Distinct precursors of the dendritic cell subtypes

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Abstract

Dendritic cells (DC) are antigen-presenting cells that are critical for the initiation and regulation of the immune response. Several DC subtypes within mouse spleen have previously been characterised and these include the plasmacytoid (pDC), and conventional DC (cDC) of the $CD8^+$ and $CD8^-$ subtypes. Each subtype appears to have a specialised role in the various arms of immunity and tolerance. Less clear is the process by which these DC develop from haematopoietic precursors, of the precursor stages and branch points from bone marrow (BM) stem cells to each of the peripheral DC subtypes.

The research described herein had the aim of identifying and isolating some of the intermediate precursors of DC, downstream of stem cells, and determining whether these differed in the steady-state versus inflammation. Particular was given to DC of the spleen. Experiments that sought the identity of such precursors involved both i) transfer of cell fractions that contained DC precursors into steady-state or inflamed recipient mice to assess their *in vivo* development at later times, and ii) analysis of an *in vitro* culture system to question whether it reflected development of the steady-state DC subtypes.

From the spleen of mice I isolated a novel DC precursor population that generated only $CD8^+$ and $CD8^-$ steady-state cDC upon transfer. These precursors had the phenotype $CD11c^{int}$ $CD43^+$ SIRP-α^{int} MHC II^- and, as they were strictly cDC-committed in their development, were termed pre-cDC. In contrast to many reports that monocytes are the precursors of DC, I found that pre-cDC were not classical monocytes. Pre-cDC were a relatively late-stage precursor population as they only transiently gave DC progeny within a few divisions. Importantly, there was already evidence of pre-commitment to either $CD8^+$ or $CD8^-$ cDC when separated by CD24, eatablishing that these subtypes represent different terminal lineages.

The DC subtypes and their precursors are rare in a mouse and so their extensive study has been hampered. I investigated an *in vitro* system in which bone marrow (BM) is cultured with flt3 ligand (FL) to generate DC. I discovered that the DC products

included, in addition to pDC, two cDC subtypes that were equivalent DC subtypes to *in vivo* steady-state $CD8^+$ and $CD8^-$ cDC. Thus this culture system reflected *in vivo* steady-state DC development.

These FL BM cultures were probed at earlier time points during culture for the precursor stages that gave rise to the DC subtypes. In this way, I identified two novel precursors that each generated pDC, $CD8^+$ cDC and $CD8^-$ cDC. These two precursors were related in a linear developmental pathway such that the upstream "pro-DC" divided and gave rise to "pre-DC", *en route* to DC generation.

As monocytes had been assumed to be precursors of steady-state DC, it was surprising that the precursors identified above were not monocytes. Therefore, I tested whether either of the characterised $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes were efficient precursors of spleen DC in the steady-state state. They were not. However, when mice were subject to inflammation, $Ly6C^{hi}$ monocytes were then able to generate DC in spleen. This was a novel DC subtype that was $CD11^{int}$ $CD11b^{hi}$ $Mac\text{-}3^+$, distinct from steady-state $CD11c^{hi}$ $CD11b^{lo}$ $Mac\text{-}3^-$ spleen cDC. These induced "inflammatory" DC appeared to actually be the *in vivo* 'myeloid' DC equivalent to those generated when monocytes or BM are cultured with granulocyte macrophage colony-stimulating factor (GM-CSF).

Thus, I have identified some of the key steps and branch points in the development of DC downstream of the early haematopoietic precursors. I have also dissected the contribution of different precursors to the DC subtypes in both the steady-state and during inflammation. The culture system I have developed will allow clonal analysis of DC development. It may also help guide the identification of the human equivalents of the various mouse DC precursors and DC subtypes, and hasten clinical applications.

Declaration

This is to certify that,

The thesis comprises the original work of the author

Due acknowledgment has been made in the text to all other material used

The thesis is less than 100,000 words in length, exclusive of figures, tables and references.

Shalin H. Naik

Contributions

My contribution to experiments in each of the Chapters was as follows:

Chapter 3: 95%

Chapter 4: 90%

Chapter 5: 80%

Chapter 6: 70%

Chapter 7: 95%

I acknowledge the important contribution of others to experiments presented here;

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Abbreviations

APC	antigen-presenting cell
APC	allophycocyanin
BM	bone marrow
BSA	bovine serum albumin
BSS	buffered balanced salt solution
$CD8^-$ cDC	$CD8^-$ conventional DC
$CD8^+$ cDC	$CD8^+$ conventional DC
cDC	conventional dendritic cell
CFA	complete Freund's adjuvant
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CLP	common lymphoid progenitor
CMP	common myeloid progenitor
CpG	cytosine (unmethylated)-phosphate-guanosine
CTL	cytotoxic T lymphocyte
DC	dendritic cell
EDTA	ethylenediamine tetra-acetic acid
FCS	fetal calf serum
Fig.	Figure
FITC	fluorescein-5-isothiocyanate
FL	flt3 ligand
FL-cDC	FL-culture-derived conventional DC
FL-DC	FL culture-derived DC
FL-pDC	FL-culture-derived plasmacytoid DC
flt3	*fms*-like tyrosine kinase
g	grams
GM-CSF	granulocyte macrophage colony-stimulating factor
GM-DC	DC derived from GM-CSF cultures
GMP	granulocyte/macrophage progenitor
hi	high

HSC	haematopoietic stem cell
i.p.	intra-peritoneal
i.v.	intravenous
IFN	interferon
IL	interleukin
int	intermediate
IRF	interferon regulatory factor
L	litre
LC	Langerhans cell
lo	low
LPS	lipopolysaccharide
mAb	monoclonal antibody
M-CSF	macrophage colony-stimulating factor
MHC	major histocompatibility complex
MHC I	MHC Class I
MHC II	MHC Class II
min	minutes
mL	millilitre
OTI	OVA-specific T cell with transgenic TCR for MHC I
OTII	OVA-specific T cell with transgenic TCR for MHC II
OVA	ovalbumin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pDC	plasmacytoid DC
PE	phycoerythrin
PI	propidium iodide
RCRB	red cell removal buffer
RPMI	Roswell Park Memorial Institute medium
TCR	T cell receptor
WEHI	The Walter & Eliza Hall Institute of Medical Research
WT	wild type

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Chapter 1 Introduction

Context

The immune system

When a pathogenic virus, bacteria or parasite infects the body, the immune system must eliminate it promptly to prevent its replication, spread, and harm to the host. The *innate* arm of the immune system is responsible for controlling the majority of infections in this way. This arm includes natural barriers such as the skin and mucosae, white blood cells such as granulocytes and macrophages, and the microbicidal products these cells secrete. As pathogens have co-evolved with the host and developed their own immune evasion mechanisms, innate immunity is often inadequate for their clearance. Enter the *adaptive* immune response. Another group of white blood cells, namely the B and T lymphocytes, are the effector cells of this arm by either producing neutralising antibody molecules (B cells), by helping this antibody generation process ($CD4^+$ helper T cells) or by killing infected cells directly (cytotoxic $CD8^+$ T cells). As a host cannot foresee exactly which pathogens it will encounter during its life, and in order to recognise the potentially infinite variations possible in the pathogen's appearance, higher vertebrates have developed a "random generator" of receptors for these lymphocytes. Of course, through this random generation, receptors that recognise the body's own tissue must also necessarily arise. A process called tolerance deletes such auto-reactive lymphocytes before they enter the circulation (*central tolerance*) as well as after (*peripheral tolerance*). Central tolerance primarily occurs in the bone marrow for B cells (Nossal, 1994) and the thymus for T cells (Gallegos and Bevan, 2006), and protects the body from the development of self-destructive lymphocytes. Thus the repertoire of lymphocytes in a steady-state individual has reactivity primarily for "non-self" and markedly reduced reactivity to "self" antigens (Janeway, 2002).

The need for antigen-presenting cells

Once *naïve* B and T lymphocytes enter the periphery they remain dormant until activated via their B cell receptor (BCR) or T cell receptor (TCR), respectively. Recognising a foreign antigen via these receptors in the context of "danger" is required to activate and then clonally expand antigen-specific lymphocytes (Matzinger, 1998). However, T cells with a TCR specific for a particular antigen, for example, are rare and in the order of 1 in 2 x 10^5 (Blattman et al., 2002). If lymphocytes relied on traversing all tissues in all locations of the body to scan for specific pathogens, the probably of a cognate reaction would be very low, such that an immune response may not be generated in sufficient time. Thus the need for compartmentalisation arises.

To restrict the area for the lymphocyte pool, the body has a concentrated 'lymphoid system', which makes up 10% of the body's volume. This includes the lymphoid organs such as the lymphatic vessels, spleen, Peyer's patches, lymph nodes and bloodstream, and is the region in which naïve lymphocytes are continuously circulating "in wait". A different mechanism to translate information about the "well-being" of the remaining 90% of the body to the lymphocyte pool is then required. This enormous task of monitoring infection in all tissues and organs peripheral to the lymphoid system (the *'periphery'*) is left to a separate subset of white blood cells, the professional antigen-presenting cells, or APC of the immune system. These cells are specialised in sampling infectious organisms in the periphery, then travelling via the lymphatic vessels or blood to the draining lymphoid organ, delivering antigen to cognate lymphocytes. Thus, by restricting the immune "headquarters" to this 10% of body volume, the probability of the right lymphocyte encountering the pathogen-bearing APC is increased by several orders of magnitude.

Enter the dendritic cell

Dendritic cells (DC) are the most potent form of T cell activating APC. DC form a vast network covering all tissues of the body but constitute less than 0.05% of total body cellularity. DC capture, process and then present both self and foreign antigen. Usually, the protein is not presented in an intact form, although there are exceptions (Dubois and Caux, 2005; Wykes et al., 1998). Rather, short peptides derived from the

antigen are bound in the groove of major histocompatability complex (MHC) molecules and deposited onto the surface of DC. Generally, intracellularly-derived antigen is presented via MHC Class I (MHC I) molecules for interaction with the TCR of cognate $CD8^+$ T cells, and exogenously-derived antigen via MHC Class II (MHC II) for interaction with the TCR of cognate $CD4^+$ T cells (Cresswell, 2005). While all cells have MHC I, only DC, B cells and some macrophages and monocytes also have MHC II (Bryant and Ploegh, 2004). These cell types are also the only cells to have T cell costimulatory molecules such as CD80 and CD86 in addition to MHC I and MHC II, and so are able to have an influence over cognate T cell fate (Greenwald et al., 2005). As such, these MHC II^+ cells are referred to as the APC.

Generally, T cells are unresponsive or tolerised when their T cell receptor (TCR) interacts with peptide-MHC complexes on DC that are in a non-activated or immature state (Forster and Lieberam, 1996; Kurts et al., 1996). This is crucial as self-peptides are continuously presented by DC in the steady-state for which T cells need to remain dormant (Wilson et al., 2004), and not all self-reactive T cells are deleted during development in the thymus. By contrast, if DC receive danger signals at the time of antigen acquisition, either from the pathogen directly or from the host inflammatory response, the levels of surface peptide-MHC II and T cell costimulatory molecules increase substantially and DC are converted to an activated or mature state. At this time, uptake of new antigen via endocytosis is shutdown to maintain a “snapshot” of the “dangerous antigen” that was encountered initially (Kampgen et al., 1991; Pierre et al., 1997; Wilson et al., 2004). Foreign peptides are found for the first time on the activated DC surface for TCR interaction. T cell activation rather than tolerisation to peptide-MHC ensues in these cases. If the mechanisms of central and peripheral tolerance have been successful during the non-infectious steady state, self-peptides that are also presented on DC during infection will have limited lymphocyte partners.

Thus, depending on the situation, DC antigen presentation can lead to either T cell tolerance, important for the prevention of autoimmunity, or to T cell activation, for the generation of adaptive immune responses (Heath and Carbone, 2001). Accordingly, DC are crucial for the fine regulation of the immune system in protecting the host not only from invading pathogens, but also from itself.

Not all DC are the same

The aforementioned biology of DC function was gathered largely by studying Langerhans cells (LC), the DC of the epidermis (Schuler and Steinman, 1985). Thus, the conversion of highly-endocytic immature DC in the periphery to poorly-endocytic mature T cell-activating DC in the lymphoid organs after activation is known as the Langerhans cell paradigm (Wilson and Villadangos, 2004). The LC paradigm has been assumed to apply to all DC of the body. As a result, surface MHC II and T cell costimulatory molecules levels have often been used to gauge the extent of *in vivo* DC maturation i.e. immature MHC II^{int} $CD80/86^{lo}$ DC versus mature MHC II^{hi} $CD80/86^{hi}$ DC.

However, the DC lifecycle appears to be more complex than this simple binary phenotype. For example, even in germ-free mice, which are free of all pathogens, it appears that lymph nodes, but not spleen, consist of both immature and already mature DC ((Huang and MacPherson, 2001) and Nick Wilson, personal communication). This observation of a subset of mature DC in the absence of infection is clearly at odds with the classic LC paradigm. Thus, it appears that not all DC follow the same rules (Wilson and Villadangos, 2004). This might reflect, and be one way to help demarcate, potentially distinct pathways of the DC lifecycle.

In fact, in recent years DC have been recognised not to be a homogeneous cell type performing all functions simultaneously. Rather, there appears to be a network of DC subtypes that are functionally, developmentally and spatially distinct for the disparate roles of the immune system (Shortman and Liu, 2002; Vremec et al., 2000; Vremec et al., 1992). Understanding this functional heterogeneity of DC is of academic interest but, as DC are such potent T cell activators or repressors, also becomes important when considering DC as a potential target of immune therapy such as for vaccination or the treatment of autoimmune disease. Several applications of DC in therapy are already in progress but have had mixed success (Hsu et al., 1996; Nestle et al., 1998; Thurner et al., 1999). An increase in knowledge of the DC subtypes and their function will be important for their clinical application.

Unlike the growing knowledge of DC function, relatively little is known about the origin of DC, about the steps in development leading from haematopoietic stem cells in the bone marrow (BM) to functional DC in the periphery. Recognition of the precise processes leading to the DC subtypes would help to further understand, and perhaps manipulate in patients, the functionally distinct DC subtypes in order to skew the immune response favourably.

Haematopoiesis

All the red and white blood cells of the body, including DC, are generated from haematopoietic stem cells (HSCs) (Spangrude et al., 1988). The very earliest self-renewing HSCs are present primarily in the BM but also exist in small numbers in the blood and spleen (Metcalf, 1971). These HSC divide and asymmetrically branch giving rise to downstream progenitors that progressively commit to a particular lineage. The capacity for a progenitor's self-renewal is concomitantly lost during this process. Ultimately, all of the individual haematopoietic lineages are differentiated in this fashion with little evidence of interconversion (Wagers and Weissman, 2004). In some cases, haematopoietic progenitors downstream of the BM HSC can seed other organs and reconstitute them with a particular set of cell types. This occurs, for example, in the case of early T cell progenitors that seed the thymus (Bhandoola and Sambandam, 2006). As far as this concept applies to DC, it is not yet clear how the many DC subtypes in different locations around the body arise from different progenitors. Nor is it clear whether DC progenitors come in a steady flux from the BM directly or are already resident and long-term within the organ itself. Furthermore, a different set of rules may apply when considering DC in the steady-state compared with situations of infection or inflammation. Knowledge of these processes would help to elucidate the developmental and functional specialisations of DC in the various arms of the immune response.

Dendritic cells of the mouse

Classification

Comparing first by the tissue location of DC, and then secondly using the level of maturity of the DC subtypes, some insight into DC lineage and lifecycle in the mouse can be achieved. DC are categorised first according to location as; 1) those that migrate from the periphery via the lymphatics to draining lymph nodes, classified as *migratory*, 2) those that are lymphoid organ-resident, or simply *resident,* and 3) those DC that are *circulating* in the blood stream (Fig. 1.1).

Migratory DC

There is a family of DC that innervate tissues peripheral to the lymphoid organs that drain them. These include the DC of the skin (Langerhans cells) and DC of the dermis, mucosae and other interstitial tissues (interstitial DC). Such DC are referred to as migratory since, even in germ-free mice, they constantly travel from peripheral tissues, via the lymphatics, to the draining lymph node ((Huang and MacPherson, 2001) and Nick Wilson, personal communication). This process occurs at a basal rate in the steady-state (Hemmi et al., 2001; Henri et al., 2001; Jakob et al., 2001) but can be enhanced during inflammation (Cumberbatch et al., 1997; Forster et al., 1999; Geissmann et al., 1999; Roake et al., 1995). Migratory DC are in an MHC II^{int} immature state in the periphery but are found in a mature MHC II^{hi} state by the time they reach the draining lymph node. Here, they are discriminated as $CD11c^{int}$ $CD45RA^{-}$ $DEC205^{+}$ MHC II^{hi} (Henri et al., 2001). Migratory DC are distinctively absent from the spleen as this organ drains the circulatory system but not any other tissues (Henri et al., 2001). This suggests that the precursors for migratory DC likely seed the peripheral tissues at some time prior to development and subsequently migrate to the draining lymphoid organ, rather than developing within the lymphoid organ itself.

Resident DC

Resident DC are located in all lymphoid organs of the mouse, including the spleen, and are found in an MHC II^{int} immature state (Wilson et al., 2003). This allows their clear distinction from the mature MHC II^{hi} migratory DC (Henri et al., 2001).

Resident DC are the CD11c^{hi} CD45RA^{lo} MHC II^{int} conventional DC (cDC), which can be further broken into two broad subsets; the CD8^{+} cDC and the CD8^{-} cDC (Vremec et al., 2000; Vremec et al., 1992). The CD8^{-} cDC can be further divided into CD4^{-} and CD4^{+} subsets, although the function and gene expression profile of these two appears very similar, and so often remain grouped as CD8^{-} cDC ((Edwards et al., 2003a) and Mireille Lahoud, personal communication). Although it has long been presumed that cDC migrate from the blood and subsequently seed the spleen and lymphoid organs [Cavanagh, 2002 #456], CD11c^{hi} cDC are actually not found in significant numbers in blood or BM of mice (O'Keeffe et al., 2003; Steinman and Cohn, 1973). Therefore, it is more likely that resident cDC develop or mature *in situ* from a phenotypically distinct form of cDC precursor.

Circulatory DC

The third general DC type is the circulating DC and includes the type-1 interferon producing plasmacytoid pre-DC (pDC). In the steady-state, pDC have a characteristic plasma cell-like morphology, not dendritic, and are found in many tissues of the mouse including blood, thymus, BM, liver, and the T cell areas of lymphoid organs(Asselin-Paturel *et al.*, 2001; Lian *et al.*, 2003; Martin *et al.*, 2002; Nakano *et al.*, 2001; Nikolic *et al.*, 2002; O'Keeffe *et al.*, 2002a; O'Keeffe *et al.*, 2003; Okada *et al.*, 2003). This cell type is CD11c^{int} CD45RA^{+} DEC205^{-} 120G8^{+} MHC II^{lo} (Asselin-Paturel et al., 2001; Grouard et al., 1997). There are phenotypically distinct subsets even within pDC, some of which are also functionally distinct, and these include subsets separated by CD4, CD8 and Ly6C expression ((O'Keeffe *et al.*, 2002a; Yang *et al.*, 2005a) and David Vremec, personal communication). pDC appear to circulate around the tissues akin to naïve lymphocytes in a CD62 ligand-dependent manner (Nakano et al., 2001). Upon activation by certain TLR ligands or infectious agents (Bjorck, 2001; Diebold et al., 2004; Jarrossay et al., 2001; Kadowaki et al., 2001; Krug et al., 2001a), and irrespective of the pDC subset, they become “dendritic” in appearance and convert uniformly to CD11c^{hi} CD45RA^{lo} CD205^{-} CD8^{hi} 120G8^{+} MHC II^{int} (O'Keeffe *et al.*, 2002a). In this CD8^{+} form they can still be discriminated from the resident CD8^{+} CD205^{+} 120G8^{-} cDC.

	Bone marrow	Blood	Spleen	Skin-draining lymph node	Non-skin-draining lymph node
Resident			$CD8^+$ cDC $CD8^-$ cDC	$CD8^+$ cDC $CD8^-$ cDC	$CD8^+$ cDC $CD8^-$ cDC
Migratory				Interstitial DC Langerhans cells	Interstitial DC
Circulating	pDC $Ly6C^{hi}$ Mo	pDC $Ly6C^{hi}$ Mo $Ly6C^{lo}$ Mo	pDC $Ly6C^{hi}$ Mo $Ly6C^{lo}$ Mo	pDC ? $Ly6C^{hi}$ Mo ? $Ly6C^{lo}$ Mo	pDC ? $Ly6C^{hi}$ Mo ? $Ly6C^{lo}$ Mo

Figure 1.1 Tissue distributions of the monocyte and DC subsets in the steady-state.

The resident conventional DC, including $CD8^+$ and $CD8^-$ subtypes are present in most lymphoid organs throughout the body. The migratory interstitial DC are found in all tissue-draining lymphoid organs, except the spleen. Migratory Langerhans cells are found only in lymph nodes that drain epidermal tissue. The circulatory pDC are found in most lymphoid organs throughout the body including BM, spleen and all lymph nodes. Monocytes also circulate, although $Ly6C^{lo}$ monocytes are generally found only in blood, while reserves of the less mature $Ly6C^{hi}$ subset are also present in BM. Whether monocytes extravasate lymphoid organs in the steady-state or are simply in circulating, is not clear.

DC function

Why so many subsets?

As an organism becomes more complex, so to does its capacity to "play host" and provide a niche for a greater variety of infectious microbes. Lower order animals have relatively basic innate immune mechanisms reflecting their limited vulnerability to pathogens. Only since the emergence of the jawed vertebrates has the adaptive immune system developed the important cytotoxic T and antibody-forming B lymphocytes (Hansen and Zapata, 1998; Matsunaga and Rahman, 1998). To accommodate for increased pathogen diversity, the immune system has had to develop its own range of mechanisms to link the unique signatures of the pathogens themselves, or their resulting pathology, to the adaptive immune response. Dendritic cells appear to be one of the most important links between the innate and adaptive arms.

DC must contend with several variables when providing such a link including 1) the distinct kinds of bacterial, viral or parasitic infections that need to be recognised, 2) the distinct routes of infection whether it be via the skin, tissue, mucosa or blood, and 3) the distinct kinds of immune response that need to develop in accordance with the pathogen and its route of infection.

Information about the nature of the infecting organism can be provided by their intrinsic set of pathogen-associated molecular patterns (PAMPs) that are distinct from the host (Janeway, 1989). Pattern recognition receptors (PRR) on DC are able to translate PAMP information accordingly (Medzhitov and Janeway, 1997). Toll-like receptors (TLR) are one such form of PRR that have been evolutionarily conserved and are present on DC (Medzhitov et al., 1997). Importantly, the DC subsets have distinct patterns of TLR and other PRR so that each subset appears to be specialised to particular types of pathogens (Diebold *et al.*, 2004; Edwards *et al.*, 2003b; Schulz *et al.*, 2005).

While subdivision is apparent, it is not clear why an animal must have so many distinct DC types. Conceivably, the one kind of DC could arm itself with all the necessary mechanisms and PRR to deal with all situations, acting only according to the profile of PAMPs and inflammatory mediators it encounters. One explanation of the subtypes is that a functionally distinct ancestral "DC" subtype developed spontaneously in addition to, and perhaps from, the pre-existing "DC". Having benefited the host its new role, and perhaps for a new pathogen, the advantageous "division of labour" between the subtypes might have then been maintained in the population. Certainly several mammalian species appear to have maintained functionally distinct and similar DC subsets (Epardaud et al., 2004; Hope et al., 2001; Liu et al., 1998). Of course, this is only one simple interpretation of many. The issue is most definitely complex and one better suited to the dedicated immune evolutionist. Nevertheless, the unique DC subtypes are present and will be briefly summarised according to their many and varied roles.

Langerhans cells

Originally identified by Paul Langerhans in 1868, Langerhans cells (LC) are stellate cells found only in the epidermis of skin, and make up to 2-4% of total cellularity (Langerhans, 1868). An immunological role for LC was not described until the 1970's and 80's with the observations that they expressed Fc and complement receptors (Stingl et al., 1977), MHC II (Klareskog et al., 1977; Rowden et al., 1977), could participate in allergic contact hypersensitivity (Silberberg et al., 1976), and were efficient APC (Green et al., 1980; Stingl et al., 1978a; Stingl et al., 1978b). LC were thus drawn in as a member of the dendritic cell family (Schuler and Steinman, 1985). However, several features apart from their location still distinguished LC from other DC. Most notably was the presence of tennis racket-shaped Birbeck granules (Birbeck, 1961), for which a function has still not been ascribed, and expression of Langerin (CD207), which colocalises with these structures (Valladeau et al., 2000).

