs (IEC-SN DCs, lower row) generated in cultures of CCR9$^-$ PDCs with IEC-SN (B).

Fig. S3 Phenotypic changes in PDCs derived from FL-DC cultures in response to IEC-SN

BST2^{high} CD11c$^+$ PDCs were sorted from day 7 FL-DC cultures and incubated with medium alone or 50 % IEC-SN for 48 hrs. Expression of the indicated markers before incubation and after exposure to medium or IEC-SN was measured by FACS. Results of one representative of 4 experiments are shown (A). Day 7 FL-DCs were

generated from BM cells of CX3CR1-eGFP reporter mice and were incubated with medium alone or 50 % IEC-SN for 48 hrs. Expression of CX3CR1 (eGFP) and BST2 was analysed by FACS (B, dot plots). Day 7 FL-DCs were labeled with CFSE and cultured with medium alone or 50 % IEC-SN for 48 hrs. CFSE staining was analysed by FACS to detect proliferation.

Fig. S4 Effect of recombinant GMCSF on phenotype and cytokine response of FL-DCs

FL-DCs were incubated with medium alone or medium supplemented with 1 ng/ml GMCSF for 48 hrs (A, B). Expression of CD11c, BST2 and MHC class II was determined by FACS (A). CpG 2216 (0.5 µM) was added after 24 h and cytokine were measured in the supernatants by ELISA after further 24 h of stimulation (B, mean ± SD, n=3). Cytokine concentration in the IEC-SN was measured by ELISA. For positive controls respective recombinant cytokines were used (C, one of two representative experiments shown). $CCR9^-$ PDCs sorted from primary BM cells were cultured with medium alone or medium supplemented with GMCSF (1 ng/ml) for 48 h. Expression of CD11b and MHC class II was measured by FACS. Results of one representative experiment are shown (D). $CCR9^-$ PDCs sorted from primary BM cells were cultured with medium alone or medium containing the indicated concentrations of recombinant GMCSF (range: 0.01 ng / ml – 10 ng / ml) for 48h. Expression of BST2 and CD11c was measured by FACS. Results of one representative experiment are shown (E).