LC are a long-lived population in the epidermis and constitutively migrate from the skin to the draining lymph nodes (Ruedl et al., 2000; Ruedl et al., 2001; Salomon et al., 1998). BrdU-labelling studies have extended these findings indicating that 25%-60% of LC in the skin and draining lymph nodes turn over every 2 weeks (Henri et

al., 2001). Lymph nodes appear to be the final port of call for LC where they probably undergo Fas/FasL-dependent apoptosis (Kawamura et al., 2000).

LC that reach the lymph node in the steady-state appear to carry skin antigens (Hemmi et al., 2001). In this capacity they may be involved in steady-state tolerance of skin-antigen-specific T cells that have escaped thymic selection (Hawiger et al., 2001). To further assess the role of LC in tolerance, the use of transgenic mice where ovalbumin (OVA) protein or peptide is under the control of the K14 keratinocyte-specific promoter have been used. These studies have produced mixed conclusions as to whether LC participate in tolerance for skin-antigens (Mayerova et al., 2004; Shibaki et al., 2004).

Conversely, whether LC are important in T cell priming for the generation of immunity is also not clear. Despite early studies demonstrating their role as APC using contact hypersensitivity as the primary readout (Pehamberger et al., 1983; Silberberg et al., 1976; Stingl et al., 1978a), demonstrating the *in vivo* contribution to the immune response is still lacking. During conditions of inflammation, LC migration is notably enhanced and is dependent on several chemokines, suggesting LC are probably important. Furthermore, LC can internalise pathogens *in vitro* and *in vivo* (Blank et al., 1996; Labuda et al., 1996; Wu et al., 2000). However, despite some indirect evidence (Moll et al., 1993; Romani et al., 1989), direct evidence for T cell priming is infectious scenarios is still lacking. In fact, in studies of cutaneous viral infection LC were not important for T cell priming (Allan et al., 2003; Zhao et al., 2003).

Interstitial DC

Interstitial DC are found throughout the body and comprise those found in all peripheral tissues excluding the epidermis. They include the well-characterised dermal DC of the skin (Nestle et al., 1993) and DC of the mucosae (MacPherson and Liu, 1999). Such DC share some characteristics with LC but also have many differences (Dupasquier et al., 2004; Duraiswamy et al., 1994; Valladeau and Saeland, 2005). An example of their similarity is that interstitial DC can also migrate, even in the steady-state and germ-free situations, to draining lymphoid organs where

they appear to be in a MHC II^{hi} mature state. Here they can be distinguished as CD11c^{int} MHC II^{hi} DEC-205^{int} DC (Henri et al., 2001; Huang and MacPherson, 2001). There are indications that interstitial DC are an immune regulating APC, based on response to injected antigen or hypersensitivity responses (Bennett et al., 2005; Itano et al., 2003). While a role for interstitial DC is more than likely, a definite role during an actual infection is not yet defined. There is some indication of their involvement in intravaginal HSV-2 infection (Zhao et al., 2003) and Leishmania (Filippi et al., 2003; von Stebut et al., 2000).

Resident CD8$^+$ DC

The initial *ex vivo* isolation of mouse splenic DC often included the removal of CD4 and CD8-expressing cells, originally presumed to deplete only T cells. However, careful examination of all CD11c^{hi} cells of the spleen and thymus revealed that a significant proportion of cells expressed either of these T cell markers. CD8 expression was found on 10-15% of CD11c^{hi} DC as an $\alpha\alpha$ homodimer rather than the $\alpha\beta$ heterodimer found on the majority of T cells (Vremec et al., 1992). CD8$^+$ cDC are found primarily in the T cell areas of the spleen and lymph nodes in the steady-state, unlike other resident cDC (De Smedt et al., 1996). They probably correspond to the interdigitating cell (IDC) that had been described previously (Agger et al., 1992; Crowley et al., 1989; Veerman and van Ewijk, 1975).

One special role for CD8$^+$ cDC is in the maintenance of tolerance to self. It is known that not all tissue-specific antigens are expressed in the thymus during T cell negative selection (Starr et al., 2003). Indeed some autoreactive T cells against several tissue antigens are present at low frequency in the periphery, so there is need for a peripheral mechanism of self-tolerance (Miller et al., 1998). Peripheral tolerance was initially found to be mediated by a haematopoietic cell (Adler et al., 1998; Kurts et al., 1996; Kurts et al., 1997) and was later confirmed that DC were responsible (Hawiger et al., 2001; Kurts et al., 2001). The proximity of CD8$^+$ cDC to T cells in the steady-state placed them in prime position as the APC involved in peripheral T cell tolerance (Steinman et al., 1997). Further evidence came from mice transgenic for the rat insulin promoter (RIP) driving pancreatic ovalbumin (OVA) expression. In these mice, injected OVA-specific CD8 T cells (OTI) transiently proliferated prior to

deletion in the pancreatic lymph node. Interestingly, only $CD8^+$ DC from this lymph node were able to drive OTI proliferation *ex vivo,* suggesting this DC subtype was the tolerogenic APC (Belz et al., 2002). More recently, the diabetes-prone NOD mouse was shown to have a specific reduction in the $CD8^+$ cDC subset. DC numbers could be boosted by prior treatment with FL treatment and was concomitant with the reversal of diabetes, implicating $CD8^+$ cDC in peripheral tolerance (O'Keeffe et al., 2005a; Vasquez et al., 2004).

As DC were thought only to be capable of presenting endogenous antigen via MHC I and exogenous antigen via MHC II, the question arose of how exogenous self-antigen could access the MHC I pathway of DC for the induction of tolerance. Interestingly, one of the unique features of $CD8^+$ DC is their ability to cross-present antigen, which occurs when exogenous antigen is diverted to the MHC Class I pathway and thus able to signal the TCR of $CD8^+$ T cells (Bevan, 1976; den Haan et al., 2000; Pooley et al., 2001). This process appears to be important not only for peripheral T cell tolerance but also other scenarios. The precise molecular mechanisms of cross-presentation are ill defined and controversial. (Groothuis and Neefjes, 2005)

In apparent contradiction to their role in T cell tolerance, $CD8^+$ DC seem to be the main T cell priming DC in several viral and bacterial infections (Allan et al., 2003; Belz et al., 2004; Bevan, 1976; den Haan et al., 2000). How can the one DC subtype perform the dual yet opposing roles of immunity and tolerance? A differential activation state and cytokine production profile within the one $CD8^+$ cDC population is probably the factor that guides CD8 T cell fate.

While $CD8^+$ cDC from the mouse express TLR4 and TLR9, they are the only DC to express TLR3, but fail to express TLR7 (Edwards et al., 2003b). In this way $CD8^+$ cDC may be specialised for dsRNA viruses (TLR3 agonist), bacteria and DNA viruses (TLR9 agonist) but not ssRNA viruses (TLR7 agonist) (Iwasaki and Medzhitov, 2004; Schulz et al., 2005). In any case, stimulation of $CD8^+$ cDC with TLR agonists or pathogens induces upregulation of MHC II and costimulatory molecules, as well as large amounts of IL-12p70 (Hochrein et al., 2001; Reis e Sousa et al., 1997). This cytokine is important for $CD4^+$ Th1 and cytotoxic $CD8^+$ T cell priming, and memory generation (Curtsinger et al., 2003; Maldonado-Lopez et al.,

1999). Interestingly, $CD8^+$ DC are specialised in this production of the active p70 form in response to several TLR agonists (Hochrein et al., 2001). Therefore, TLR or other danger signals probably provide the switch for $CD8^+$ DC from a tolerogenic to an immunogenic state.

There has yet to be a function ascribed for CD8 in mouse DC (Kronin et al., 1997). In fact, most other animals to date do not express CD8 on their DC. Rat, human, and bovine DC fail to express this molecule on their splenic DC (Howard et al., 1997; Liu et al., 1998; MacDonald et al., 2002), whereas sheep DC (Epardaud et al., 2004) do, suggesting expression is species-specific. Does this mean that mouse and sheep $CD8^+$ cDC are some evolutionary anomaly? Or do all other animals, including humans, have an equivalent DC type that happens not express CD8? This will be very important to determine considering the importance of $CD8^+$ DC in the various arms of the murine immune response.

Resident $CD8^-$ cDC

(includes $CD4^+CD8^-$ and $CD4^-CD8^-$)

$CD4^+CD8^-$ and $CD4^-CD8^-$ lymphoid tissue-resident cDC are often regarded together as the one $CD8^-$ cDC subtype due to their relatedness (Edwards et al., 2003a; Vremec et al., 2000). They are the most numerous of the lymphoid organ-resident cDC, comprising roughly 80% of the total. By contrast to T cell-resident $CD8^+$ cDC, $CD8^-$ cDC tend to be found in the marginal zones (Agger et al., 1992; De Smedt et al., 1996; Metlay et al., 1990). They uniquely express the chemokine receptor CCR6, which appears to colocalise them to this region where its binding partner MIP-3α is also expressed ((Iwasaki and Kelsall, 2000) and Anna Proietto, personal communication), although some are also seen in the T cell areas in the steady-state. However, upon stimulation by LPS or other TLR agonists, $CD8^-$ cDC relocalise to the T cell areas of lymphoid organs (De Smedt *et al.*, 1996; Reis e Sousa *et al.*, 1997). They also secrete large amounts of the inflammatory chemokine RANTES, MIP-1αβ and MIP-1β (Proietto et al., 2004). However, where $CD8^-$ cDC are crucial for the control of a particular infection is yet to be determined.

Circulatory pDC

The best-described role of pDC is in pathogen surveillance. They become activated upon exposure to viruses (Siegal et al., 1999), bacteria (Bjorck, 2001; O'Keeffe *et al.*, 2002a) and certain pathogen-associated molecular patterns including CpG-containing DNA (a TLR-9 agonist) and ssRNA (a TLR-7 agonist) (Diebold et al., 2004; Jarrossay et al., 2001; Kadowaki et al., 2001; Krug et al., 2001b). This activation results in the production of inflammatory chemokines (Proietto et al., 2004) and cytokines, including a characteristic interferon alpha (IFN-α) burst, important for the control of viral replication and activation of nearby DC. pDC also have an overlapping role as antigen-presenting cells expressing low levels of MHC I, MHC II and costimulatory molecules in the steady state, all of which are upregulated upon activation (Asselin-Paturel et al., 2001; Grouard et al., 1997). At such time, pDC concomitantly acquire dendritic processes and are thus instilled with the potential for T cell activation.

While it appears pDC are poor at presentation of exogenous antigen and cross-presentation, they do seem to be effective at $CD8^+$ T cell activation if infected directly (Salio et al., 2004) or when encountering an antigen-experienced T cell (Krug et al., 2003). Moreover, a role for IFN-α production by pDC has been ascribed as an essential 'third signal' for the generation of CD8 T cell memory, in addition to the two signals proved by cDC peptide-MHC and costimulation (Curtsinger et al., 2005; Yoneyama et al., 2005). Collectively, these recent studies of pDC enlist them as crucial mediators linking the innate and adaptive arms of the immune system. As pDC are found in most organs of the body, the location of pDC precursors has been difficult to pinpoint.

DC development

Introduction

DC are of haematopoietic origin. Any further conclusions about DC development are met with great debate. This is partly due to confusion about the numerous DC subsets, their different locations, their maturation states, the distinct assumptions in

human versus mouse studies, and whether the many *in vitro* conclusions about their development actually apply *in vivo*.

A simplified view of haematopoiesis is that all lineages derive from BM-resident precursors, continuously migrating into the bloodstream then seeding their respective organs before giving rise to progeny. In support of this are the numerous studies whereby a chosen BM precursor or blood-derived population is extracted from its natural state and transferred *in vivo*, conclusions being drawn about the subsequent product cells. However, haematopoietic process may not be as simple as this.

Early progenitors

The initial descriptions on the origin of splenic DC by Steinman and Cohn had suggested that DC were radiosensitive and that they could be reconstituted by precursors in both bone marrow and spleen, but not thymus (Steinman and Cohn, 1973, 1974; Steinman *et al.*, 1974). A BM origin for Langerhans cells was also demonstrated (Katz et al., 1979). Subsequent to these studies, it had only been established that DC precursors were MHC II^- (Inaba et al., 1992a). Since these initial observations, information about the precise precursor populations and steps in development of DC has been obscure.

Studies of T-cell development found that the earliest thymic-resident $CD4^{lo}$ progenitor of T cells (Wu et al., 1991) could, unexpectedly, reconstitute the $CD8^+$ DC lineage of the thymus when injected intra-thymically (*i.t.*), or splenic DC when injected intravenously (*i.v.*) (Ardavin et al., 1993). Some but not all T cell progenitor stages downstream of $CD4^{lo}$ T cell precursors could also reconstitute the thymic and splenic $CD8^+$ DC lineage (Wu et al., 1996). This led to the notion that $CD8^+$ DC were of lymphoid origin while the $CD11b^+$ DC lineages ($CD8^-$ cDC and LC) were of myeloid origin.

This concept was challenged with the isolation of clonogenic precursors of either the lymphoid (Kondo et al., 1997) or myeloid (Akashi et al., 2000) lineage. Common lymphoid precursors (CLP) were identified as BM-resident lin^- $Sca\text{-}1^-$ $c\text{-}kit^+$ $IL\text{-}7R^+$ BM cells able to reconstitute the T, B and NK lineages both *in vitro* and upon transfer

into irradiated recipients. When tested for DC potential, CLP could reconstitute not only the $CD8^+$ DC and pDC of thymus and spleen (D'Amico and Wu, 2003), but also splenic $CD8^-$ cDC (Manz *et al.*, 2001b; Wu *et al.*, 2001). However, in some (Wu et al., 2001) but not all (Traver et al., 2000) studies, there seemed to a bias towards splenic $CD8^+$ DC generation by CLP.

Similarly, a clonogenic common myeloid precursor (CMP) ($lin^-CD34^+Fc\gamma R^{lo}$) in BM was defined that gave rise to the myeloid lineages via two distinct intermediates – the granulocyte/macrophage precursor (GMP) ($lin^-CD34^+Fc\gamma R^{hi}$) and the megakaryocyte/erythroid precursor (MEP) ($lin^-CD34^-Fc\gamma R^{lo}$) (Akashi et al., 2000). Interestingly, CMP but not the GMP or MEP intermediates, could directly give rise to pDC, $CD8^-$ and $CD8^+$ cDC upon transfer to irradiated recipients *in vivo* (Manz *et al.*, 2001b; Traver *et al.*, 2000; Wu *et al.*, 2001). The observation of $CD8^+$ cDC and pDC generation from myeloid precursors changed the dogma that the these two lineages were "lymphoid". Moreover, that DC were generated independent of the defined GMP/MEP intermediates suggested that there may be a unique route for a subset of CMP to DC directly.

To seek out a potential direct route, heterogeneity was first sought within the total CMP population. In light of the importance in DC generation of surface fms-like tyrosine kinase 3 (flt3), also known as flk-2, various progenitor populations were assessed for expression of this molecule. Within CMP only 30% were found to be $flt3^+$ (D'Amico and Wu, 2003). Interestingly, only the $flt3^+$ fraction was able to generate all cDC and pDC lineages upon transfer *in vivo* (D'Amico and Wu, 2003; Karsunky et al., 2003). Similarly, when assessed on CLP only the $flt3^+$ fraction, which constituted 70% of the total CLP pool, could generate the DC lineages. Thus not only was the heterogeneity of CMP established, but also a potential GMP/MEP-independent route of myeloid generation.

Collectively, these studies showed that pDC, $CD8^+$ and $CD8^-$ cDC were unique within the haematopoietic system in that they could be derived from both myeloid and lymphoid-restricted progenitors. Why this has evolved in not clear. Considering the importance of DC in linking the innate and adaptive arms, perhaps it is beneficial for

the host to allow development from both of the evolutionarily distinct lymphoid and myeloid lineages.

However, whether the CMP, CLP, both, or perhaps a different precursor contribute to the steady-state situation is still not clear. Although it has been proposed that CMP are 10-fold more numerous and therefore the major contributing DC precursor (Manz *et al.*, 2001b), this says nothing about any fine control in the release, if any, of these precursors from BM into the bloodstream during the steady-state.

More recently, the classical model of cell fate deriving from either CMP or CLP was shown to be an oversimplification. A lin$^-$ Sca-1^{hi} c-kit$^+$ (LSK) flt3$^+$ clonal precursor was identified in BM that had already lost its megakaryocyte and erythroid (Mk/E) potential, but retained granulocyte, macrophage, T and B cell development (Adolfsson et al., 2005). This "lymphoid-primed" multipotent progenitor (LMPP) was proposed to occur prior to any lineage divergence into either lymphoid or myeloid fates, but downstream of the long-term HSC and the Mk/E branch. This model has support from several other studies that suggest Mk/E diverge relatively early (Nutt et al., 2005; Singh, 1996; Yang et al., 2005b). LMPP appear to be upstream of CLP, but the precise relationship of this population to CMP is unknown (Akashi et al., 2005). Considering DC only derive from flt3$^+$ progenitors, flt3$^+$ LMPP may be a better candidate and model population to address subsequent branching of DC development, rather than the dual considerations of flt3$^+$ CMP or CLP.

Late progenitors.

Irrespective of the model for the early progenitors, the precise steps in DC development downstream are even less clear. Continuous BrdU labelling experiments, in which BrdU only incorporates into the DC of dividing cells, were performed in mice to determine if the DC subsets were simply developmental steps in a linear progression or whether they developed independently from separate precursors (Kamath et al., 2000). As most DC are post-mitotic, the assumption was that if, for example, CD8$^+$ cDC arose from the CD8$^-$ cDC then there would be a lag in CD8$^+$ cDC labelling concomitant with the time required for CD8$^-$ to CD8$^+$ cDC conversion. This was found not to be the case, and all DC subsets labelled almost immediately,

indicating that each DC subset developed independently, at least as far back as the last dividing precursor. In addition, the labelling kinetics suggested that most splenic DC turned over within 3-5 days with $CD8^+$ cDC turning over the fastest. However, the immediate precursors that give rise to the many DC subtypes with these kinetics are poorly characterised.

Langerhans cells

Langerhans cells (LC) are the prototypic epidermal DC. Whether LC develop from precursors continuously migrating from the BM or whether they arise form long-term skin-resident precursors was not clear. Initial suggestions of a blood-derived precursor came from mice analysed at late timepoints after irradiation (Frelinger et al., 1979; Katz et al., 1979; Stingl et al., 1980). However, other studies of skin transplants and ^{3}H Thymidine incorporation after UV irradiation (Giacometti and Montagna, 1967; Krueger et al., 1983), suggested that LC either proliferated themselves or could develop from skin-resident precursors. More recent studies of parabiotic mice (which is when two mice are surgically joined by the skin and so share their bloodstream) demonstrated that neither mouse could develop partner-derived LC even after 24 weeks (Merad et al., 2002), even though LC turnover every 2-4 weeks (Kamath et al., 2002). This suggested that LC developed from a long-term skin-resident precursor and not an immediate blood or BM-derived precursor in the steady-state (Merad et al., 2002). Surprisingly, even after radioablation and reconstitution with donor BM, the majority of LC remained of host origin while all other DC were of donor origin, even out to 1.5 years. Only upon exposure to UV light were LC depleted from skin and reconstituted from circulating precursors, a process largely dependent on CCR2 expression by LC precursors and CCR2 ligand expression by inflamed skin (Merad et al., 2002). More recently, there has been direct demonstration that CCR2-expressing monocytes could act as LC precursors (Ginhoux et al., 2006).

LC have long been thought of as related to the mononuclear-phagocyte series (Berman and Winkelmann, 1978; de Fraissinette et al., 1988; Stingl et al., 1987). Certainly, many *in vitro* protocols exist where LC-like cells that express CD1a and Birbeck granules can be generated from blood precursors or monocytes in the

presence of GM-CSF (Caux et al., 1992), and enhanced in the presence of TGF-β (Geissmann et al., 1999). These studies demonstrate that $CD14^+$ monocytes down-regulate CD14 and upregulate CD1a and MHC II on their way to a LC phenotype. *In vivo* studies suggest similar requirements for a $CCR2^+$ monocyte precursor with the observations that newborn *op/op* mice (deficient for M-CSF and monocytes) have reduced LC (Cecchini et al., 1994), that $CCR2^{-/-}$ cells have slower reconstitution of LC (Merad et al., 2002), and that skin-resident $CD14^+$ cells can be LC precursors *ex vivo* (Larregina et al., 2001).

$CD8^+$ and $CD8^-$ cDC

While transfer of $CD4^{lo}$ precursors from the thymus could reconstitute $CD8^+$ DC in the thymus and spleen, it was unlikely that this rare precursor was responsible for $CD8^+$ cDC in all lymphoid organs (Ardavin et al., 1993). Several candidates of $CD8^+$ cDC have since been examined.

In the BM, Bruno *et al.* identified $CD11c^+$ $CD31^{hi}$ $Ly6C^{hi}$ $B220^+$ cells that could differentiate into DC-like cells in vitro with GM-CSF, or $CD8^+$ and $CD8^-$ DC in fetal thymic organ cultures (FTOC) (Bruno et al., 2001). No *in vivo* differentiation data was shown for this precursor. Wang *et al.* described a $CD11c^-$ $CD8^+$ cell type in spleen, lymph nodes and spleen that generated exclusively $CD8^+$ cDC in the spleen and thymus (Wang et al., 2002). Peak $CD8^+$ cDC generation was at 7-14 days post transfer into irradiated recipients, with donor progeny undetectable by 28 days. However, no reports have since confirmed this finding and attempts at recapitulation have failed (Li Wu, personal communication).

Kamath *et al.* demonstrated using BrdU labelling that $CD8^-$ cDC turned over more slowly and, therefore, could not be the primary direct precursor of the $CD8^+$ cDC, (Kamath et al., 2000). However, Martinez del Hoyo *et al.* suggested these two DC subsets were related developmentally in a one-way linear fashion (Martinez del Hoyo et al., 2002). They transferred magnetic bead-purified $CD8^-$ cDC into non-irradiated recipients and, four days later, detected $CD8^+$ cDC progeny. This was concomitant with DEC-205 upregulation and Mac-1 downregulation. However, recoveries in these assays were poor and, at best, only 0.14% of transferred $CD8^-$ cDC population

converted to $CD8^+$ cDC. This figure was well within the realms of a high-efficiency contaminant precursor.

The same group also claimed identification of a common DC precursor, able to reconstitute pDC, $CD8^+$ cDC and $CD8^-$ cDC lineages (del Hoyo et al., 2002). This precursor was identified as $CD11c^+$ $B220^+$ $CD11b^+$ MHC II^-. Upon transfer into irradiated mice these precursors developed, with division, into all DC subsets in the spleen. Peak DC generation was observed at 14 days with low levels by 7 days, suggesting these were not the immediate precursors of DC, rather an earlier form. However, upon further investigation, it was recognised that the majority of these cells were actually blood NK cells and so this preparation could not be confirmed as a homogeneous common DC precursor population (del Hoyo et al., 2004). The issue of whether there are common or separate precursors of the DC subsets is still an open question.

Considering all the *in vitro* evidence pointing towards monocytes as being precursors of DC (see below), another study attempted to go one step further and confirm this *in vivo.* Purified $Ly6C^{hi}$ monocytes from the BM were suggested to differentiate into $CD8^-$ and $CD8^+$ cDC upon transfer into irradiated recipients by 7-14 days (Leon et al., 2004). At the 7-day timepoint some $CD11c^+$ MHC II^+ DC progeny could be detected, although no CD8 expression was shown. By 14 days, the majority of donor-derived light density cells were DC, although recovery was poor with only 0.3% of light density cells being donor-derived. This finding is also yet to be confirmed in the literature.

Most but not all of the above studies have relied on the transfer of cells into lethally irradiated recipients. Irradiation is used to clear “niches” for the enhanced expansion of small numbers of early haematopoietic precursors that would otherwise not seed and proliferate. However, for that very reason, small amounts of contaminating but *bona fide* precursors within impure non-precursor fractions can give false results. Furthermore, significant DC generation was not seen at early timepoints after transfer for any of the studies (ie 2-5 days) only at later times (7-14 days) Such assays would preferentially address early precursors rather than immediate precursors.

The clearest evidence of resident DC development has also come from the study of parabiotic mice. Such mice were shown not to develop significant partner-derived splenic cDC even after 6 weeks (Kabashima et al., 2005) even though splenic resident cDC turnover every 3-5 days in the steady-state (Kamath et al., 2000). The authors attributed this to homeostatic proliferation of the $CD8^-$ cDC compartment. $CD8^+$ cDC, by contrast, did not appear to homeostatically proliferate. Another possibility for the independence of DC development from the bloodstream, not mutually exclusive from homeostatic proliferation, is that splenic cDC derive from a long-term spleen-resident precursor, not one that leaves the BM and seeds the spleen via the blood. The identity of such a precursor and its downstream intermediates are unknown. However, the relative independence from the bloodstream of spleen DC generation could change during conditions of inflammation when precursors might be called in from the BM.

pDC

pDC are BM-derived and appear very early during mouse development. Detectable numbers of pDC can be found in the thymus as early as embryonic day 17 and by day 1 in neonatal spleen (Dakic et al., 2004). pDC probably develop in the bone marrow and/or blood then traffic to the lymphoid organs via the blood as developed pDC, similar to B cells. Evidence for this comes from several sources. First, unlike cDC, pDC can be found in significant numbers in the blood and bone marrow, and in a form that is already capable of IFN-α production and transformation to DC (Corcoran et al., 2003; Martin et al., 2002; Siegal et al., 1999), although blood pDC appear to be at a less mature stage (O'Keeffe et al., 2003). Second, pDC are known to circulate between these tissues in a CD62L (L-selectin)-dependent manner, and to enter lymphoid organs via high endothelial venules (Nakano et al., 2001; Penna et al., 2001; Yoneyama et al., 2004). This is akin to circulating T and B-lymphocytes, but not like monocytes and migratory DC. Third, while peripheral MHC II^+ pDC have recently been shown to uniquely express Ly49Q in the steady state, a precursor form has been identified in the BM, blood and liver that is also $CD11c^{int}B220^+$ but negative for Ly49Q and MHC II (Diao et al., 2004; Kamogawa-Schifter et al., 2005; Omatsu et al., 2005; Toyama-Sorimachi et al., 2005). Interestingly, none of the precursor $Ly49Q^-$ pDC are detected in the peripheral lymphoid organs. Upon culture with FL for 4 days, $Ly49Q^-$ pDC could give rise to $Ly49Q^+$ pDC without division. This further suggests

that the last step of pDC differentiation occurs at sites outside of peripheral lymphoid organs, such as the BM, or perhaps upon extravasation into organs. Despite this *in vitro* evidence of conversion, transfer assays *in vivo* are still needed to formally demonstrate this step in development and to ask whether Ly49Q$^+$ pDC are the only cells produced, especially considering the description of a phenotypically overlapping CD11c$^+$CD11b$^-$B220$^+$MHC II$^-$ precursor, which could generate both pDC and cDC *in vivo* (Diao et al., 2004).

Downstream of the early lymphoid precursors, B and T cells diverge and express tissue-restricted molecules that enable their activation by antigen. These include the antigen receptor genes themselves and gene rearrangements that permanently mark the genome of the cell. Interestingly, both thymic and peripheral pDC expressed a range of these lymphoid gene products including pre-Tα, and D-J but not V-D-J rearrangements of IgH genes(Bendriss-Vermare et al., 2001; Corcoran et al., 2003; Res et al., 1999; Shigematsu et al., 2004; Wang and Liu, 2004). Perhaps unexpectedly, in light of their 'myeloid' origin, pDC derived from CMP also carried D-J rearrangement of IgH genes (Shigematsu et al., 2004).

One study has suggested that BM CD11c$^+$CD11b$^-$B220$^+$ cells, said to be pDC, could transform into CD11b$^+$ 'myeloid' DC with virus infection when cultured with FL (Zuniga et al., 2004). This may be a mechanism of pDC conversion to a cell type that is more efficient at antigen presentation of endogenous virus. However, the exact developmental state of the cell considered as a BM pDC is not clear. The observation may also be explained by a virus-induced conversion of BM-resident CD11c$^+$CD11b$^-$B220$^+$ cDC precursors that are not pDC (Diao et al., 2004; Omatsu et al., 2005) into CD11b$^+$ myeloid DC, rather than conversion of *bona fide*. Additional studies involving cell sorting for Ly49Q$^+$MHC-II$^+$ BM pDC and subsequent transfer *in vivo* would be required to conclusively demonstrate the biological importance of such an *in vitro* phenomenon.

Molecular requirements of DC development

Cytokines

There are several cytokines that are important for DC development, and some of these are specific for particular subtypes. Certain cytokines appear to be obligatory for DC development, some enhance DC generation and/or survival, and some are produced during certain infections to boost numbers.

FL is a haematopoietic cytokine that was first described to have a role for mutlipotential stem cell and lymphoid differentiation (Mackarehtschian et al., 1995; Rasko et al., 1995). Genetic knockouts of FL and its associated signalling pathways have the most striking defects in DC development. $FL^{-/-}$ mice have drastically reduced numbers of pDC, cDC and NK cells (Brawand et al., 2002; McKenna et al., 2000). Mice genetically deficient for STAT3, a downstream signalling molecule of FL, also have reduced pDC and cDC numbers, but normal NK cell numbers (Laouar et al., 2003). The necessity of FL for DC generation *in vivo* also correlates with the observation that injection of recombinant FL could boost numbers of pDC and all cDC (Maraskovsky *et al.*, 1996; Pulendran *et al.*, 2000). Moreover, large numbers of pDC and cDC can be generated when BM is cultured with FL as a single addition cytokine (see below) (Brasel et al., 2000; Brawand et al., 2002, Gilliet, 2002 #100).

GM-CSF was originally identified as a cytokine able to promote the generation of granulocyte and macrophage colonies in soft agar cultures (Burgess et al., 1977). An important boon for DC research was the realisation that large numbers of MHC II^+ DC could be generated *in vitro* when monocytes or BM were cultured with GM-CSF, with or without IL-4 (see below). However, it was later realised that while GM-CSF was important for 'myeloid' DC generation *in vitro* (Sallusto and Lanzavecchia, 1994, inaba), it did not seem to be required for steady-state pDC or cDC development as mice deficient for GM-CSF or its receptor have normal numbers and function of pDC and cDC ((Hikino et al., 2000; Vremec et al., 1997) and my unpublished observations). Interestingly, injection of pegylated GM-CSF has no reported effects on pDC or $CD8^+$ cDC numbers but did increase $CD8^-$ cDC numbers. GM-CSF is also found to enhance pDC and cDC survival *in vitro* (O'Keeffe *et al.*, 2002a).

G-CSF, a myeloid cytokine that can immobilise haematopoietic progenitors, is a potent inducer of numbers of pDC but not cDC. This effect has been observed either when administered alone or in combination with FL or thrombopoietin (Chen et al., 2004; Kared et al., 2005; Koopman et al., 2004; Pulendran et al., 2000). Whether this cytokine exerts its affect by boosting numbers of the early precursors and/or specific pDC-restricted precursors is not clear.

IL-6 is well established as an important haematopoietic regulator (Hirano, 1998), although the role for this cytokine in DC development has only recently been questioned. Initial indications came from the first descriptions of FL BM cultures. The addition of blocking IL-6 antibody or the use of IL-6$^{-/-}$ BM abrogated significant CD11c^{+} DC generation (Brasel et al., 2000). In contrast, another study stated that numbers of splenic pDC and cDC in IL-6$^{-/-}$ mice were unchanged, although data was not shown (Park et al., 2004). Rather, the role for IL-6 was suggested to be suppressive in that study as CD11c^{+} MHC II^{int} cells in the lymph nodes of IL-6$^{-/-}$ mice, corresponding to immature cDC and pDC, had spontaneously developed *in vivo* into activated MHC II^{hi} cells. Both IL-6 and FL act through STAT3 so it is interesting to note that STAT3$^{-/-}$ mice also have reduced numbers of pDC (Laouar et al., 2003). There may be a more complex relationship between these factors in DC development that has still to be identified.

Transcription factors

In recent years, the generation of mice genetically deficient for specific transcription factors has highlighted the role of such factors in the development of particular cell types, including DC. Significant progress has been made in delineating the molecular mechanisms and families of transcription factors that control the differentiation of particular DC subtypes.

The interferon regulatory factor (IRF) family of proteins (IRF-1 to -9) were originally identified as transcription factors involved in gene regulation in response to type I and II interferons (Taniguchi et al., 2001). IRF8, also known as interferon consensus sequence binding protein (ICSBP), is involved in several developmental and

functional roles of the immune system. IRF8$^{-/-}$ have a chronic myelogenous leukaemia-like phenotype and are severely immuno-compromised (Driggers et al., 1990; Holtschke et al., 1996). Interestingly, these mice have reduced splenic CD8^{+} cDC, pDC and LC numbers (Aliberti et al., 2003; Schiavoni et al., 2004; Schiavoni et al., 2002; Tsujimura et al., 2003). IRF4$^{-/-}$ mice have a converse phenotype, with an absence of most CD4^{+} cDC, some pDC, but no effect on CD8^{+} cDC (Suzuki et al., 2004). IRF4$^{-/-}$ IRF8$^{-/-}$ double knockout mice have further reductions in these subsets (Tamura et al., 2005). IRF2$^{-/-}$ mice also have reduced CD8^{-} cDC numbers, but normal numbers of pDC and CD8^{+} cDC (Honda et al., 2004). Collectively, these studies place IRFs as crucial drivers of the DC compartments. Perhaps the observed susceptibility to infections in IRF null mice may not simply be due to defective interferon responsive gene induction after infection, but also a result of DC and other immune cell deficiencies in the first place, prior to infection.

The nuclear factor of kB (NF-kB) contain several subunits and, like the IRFs, are crucial in both steady-state and induced immune responses. NF-kB is downstream of a host of signalling pathways (Baeuerle and Henkel, 1994; Gilmore, 1999). Recently, defects in steady-state pDC and cDC development have been observed in *Nfkb1*$^{-/-}$ and/or c-*Rel*$^{-/-}$ (O'Keeffe et al., 2005b). To add complexity, it appears that different members of this family can contribute independently to processes that govern DC development, cytokine secretion and morphological activation.

Id2 and Id3 are members of the helix-loop-helix (HLH) transcription factors and exert inhibitory effects on the transcriptional activities of other HLH transcription factors. A transcriptional profiling study revealed that Id2 was upregulated during DC development and that mice deficient for Id2 lacked Langerhans cells, and had markedly reduced numbers of splenic CD8^{+} cDC, but no change in pDC numbers (Hacker et al., 2003). However, ectopic expression of Id2 and Id3 can strongly inhibit the development of pDC without effect on the development of cDC, at least when DC are derived from human CD34^{+} progenitors (Spits et al., 2000). These findings suggest that Id2 and Id3 are obligatory for cDC development, and inhibitory for pDC.

There has been solid progress in understanding the role of many individual as well as families of transcription factors in DC development. Identification of yet more

players as well as the biochemical and bioinformatic study of their interactions and relationships to each other should illuminate the developmental programming of the DC subtypes.

In vitro models of DC differentiation

Since the first description of DC from the spleen and lymphoid organs of mice, a culture method for their generation was long sought. Several methods for the generation of DC have been established using various precursors and combinations of cytokines.

"Myeloid" DC

Monocytes are phagocytic cells of the myeloid lineage and are found in several organs including blood, bone marrow and spleen. They represent a relatively terminal stage in the myeloid lineage and are a cell in what is often referred to as the mononuclear phagocyte system, or MPS (Gordon and Taylor, 2005). In both mice and humans, monocytes have the capacity to differentiate into macrophages in response to M-CSF (Becker et al., 1987) and into $CD11c^{+}$ $MHCII^{+}$ DC in the presence of GM-CSF, with or without IL-4 (Inaba et al., 1992a; Inaba et al., 1992b; Lu et al., 1995; Peters et al., 1993; Sallusto and Lanzavecchia, 1994; Sanchez-Torres et al., 2001; Scheicher et al., 1992; Zhou and Tedder, 1996). More recently separate $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes have been identified, and both could generate DC *in vitro,* with some reports of *in vivo* generation (Geissmann et al., 2003; Ginhoux et al., 2006; Leon et al., 2004; Randolph et al., 1999; Sunderkotter et al., 2004). As such, monocytes, or their earlier myeloid precursors (Nikolic et al., 2003), have long been regarded as the definitive precursors of DC, and the DC derived in vitro with GM-CSF as representative of DC found normally in a mouse or patient. This has since become the standard method for generation of DC *in vitro* for subsequent assessment of any effects on DC function by genetic, synthetic, biological or pathogenic factors.

Correlation between monocyte-derived and *in vivo* DC

However, GM-CSF levels are actually undetectable in a steady-state mouse and in healthy humans and so are unlikely to contribute to significant steady-state DC generation from monocytes (Baiocchi et al., 1993; Cebon et al., 1994; Cheers et al., 1988; Metcalf, 1988). There are two possibilities for this discrepancy. There may be a factor *in vivo* that could substitute for GM-CSF to generate DC, or alternatively, steady-state DC may actually be a distinct lineage from those that are monocyte-derived. Although not proof in their own right, there are several genetic observations that suggest monocytes give rise to a distinct DC lineage than is found in the steady-state.

Genetic knockouts for both GM-CSF and its receptor would never be able to make DC, if this cytokine was obligatory. However, both knockouts have normal splenic resident DC subtypes in their expected ratio and number and with no difference in function (Hikino et al., 2000; Vremec et al., 1997). This suggests that a separation of monocyte-derived versus the steady-state DC lineage may exist. M-CSF null mice (*op/op*) and M-CSF receptor$^{-/-}$ have severely perturbed monocyte development in early life and an absence of some macrophage subsets (Ginhoux *et al.*, 2006; Wiktor-Jedrzejczak *et al.*, 1990; Wiktor-Jedrzejczak *et al.*, 1982; Yoshida *et al.*, 1990). While total DC numbers are down in these mice (MacDonald et al., 2005) it is in accordance with total spleen size and so probably does not reflect a specific defect in the DC compartment ((Witmer-Pack et al., 1993) and David Vremec, personal communication). Of the DC that are present, the splenic subtypes are also in their expected ratio and density and exhibit normal function, suggesting steady-state DC precursors are unaffected and are not monocytes, although this has never formally been demonstrated.

When would GM-CSF become important for monocyte-derived DC generation *in vivo* if it is not present at significant levels in the steady-state? Infections such as *Listeria* (Cheers et al., 1988) and other situations induce higher serum levels of the cytokine (Nicola and Metcalf, 1995). Indeed, a novel DC subtype was recently identified in the spleen of mice 1-2 days after infection with *Listeria monocytogenes* (Serbina et al., 2003). This DC was unique in phenotype in that they were CD11c^{int}

CD11b^{hi} MAC-3^{+} unlike splenic steady-state cDC, which are CD11c^{hi} CD11b^{low} MAC-3^{-}. In mice deficient for the chemokine receptor CCR2, this DC subset failed to develop in spleen, but CD11c^{hi} cDC numbers were normal. Interestingly, the Ly6C^{hi} monocyte subset selectively expresses CCR2 (Geissmann et al., 2003), and so was presumed to be the contributing precursor to this kind of DC. Since the description of Tip DC, other CD11c^{int} CD11b^{hi} DC have been described that arise during infections and autoimmune sequelae (Balazs et al., 2002; Gonzalez-Juarrero et al., 2003; Homann et al., 2004; Lowes et al., 2005; Peters et al., 2004; Sundquist and Wick, 2005; Turley et al., 2003).

Flt3 ligand BM cultures

In view of the observations that injection of the cytokine FL increased DC numbers (Maraskovsky et al., 1996; Pulendran et al., 1999), and that mice deficient for FL had abrogated DC numbers (McKenna et al., 2000), this same research group devised a culture system to generate DC from BM (Brasel et al., 2000). They cultured this cytokine with BM for 9 days and generated DC to roughly the same number as total BM cells initially seeded. These DC were morphologically and phenotypically distinct from GM-DC. However, the lack of CD8 or CD4 expression meant that establishing their relationship to the in vivo subtypes was not straightforward. Even so, they were shown to have CD11c^{hi} CD11b^{lo} “lymphoid” DC, and CD11c^{low} CD11b^{hi} myeloid” DC subtypes. The “lymphoid” DC could upregulate CD8 in response to LPS, and produce IL-12 p70 in response to heat killed S. aureus, thus were proposed to be similar to CD8^{+} cDC of the spleen. Subsequent characterisation of this culture system demonstrated that they also contained a type I interferon-producing B220^{+} component, which were proposed to be equivalent to pDC (Brawand et al., 2002; Gilliet et al., 2002). Considering the differential expression of several surface markers, the precise relationship of FL-DC to the in vivo subtypes is still unknown.

Human DC comparisons

Comparison of human DC with their mouse equivalents has been difficult. Like for mouse DC, the tissue source and methods for deriving human DC have been varied.

Human DC can be characterised in the first instance according to freshly isolated versus precursor derived.

Monocyte-derived DC

Most human DC studies have focused on monocyte-derived DC. Human monocytes are able to convert into macrophages with M-CSF (Becker et al., 1987) or to DC upon culture with GM-CSF & IL-4 (GM-DC) (Sallusto and Lanzavecchia, 1994; Sanchez-Torres et al., 2001; Zhou and Tedder, 1996). They can also be separated, usually as $CD14^+$ or $CD16^+$ monocytes (Passlick et al., 1989). On the basis of chemokine receptor expression, CX3CR1 in particular, $CD14^+$ human monocytes are proposed to be equivalents of the $Ly6C^{hi}$ $Gr1^+$ $CX3CR1^{lo}$ mouse monocytes, and the $CD16^+$ human monocytes equivalent to the $Ly6C^{lo}$ $Gr1^-$ $CX3CR1^{hi}$ monocytes. The human monocyte subsets and their progeny thus correlate well to the mouse studies, at least when studies *in vitro*

$CD34^+$ progenitor-derived DC

$CD34^+$ progenitors can also give rise to various DC subtypes when cultured under various conditions (Strobl, 2003). In the presence of GM-CSF and IL-4, the DC progeny resemble monocyte-derived DC. However, if FL, IL-3 and/or TNF-α are in addition, different subsets can emerge. For example, culture with FL and thrombopoietin can yield pDC and myeloid DC (Chen et al., 2004).

Blood DC

As blood has been the organ of greatest accessibility, it has been the main source of freshly isolated human DC. Human blood DC are initially categorised as "lymphoid" and "myeloid" DC. Lymphoid DC are the human equivalents of mouse plasmacytoid, and are also known as natural interferon producing cells (NIPC) (Grouard et al., 1997; Olweus et al., 1997; Siegal et al., 1999). Human pDC are characterized as $CD11c^-$, $CD4^+$, $IL\text{-}3R^{hi}$, $B220^-$ and $CD45RA^+$. The remaining "myeloid" DC in the human contain at least 4 $CD11c^+$ $HLA\text{-}DR^+$ subsets including $CD16^+$, $CD1b/c^+$, and BDCA-3^+ and $CD34^+$ DC populations (MacDonald et al., 2002). The relationship of these subsets to each other, or to the $CD14^+$ or $CD16^+$ monocyte subsets is still unclear

(Dzionek et al., 2000; Ito et al., 1999; MacDonald et al., 2002). While mouse blood also contains pDC, they have few cells that can be considered developed DC. Rather there is a $CD11c^{lo}$ MHC II^{lo} cell type that requires the addition of cytokines to develop before it can be considered *bona fide* DC (O'Keeffe *et al.*, 2003). Thus the relationship of blood DC between the two species is still hard to pinpoint.

Lymphoid organ DC

Only a few studies have focussed on DC of human spleen. Warnke and colleagues identified discrete DC subsets in lymphoid follicles that contained $CD21^{+}$ follicular DC, a $CD1a^{+}$ population likely to be Langerhans cells, and another $CD1a^{-}$ $S100^{+}$ DC subset that was an interdigitiating cell (IDC) in the T cell areas (Wood et al., 1985). Hosmalin and co-workers analysed human splenic DC and found marginal zone DC, as well as IDC (McIlroy et al., 2001). These IDC all expressed CD83 and CD86. Interestingly, when total spleen DC were analysed, there was heterogeneous expression of CD4, CD40 and CD32 (McIlroy et al., 2001), perhaps pointing to the presence of subsets. One important challenge in the DC field will be to correlate findings for the mouse DC subsets with their putative human counterparts.

Chapter 2
Materials and Methods

Abstract

This chapter describes materials and methods that were central to experiments described in this thesis. For the purpose of concision and clarity, well-established methods are mentioned only briefly and the relevant references cited. A detailed list of manufacturers is provided in Table 2.1, and a complete list of antibodies and conjugates is provided in Tables 2.2 and 2.3, and the channels used for fluorescent conjugates and markers for the flow cytometry machines specified in Table 2.4.

Media

Culture and suspension media, prior to the addition of supplements, were prepared by the Walter & Eliza Hall Institute of Medical Research (WEHI) Media Department. All media were sterilised by filtration through a 0.22μm membrane filter and stored in the dark at 4 ^{o}C. Media were modified to mouse tonicity (308 mOsM equivalent to 0.168M NaCl)

Culture medium

RPMI 1640 (modified for mouse tonicity) - was prepared by dissolving RMPI 1640 powder (Gibco BRL), 0.015 M NaCl, 0.0238 M $NaHCO_3$, 1mM sodium pyruvate, 2mM L-glutamine (Gibco BRL), 100 U/mL penicillin, 100 μg/mL streptomycin and β-mercaptoethanol in Milli Q water Final pH 7.2

Culture medium - was modified RPMI 1640 supplemented with 10% fetal calf serum (FCS).

FL conditioned medium (day 3 or day 10) – BM was cultured with 200-300 ng/mL of FL (see below). Either 3 days or 10 days after culture, the cells were removed by centrifugation the supernatant FL conditioned medium was collected, passed through a 0.22 μM filter and retained at –70° for subsequent use.

Suspension media

Buffered Balanced Salt Solution (BSS) (HEPES buffered, mouse tonicity) **-** was prepared by mixing the following stock solutions: 17.052 L of Milli Q water, 1.694 L of 1.68 M NaCl, 42 mL of 16.8 M KCL, 42 mL of 1.12 M $CaCl_2$, 14 mL of 1.68 M $MgSO_4$, 28 mL of potassium phosphate buffer (prepared by mixing 1.68 M KH_2PO_4 with 1.12 M K_2PO_4), 168 mL of 1.68 M HEPES (N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid, Gibco BRL) buffer (pH to 7.22 at 20 ^{o}C with NaOH). Final pH = 7.22

BSS with EDTA (EDTA-BSS) - consisted of 97.5 mL BSS media supplemented with 5 mM ethylenediamine tetra acetic acid (EDTA).

RPMI-2% - was modified RPMI 1640 medium supplemented with 2% FCS

Phosphate Buffered Saline (PBS) (mouse tonicity) **-** was prepared as a 10 x concentrate by preparing 0.16 M of $Na_2HPO_4.2H_2O$ (0.016 M HPO_4), 0.0443 M of $NaH_2PO_4.2H_2O$ (4.43 mM PO_4) and 0.149 M of NaCl in Milli Q water. This stock was then diluted 1:10 in MilliQ water before use. Final pH = 7.3.

PBS/BSA - was 97.7 mL of PBS (Gibco BRL) with 1.3 mL of 7.5% w/v BSA (Gibco BRL) (0.1 % BSA final concentration)

Supplements, buffers and solutions

Ethylenediamine tetra acetic acid (EDTA) pH 7.2 – A mouse osmolarity 0.1M solution of EDTA was made by dissolving disodium salt (Sigma) in Milli Q water, and then neutralised with NaOH to pH 7.2.

Fetal Calf Serum (FCS) - was supplied by CSL Limited and was heat-inactivated by immersion in a 56°C water bath for 60 min. The heat-inactivated FCS was stored at -20°C.

EDTA/FCS - was FCS supplemented with 10% v/v of 10 mM EDTA.

Red cell removal buffer (RCRB) - was 0.168 M NH_4Cl in Milli Q water.

Nycodenz - EDTA – was used as a medium for density centrifugation. It was made using iohexol carbohydrate (Nycomed Pharma). The powder was dissolved in water to make a 0.37 M stock and diluted stocks were made in BSS, adjusting to the final required osmolarity and density at 4°C.

Enzyme-linked immunosorbent assay (ELISA) substrate solution – was made by adding 10µL 30% H_2O_2 (Sigma), 200 µL of a 50x concentration stock of ABTS (2.2'-azinobis (3-ethyIbenz-thi-azoline-6-sulphonic acid)) (Sigma) in 10 mL 0.1M Citric acid (pH 4.2). This solution was made fresh every time.

ELISA wash buffer – contained PBS with 0.05% Tween-20 (C_{58} H_{114} O_{26}).

ELISA blocking buffer – contained PBS with 1% FCS and 1% BSA.

Miscellaneous reagents

CpG 2216 Phosphorothioate-stabilised, oligodeoxynucleotide **-** 5′- GGG GGA CGA TCG TCG GGG GG -3′ was purchased from Geneworks. For *in vitro* culture CpG 2216 was dissolved in RPMI-2% and stored at 4°C.

Lipopolysaccharide (LPS) - was purchased from Sigma. The *E. coli* source was 0111:B4. LPS was made up in PBS at 500μg/mL, and stored at -20°C. Each aliquot was thawed once only and stored at 4°C.

Ovalbumin protein grade V (OVA) - 98% purity, was purchased from Sigma. OVA was dissolved in PBS or RPMI-2% for additions to culture.

Ovalbumin peptide - with the amino acid sequence corresponding to residues 257-264 in OVA protein ($OVA_{257\text{-}264}$ – SIINFEKL) is a H-2k^b MHC class I-restricted epitope. The peptide with the amino acid sequence corresponding to residues 323 to 339 in OVA protein ($OVA_{323\text{-}339}$) is a I-A^b MHC class II restricted epitope. Both peptides were purchased from Auspep.

Mice

All experimental mice used were between 7-14 weeks of age. All mice were bred and maintained under specific pathogen-free conditions at the WEHI animal breeding facility, according to institutional guidelines. Mice were killed using $C0_2$ asphyxiation or cervical dislocation.

Strains

C57BL/6J Wehi mice (Ly5.2) were used for most experiments, and as donors in cell transfer experiments. C57BL/6 Pep^{3b} (Ly5.1) mice of similar age were used as recipients in such transfer experiments. CBA CaH WEHI (CBA) were used as a source of alloreactive T cells in mixed leukocyte reations. Transgenic mice used include OT-I H-2k^b (restricted TCR (Vα2Vβ5) specific for $OVA_{257\text{-}264}$) (Hogquist et

al., 1994), OT-II I-A^b (restricted TCR (Vα2Vβ5) specific for $OVA_{323-339}$) (Murphy et al., 1990) and mice with an eGFP knock-in into the MHC II region (MHC II-eGFP) (Boes et al., 2002). Mice genetically deficient for IRF8 ($IRF8^{-/-}$) were also used in some experiments (Holtschke et al., 1996).

Injections

All intravenous (*i.v*) and intraperitoneal (*i.p.*) injections were with 27 gauge needles. For *i.v.* injections, mice were left under a heat lamp for 15 min prior to injection to dilate the tail vein. The volume injected was between 100 and 250 µL for *i.v.*and 100 µL to 1 mL for *i.p.* injections.

Irradiation

For mice that required lethal irradiation prior to cell transfer experiments, γ-irradiation was performed using ^{60}Co source on an Eldorado 8 instrument. Mice were left in their box and given 5.5 Gy for two 30 min periods, 3 hours apart, and cell transfers performed within 4 hours. Mice were always reconstituted with 1 x 10^4 host strain BM cells in addition to any donor cell transfers to prevent radiation sickness. They were also supplied water containing neomycin sulphate (0.1% w/v) (Sigma) as a precaution against bacterial infection for the duration of the experiment.

Flow Cytometry

Cell preparation – Cells from various preparations (see next section) to be analysed or sorted by flow cytometry were first labelled with antibodies against surface markers. Cells were usually resuspended at 1 x 10^6 cells in 10 µL of staining solution containing conjugated mAbs (Table 2.2) for 25 min, then washed with a large volume of EDTA-BSS.

Monoclonal antibodies (mAbs) - Unless stated, all antibodies were generated after culture of hybridomas and then concentration of supernatants (made entirely by David Vremec, Jo Pooley, Angela D'Amico and Michael Bradtke). Each purified mAb was conjugated to either biotin (Molecular Probes), allophycocyanin (APC)

(Prozyne), Cy5-bis-OSU N,N'-biscarboxypentyl-5,5'-disulfonatoindodicarbocyanine (Cy5) (Amersham Life Science), R-phycoerythrin (PE), Alexa Fluor 594 (A594) (Molecular Probes), or fluorescein-5-isothiocyanate (FITC) (Molecular Probes) fluorochromes. Monoclonal antibodies were titrated on 10^6 spleen or LN cells or purified DC to determine the optimal staining concentration for detection by flow cytometry. The conjugated antibodies used in this thesis are listed in Table 2.3.

Cells for analysis - 20 – 40 x 10^6 cells per mL were analysed in 5 mL tubes (Becton Dickinson - polypropylene for MoFlo and polystyrene for all other machines) and kept at 4°C. Prior to analysis, propidium iodide (Calbiochem) was added to the sample at a final concentration of 0.5 μg/mL to label dead cells for subsequent for dead cell exclusion.

Instruments and software - Analytical and preparative flow cytometry was carried out employing a FACStar Plus, FACScan, FACS DiVa, LSR (Beckton Dickinson), or Mo-Flo (Cytomation) instruments. Analysis of data was performed using WEASEL software (Dr. F. Battye, WEHI).

Compensation – A small portion of cells that were to be analysed were first used in single stain controls to allow for the spillover of fluorescence from one conjugate into the detection channels for other conjugates. The mAb-conjugate to be used in the experiment that provided the highest level of fluorescence in its respective channel was tested in each fluorescence channel. Using the instrument software, leakage of fluorescence from each conjugate into other channels was compensated. The settings were then saved and used for all subsequent analysis. This compensation procedure was performed on every occasion prior to flow cytometric sorting or analysis.

Cell suspensions

All cellular preparations were performed at 4°C, except where indicated.

Splenocytes

Splenocytes were prepared as previously described (Vremec et al., 2000; Vremec and Shortman, 1997). For every 8 mice, spleens were chopped with scissors then digested at for 20 min at room temperature in a mixture of 6mL RPMI-2% containing 7 mg Collagenase (121 U/mg - Type III, Worthington Biochemicals) with a further addition of 1 mL of DNase (1mg/mL) (Roche). Pipetting the tissue through a wide bore pipette tip repetitively during digestion facilitated dispersion to single cell suspension. EDTA (600 μl of 0.1 M solution) was then added and mixed by pipetting for a further 5 min to ensure DC-T cell clusters were disrupted. Undigested fragments were removed by filtering through a sieve.

Bone Marrow (BM)

BM was extracted from the tibia and femur with RMPI-2% using a 3 mL syringe and 21-gauge needle. This single cell suspension was then centrifuged and the pellet resuspended in a small volume (0.5-2 mL) RCRB for 1 minute to lyse erythrocytes. After this time, cells were diluted up to 10 mL in RPMI-2% and washed 2-3 times. Pellets were then resuspended in RPMI-2% and passed through a sieve to remove bone fragments and dead cell clumps.

Blood mononuclear cells

To isolate blood mononuclear cells, all steps were performed at room temperature. Blood was collected by lumbar puncture into heparinized syringes using a 25-gauge needle and collected into heparinized tubes. Blood was then diluted 5 fold in BSS (no FCS) and layered onto an equal volume of 1.083 g/cm^3 Histopaque (Sigma). Tubes were then centrifuged at 2,500 rpm for 25 min. Buffy coats were collected using a plastic pasteur pipette with care to ensure minimal collection of the lower layer of histopaque. These cells were then diluted in cold BSS and washed.

Lymph node cells

Single cell suspensions were made by forcing pooled subcutaneous and mesenteric lymph nodes through a sieve using a syringe bore. Cells were then diluted in EDTA-BSS, underlaid with EDTA-FCS and centrifuged at 4 ^{o}C at 1500 g for 7 min.

Peritoneal cells

Sacrificed mice were injected with 10 mL of EDTA-BSS (no FCS) into the peritoneum, in an area where peritoneal fat could block the hole created by the syringe after withdrawal. Mice were rolled side-to-side for 30 s to suspend cells in the EDTA-BSS. The solution containing cells was then extracted from the peritoneum using the same syringe but at a different site to the initial injection.

Cellular labelling

CFSE-labelling

Carboxyfluorescein diacetate succinimidyl ester (CFSE) labelling was performed as originally described by Lyons and Parish 1994 (Lyons and Parish, 1994). Enriched T cells or BM was pelleted and washed twice with PBS/BSA and resuspended at 1 x 10^7 cells/mL in PBS/BSA and transferred to a new tube with dry sides. A 1 μL aliquot of 0.5 μM CFSE (Molecular Probes) per 1 x 10^7 cells was applied to the dry inside of the tube and mixed thoroughly by vortexing to ensure consistent labelling. Cells were the incubated with CFSE for 10 min at 37 ^{o}C. Cells were then washed three times in RPMI-2%, counted and resuspended in culture medium.

PKH-labelling

To label cells with the membrane dye PKH-26 (Sigma), cells were washed in BSS without FCS and resuspended at a concentration of 1 x 10^7 per mL of diluent C (Sigma). An equal volume of diluent C containing a 1/250 dilution of PKH-26 membrane label (Sigma) was added with rapid mixing and incubated for 3-5 min at room temperature. An equal volume of FCS was added and mixed for 1 minute to stop the reaction, followed by 3 washes in EDTA-BSS. Donor cells were analysed in the FL-2 channel of the LSR either prior to, or at a time after, transfer *in vivo* or culture *in vitro*. Fluorescence levels on undivided PKH-26 labelled T cells after transfer *in vivo* were found not to change significantly over this time.

Dendritic cells (DC)

Splenic DC isolation

To isolate dendritic cells, a cell suspensions from the spleens of 4 mice (see above) were first pelleted, then resuspended in 5 mL of cold 1.077g/cm^3 Nycodenz and layered onto 5 mL of fresh Nycodenz. A further 0.5 mL of EDTA/FCS was then layered on top the spleen cells. Density separation was performed in a swing-out head centrifuge (Beckman) at 1700g for 10 min at 4 ^{o}C. The supernatant containing light density cells (3-5% of total spleen cells) and most splenic DC (90%) was collected and diluted in at least 3-fold the volume of EDTA-BSS. The pellet containing dense non-DC was discarded.

The light density cells were resuspended in depletion cocktail (10μl per 10^6 cells). The antibody cocktail contained rat antibodies against mouse CD3 (KT3-1.1), CD90 (T24/31.7), CD19 (ID3), Ly6G (1A8), and erythrocytes (Ter-119). Cells were incubated with the cocktail for 25 min at 4°C then diluted to 9 mL with EDTA-BSS, underlaid with EDTA-FCS and centrifuged for 7 min at 1500g. The cell pellet was resuspended in 300 μl EDTA-BSS and transferred to a pellet of pre-washed sheep anti-rat Ig coupled magnetic beads (M450 Dynalbeads, Dynal) at 5 beads/cell or Biomag beads (Qiagen) at 10 beads/cell. The cells were mixed and rotated at a 45° angle for 20 min at 4 ^{o}C. Following this incubation, cells and beads were diluted to 2 mL with EDTA-BSS and placed against a magnet (Dynal). The supernatant containing the DC-enriched population was recovered, and the magnetically bound cells (non-DC) were discarded. These were the enriched DC preparation.

Where purified DC subsets were required, flow cytometric sorting was performed on a MoFlo or DiVa instrument using up to four fluorochromes. The FL3 or FL5 channel was used to exclude PI$^+$ (dead) cells. Live cells that were CD11c$^+$ were gated as DC. These cells were further divided based on their staining with fluorochrome-conjugated mAbs against CD8, CD4, and CD45RA. DC purity was routinely between 92-99 %.

DC generation from FL BM cultures

FL-DC were generated in culture as described (Brasel et al., 2000; Brawand et al., 2002; Hochrein et al., 2004), with some modifications. BM cell suspensions were

prepared as above. Cells were then resuspended at 1.5-3 x 10^6 cells/ml of culture medium containing an optimised concentration of 200-300 ng/ml murine FL (generated in house from a Chinese Hamster Ovary cell lines that expressed FLAG-tagged FL) and seeded into sterile non-pyrogenic filter-capped tissue culture flasks (Iwasaki or Nunc) of a size appropriate for the volume. Culture was for 8-10 days at 37°C in 10% CO_2. DC were then dislodged by vigorous tapping of flasks and the media containing DC collected. DC were spun and resuspended in EDTA-BSS for 5-10 min then passed through a 27-gauge needle to disrupt clusters. Four-colour staining with mAbs to CD11c, CD45RA, CD24 and CD11b, or alternatively CD11c, CD45RA and SIRP-α, was used to segregate the FL-DC subsets.

DC precursor isolation

Pre-cDC (Chapter 4)

Determination of density characteristics

For experiments that determined the buoyant density characteristics of intrasplenic DC precursor cells, splenocytes were loaded onto a series of tubes containing Nycodenz medium ranging in density from 1.070 g/cm^3 to 1.091 g/cm^3 and centrifuged at 1700 *g* for 10 mins. The light density supernatant was collected, diluted in BSS, counted and washed prior to injection of a sample of these cells. The pellets of dense cells were then subjected to a second round of centrifugation in 1.091 g/cm^3 Nycodenz, to collect the upper zone of viable cells for injection while excluding dead cells and erythrocytes in the pellet.

After determining that precursor cells were of intermediate density, a one-step dual density procedure was adopted to exclude the very light density DC, and the dense splenocytes, erythrocytes and dead cells. Splenocytes from 4 mice were resuspended in 4 ml 1.084 g/cm^3 Nycodenz medium and layered onto 4 ml fresh Nycodenz medium of the same density. Layered on top of this was 4 ml 1.076 g/cm^3 Nycodenz medium followed by 1 ml FCS. After centrifugation at 1700*g* for 10 minutes at 4°C two bands appeared. The uppermost band of cells, lighter than 1.076 g/cm^3, was discarded. The remaining ‘medium density’ band of cells that was lighter than 1.084

g/cm^3 but denser than 1.076 g/cm^3 was collected for injection or for further DC precursor enrichment. The pellet was discarded.

Immunomagnetic bead depletion

Medium density cells, enriched for DC precursor activity, were normally coated with biotinylated mAb against CD19, CD3, NK1.1 and TER-119 for 25 minutes on ice. In the alternative protocol, mAbs were used against MHC II, Ly6G, CD3, TER-119 and CD49b instead of the above. Cells were washed then resuspended with MACS anti-biotin magnetic beads (Miltenyi Biotec) using 1 µL beads and 9µL of EDTA-BSS (0.5% FCS) per 4 x 10^6 cells. After washing, cells were resuspended in BSS (containing 0.5% FCS) and passed over a MACS LS column using a VarioMACS (Miltenyi Biotec). Both the eluent (lymphocyte-depleted) and bound fraction (lymphocytes) were collected, counted and a small fraction of cells injected for assay of DC precursor activity. Alternatively, the flow through was kept for the next round of enrichment by flow sorting.

Immunofluorescent labelling and sorting

In the steps of pre-cDC isolation, medium density, lymphocyte-depleted cells were stained with mAb for CD11c, CD45RA, CD43 and SIRP-α. In the alternative isolation protocol, staining for CD43 was omitted as all cells were CD43int after pre-depletion of cDC. Precursors were gated as being low for orthogonal light scatter (side scatter – SSC), CD11cint CD45RAneg/low SIRP-αint CD43+. Additional stains used to characterise the pre-cDC included antibodies against CD24, MHC II, CD4 and CD8. All cell sorting or analysis was performed on a DiVa, a MoFlo or an LSR instrument. Cells were sorted at low speed and low pressure, and the time between precursor isolation and injection or culture minimised to ensure good recoveries.

Pro-DC and pre-DC from FL cultures (Chapter 6)

Wildtype BM was labelled with CFSE (see above), prior to culture with FL, as above. After 3.5 days, cells were dislodged from flasks by pipetting and centrifuged. The cell pellet was resuspended in EDTA-BSS for 10 minutes at 4°C followed by passage through a 27 gauge needle, to disrupt clusters and create a single cell suspension. Cells were spun and pellets resuspended in 1.086 g/cm^3 Nycodenz medium and

centrifuged at 1700g for 10 min. The supernatant containing buoyant DC precursors was collected, diluted in EDTA BSS, washed and retained. The pellet containing dense non-precursors and dead cells was discarded. The cells of the precursor-enriched fraction were then coated with biotinylated antibodies against CD19, Ly6G, IL-7R-α and MHC II. Biotinylated cells were then incubated with anti-biotin magnetic beads (Miltenyi) prior to depletion on a magnetic column (Miltenyi). The column-bound non-DC precursor cells were discarded and the flow through, which contained DC precursors, was retained. The DC-enriched fraction was then labelled with anti-CD11c and anti-Ly6C-biotin, with second stage staining by streptavidin-phycoerythrin. The final purification involved flow cytometry. All cells were gated for viable cells that had undergone division ($CFSE^{low}$), and pro-DC were sorted as large $CD11c^-$ $Ly6C^-$ cells, while pre-DC were gated as large $CD11c^+$ cells.

Monocyte isolation

Monocytes were isolated based on a procedure describe previously (Geissmann et al., 2003; Lagasse and Weissman, 1996; Sunderkotter et al., 2004; Taylor et al., 2003). Monocytes were isolated from whole BM suspensions, from whole spleen, or from blood mononuclear cells, as first prepared above. When isolating from BM, cells were first depleted of $Ly6G^+$ cells (granulocytes) and $B220^+$ cells (B cells and pDC). BM was incubated with rat mAbs for Ly6G and B220 for 30 minutes at 4°C and washed in EDTA-BSS. Cells were then incubated with pre-washed polyclonal anti-rat Ig Biomag beads (QIAGEN) at a ratio of 10 beads per cell for 20 min at 4°C on rotation at an angle of 45°. After placing on a magnet, the unbound cells in the supernatant containing monocytes were collected and the magnetically bound cells discarded. Monocytes for all sources were then isolated using flow cytometry, by staining for Ly6C and CD11b, as well as with NK1.1 in the case of blood monocytes. Monocytes were gated for low side scatter (SSC^{lo}), $CD11b^+$ and $NK1.1^-$ (in the case of blood), and then sorted for either $Ly6C^{hi}$ or $Ly6C^{lo}$ subsets.

Preparation of T cells

OT-I CD8$^+$ T cells

For OT-I CD8$^+$ T cells, single cell suspensions were made from pooled subcutaneous and mesenteric lymph nodes of OT-I transgenic mice, as above. The cell pellet was resuspended in 1 mL of RPMI-FCS containing the rat anti-mouse monoclonal antibodies against CD11b, F4/80, erythrocytes, Gr-1, MHC class II, and CD4. After 30 min at 4°C cells were diluted to 9 mL with BSS-FCS, underlaid with FCS and centrifuged for 7 min at 1500g. The cell pellet was resuspended in 300 μl BSS-FCS and transferred to a pellet of pre-washed sheep anti-rat Ig magnetic beads (Dynal) at 5 beads/cell. The cells were mixed and rotated at a 45° angle for 20 min at 4 ^{o}C. Following the incubation, the cells and beads were diluted to 2 mL with BSS-FCS and placed against a magnet (Dynal). The supernatant containing the naïve CD8$^+$ T cell-enriched population was then recovered and bound (non-CD8$^+$ T cells) cells discarded. The purity of OT-I cells was determined by staining a small sample of enriched cells with anti-CD8 and anti-Vα2 and assessing by flow cytometry. T cells were between 85-98 % pure after enrichment. Cells were then labelled with CFSE, as above.

OT-II CD4$^+$ T cells

OT-II naïve CD4$^+$ T cells were purified from OT-II transgenic mice using the protocol described for OT-I T cells, except in the antibody cocktail anti-CD4 was replaced with anti-CD8 in the depletion cocktail. All CD4$^+$ T cell preparations were between 85-98% pure, as determined by flow cytometry using the antibodies for anti-CD4 and anti-Vα2. Cells were labelled with CFSE, as above.

CBA T cells

Naïve T cells were purified from CBA mice using the protocol described for OT-I T cells, except in the antibody cocktail, mAbs for CD4 or CD8 were not included. All CBA T cell preparations were between 95-98% pure, as determined by flow cytometry using anti-CD3. Cells were labelled with CFSE, as above.

Assays of DC development

Steady-state DC development *in vivo*

An *in vivo* assay was developed to allow detection of cDC precursor activity, and thereby identification of putative DC precursors, from a given tissue source without the need for prior knowledge of the precursor itself. It involved the *i.v.* transfer of cells from a Ly5.2 mouse into at least 2 Ly5.1 recipient mice, which were usually non-irradiated. Transferred cells were from different organs, or from fractions of spleen, during precursor purification. Subsequent analysis of recipient spleen DC by flow cytometry allowed detection of donor-derived Ly5.2$^+$ DC generation and thus allowed calculation of the DC precursor activity of the transferred fraction. This required monitoring of two measures of precursor activity; 1) the 'per cell' activity and 2) the 'total' activity.

'Per cell' DC precursor activity

Various fractions from spleen, BM, blood or FL cultures were isolated and the number of cells recovered per mouse determined (for subsequent calculation of 'total' DC precursor activity). Then, a sample of this fraction was transferred intravenously (0.1-10x10^6 cells per recipient) into 2 or more non-irradiated Ly5.1 recipients. At various times after transfer, mice were sacrificed and the spleens of recipient mice pooled. Recipient DC were then enriched and counted. The DC-enriched preparation was blocked for 15 min with rat Ig and anti-F$_C$ receptor (2.4G2), followed by staining for donor-type Ly5.2, the DC marker CD11c, and for CD8α, CD4, CD45RA, MHC II or CD205. The number of donor derived DC produced per 10^6 cells of the fraction transferred was then determined as a measure of per 10^6 cell DC precursor activity, usually expressed as the 'per cell' precursor activity. This was achieved using the following calculation.

$$\frac{\text{\% Ly5.2}^+ \text{ DC} \times \text{No. of enriched recipient DC}}{\text{No. of Ly5.2}^+ \text{ cells initially transferred } (\times 10^{-6})} = \textbf{Per } \mathbf{10^6} \textbf{ cell DC precursor activity}$$

This figure was used to gauge precursor *enrichment* during its isolation and was expected to rise concomitant with DC precursor isolation.

'Total' DC precursor activity

As only a sample of each fraction was transferred into mice to calculate the 'per cell' figure, a means of accounting for the activity of the entire transferred fraction, as originally present in one mouse, was required. To calculate this 'total' DC precursor activity per mouse, the 'per cell' cDC precursor activity of each fraction was multiplied by the total number of cells of the injected fraction that was originally recovered from one mouse prior to transfer. This was calculated as;

Total cell # of transferred fraction ($x10^{-6}$) × per 10^6 cell DC precursor activity of that fraction

= **'Total' DC precursor activity**

This figure was used to gauge precursor *recovery* and so determine any loss during purification. This figure would ideally remained constant, concomitant with total DC precursor isolation, but some loss during the process was inevitable.

Inflammatory DC development *in vivo*

We adopted the antigen-induced GM-CSF-dependent inflammation system as described elsewhere (Cook et al., 2004). Briefly, each Ly5.1 mouse was injected intradermally in two separate regions at the base of tail with a total of 100 μL of 1 mg/mL methylated bovine serum albumin (mBSA) in complete Freund's adjuvant (CFA), and boosted two weeks later with the same treatment. At least 7 days later, mice were injected *i.v.* with sorted Ly6C^{hi} monocytes. One day after cell transfer, mice were injected *i.p.* with 100 μg mBSA in BSS to induce inflammation. A further day later, spleen suspensions, blood, BM and peritoneal cells were analysed or sorted for donor-derived cells by flow cytometry.

Short-term DC development *in vitro*

For DC development of pre-cDC, pre-DC or pro-DC, a fixed number of cells were cultured in 200 μL of either day 3 (for pre-DC and pro-DC) or day 10 (for pre-cDC) FL conditioned medium (medium described above) for different times in wells of a

96-well U-bottom plate. After this period, a fixed number of latex beads were added for subsequent quantitation, plates spun at 1500 g for 5 min, and cells stained for CD11c, CD45RA, MHC II and SIRP-α. DC progeny segregated into plasmacytoid DC (CD45RA^{hi} SIRP-α^{int}), CD8^{+} cDC equivalents (CD45RA^{-} SIRP-α^{lo}) or CD8^{-} cDC equivalents (CD45RA^{-} SIRP^{hi}) and their number deteremined.

Antigen Presentation Assays

Presentation to OTI and OTII *in vitro*

Assays for the presentation of antigen by DC to naïve T cells were based on those described (Wilson et al., 2003). For the experiments using soluble OVA or synthetic peptides, 5×10^3 cells of DC populations were plated in 96-well U-bottom plates (Costar) in each well. DC were incubated with the indicated concentrations of OVA protein, $OVA_{257\text{-}264}$ synthetic peptide (OT-I) or $OVA_{323\text{-}339}$ synthetic peptide (OT-II) for 45 min at 37°C in complete medium. The DC were then washed three times and resuspended in 200 μl of complete medium containing 5×10^4 CFSE-labelled OT-I or OT-II cells. Proliferation was analysed after 60 h of culture. Cells were resuspended in 100 μl of BSS/FCS containing a known number of latex beads (BD Biosciences). Numbers of CFSE^{low} PI^{-} T cells that undergone division were determined relative to the number of latex beads added per well. Each determination was performed in duplicate. For experiments using cell-associated OVA, 2-5 × 10^4 purified DC were seeded into 96-well U-bottom plates with 2-5 × 10^5 OVA-coated bm1 mutant (H-2k^{bm1}) and 5×10^4 naïve CFSE-labelled OT-I or OT-II cells and incubated for 60 h. Wells containing cells were harvested and stained in 25 μl with mAbs against Vα2 and either CD4 or CD8 for 30 min. T cell proliferation was determined as described for the experiments using soluble OVA.

Mixed leukocyte reaction (MLR)

CBA T cells were prepared and CFSE-labelled, as above. DC derived *in vivo* from either pre-cDC or monocytes, as well as freshly isolated splenic cDC, were sorted and cultured with 2 x 10^4 CBA T cells for 5 days in culture medium at 37°C and 10% CO_2 in air. The number of divided T cells was calculated as for OTI and OTI (above).

Miscellaneous protocols

Real-time PCR

RNA was prepared from cell pellets of purified DC, monocyte or DC precursor populations using the RNeasy Mini Kit (QIAGEN) as per manufacturer's instructions. RNA (up to 2μg) was DNase treated with RQ1 DNase (Promega) then reverse transcribed into cDNA using random primers (Promega) and Superscipt II reverse transcriptase (Gibco BRL). Real-time PCR was performed to determine the expression of *TLR3, TLR4, TLR7 & TLR9*, and *Gapdh* in splenic DC subsets using the QuantiTect SYBR Green PCR Kit (QIAGEN) and a Light cycler (Roche), as per manufacturer's instructions. The specific primers for real-time PCR were as follows:

TLR3 ;	L	5'- AAAAACTCAGCGGCCGGAATG -3';
	R	5'- AGTTACGAAGAGGGCGGAAAGG -3'
TLR4 ;	L	5'- CCATCGGTTGATCTTGGGAGAA -3'
	R	5'- TGCCAGAGACATTGCAGAAACA -3'
TLR7:	L	5'- ATCGTGGACTGCACAGACAAGC -3'
	R	5'- TTGGCTTTGGACCCCAGTAGAA -3'
TLR9 ;	L	5'- GCCTTCGTGGTGTTCGATAAGG -3'
	R	5'- GAGGTTCTCGAAGAGCGTCTGG -3'
Gapdh;	L	5'- CATTTGCAGTGGCAAAGTGGAG -3'
	R	5'- GTCTCGCTCCTGGAAGATGGTG -3'
CXCR3	L	5'- CATTGCCAGTACAACTTCCCA -3'
	R	5'- CAAAGGCTGCCACCACCACTA -3'
CCR9	L	5'- ATGATGCCCACAGAACTCACAAG -3'
	R	5'- CAAGCCAGTACAGAGGTGGAAGA -3'
CCR6	L	5'- TCCATCATCATCTCAAGCCCTACA -3'
	R	5- AGGGGTGAAGAACCCAAAGAACA -3'

CX3CR1	L	5'- GGTCTGGTGGGAAATCTGTTG -3'
	R	5'- GAAGAAGGCAGTCGTGAGCTT -3'

An initial activation step for 15 mins at 95^0C was followed by 40 cycles of: 15 secs at 94^0C (denaturation), 20-30 secs at 50-60^0C (annealing) and 10-12 secs at 72^0C (extension), followed by melting point analysis. The expression level for each gene was determined using a standard curve prepared from 10^{-2}-10^{-6} pg of specific DNA fragment, then expressed as a ratio to *Gapdh.*

Western blot analysis

For Western blot analysis, frozen cell pellets were resuspended in sample buffer and subjected to several cycles of vortexing and heating at 95°C. Samples were centrifuged for 2 min (3000 g), and resolved by 11.5% SDS-polyacrylamide Gel Electrophoresis. All Western blots were performed as described (Villadangos et al., 1997). Poteins were then electroblotted using a Hoefer™ semi-dry transfer unit (Amersham) onto an Hybond-P™ PVDF membrane (Amersham) and blocked for 2 h in PBS/Tween and 5% (w/v) skim milk powder. The membranes were rinsed in PBS/Tween and incubated with an anti-human Cystatin C rabbit serum (DAKO) for 1.5 h. Bound proteins were detected using anti-rabbit antibodies conjugated to horseradish peroxidase (HRPO) (Amersham). Detection was performed using SuperSignal® chemiluminescence substrate (Pierce). All Western blot quantitation was performed with a Molecular Dynamics densitometer using ImageQuant™ 5.0 software.

DC activation

Sorted DC (1 x 10^5) were cultured in duplicate in the wells of a 96-well round bottom plate, either in 200 µL culture medium alone or with 1 µg/ml R848 (Invitrogen), 1 µM CpG 2216 (Geneworks) or 1µg/ml LPS (Sigma) for 24 hours. For IL-12 p40/p70 and IFN-α production, DC were cultured under the same conditions as above but also with 100 ng/mL GM-CSF, 20 ng/ml IL-4 and 20 ng/ml IFN-γ, for 48 hours.

ELISA

ELISAs were performed as previously described (Hochrein et al., 2001). All incubation steps were either at 2-4 hr at room temperature or overnight at 4°C, and each wash step involved 3 washes in PBS/Tween. The capture mAbs are listed in Table 2.3. Each capture mAb was diluted in the appropriate concentration in PBS and 50 μL added per well of an ELISA plate (Dynal) and incubated. Plates were then washed and incubated with 150 μL of ELISA blocking buffer. Plates were washed and the supernatant samples from cultures of DC activation (see above), or cytokine standards, were titrated in blocking buffer and 50μL incubated per well. Plates were washed prior to addition of secondary mAbs for detection (Table 2.3). These mAbs were diluted in blocking buffer and 50μL incubated per well. Plates were washed prior to cytokine detection by biotinylated mAbs, where 50μL of a 1/5000 dilution of streptavidin-horseradish peroxidase (HRPO) (RPN4401 conjugate) (Amersham) in blocking buffer was added per well for 30 min - 1 hr. For cytokine detection by polyclonal anti-rabbit Ig, 50 μL of 1/3000 dilution of donkey anti-rabbit Ig-HRPO **(SUPP)** in blocking buffer was added for 30 min - 1 hr. Plates were then washed thoroughly >5 times with PBS/Tween. ELISA substrate (100 μL) was then added for 5-10 min or until sufficient colour developed. The optical density of plates was read with a Kinetic microplate reader set to 405-490 nm (Molecular Devices). Sample cytokine concentrations were interpolated from the standard curve calculated for each cytokine.

Cell cycle analysis

For cell-cycle analysis, pre-cDC were washed in BSS without FCS and the pellet resuspended in a solution containing 0.1% sodium citrate, 0.2% Triton X-100, 10 μg/mL RNase A and 100 μg/mL PI. Cells were left on ice for 15 minutes prior to analysis. Fluorescence of PI was detected in the FL-3 channel of the LSR, set to linear scale, after gating on single nuclei.

May Grünwald Giemsa stains

May Grünwald stain was made by dissolving 0.3 g of May-Grünwald eosin methylene blue (Merck) in 100 mL methanol, stirring for 1 hr, then filtering with filter paper (Whatman). This staining solution was kept at room temperature for subsequent use long-term. Giemsa stain was made fresh for each use by diluting a solution of

Giemsa's azure eosin methylene blue solution (Merck) 4% v/v in tap water. Purified pre-cDC or Ly6C^{hi} monocytes were prepared by cytospin centrifugation onto glass slides and dried for at least 24 hours. Slide were then dipped into May-Grünwald stain in Coplin jars for 4 min then dipped directly into a separate Coplin jar of Giemsa for 4 min, then rinsed numerous times in tap water also in a coplin jar. Slides were viewed and photographed using a compound microscope (Zeiss) and a digital camera (Axiocam, Zeiss).

M-CSF cultures

Cell populations were cultured in duplicate at 1 x 10^4 per well in a 96-well round bottom plate in culture medium containing a titration of rhM-CSF at 37°C and 10% CO_2 in air for 48 hrs. At this time, cell morphology was determined by phase contrast microscopy and the slides photographed using an attached digital camera (Zeiss). To quantify cell numbers at this time, a known number of latex beads to each well, the cells removed by trypsinisation for 3 min at 37°C then the number of viable cells relative to beads assessed using flow cytometry.

Phagocytosis

For phagocytic capacity, sorted pre-cDC or monocytes were incubated with fluoresbrite beads (Polysciences Inc) in culture medium for 45 minutes at 37°C or at 4°C. Cells were then washed and assessed by flow cytometry for bead uptake in the FL-1 channel of an LSR. Percentage uptake was determined by deducting the background uptake at 4°C from the value at 37°C.

Cell-associated antigen

To prepare cell-associated OVA spleen suspensions from bm1 mice were first made as above. The cell suspension was then irradiated at 1500 cGy. Following irradiation, the cells were washed and resuspended at 2 x 10^8 cells/mL. Coating with OVA protein was achieved by incubating the cell suspension in the presence of 10 mg/mL OVA protein in RPMI for 10 min, at 37°C. Following the incubation, cells were washed three times in RPMI-2%, counted and then resuspended in culture medium. The amount of OVA associated to the cells after the procedure has been quantitated to be 8 ng OVA protein/10^6 cells (Li et al., 2001).

Table 2.1 Manufacturers of Reagents

Manufacturer	**Local address for order**
Amersham Pharmacia Biotech	Castle Hill, Australia
Auspep	Melbourne, Australia
BD Pharmingen	San Diego, CA, USA
Beckton Dickinson	Franklin Lakes, NJ, USA
Caltag	Burlingame, CA, USA
Costar	Broadway, Cambridge, UK
CSL Limited	Victoria, Australia
Cytomation	Colorado, USA
Dako	Glostrup, Denmark
Dynal	Oslo, Norway
Geneworks	Adelaide, Australia
Gibco BRL	Geithersburg, MD, USA
Hybaid	NSW, Australia
ICN Biomedicals	Costa Mesa, CA, USA
Invitrogen	Melbourne, Australia
Merck	Melbourne, Australia
Millipore	Billerica, MA, USA
Miltenyi	North Ryde, NSW, Australia
Molecular Dynamics	Victoria, Australia
Molecular Probes	Portland, OR, USA
Nycomed Pharma	Oslo, Norway
Perkin-Elmer	Boston, MA, USA
Pierce	Rockford, IL, USA
Polysciences Inc	Warrington, PA, USA
Promega	Madison, WI, USA
Prozyne	San Leandro, CA, USA
QIAGEN	Victoria, Australia
R&D systems	Mineapolis, MN, USA
Roche	Victoria, Australia

Sigma	Castle Hill, NSW, Australia
Upstate Biotechnology	Lake Placid, NY, USA
WEHI	Victoria, Australia
Whatman	Mt Eliza, VIC, Australia
Worthington	Freehold, NJ, USA
Carl Zeiss	North Ryde, NSW, Australia

Table 2.2 Antibodies for staining and flow cytomtery

Surface molecule	Alternative Name	mAb Clone	Source
MHC-II		M5/114	In house
CD1d		1B1	In house
CD3		KT3	In house, Pharmingen
CD4	Ly-4	GK1.5	In house
CD8	Ly-2	YTS169.4	In house
CD11a	LFA-1α	121/7.7	In house
CD11b	Mac-1	M1/70	In house
CD11c	Integrin-α_X	N418	In house
CD16/32	FcgRIII	2.4G2	In house
CD18	Integrin-β2	M18/2.a.12.7	In house
CD19	B4	ID3	In house
CD24	Heat Stable Ag	M1/69	In house
CD25	IL-2Rα	PC61	In house
CD34	Mucosialin	RAM34	In house
CD40	gp39	FGK45.5	In house
CD43	Leukosialin	S7	In house
CD45R	B220	RA36B2	In house
CD45.1	Ly5.1	A20-1.1	In house
CD45.2	Ly5.2	S450-15.2, ALI-4A2	In house
CD45RA	Exon A isoform	14.8	In house
CD49b	Pan NK	DX5	Pharmingen, USA
CD51	Integrin-αV	H92B8	Pharmingen, USA
CD54	ICAM-1	YN1/1.7.4	In house
CD62L	L-selectin	MEL-14	In house
CD69	Early Activ. Ag	H1.2F3	In house
CD80	B7-1	16-10A1	In house
CD86	B7-2	GL-1	In house
CD103	Integrin-a_{IEL}	M2/90	In house
CD122	IL-2 Rβ		Pharmingen, USA
CD127	IL-7 R	A7R34-2.2	In house
CD154	ICAM-1	YN1/1.7.4	In house
CD161c	NK1.1	PK136	In house
CD172α	SIRP-α	P84	In house
CD205	DEC 205	NLDC-145	In house
Mac-2	Galectin 3	M3/38	In house
F4/80	Ly-71	F4/80	In house
Mac-3	Lamp-2/CD107b related	M3/84	In house
Ly6A/E	Sca-1	D7	In house
Ly6C		5075-3.6	In house
Ly6G		1A8	In house
Vα2		B20.1	PharMingen, USA

Table 2.3 Monoclonal antibodies for depletion and ELISA

Antibody	Clone name	Source/Reference
CD90	JIJ	In house
CD90	T24/31.7	In house
CD4	RL172	In house
CD4	GK1.5	In house
CD8	3.168	In house
CD45R (B220)	RA36B2	In house
CD3	KT3-1.1	In house
Gr-1	RB68C5	In house
CD11b	M1/70	In house
F4/80	F4/80	In house
erythrocyte	TER119	In house
CD19	ID3	In house
F4/80 antigen	F4/80	In house
Ly6G	1A8	In house
IL-12 p40/p70 (capture)	C15.6 or R29A5	In house
IL-12 p40/p70 (detection)	C17.8	In house
IL-6 (capture)	MP5-20F3	In house
IL-6 (detection)	M35-32C11	In house
RANTES (capture)	53433	R&D, MN
RANTES (detection)		R&D, MN
MIP1α (capture)	39624.11	R&D, MN
MIP-1-α (detection)		R&D, MN
IFN-α (capture)	RMMA-1	PBL, NJ
IFN-α (detection)	polyclonal rabbit	PBL, NJ

In house – hybridomas were grown, the supernatants concentrated and the antibodies conjugated to various fluorochromes. David Vremec, Angela D’Amico, Michale Bradtke and Jo Pooley prepared all mAb reagents.

Table 2.4 Channels for fluorescence in flow cytometry instruments

Channel	**MoFlo**	**FACStar**	**DiVa**	**LSR**
FL1	FITC, CFSE	FITC, CFSE	FITC, CFSE	FITC, CFSE
FL2	PE, PKH-26	PE, PKH-26	PE, PKH-26	PE, PKH-26
FL3	PI	Cy5, APC	PerCpCy5.5, PE-Cy7	PI, PerCpCy5.5, PE-Cy7
FL4	Cy5, APC	A594	A594	
FL5		PI, PerCpCy5.5, PE-Cy7	Cy5, APC	
FL6			PI	
SSC-W				Cy5, APC

Chapter 3:
$CD8^-$ cDC do not generate $CD8^+$ cDC

Abstract

Although previous studies had indicated that the $CD8^-$ and $CD8^+$ subtypes of murine dendritic cells (DC) differ in immediate origin, a recent study found that intravenous transfer of $CD8^-$ cDC led to $CD8^+$ cDC in the spleen several days later, suggesting a direct precursor-product relationship. In this Chapter, I repeated these experiments are with a balance-sheet approach. Although a few $CD8^+$ cDC could be generated in such experiments, this was a rare event and could have been due to a contaminant precursor. The vast majority of the immediate precursors of $CD8^+$ cDC were cells that lack the phenotype of a recognisable DC. Therefore, $CD8^-$ cDC and $CD8^+$ cDC are not precursor-product related, although these sublineages may be connected further upstream.

Introduction

The CD8$^+$ and CD8$^-$ subsets of dendritic cells (DC) from mouse spleen are of special interest, since as freshly isolated they differ in functional characteristics (Shortman and Liu, 2002). However the developmental origin of these DC subtypes and their relationship has been controversial. The original view that splenic CD8$^+$ cDC were of lymphoid origin and that splenic CD8$^-$ cDC were of myeloid origin has been shown to be incorrect (Manz et al., 2001b; Wu et al., 2001). However, differences in the cytokine and transcription factor requirements still suggest that the developmental pathways differ. Culture of purified splenic CD8$^+$ cDC or splenic CD8$^-$ cDC has failed to show any transformation from one into the other (Kamath et al., 2000), although there is evidence that CD8 can be induced to at least moderate levels on some DC subtypes (Merad *et al.*, 2000). An *in vivo* kinetic study, using continuous BrdU labeling, showed no sign of a precursor to product relationship between CD8$^-$ and CD8$^+$ cDC (Kamath et al., 2000).

In direct conflict with this is the conclusion of a recent paper by Martinez del Hoyo et al. (Martinez del Hoyo et al., 2002). They isolated CD8$^-$ cDC, transferred them intravenously into a Ly 5 disparate non-irradiated recipient, and found 3-4 days later CD8$^+$ cDC of donor origin. They concluded that CD8$^+$ cDC normally originate from CD8$^-$ cDC, the two being merely maturation stages of the same lineage. In view of the conflict, similar experiments were carried out and are reported in this Chapter. Although some CD8$^+$ cDC were obtained on transfer of a CD8$^-$ cDC preparation, recoveries were extremely low, suggesting that the production of CD8$^+$ cDC was due to a contaminant precursor. The majority of the immediate precursors of CD8$^+$ cDC were found to be cells lacking normal DC characteristics.

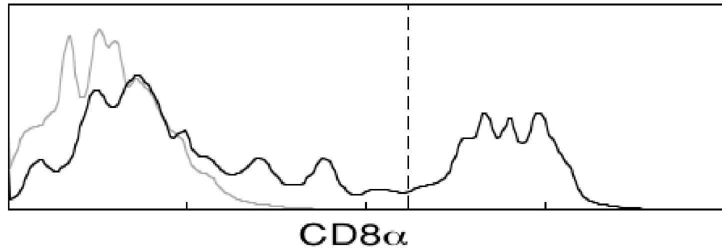
Results

Minimal $CD8^+$ cDC generation from purified $CD8^-$ cDC.

A repeat of the experiment where transfer of purified $CD8^-$ splenic cDC produced $CD8^+$ cDC was attempted (Martinez del Hoyo et al., 2002). However, to obtain high purity and to segregate by different levels of marker expression, we used fluorescence activated cell sorting rather than immunomagentic particle separation in the final separation of $CD8^-$ cDC. Cells bearing relatively high levels of MHC II were additionally selected, so defining more tightly the DC population. The purity was close to 99% on reanalysis, so around 1% of the preparation represented possible contaminants. These cells (3-5 x 10^6) were then transferred intravenously into non-irradiated Ly5.1 recipients. After 3.5 days, an enriched cDC preparation was made from the pooled recipient spleens and the donor-derived ($Ly5.2^+$) cells gated and analysed.

Donor-derived cDC were detected in the recipient spleens in 3 out of 7 experiments. An example of one of these is given in Fig. 3.1a. From 5 to 30% of these donor-derived cDC were $CD8^+$ and also DEC-205^+ (Fig. 3.1a), whilst the rest remained $CD8^-$ or stained at only low levels for CD8. Similar results were obtained at days 2 and 4. Thus the generation of $CD8^+$ cDC from a $CD8^-$ cDC fraction was sometimes observed, although in contrast to the previous study (Martinez del Hoyo et al., 2002) only a minority of the recovered donor-derived DC showed acquisition of CD8. Since most splenic DC turnover in 3 days (Kamath et al., 2000), the majority of donor-derived DC should have been $CD8^+$ if they originated from the $CD8^-$ cDC subset.

(a) Surface phenotype of recovered transferred cells



(b) Recovery of transferred cells

All donor-derived cells	0.33%
Donor-derived DC	0.24%
Donor-derived CD8α$^+$DC	0.08%

Figure 3.1 **Characterisation and recovery of donor-derived cDC following transfer of purified CD8$^-$ cDC.**

Splenic CD8$^-$ CD11c^{hi} MHC II^{hi} cDC were isolated and sorted from Ly5.2 mice and transferred intravenously into non-irradiated Ly 5.1 recipients (5x10^6 per mouse). After 3.5 days cDC were isolated from the pooled recipient spleens and Ly5.2$^+$CD11c$^+$ cDC analysed for CD8 expression. The experiment presented is the one showing the highest donor-cell recovery and the highest proportion of Ly5.2$^+$ CD8$^+$ cDC. Of 7 transfer experiments 4 gave no detectable donor-derived cDC while 3 gave donor-derived cDC containing from 5 to 33% CD8$^+$ cDC. In a) the flow cytometric analysis of CD8 expression on the recovered donor-type cDC is presented and in b) the measured recoveries of the donor cells transferred is given for this same experiment.

Recoveries of cDC on transfer of the $CD8^-$ cDC fraction

In addition to the low proportion of DC becoming $CD8^+$, a disturbing aspect of the experiment was the low overall recovery of the transferred cDC in the spleens of the recipient mice (Fig. 3.1b). This was true at all time points studied. The highest recovery measured in these experiments was 0.33% of the $CD8^-$ cDC transferred, of which only around one quarter were $CD8^+$ cDC (Figure 3.1b). Even allowing for a 50% loss of DC in the enrichment procedure preceding analysis, at most 0.16% of the transferred DC produced $CD8^+$ cDC in the recipient spleens.

This is in line with the reported values of Martinez del Hoyo et al. (Martinez del Hoyo et al., 2002), from which it can be calculated that only 0.3% of the transferred cells were recovered as $CD8^+$ cDC. Since this is within the contaminant level of the transferred $CD8^-$ cDC it was not proven that it was the $CD8^-$ cDC themselves which produced the $CD8^+$ cDC.

Distribution of $CD8^+$ cDC precursors during DC enrichment.

It could still be argued that this observed production of $CD8^+$ cDC, although derived from only 0.3% of the $CD8^-$ cDC fraction injected, was representative of the other 99.7% transferred but lost. If all the immediate precursors of $CD8^+$ cDC were indeed $CD8^-$ cDC, such precursor activity, as measured by the ability to produce $CD8^+$ cDC in the spleen 3 days after transfer, should separate with $CD8^-$ cDC and therefore be over 100-fold enriched in a pure $CD8^-$ cDC fraction. The distribution of such immediate $CD8^+$ DC-precursor activity in fractions sampled during the steps of DC isolation was therefore determined, following intravenous transfer of a known number of cells. Precursor activity was determined as 'per cell' activity (donor-derived $CD8^+$ cDC per recipient spleen per 10^6 donor cells transferred) and then calculated as "total activity" ('per cell' activity x 10^{-6} multiplied by the number of cells of each fraction originally recovered from one donor spleen prior to transfer) (Table 3.1). 'Per cell' activity allows a measure of precursor enrichment, total activity a measure of precursor recovery (see Materials & Methods for detailed formula).

In order to gauge the enrichment and recovery of $CD8^+$ cDC precursors concomitant with $CD8^-$ cDC enrichment, the total $CD8^+$ cDC-precursor potential of a spleen was first determined. A suspension of spleen cells with dead cells and erythrocytes removed was counted and injected intravenously and the number of donor-type $CD8^+$ cDC arising after 3.5 days determined. The 'per cell' activity of splenocytes was then multiplied by the number of viable nucleated cells originally obtained from each donor spleen, to give an initial 'total' precursor activity figure for balance sheet purposes: Approximately 5,500 $CD8^+$ cDC generated per spleen transferred after 3.5 days (Table 3.1).

The first step of DC purification involved separating a light-density ($<1.077 g/cm^3$) fraction, which included only around 5% of the viable nucleated spleen cells, but virtually all of the fully developed DC. Although this fraction had a higher per cell $CD8^+$ cDC precursor activity (around 5-fold) this was less than the 20-fold enrichment of the DC themselves. More importantly, the balance sheet of $CD8^+$ cDC total precursor activity showed that 70% was eliminated with the dense cells (Table 3.1). Further studies showed that most of the immediate precursors of $CD8^+$ cDC were within the 28% of spleen viable nucleated cells of density between $1.083 g/cm^3$ and $1.077 g/cm^3$.

Further enrichment of fully developed cDC, by successive immunomagnetic bead depletion of non-DC lineage cells and then sorting to remove autofluorescent macrophages, enriched the DC to 50-70% purity, but gave little further enrichment of per cell $CD8^+$ cDC precursor activity (Table 3.1). The final DC-enriched fraction was only 5-fold enriched in 'per cell' $CD8^+$ precursor activity and represented only 11% of the total precursor activity in the original spleen suspension. Much of this apparent activity could have been due to seeding and persistence of preformed $CD8^+$ cDC rather than their generation from precursors. Thus most of the splenic $CD8^+$ cDC precursors were not $CD8^-$ cDC.

Table 3.1 Immediate precursors of CD8$^+$ cDC are not all recovered in the DC-enriched fractions.

Fraction	Immediate CD8$^+$ cDC precursor activity	
	'per cell' activity (per 10^6 donor cells)	**Total activity (per donor spleen)**
Spleen suspension	31 ± 6	5490 ± 910
Light density cells	169 ± 93	1660 ± 260
Enriched DC + Autofluorescent cells	254 ± 41	730 ± 90
Autofluorescent cells	39	10
Enriched DC – Autofluorescent cells	162 ± 67	630 ± 150
Enriched DC, CD11c labeled	160 ± 68	620 ± 180
Enriched DC, CD8 labeled	161 ± 70	620 ± 110

Successive stages in the isolation of DC from C57BL/6 mouse spleen (Ly5.2) were sampled and assayed for the ability to produce CD8$^+$ cDC in the spleens of non-irradiated recipient Ly5.1 mice 3.5 days after intravenous transfer. Immediate CD8$^+$ cDC precursor activity is expressed either on a 'per cell' activity basis (Ly5.2$^+$ CD8$^+$ CD11c$^+$ cDC per recipient spleen per 10^6 cells transferred), or on a total activity per original donor spleen basis (calculated as the per cell activity of transferred cells multiplied by the transferred cells originally obtained from one donor spleen). An average of 5% of the original viable nucleated spleen cells were found in the light-density fraction and 2% in the enriched DC fraction. Autofluorescent cells constituted on average 10% of the DC-enriched fraction. Results are means from 4 experiments ± the range.

The distribution of CD8$^+$ cDC-precursor activity during sorting for CD8$^-$ cDC.

Since some apparent CD8$^+$ cDC precursor activity did persist in the DC-enriched fraction, we asked if CD8$^-$ cDC could be a real if minor source of CD8$^+$ cDC. The enriched fraction (50-60% DC) was therefore segregated according to surface expression of the three markers used to define CD8$^-$ cDC, namely CD11c, MHC II and CD8. Labeling the DC with antibodies to these markers did not result in loss of CD8$^+$ cDC precursor activity (Table 3.1). On sorting the labeled cells, precursor activity was found distributed over many fractions (Table 3.2) and not just in the CD8$^-$ or CD11c^{hi} or MHC II^{hi} fractions, which would be the case if CD8$^-$ cDC were precursors. Upon separation by CD8 expression levels, some 'activity' was associated with the CD8 high fraction, and this presumably represented the seeding and persistence of pre-existing CD8$^+$ cDC. However, even more activity, both 'total' and on a 'per cell' basis was found in the CD8 intermediate fraction, suggesting the presence of less mature cells en route to producing CD8 high cDC (Table 3.2). On separation according to CD11c or MHC II expression, the greatest enrichment of activity was in the fractions expressing levels of CD11c and MHC II that were lower than that of fully developed DC, and might therefore contain early DC forms (Table 3.2). In no case was the precursor activity enriched along with the markers of CD8$^-$ cDC (MHC II^{hi}, CD11c^{hi}, or CD8$^-$), although some measurable activity was usually associated with these markers, as in Figure 3.1. When all three markers were used together to isolate CD8$^-$ CD11c^{hi} MHC II^{hi} cDC (as in Figure 3.1), a mean of 1.1% of the total initial spleen precursor activity was recovered in the 3 of 7 experiments when any CD8$^+$ cDC were found in the recipient spleens (data not shown).

Table 3.2 Immediate precursors of CD8$^+$ cDC in cDC-enriched spleen preparations do not segregate with the markers of mature CD8$^-$ cDC.

Sorted fraction	Immediate CD8$^+$ cDC precursor activity	
	'Per cell' activity (per 10^6 transferred)	'Total' activity (per donor spleen)
CD8 negative	172	419
CD8 intermediate	1059	1231
CD8 high	485	478
CD11c negative	738	442
CD11c intermediate	230	139
CD11c high	80	263
MHC II negative	1289	562
MHC II intermediate	719	1221
MHC II high	147	441

After preparing an enriched DC preparation, as in Table 3.1, the preparation was fluorescent-labeled with one marker-specific mAb and sorted into fractions negative, intermediate or high for the chosen marker, eliminating autofluorescent and dead cells in a separate channel. Fully developed CD8$^-$ cDC should be found in the CD8 negative, the CD11c high and MHC II high fractions. Assay for immediate precursor activity and expression of results is as for Table 3.1. Results represent 3 individual experiments, each typical of 2 such experiments undertaken.

Discussion

In at least some of the experiments $CD8^+CD205^+$ splenic cDC were generated when purified samples of $CD8^-$ cDC were transferred intravenously, in accordance with the observations of Martinez del Hoyo et al. (Martinez del Hoyo et al., 2002). However the conclusions of this study differ radically from theirs, since the majority of immediate precursors of $CD8^+$ cDC were found not to be $CD8^-$ cDC at all, but were cells lacking the characteristics of mature DC and so were lost on DC enrichment. The results reported here highlight the importance of a balance-sheet approach, examining activity at all enrichment steps, rather than only determining the activity of an enriched candidate cell.

A major limitation of the earlier study was the extremely low recovery of DC in the spleen following intravenous transfer. The present study gave comparable recoveries with at most only 0.16% of the sorted $CD8^-$ cDC transferred forming $CD8^+$ cDC in the recipient spleens. This poor recovery of DC following intravenous transfer is compounded by the separate problem of a poor recovery of $CD8^-$ cDC-precursor activity during DC enrichment and purification. When any $CD8^+$ cDC precursor activity was recovered in a highly purified, sorted $CD8^-$ $CD11c^{hi}$ MHC II^{hi} fraction, it represented only 1.1% of the total initial spleen activity. Even allowing for some DC loss during enrichment, this is a very low recovery of activity.

However, this final result is in line with the results of Tables 1 and 2 indicating most $CD8^+$ cDC precursor activity did not segregate with individual markers for the $CD8^-$ fully developed cDC. Although this low level production from the sorted $CD8^-$ cDC might point to one minor route of $CD8^+$ cDC production, it could also arise from a high efficiency precursor contaminant. Rather than resolve this issue, it is proposed that future investigation should focus on the major pathways leading to $CD8^+$ (and $CD8^-$) cDC.

What then are the majority of immediate precursors of the $CD8^+$ cDC found in normal laboratory mice? It was demonstrated in this Chapter that they are lighter in density than most spleen lymphocytes but more dense than mature DC. One candidate

precursor, the plasmacytoid pre-DC, has recently been eliminated, since it produces a subtype of CD8$^+$ DC differing in other markers, and only after microbial stimulation (O'Keeffe et al., 2002a). Other kinetic studies (Kamath et al., 2000; O'Keeffe et al., 2002a) indicate that the three mature DC subsets and the plasmacytoid cells of normal mouse spleen represent separate development streams at least as far back as the last dividing precursor. However this may not be very far back. The relationship between all these DC subtypes, and the nature and lineage commitment of their immediate precursors remains to be elucidated, and will be the focus of subsequent Chapters.

Chapter 4:
An intrasplenic precursor of cDC

Abstract

The nature of the immediate precursors of the spleen-resident steady-state DC subtypes has been obscure. In Chapter 3 I showed that $CD8^-$ cDC are not precursors of $CD8^+$ cDC. In this chapter, I describe my purification of an intrasplenic precursor population that produces all steady-state splenic $CD8^+$ and $CD8^-$ conventional DC (cDC) subtypes, but not plasmacytoid cells or other cell lineages. This 'pre-cDC' population included some cells pre-committed to form either $CD8^+$ or $CD8^-$ cDC only, when further separated by CD24, indicating there was already a degree of pre-commitment. Pre-cDC were rare, representing only 0.05% of splenocytes. They had the surface phenotype $CD11c^{int}$ $CD45RA^{lo}$ $CD43^{int}$ Sirp-α^{int} MHC II^- $CD4^-$ $CD8^-$, but were not classical monocytes. Pre-cDC were a late-stage precursor able to generate DC within only 3 divisions, with peak production by 5 days and low to undetectable numbers of progeny by 8 days. Thus I have identified a spleen-resident immediate precursor of all splenic cDC subsets.

Introduction

The antigen-presenting dendritic cells (DC) of the immune system have two roles. One is to maintain T-cell tolerance to self-components in the non-infected steady-state, the other is to initiate and regulate the response of T-cells to invading pathogens (Steinman, 2003). Multiple subtypes of DC have been described, with specialised but overlapping functions (Shortman and Liu, 2002). The primary division of mouse spleen DC is into type-1 interferon producing plasmacytoid cells (pDC) and lymphoid organ-resident conventional DC (cDC). The resident cDC of mouse spleen can in turn be subdivided into three main subtypes ($CD4^-8^+$, $CD4^+8^-$ and $CD4^-8^-$) (Vremec et al., 2000). These resident cDC are distinct from the migratory DC, such as Langerhans cells and interstitial DC, that migrate from peripheral tissues to the lymph nodes via the lymphatics (Henri et al., 2001; Villadangos and Heath, 2005). The developmental origin of all these multiple DC subtypes has been obscure.

DC show more developmental flexibility at early stages of haematopoiesis than other blood cell lineages, since pDC and all subtypes of splenic cDC can be generated from either common myeloid or common lymphoid precursors (Traver et al., 2000; Wu et al., 2001). Thus the key developmental events leading to the different cDC subtypes must be downstream of these early precursors. Cell kinetic studies indicate that, at some point, the lineages leading to these cDC subtypes branch and develop independently (Kamath et al., 2000). This is supported by studies showing that different transcription factors are required for the development of the different DC subtypes (Hacker et al., 2003; Schiavoni et al., 2002; Spits et al., 2000; Suzuki et al., 2004). Recent studies have suggested that DC may not be end cells, but are themselves capable of division and self-renewal (Kabashima et al., 2005; Shortman and Wu, 2004; Zhang et al., 2004). Nevertheless, downstream of the early precursors but upstream of these end product DC, there must be precursor cells that progressively become DC-restricted, then restricted to particular subtypes. Identification of these immediate cDC precursors has proven to be a contentious area.

Past attempts at direct isolation of cDC precursors from mice have given a range of results (del Hoyo et al., 2004; del Hoyo et al., 2002; Diao et al., 2004; Leon et al., 2004; Wang et al., 2002). Common to these studies was the transfer of precursors into

irradiated recipients, with the emergence of DC being measured at relatively late time points. Such conditions perturb the steady-state and select for early rather than late precursors. In a recent study a clonogenic precursor cell able to produce macrophages and DC, but not other myeloid or lymphoid cells, was isolated from bone marrow (BM) (Fogg et al., 2006) and shown to generate splenic DC even in non-irradiated recipients. However, in Chapter 3 it was reported that immediate precursors of DC did exist within spleen itself. Therefore, experiments reported in this Chapter focussed on spleen rather than BM as a precursor source. The precursors of spleen DC were assayed by transfer into non-irradiated mice and assessment of DC progeny relatively early after transfer. Reported in this Chapter is the identification within the spleen itself of a late precursor population, distinct from monocytes, that produces all steady-state splenic cDC subsets but not other haematopoietic cells.

Results

The DC precursor assay

The main considerations when designing an assay for the immediate precursors of splenic cDC were: (1) Precursors should initially be sought in the spleen itself, rather than BM. (2) The precursors should produce DC *in vivo* following transfer into non-infected, non-irradiated recipients, which minimise disturbances to the steady-state. (3) The readout of cDC production in recipient spleen should be after a time long enough to allow turnover of most pre-existing cDC but within a time short enough to detect the most immediate precursors (Kamath et al., 2000). The assay involved intravenous (*i.v.*) transfer of spleen cell fractions from Ly5.2 mice into non-irradiated Ly5.1 recipients. It allowed the clear delineation of $Ly5.2^+$ $CD11c^+$ donor-derived cDC in recipient spleens 5 days later (Fig. 4.1a).

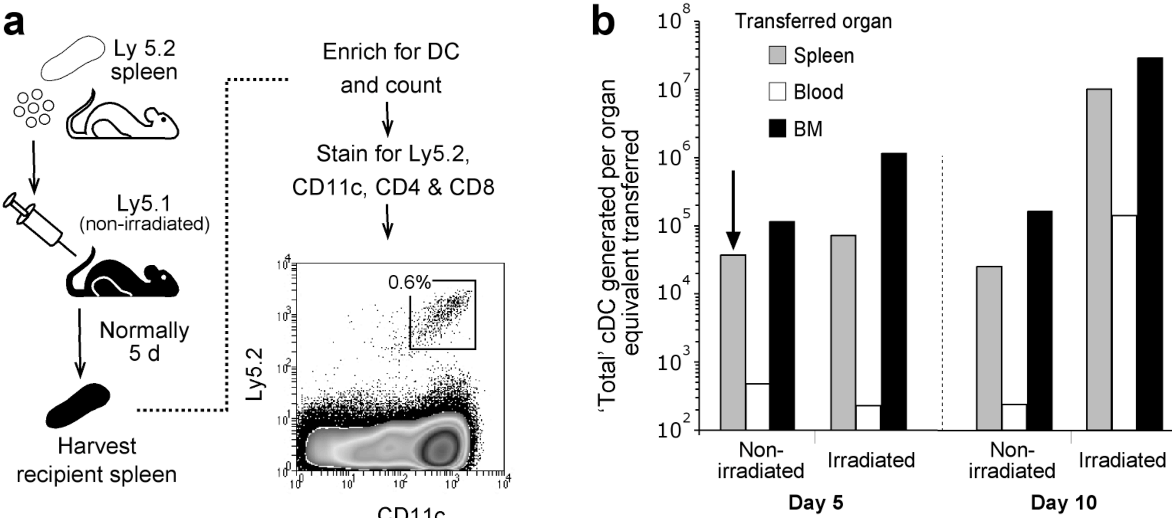
This assay was used to measure the immediate cDC precursor activity of different organs or of different fractions during precursor purification. Quantitation of the total number of donor-derived cDC within one recipient spleen relative to the number of cells injected gave a measure of the cDC precursor activity on a per cell basis (presented as spleen cDC generated per 10^6 transferred cells) and as used in Chapter 3 (see Materials & Methods for detailed calculation). This enabled us to gauge the enrichment of precursor activity during purification from spleen. The total recovery in each fraction of the initial spleen precursor activity was also tracked. This figure for total cDC precursor activity of an organ or a fraction obtained during purification was calculated by multiplying the per cell precursor activity of the cells transferred by the number of such cells originally isolated from the organ of one mouse (as per Chapter 3 and detailed in Materials & Methods). This enabled us to keep track of total cDC precursor recovery during purification.

Figure 4.1. *In vivo* assay for cDC precursor activity and spleen as a source of precursors.

a) The standard assay for immediate cDC precursor activity. Spleen cells from Ly5.2 mice were transferred *i.v.* into non-irradiated Ly5.1 recipient mice. Five days later, recipient spleens were pooled, enriched for DC and enumerated. The proportion of $CD11c^{hi}$ $Ly5.2^+$ cDC was determined by flow cytometry, for calculation of the total number of donor-derived splenic cDC. Expressing this value relative to the number of cells transferred gave the 'per cell' cDC precursor activity (expressed as per 10^6 cells transferred). **b)** To determine the relative levels of immediate cDC precursors in spleen, blood or BM, between 2-10 x 10^6 nucleated cells from each source was transferred *i.v.* into irradiated or non-irradiated Ly5.1 recipients. Five or 10 days later, DC were enriched from recipient spleens and the 'total' cDC precursor activity determined for each organ. 'Total' activity was calculated as the 'per cell' activity for the transferred fraction, as calculated in a), multiplied by the total number of transferred cells that were originally recovered from the organ of one mouse (in this case, ~200 x 10^6 for spleen, ~2.2 x 10^6 for blood and ~55 x 10^6 for BM femurs and tibias). The arrow indicates the parameters used for subsequent assay of DC precursor activity: the transfer of fractions of spleen into non-irradiated recipients and measurement of DC precursor activity 5 days after transfer.

DC precursor activity of spleen, blood and BM

To assess the importance of the spleen itself as a source of immediate precursors of spleen cDC, unfractionated cells from spleen, blood or BM were transferred and their total DC precursor potential compared (Fig. 4.1b). Spleen was found to be a good source of immediate DC precursor activity as measured at 5 days post transfer into non-irradiated recipients. BM was also a rich source of DC precursor activity, 3-fold greater than that of spleen. However, blood nucleated cells showed a much lower 'total' immediate DC precursor activity, and even on a 'per cell' transferred basis they were 40-fold less efficient than spleen. Using this assay, cDC yield was only marginally increased 10 days after transfer. Irradiation of recipients marginally enhanced cDC recovery at 5 days, but much higher levels of donor-derived cDC were obtained from all sources, including blood, after 10 days. This latter set of conditions would have favoured the generation of cDC from earlier multipotent haematopoietic precursors (Wu et al., 2001) rather than immediate precursors. As a result of these tests, spleen appeared to be the source of the most immediate precursors of splenic DC.



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a) The standard assay for immediate cDC precursor activity. Spleen cells from Ly5.2 mice were transferred *i.v.* into non-irradiated Ly5.1 recipient mice. Five days later, recipient spleens were pooled, enriched for DC and enumerated. The proportion of $CD11c^{hi}$ $Ly5.2^{+}$ cDC was determined by flow cytometry, for calculation of the total number of donor-derived splenic cDC. Expressing this value relative to the number of cells transferred gave the 'per cell' cDC precursor activity (expressed as per 10^6 cells transferred). **b)** To determine the relative levels of immediate cDC precursors in spleen, blood or BM, between 2-10 x 10^6 nucleated cells from each source was transferred *i.v.* into irradiated or non-irradiated Ly5.1 recipients. Five or 10 days later, DC were enriched from recipient spleens and the 'total' cDC precursor activity determined for each organ. 'Total' activity was calculated as the 'per cell' activity for the transferred fraction, as calculated in a), multiplied by the total number of transferred cells that were originally recovered from the organ of one mouse (in this case, ~200 x 10^6 for spleen, ~2.2 x 10^6 for blood and ~55 x 10^6 for BM femurs and tibias). The arrow indicates the parameters used for subsequent assay of DC precursor activity: the transfer of fractions of spleen into non-irradiated recipients and measurment of DC precursor activitiy 5 days after transfer.

Enrichment of spleen DC precursors by density separation

The density characteristics of the spleen cells with cDC precursor activity were first determined in order to segregate them from preformed DC and devise an initial enrichment. Splenocytes were suspended in isosmotic Nycodenz media of different densities and centrifuged. The total recovery of cDC precursor activity, and the number of preformed DC and of nucleated cells in the supernatant and in the pellet, was determined. From this the density distribution of the cDC precursors was calculated and compared to that of DC, and of all spleen cells (Fig. 4.2a). Most cells with cDC precursor activity were lighter than 1.084g/cm^3, but denser than most DC. However, about 30% of the apparent total cDC precursor activity was associated with cells less dense than 1.076g/cm^3, the region where preformed DC were concentrated. Much of this apparent precursor activity was attributed to those few pre-existent cDC which re-seeded the spleen and then persisted for 5 days, for the following reasons: (1) Around 0.2% of purified cDC had been found to reseed the spleen and persist for a similar period (Chapter 3). (2) About 50% of the donor-derived DC showed low expression of CD11c and Ly5.2, a feature of aging cDC; this was verified by purifying spleen cDC, transferring them then analysing the few donor-type cDC recovered after 5 days (data not shown). When these aging donor-derived CD11c^{lo} Ly5.2^{lo} cDC were eliminated from the cDC precursor readout, only 15% of the true cDC precursor activity overlapped the density characteristics of cDC (Fig. 4.2a). These few precursors which had already acquired the light density characteristics of cDC were eliminated from further enrichment steps.

Based on these results a single step, two density zone centrifugation procedure was developed that excluded cells lighter than 1.076g/cm^3 (including most preformed DC) as well as all cells denser than 1.084g/cm^3 (including small lymphocytes, dead cells and erythrocytes) (Fig. 4.2b). This step enriched cDC precursor activity in the medium density zone 3-fold (Table 4.1).

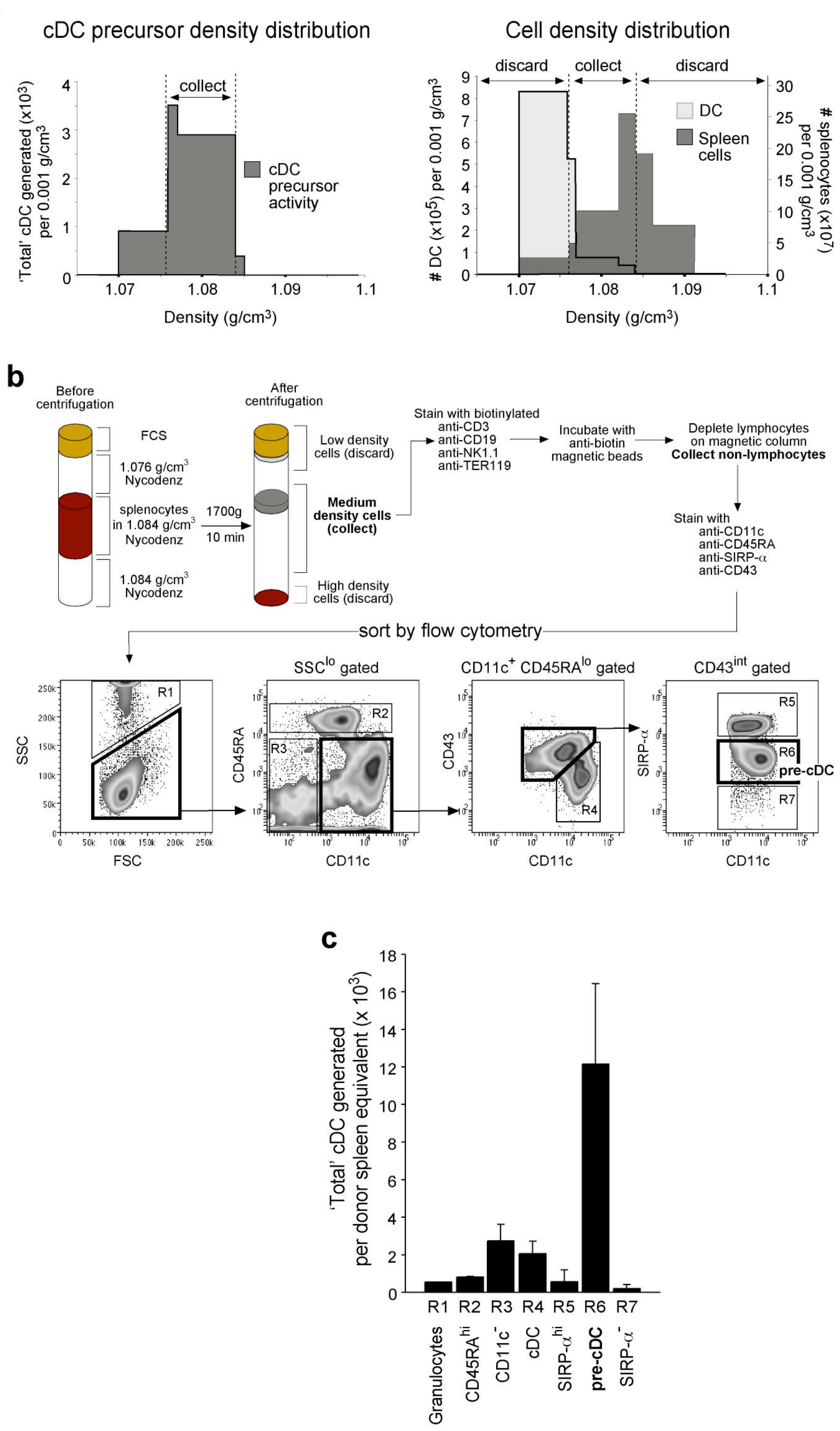
Table 4.1 Balance sheet for cDC precursor purification.

Fraction	% Total splenocytes	DC precursor activity (Per 10^6 transferred)	Fold Enrichment	Recovery (%)
Whole spleen	100.0	182± 70	1	100
Medium density	31.0	501± 242	3	85
Lymphocyte-depleted medium density	2.8	3,420± 1,270	19	53
pre-cDC	0.05	108,904± 38,025	600	30

The successive fractions during pre-cDC enrichment from spleen suspensions of Ly5.2 mice were assayed for their capacity to produce Ly5.2$^+$ cDC in recipient spleens 5 days after transfer *i.v.* into non-irradiated Ly5.1 recipients. Shown for each fraction is (i) their numerical representation as a percentage of total nucleated spleen cells, (ii) their 'per cell' cDC precursor activity, (iii) their enrichment of 'per cell' cDC precursor activity compared to whole spleen, and (iv) the 'total' precursor activity for each fraction and related back to the figure for original spleen suspension, adnd expressed as % recovery. The mean of individual experiments ± range is presented.

Figure 4.2 Successive steps in the purification of cDC precursors from spleen

a) Density separation. Spleen suspensions from Ly5.2 mice were centrifuged in a series of iso-osmotic Nycodenz media of different densities. The supernatant and pellet fractions from each density cut were collected and 5-10 x 10^6 cells of each fraction were then transferred *i.v.* into Ly5.1 recipient mice and the number of donor-derived spleen cDC generated 5 days later determined as in Fig.1. Shown is the distribution of 'total' apparent cDC precursor activity per density increment. The filled zones represent the precursor activity based only on donor-derived cDC with the typical $CD11c^{hi}$ $Ly5.2^{hi}$ phenotype. The total number of splenocytes and pre-existing $CD11c^{hi}$ cDC within in each density separation was also determined, and is also expressed per density increment. Based on this data, the medium density zone (1.076–1.084g/cm^3) was chosen for further cDC precursor purification. **b)** The normal final purification steps in isolating pre-cDC. A single dual-density centrifugation was used to select medium density cells. Lymphocytes and erythroid cells were coated with mAb and depleted with immunomagnetic beads. The lymphocyte-depleted, medium density cells were then stained in 4 fluorescent colours and sorted for forward light scatter (FSC), side light scatter (SSC) as well as fluorescence intensity, using the successive gates outlined by the heavy lines in the lower panel, to produce the final "pre-cDC" population. The activity and recoveries are shown in Table 4.1. **c)** The 'total' cDC precursor activity recovered in each of the sorted fractions of b). The activity and enrichment levels for the pre-cDC (R6) are given in Table 4.1.


a
cDC precursor density distribution
collect
'Total' cDC generated (x10³) per 0.001 g/cm³
cDC precursor activity
Density (g/cm³)
Cell density distribution
discard
collect
discard
DC (x10⁵) per 0.001 g/cm³
DC
Spleen cells
splenocytes (x10⁷) per 0.001 g/cm³
Density (g/cm³)
b
Before centrifugation
FCS
1.076 g/cm³ Nycodenz
splenocytes in 1.084 g/cm³ Nycodenz
1.084 g/cm³ Nycodenz
1700g
10 min
After centrifugation
Low density cells (discard)
Medium density cells (collect)
High density cells (discard)
Stain with biotinylated anti-CD3 anti-CD19 anti-NK1.1 anti-TER119
Incubate with anti-biotin magnetic beads
Deplete lymphocytes on magnetic column
Collect non-lymphocytes
Stain with anti-CD11c anti-CD45RA anti-SIRP-α anti-CD43
sort by flow cytometry
SSClo gated
CD11c+ CD45RAlo gated
CD43int gated
SSC
FSC
CD45RA
CD11c
CD43
SIRP-α
R1
R2
R3
R4
R5
R6
R7
pre-cDC
c
'Total' cDC generated per donor spleen equivalent (x 10³)
Granulocytes
CD45RAhi
CD11c-
cDC
SIRP-αhi
pre-cDC
SIRP-α-

Depletion of irrelevant cells by immunomagnetic particle separation

In the standard procedure, the medium density fraction containing the immediate cDC precursors was then depleted of B, T, NK and erythroid cells by first coating these cells with biotinylated mAb recognizing CD19, CD3, NK1.1 and the Ter-119 antigen, and then removing them with anti-biotin magnetic beads (Fig. 4.2b). Most of the activity was retained in this lymphocyte-depleted fraction, which was then over 20-fold enriched compared to the spleen suspension (Table 4.1). In an alternative protocol designed to additionally remove residual pDC, cDC and granulocytes, a depletion cocktail of mAb against MHC II, CD3, CD49b, Ter-119 and Ly6G was used instead of the above.

Purification by fluorescence activated cell sorting

The final purification of the cDC precursors involved immunofluorescent staining and cell sorting. Segregation of the lymphocyte-depleted, medium density fraction was based on multiple parameters, including light scatter characteristics and expression of CD11c, CD45RA, CD43 and Sirp-α. The successive gatings used to select the fraction with cDC precursor activity is shown in Fig. 4.2b, and the total recovery of cDC precursor activity in the finally selected and the discarded fractions is shown in Fig. 4.2c. Precursor activity was present in cells with low to moderate, but not high, side light scatter. Segregation by CD11c versus CD45RA expression showed no activity associated with $CD45RA^{hi}$ cells, which included residual pDC, and low activity associated with $CD11c^-$ cells; activity was concentrated with $CD11c^{int}$ $CD45RA^{lo}$ cells but overlapped with the $CD11c^{hi}$ region. Clear separation from residual cDC was then obtained by including staining for CD43. The $CD11c^{hi}$ $CD43^{lo}$ residual cDC fraction showed low precursor activity, and levels of their progeny were lower for CD11c and Ly5.2, therefore categorised as aging DC that survived transfer (data not shown). The active $CD11c^{int}$ $CD43^{int}$ fraction was further segregated by Sirp-α expression, removing the inactive Sirp-α^{hi} and Sirp-α^- components and leaving a distinct Sirp-α^{int} population, which concentrated all the cDC precursor activity. When the alternative protocol for depletion was used, where residual $CD43^{lo}$ cDC had been removed, staining and sorting based on CD43 expression could be omitted.

The $CD11c^{int}$ $CD45RA^{lo}$ $CD43^{int}$ Sirp-α^{int} fraction, now referred to as pre-cDC, represented the final immediate cDC precursor preparation. The standard and the alternative isolation protocols produced an identical pre-cDC population. Although there was some inevitable loss of cDC precursor activity during purification, it was important that all the cells that were discarded during bead depletion or cell sorting had low or negligible cDC precursor activity. Thus most of the capacity of the original spleen to generate cDC in the 5 day assay could be ascribed to this pre-cDC population. The pre-cDC population represented about 0.05% of spleen cells and cDC precursor activity was enriched over 600-fold in this preparation (Table 4.1**)**.

Surface antigenic phenotype of the pre-cDC population

The pre-cDC cells were sorted as $CD11c^{lo}$ $CD45RA^{int}$ $CD43^{int}$ Sirp-α^{int}. Importantly, they were negative for surface expression of CD4, CD8 and MHC II, providing clear segregation from preformed splenic cDC (Fig. 4.3a) and being low for expression of CD45RA, were distinct from pDC. The pre-cDC were homogeneous in expression of 35 surface markers tested, but were heterogeneous in expression of CD24 (Fig. 4.5a and Table 4.2). These pre-cDC represent a previously unrecognised spleen cell type.

The DC subtype potential of the pre-cDC

Pre-cDC generated a population of $CD11c^{hi}$ MHC II^{hi} DC after 5 days *in vivo* (Fig. 4.3b). Further flow cytometric analysis showed that none of the progeny were $CD45RA^+$, so no pDC were produced. Thus these precursors were appropriately termed pre-cDC. Of the cDC, all three subtypes normally found in spleen, $CD4^+8^-$, $CD4^-8^-$ and $CD4^-8^+$, were produced, although the proportion of $CD8^+$ DC was higher than found in a steady-state spleen (Fig. 4.3a, Fig. 4.5c).

Precursor frequency

While good recovery of the initial total cDC precursor activity had been maintained (Table 4.1), the final efficiency of splenic cDC generation was approximately 10% of pre-cDC transferred. This low efficiency was expected, in view of the competition from endogenous precursors in the non-irradiated recipients and in view of the likelihood of pre-cDC seeding tissues other than spleen following i.v. transfer; indeed

in preliminary experiments some DC progeny were also found in lymph nodes. However, this figure could also indicate that not all cells in the pre-cDC preparation were DC precursors. To check this, the pre-cDC were first labelled with PKH-26, a membrane dye that dilutes with successive divisions. Precursors were then incubated 24hr in conditioned medium from day 9 of flt-3 ligand stimulated BM cultures, a medium known to support the generation of spleen DC equivalents (Chapter 5). Cell survival was 82% (range 75-88%) of the pre-cDC cultured, and of these surviving cells 81% (range 72-96%) increased in size, upregulated MHC II and became DC (Fig. 4.3b). PKH-26 levels were conserved, so no there was no detectable cell division over this time. Thus the mean DC precursor frequency in the pre-cDC population was at least 67% (range 57-84%). Since such culture systems are unlikely to be fully efficient, this indicates that the majority if not all cells in the preparation were cDC precursors.

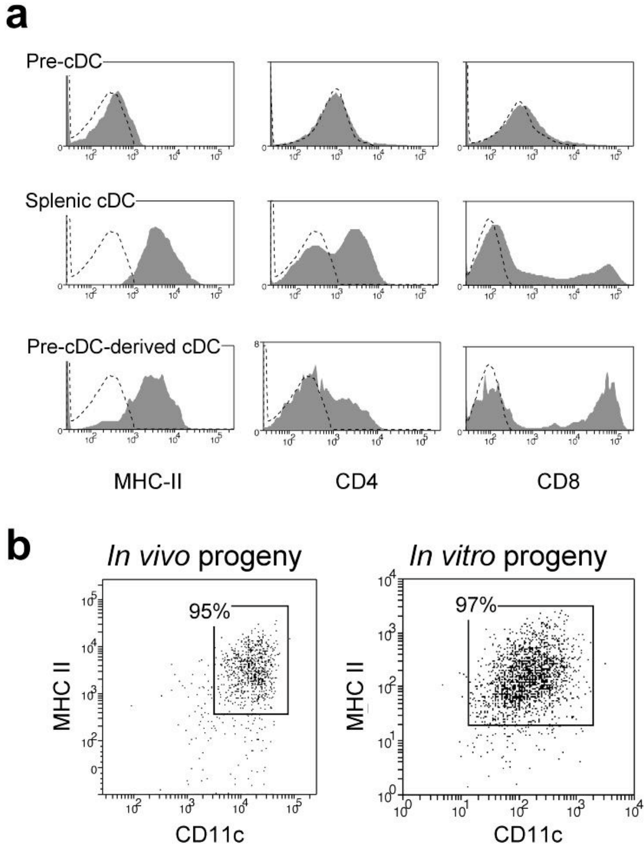


Figure 4.3 Characterisation of the pre-cDC population and its cDC progeny.
Results are typical of 3-5 experiments. **a)** Pre-cDC, freshly isolated splenic cDC or donor-derived DC isolated from recipient spleens following pre-cDC transfer, were all analysed for expression of MHC II, CD4 and CD8. **b)** DC derived from pre-cDC were analysed for the expression of MHC II and CD11c, either using the standard 5 day *in vivo* readout, or after culturing 1 x 10^4 pre-cDC for 24 hrs in conditioned medium from 9 day cultures of BM with Flt-3 ligand.

Table 4.2 Surface phenotype of pre-cDC, cDC and pDC.

Surface molecule	Alt. Name	mAb Clone	Expression by pre-cDC	Expression by cDC	Expression by pDC
MHC II		M5/114	–	+	–/+
CD3		KT3	–	–	–
CD4	Ly-4	GK1.5	–	⊕	⊕
CD8	Ly-2	YTS169.4	–	⊕	⊕
CD11b	Mac-1	M1/70	–/+	⊕	–
CD16	FcγRIII	2.4G2	–/+	⊕	+
CD19	B4	ID3	–	–	–
CD24	Heat Stable Ag	M1/69	⊕	⊕	–
CD25	IL-2Rα	PC61	–	+	–/+
CD34	Mucosialin	RAM34	–	–	–
CD40	gp39	FGK45.5	–	+	–
CD43	Leukosialin	S7	+	–	+
CD45R	B220	RA36B2	–/+	–	++
CD45RA	Exon A isoform	14.8	–/+	–	++
CD49b	Pan NK	DX5	–	–	–
CD54	ICAM-1	YN1/1.7.4	–	⊕	⊕
CD62L	L-selectin	MEL-14	–/+	⊕	+
CD69	Early Activ. Ag	H1.2F3	–	–	–
CD80	B7-1	16-10A1	–/+	+	–/+
CD86	B7-2	GL-1	–/+	–/+	–/+
CD103	Integrin-α_{IEL}	M2/90	–	⊕	–
CD127	IL-7 R	A7R34-2.2	–	–	+
CD161c	NK1.1	PK136	–	–	–
CD172a	SIRP-α	p84	+	⊕	+
CD205	DEC 205	NLDC-145	–/+	⊕	–
Mac-2	Galectin 3	M3/38	–	–	–
F4/80	Ly-71	F4/80	–/+	⊕	–
Mac-3	Lamp-2/(CD107b)	M3/84	+	–	–
Ly6A/E	Sca-1	D7	–	–	+
Ly6C		5075-3.6	+	–	+
Ly6G		1A8	–	–	–

Table 4.2

–/+ Continuous negative to low expression

⊕ Expression by a subset only

Lymphocyte-depleted, medium density spleen cells were blocked with rat Ig and anti-FcγRIII for 15 minutes then stained with antibodies against CD11c, CD45RA, CD43, SIRP-α and each of the indicated mAb (either biotinylated or directly conjugated to PE). Four-colour flow cytometric analysis was then performed on samples gating on SSC^{lo} cells, then pre-cDC ($CD11c^{int}$ $CD45RA^{lo}$), cDC ($CD11c^{hi}$ $CD45RA^{lo}$) or pDC ($CD11c^{int}$ $CD45RA^{hi}$).

Kinetics of DC generation

The cDC precursor assay readout was at day 5, providing a bias for immediate DC precursors. However this chosen time for read-out may not have represented the peak of cDC production by pre-cDC. Accordingly, the production of cDC was assessed from 2 to 13 days post transfer of pre-cDC (Fig. 4.4a). This showed the peak of cDC generation was indeed close to 5 days, a clear distinction from earlier haematopoietic precursors where the peak cDC production is around 14 days (Wu et al., 2001).

Cell division during DC generation

Despite the lack of division over 1 day *in vitro*, an important question was whether the pre-cDC proliferated to produce cDC over the 5 days *in vivo*, or whether the process involved a one-for-one conversion. By propidium iodide (PI) staining of the nucleus of freshly isolated pre-cDC, 18% were estimated to be in G_2, S and M-phase in the spleen (Fig. 4.4b). To gauge the extent of proliferation during cDC generation, pre-cDC were labelled with PKH-26 prior to transfer. Analysis of the donor-derived cells 5 days after transfer indicated that only a limited number of divisions (0 to 3) were involved in the production of cDC from pre-cDC *in vivo* (Fig. 4.4c). MHC II^{hi} cDC were found in every division peak.

Ability of pre-cDC derived DC to activate naïve T cells

To test whether the $CD11c^{hi}$ MHC II^{hi} cells that developed from pre-cDC *in vivo* were functional antigen-presenting DC, they were sorted and cultured with naïve T cells in a mixed leukocyte reaction. Precursor-derived cDC were able to stimulate allogenic T cell proliferation to an extent comparable with normal splenic cDC (Fig. 4.4d).

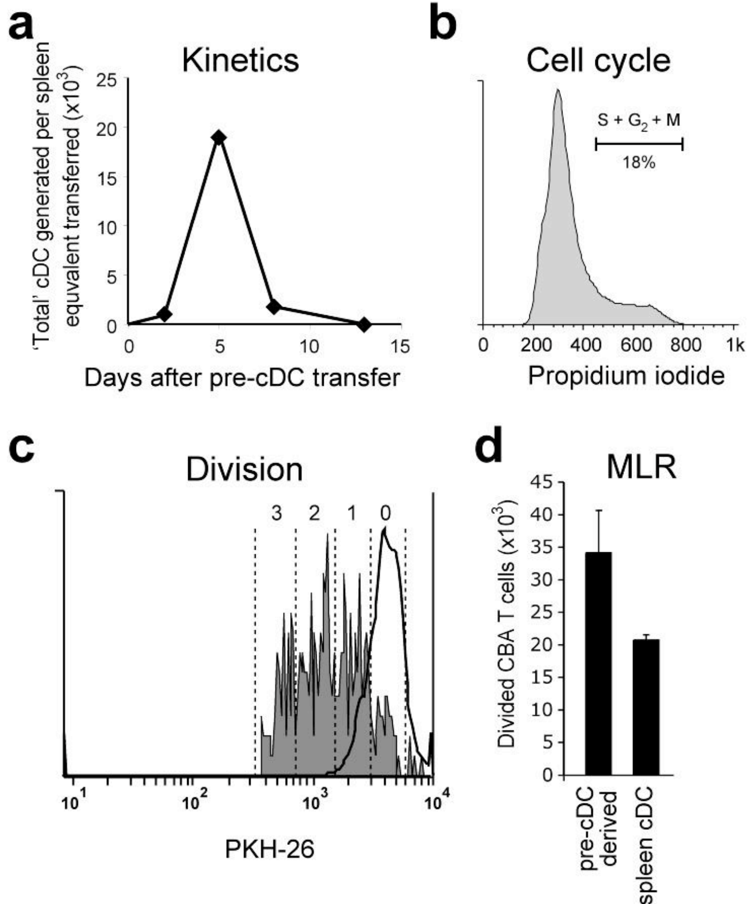


Figure 4.4 Kinetics, division and T cell activation

a) Pre-cDC were transferred into non-irradiated recipients and analysed for cDC generation at day 2, 5, 8 and 13. **b)** Cell cycle analysis of pre-cDC using propidium iodide (PI) staining of the nucleus. **c)** Pre-cDC were labelled with PKH-26 membrane dye prior to transfer. Recipient cDC were enriched after 5 days, stained and analysed for dilution of PKH-26 in the FL-2 channel after gating on $Ly5.2^+$ donor-derived cells. Undivided pre-cDC and donor-derived cDC (grey area) histograms are shown. **d)** Donor-derived cDC and host cDC were sorted at day 5 following pre-cDC transfer and cultured at 930 cells per well with 2 x 10^4 CFSE-labelled CBA T cells. Five days later, the total number of divided (low CFSE fluorescence) T cells was quantitated. The mean ± the range is shown.

cDC restricted potential of pre-cDC

The capacity of the pre-cDC to form other cell lineages on transfer was assessed by determining the phenotype of all the host-derived $Ly5.2^+$ cells in the spleen. Over 90% of the host-derived cells were less dense than $1.077 g/cm^3$, so possessed the buoyant density of DC. In all experiments over 99% of the total Ly5.2 cells were $CD11c^+$, and of these 90-95% were $CD11c^{hi}$ DC (Fig. 4.3b). The 5-10% cells expressing intermediate levels of CD11c were assumed to be precursors *en route* to becoming cDC. Clearly, the major product cells were cDC. No T cells, B cells, pDC, monocytes or macrophages were detected in this *in vivo* assay (data not shown).

The potential of the pre-cDC to form other cell lineages under the influence of various myeloid generating cytokines was assessed by their ability to form colonies in soft agar cultures (Metcalf et al., 2002). The pre-cDC were unable to produce any colonies in response to any of the cytokine combinations tested (Table 4.3).

Table 4.3 Lineage potential of pre-cDC and monocytes

		Mean number of colonies						Clusters	Number of experiments
		Blast	G	GM	M	Eo	Meg		
pre-cDC	GM-CSF	0	0	0	0	0	0	0	2
	M-CSF	0	0	0	0	0	0	<5	2
	SCF+IL-3+Epo	0	0	0	0	0	0	0	2
	FL+LIF	0	0	0	0	0	0	0	1
	Saline	0	0	0	0	0	0	0	2
Monocyte	GM-CSF	0	0	0	0	0	0	0	2
	M-CSF	0	0	0	1±1	0	0	95±62	2
	SCF+IL-3+Epo	0	0	0	0	0	0	0	2
	FL+LIF	0	0	0	0	0	0	0	1
	Saline	0	0	0	0	0	0	0	2
BM	GM-CSF	0	18±1	11.5±2.5	21±1	1.5±1.5	0		2
	M-CSF	0	2.5±0.5	3±0	31.5±2.5	0	0		2
	SCF+IL-3+Epo	6.5±1.5	20±7	13±6	14±0	1±1	21.5±6.5		2
	FL+LIF	4	2	0	0	0	0		1
	Saline	0	0	0	0	0	0		2

Soft agar colony assays were performed on sorted pre-cDC, Ly6C^{hi} monocytes or control BM under various conditions. Cells from each population (1x10^{3}) were cultured in 1 ml of Dulbecco's modified Eagle medium containing 0.3% agar and analyzed as described previously (Metcalf et al., 2002). The recombinant cytokines were used at the following final concentrations: GM-CSF, IL-3, M-CSF, LIF (10 ng/ml) and SCF (100 ng/ml), flt3L (500 ng/ml) and erythropoietin (EPO) (2I U/ml). After 7 d of incubation, differential colony counts were performed on fixed whole mount preparations that were stained for acetylcholinesterase, Luxol fast blue, and hematoxylin.

Separation of precursors for $CD8^+$ or $CD8^-$ cDC based on CD24 expression

That all three cDC subtypes could be generated from the pre-cDC in only a few divisions was surprising. This raised the issue of whether the pre-cDC population included cells pre-committed to particular cDC subtypes. The pre-cDC were heterogeneous in expression of CD24 (Fig. 4.5a), which has proved to be a useful marker to segregate $CD8^+$ from $CD8^-$ cDC sublineages (Crowley et al., 1989; Shortman and Liu, 2002; Vremec et al., 2000). Accordingly, the $CD4^-$ $CD8^-$ MHC II^- pre-cDC population were separated based on low, intermediate and high expression of CD24 and assessed for which DC subtypes they produced after transfer. The $CD24^{hi}$ pre-DC generated almost exclusively $CD8^+$ DC (Fig. 4.5b and Fig. 4.5c), the $CD24^{lo}$ pre-cDC generated mainly $CD8^-$ DC, whereas the well resolved $CD24^{int}$ population produced both subtypes (Fig. 4.5c). No other cell types apart from $CD11c^+$ DC were generated. Thus at least some proportion of the pre-cDC population had already undergone commitment to cDC subtype.

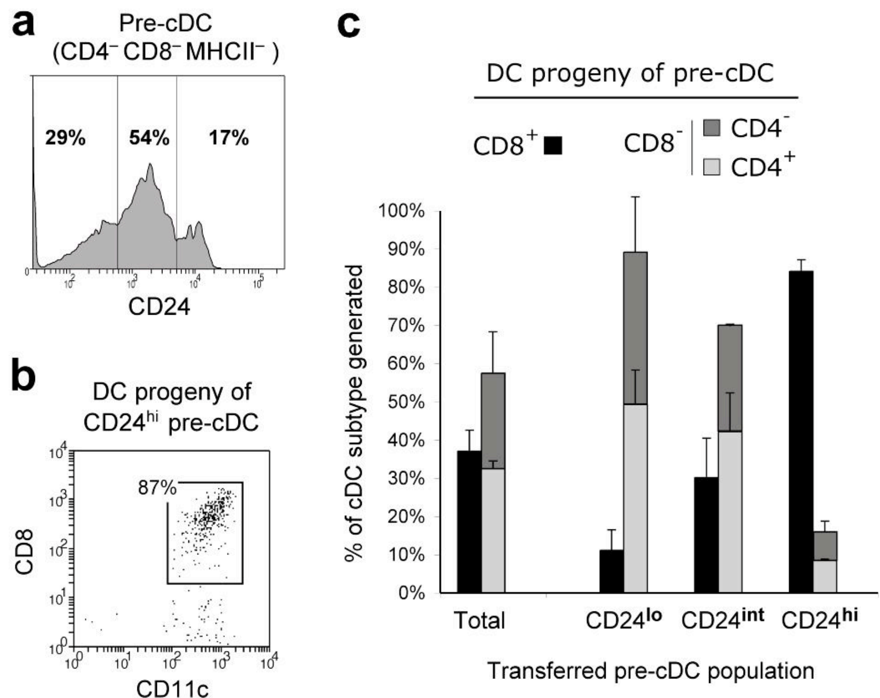


Figure 4.5 CD24 expression on pre-cDC segregates $CD8^+$ from $CD8^-$ cDC precursors.

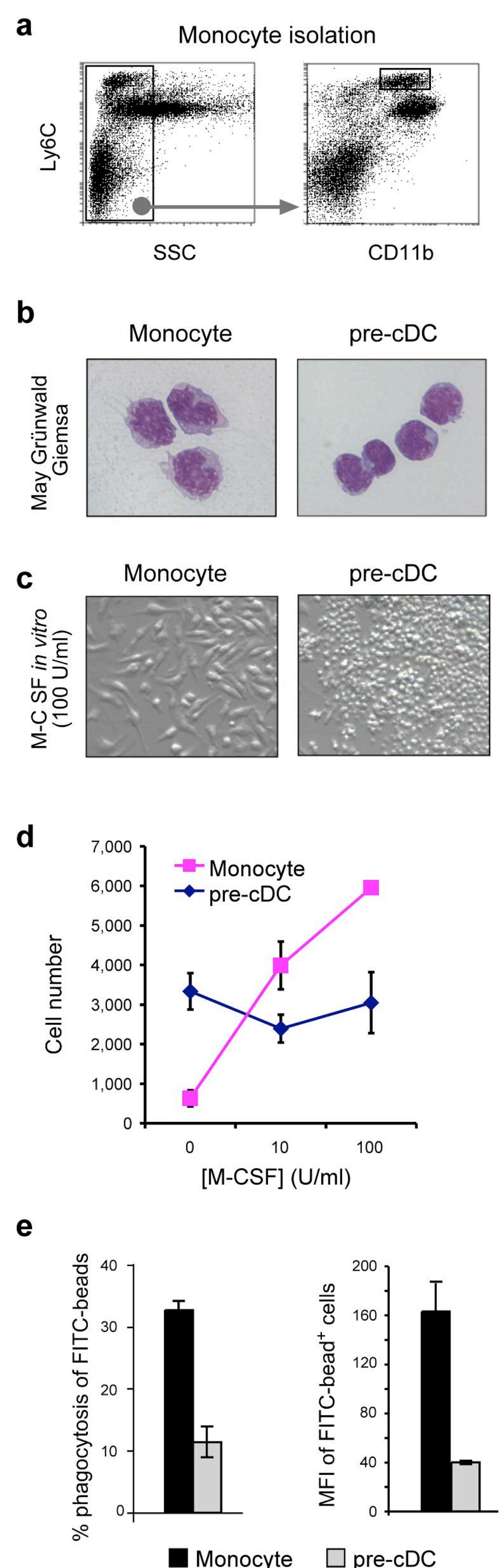
a) Pre-cDC were isolated and sorted according to the flow cytometric method of Fig. 4.2, with additional gating for cells negative for CD4, CD8 and MHC II. The pre-cDC were stained and analysed for CD24 expression. **b)** After transfer of the $CD24^{hi}$ pre-cDC from a), donor-derived DC were isolated and assessed for CD8 and CD11c expression. **c)** Either unseparated pre-cDC or fractions sorted according to CD24 expression from a) were assessed for production of different cDC subtypes in the standard 5 day *in vivo* assay. Each donor-derived DC subtype is expressed as a proportion of total donor-derived DC recovered. Data are derived from 3 independent experiments.

Distinguishing DC precursors from monocytes

Since monocytes have a potential to produce DC in culture (Leon et al., 2004; Sallusto and Lanzavecchia, 1994), and have been claimed to be the source of some DC *in vivo* (Geissmann et al., 2003; Leon et al., 2004; Randolph et al., 1999), it was important to test if pre-cDC were classical monocytes. $Ly6C^{hi}$ monocytes were isolated from BM for comparison to pre-cDC (Fig. 4.6a). Morphologically, pre-cDC were distinct from typical monocytes being smaller and having less cytoplasm (Fig. 4.6b). When cultured with M-CSF for 48hr most pre-cDC died; in contrast, purified monocytes survived well, adhered to the plastic surface of culture vessels and assumed macrophage morphology (Fig. 4.6c and Fig. 4.6d). This was in accordance the poor soft-agar cluster-forming ability of pre-cDC in response to M-CSF compared to purified monocytes (Table 4.3). Finally, the phagocytic capacity of pre-cDC and monocytes was compared, using fluorescent beads. A lower proportion of pre-cDC were actively phagocytic, and those pre-cDC that were phagocytic took up a lower number of beads than each phagocytic monocyte (Fig. 4.6e). Overall, pre-cDC did not fit the accepted profile of monocytes.

Figure 4.6 Comparison of pre-cDC to monocytes.

Results are representative of at least 2-5 individual experiments. **a)** Monocyte isolation. BM suspensions were stained with Ly6C and CD11b. Using flow cytometry, cells were gated for low side scatter (SSC). $CD11b^+$ $Ly6C^{hi}$ monocytes were sorted to purity **b)** The freshly isolated pre-cDC or $Ly6C^{hi}$ BM monocytes were stained with May Grünwald Giemsa. **c)** These cell types were also cultured at 1 $x10^4$ per well in duplicate for 48 h with 0, 10 or 100 U/ml rhM-CSF then examined by phase-contrast microscopy or **d)** their survival enumerated after harvesting with trypsin. **e)** The pre-cDC or monocytes were incubated with FITC-labelled beads for 45 minutes at 37°C or 4°C, then bead uptake assessed by flow cytometry deducting background of the 4°C control. The left panel compares the percentage of cells with any phagocytic capacity and the right panel compares the extent of uptake of those that are phagocytic, expressed as mean fluorescence intensity of fluorescent beads (MFI).


a
Monocyte isolation
Ly6C
SSC
CD11b
b
Monocyte
pre-cDC
May Grünwald Giemsa
c
Monocyte
pre-cDC
M-C SF in vitro (100 U/ml)
d
Monocyte
pre-cDC
Cell number
7,000
6,000
5,000
4,000
3,000
2,000
1,000
0
0
10
100
[M-CSF] (U/ml)
e
% phagocytosis of FITC-beads
40
30
20
10
0
MFI of FITC-bead+ cells
200
160
120
80
40
0
Monocyte
pre-cDC

Discussion

Rather than exploring the DC developmental potential of the clonogenic haematopoietic precursor cells in BM, this study set out to characterise the immediate precursors of steady-state splenic DC, and designed an assay selective for such late DC precursors. Using this assay, it was demonstrated that spleen itself contains a major reservoir of immediate cDC precursors. This suggests that the spleen is capable of substantial cDC generation without immediate replenishment by precursors migrating from BM, although eventually such replenishment may be essential.

The findings are in line with studies demonstrating the generation of DC from spleen cells in culture (Berthier et al., 2000; O'Neill et al., 2004; Winzler et al., 1997). The low cDC precursor activity found of blood cells using the *in vivo* assay is further support for this concept and agrees with recent studies using parabiotic mice demonstrating a degree of independence from the bloodstream in splenic cDC generation (Kabashima et al., 2005). This independence may in part be explained by the recent finding that steady-state splenic $CD8^-$ cDC are not end cells, but are capable of a degree of homeostatic proliferation (Kabashima et al., 2005). Thus DC themselves could be considered as immediate DC precursors. It is possible that part of the apparent $CD8^-$ cDC precursor activity obtained in the DC-enriched light density fraction of spleen represented such proliferation of donor cDC that seeded the recipient spleen. However, it is important to note that only a small proportion of the cDC generation potential of the spleen could be ascribed to preformed cDC. Most of the immediate spleen cDC generation potential of the spleen could be ascribed to the pre-cDC identified in this Chapter, which are MHC II^- and more dense than preformed cDC.

The immediate cDC precursors found in spleen, the pre-cDC, have a $CD11c^{int}$ $CD4RA^{lo}$ $CD43^{int}$ Sirp-α^{int} $CD4^-$ $CD8^-$ MHC II^- surface phenotype. This phenotype differs from the cDC precursors described by some investigators (Bruno et al., 2001; del Hoyo et al., 2004; del Hoyo et al., 2002; Leon et al., 2004; Wang et al., 2002), but resembles in some features one population of cDC precursors found in BM (Diao et al., 2004). The latter may represent a BM progenitor of spleen pre-cDC. Similarly, the

recently described clonogenic progenitor of macrophages and DC found in BM (Fogg et al., 2006) may represent an earlier step in DC development. Most of the differences between other studies and the results in this Chapter can be ascribed to several parameters in this study; the selective assay for immediate rather than early DC precursors, by seeking precursors of spleen DC in the spleen rather than BM, and as a result of the assay which involved steady-state rather than inflammatory conditions *in vivo*. It is emphasised that the pre-cDC described herein are precursors of the cDC found in steady-state spleen, and these could differ from the immediate precursors of other DC subtypes, particularly of the migratory DC found in lymph nodes (Ginhoux *et al.*, 2006; Merad *et al.*, 2002; Villadangos and Heath, 2005).

An important question is whether the pre-cDC precursor population is pure, whether every cell present is a pre-cDC. This has been a crucial issue in previous studies (del Hoyo et al., 2004; del Hoyo et al., 2002). The extent of activity enrichment indicates a high degree of purification. The limit-dilution or single cell clonal assays used for determining the precursor frequency of early progenitor populations are not applicable here, since pre-cDC are close to end-stage cells with only a few remaining divisions, and "clone" sizes of 1-8 DC are not detectable in most assays. Instead, the proportion of the pre-DC population able to form cDC without significant cell division after short-term culture in an appropriately conditioned medium was determined. This demonstrated that at least 70% of the pre-DC were able to form cDC. Thus, this study was dealing with a nearly pure, if not completely pure, DC precursor population and the surface phenotype presented must correspond to that of cDC precursors.

It was important to demonstrate whether the immediate precursors of steady-state splenic cDC were monocytes, as has been suggested (Geissmann et al., 2003; Leon et al., 2004). The pre-cDC population was distinct from monocytes, as they were not responsive to M-CSF, were poorly phagocytic, differed in morphology and by phenotype. In particular, pre-cDC were $CD11b^{low}$ $F4/80^{low}$ SIRP-α^{int} as opposed to monocytes which are $CD11b^{hi}$ $F4/80^{+}$ SIRP-α^{hi}.

How then do the various precursors of DC fit into the overall programme of splenic DC generation? Early $flt3^+$ BM precursors, whether lymphoid or myeloid-restricted, are able to generate both cDC and type-1 interferon producing pDC (Adolfsson et al., 2005; D'Amico and Wu, 2003; Karsunky et al., 2003) (Fig. 4.7). However, downstream of these precursors there must exist a developmental branch that separates the two DC lineages, since the intrasplenic pre-DC reported in this Chapter are unable to generate pDC. This is likely to occur in BM as both developed pDC and their precursors are found in this site, whereas only the developed form of pDC is found in spleen (Fig. 4.7 and (Kamogawa-Schifter et al., 2005; Omatsu et al., 2005)). In contrast, fully formed cDC are not detectable in large numbers in BM or blood (O'Keeffe et al., 2003), but are found in spleen. This suggests that a form of cDC precursor leaves the BM and seeds the spleen prior to the final stages of cDC generation from pre-cDC within this organ. A point of particular interest from the studies in this Chapter is that pre-commitment to the separate $CD8^+$ versus $CD8^-$ cDC subtypes can already be seen within the pre-cDC population, and is revealed by differences in CD24 expression well before CD8 itself appears (Fig. 4.7). This reinforces the view that these cDC subsets should be considered as products of separate cDC sublineages (Chapter 3), rather than the $CD8^+$ cDC developing from $CD8^-$ cDC (Martinez del Hoyo et al., 2002).

It is proposed pDC and the pre-cDC identified in this study can both be precursors of DC. When there is a microbial stimulus, a form of DC is generated from pDC (Grouard et al., 1997; O'Keeffe et al., 2002a; Zuniga et al., 2004). However, only pre-cDC develop further into splenic DC in the steady-state (Fig. 4.7).

Chapter 5:
Monocytes are precursors of inflammatory DC

Abstract

In Chapter 4, I reported the identification of a unique intrasplenic precursor of conventional $CD8^+$ and $CD8^-$ cDC, termed pre-cDC. This pre-cDC population was not a classic monocyte. This finding was in contrast to the extensively characterised role of monocytes, both $Ly6C^{hi}$ and $Ly6C^{lo}$ subsets, as immediate precursors of DC *in vitro* when cultured with GM-CSF. Therefore, in this Chapter I have assessed the role of the monocyte subsets as precursors of DC *in vivo*. I ran a series of tests to assess monocyte to DC differentiation both in the steady-state and during inflammation. Upon transfer into steady-state mice, $Ly6C^{lo}$ monocytes gave a low output of DC, none of which were $CD8^+$ cDC. On a per cell basis, they were 50-fold less effective than the pre-cDC identified in Chapter 4. $Ly6C^{hi}$ monocytes were also poor at DC production, and no DC progeny were detected either 2 or 5 days after transfer into steady-state mice, nor when examined 10 days after transfer into irradiated recipients. However, when recipient mice were subjected to a GM-CSF-dependent inflammation, $Ly6C^{hi}$ monocytes could then produced a distinct type of $CD11c^{int}$ $CD11b^{hi}$ Mac-3^+ splenic DC. Thus the origin and type of DC present in the spleen is determined by the inflammatory status of the host.

Introduction

Monocytes are typically characterised by their 'monocytic' morphology, high capacity for endocytosis, and responsiveness to M-CSF to generate macrophages. Within the monocyte population, separate $Ly6C^{hi}$ and $Ly6C^{lo}$ monocyte subsets have recently been identified (Geissmann et al., 2003; Lagasse and Weissman, 1996; Sunderkotter et al., 2004; Taylor et al., 2003) and are proposed to be the equivalents of human $CD14^{+}$ and $CD16^{+}$ human monocytes, respectively (Geissmann et al., 2003). In addition to being precursors of macrophages, monocytes have been an attractive candidate for a precursor of cDC due to their ability to produce DC *in vitro* under the influence of GM-CSF with or without IL-4 (GM-DC) (Inaba et al., 1992a; Inaba et al., 1992b; Lu et al., 1995; Peters et al., 1993; Sallusto and Lanzavecchia, 1994; Scheicher et al., 1992). This is true for both monocyte subsets (Geissmann et al., 2003; Leon et al., 2004). However, there are mixed reports regarding their capacity to produce DC *in vivo.* In most of such studies a phagocytic or inflammatory stimulus was required, and the resultant DC did not correspond well to the described steady-state DC subsets isolated from spleen (Geissmann et al., 2003; Ginhoux et al., 2006; Leon et al., 2004; Randolph et al., 1999). Thus, the contribution of the monocyte populations to DC generation both in the steady-state versus conditions of duress, and the nature of the progeny DC, was still unclear.

As monocytes generate a relatively homogeneous population of $CD11c^{+}$ $CD11b^{+}$ $CD8^{-}$ MHC II^{+} 'myeloid' DC in culture, they have often been referred to as the equivalent of $CD8^{-}$ cDC of the spleen and lymphoid organs, which share this pattern of marker expression. However, formal proof of such a correlation is lacking. Indeed several lines of evidence suggest monocyte-derived DC may not be related to the development of steady-state DC. Firstly, mice deficient for M-CSF or GM-CSF, or their receptors, which have defective monocyte development or transformation to DC, respectively, actually have the expected numbers, ratio and function of steady-state splenic DC (Ginhoux et al., 2006; Hikino et al., 2000; Usuda, 1994; Vremec et al., 1997; Witmer-Pack et al., 1993). Secondly, GM-CSF levels are low to undetectable in the steady-state (Baiocchi et al., 1993; Cebon et al., 1994), further suggesting this cytokine is not necessary for steady-state development. Of note, GM-CSF levels do

increase with infection and inflammation (Cheers et al., 1988; Metcalf, 1988). Thirdly, the major cytokine driving the generation of the steady-state cDC subtypes *in vivo* appears to be flt-3 ligand (FL) as evidenced by an absence of DC in genetic knockout of FL, or members of it signalling pathway, such as STAT3 (Laouar et al., 2003; McKenna et al., 2000). By contrast, mice deficient for FL have normal monocyte development (Walzer et al., 2005).

In this Chapter, DC production was assessed after transfer of the monocyte subsets into steady-state, irradiated, or inflamed recipients. The contribution of monocytes to steady-state development appeared to be low to negligible. By contrast, mice that had undergone a GM-CSF-dependent inflammation could convert monocytes to a unique subset of inflammatory DC. This suggests monocyte to DC transformation is determined by the inflammatory situation of the host.

Results

Monocytes do not generate splenic DC in steady-state

Although the pre-cDC population identified in Chapter 4, which were not monocytes, accounted for most of the 'total' cDC precursor activity of spleen, this did not exclude the possibility that monocytes have some DC-precursor potential. Therefore, purified Ly6C^{hi} and Ly6C^{lo} monocytes from blood, spleen and BM (Fig. 5.1) were assessed for their capacity to form splenic DC in the 5 day steady-state *in vivo* assay (Table 5.1, as described in Chapters 3 and 4. None of the Ly6C^{hi} monocytes gave rise to significant numbers of spleen DC and the rarer Ly6C^{lo} monocytes gave only a low yield of DC, which were all CD8^{-}. On a per cell basis, blood Ly6C^{lo} monocytes were 50-fold less effective, spleen Ly6C^{hi} monocytes over 500-fold less effective, and Ly6C^{hi} monocytes from blood and BM over 1000-fold less effective than the pre-cDC population in generating DC *in vivo*.

To test whether using later time points or irradiated recipients would allow monocyte to DC conversion, as has been claimed previously (Leon et al., 2004), either BM Ly6C^{hi} monocytes or all BM non-monocytes (Fig. 5.1) were transferred into either irradiated or non-irradiated recipients, their progeny assessed after 10 days (Table 5.2). No donor phenotype Ly6C^{hi} monocyte progeny could be detected in non-irradiated recipients and only a few cells that could be considered DC were generated in irradiated recipients, a figure 1000-fold lower than all BM non-monocytes under the same conditions. This low-level monocyte to DC conversion in irradiated recipients could represent a legitimate event, or else be the result of contamination by early BM-resident DC precursors that were not monocytes. As all BM non-monocytes were 1000-fold more effective in DC production, a contamination of only 0.1% of the monocyte preparation by such cells would be necessary. In any case, the results indicated that Ly6C^{hi} monocytes were not precursors of DC under steady-state conditions, even when irradiated 'empty' hosts were provided.

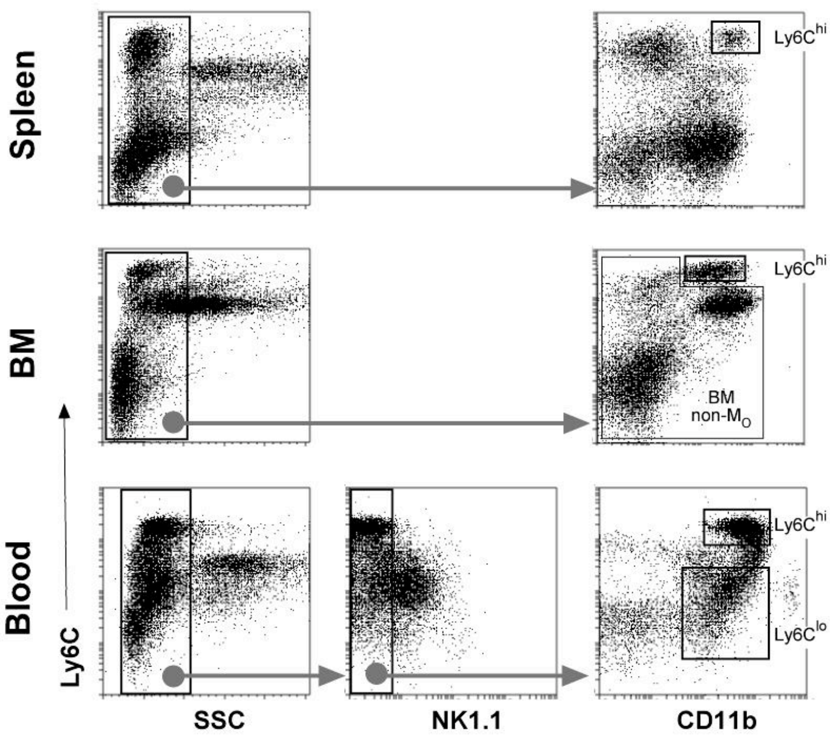


Figure 5.1 Isolation of monocyte subsets

Medium density, lymphocyte-depleted (<1.082 g/cm^3) spleen, blood mononuclear fractions or BM suspensions were stained with Ly6C and CD11b, as well as NK1.1 for blood preparations. Using flow cytometry, cells were gated for low side scatter (SSC) and NK1.1$^-$ cells (for blood). Either CD11b$^+$ Ly6C^{hi} monocytes from all tissues, all non-monocytes from BM, or CD11b$^+$ Ly6C^{lo} monocytes from blood were sorted to purity, in all cases >98% purity.

Table 5.1. cDC precursor activity of monocyte subsets compared to purified spleen pre-cDC.

	'Per cell' activity (cDC recovered per 10^6 cells transferred)
Spleen pre-cDC ($CD11c^{int}$ $CD43^{int}$)	108,904 ± 38,025
Blood $Ly6C^{lo}$ monocytes	2,260 ± 990
Spleen $Ly6C^{hi}$ monocytes	205 ± 103
Blood $Ly6C^{hi}$ monocytes	None detected
BM $Ly6C^{hi}$ monocytes	None detected

Spleen pre-cDC were purified as described in Fig. 4.2b and monocytes as per Fig. 5.1. Samples (1 x 10^5 to 2 x 10^6 cells) were transferred *i.v.* into Ly5.1 recipient mice (non-irradiated) and the production of donor-derived cDC in the spleen determined 5 days later. Values are the means of individual experiments ± the range or standard deviation.

Table 5.2 cDC precursor activity of Ly6C^{hi} monocytes and non-monocytes after 10 d

	Recipient irradiation	% Ly 5.2 cells in recipient spleen	'Per cell' activity (cDC generated per 10^6 cells transferred)	Subset distribution of donor derived cDC (%)		
				$CD8^+$	$CD4^+$	$CD8^-$ $CD4^-$
BM monocytes	-	0.00	0	-	-	-
	+	0.05	9,790	2	49	49
BM all non-monocytes	-	0.10	20,820	50	32	18
	+	39.00	10,339,690	67	19	14

Purified BM Ly6C^{hi} monocytes, or all other BM non-monocytes were purified and between 0.5-1 x 10^6 cells injected into either non-irradiated (0 Gy) or lethally irradiated (11 Gy) Ly 5.1 recipients, in each case along with 5 x 10^4 host Ly5.1 BM cells. Ten days later splenocytes were harvested, pooled and the level of donor-derived DC determined.

Monocytes can generate splenic DC during inflammation

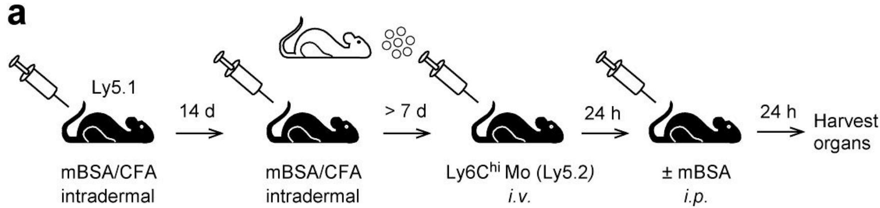
In light of reports that monocytes can act as precursors of peritoneal and lymph node DC duration inflammation (Geissmann et al., 2003; Randolph et al., 1999), a series of tests was performed to determine if transferred monocytes could generate DC of the spleen, with or without various induced experimental conditions of inflammation. With thioglycolate-induced inflammation of recipient mice, donor-derived cells from Ly6C^{hi} monocytes could not be detected in the spleen, although some cells were present in the peritoneum. However, none of these cells were MHC II^{hi} DC under these conditions (data not shown). As thioglycolate can induce an inflammatory response independent of GM-CSF (Cook et al., 2004), and considering that GM-CSF is a key cytokine for monocyte to DC conversion *in vitro*, a different inflammatory model that was dependent on GM-CSF was tested (Cook et al., 2004). This established protocol involved priming then boosting recipient mice with methylated bovine serum albumin (mBSA) in complete Freund's adjuvant (CFA), then finally inducing inflammation with mBSA alone (Fig. 5.2a).

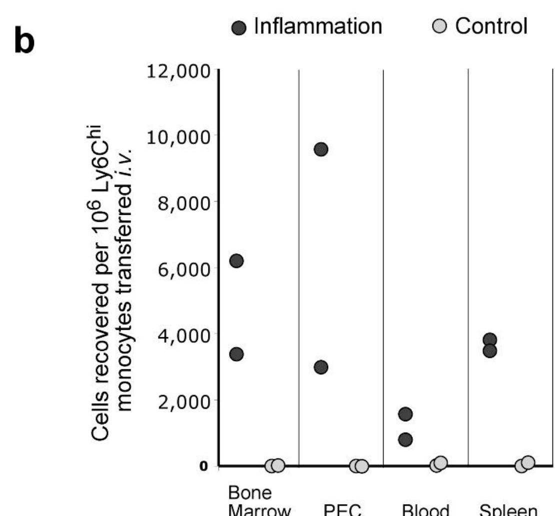
Ly6C^{hi} monocytes were transferred *i.v.* into the fully primed mice, and then inflammation induced one day later with mBSA alone. A further day later spleen, blood, BM and peritoneal cells were assayed for donor monocyte-derived DC. In control mice without inflammation, no donor-derived cells could be found in spleen (Fig. 5.2b), in accordance with the experiments of Table 5.1, with the rapid turnover of monocytes (Kabashima et al., 2005; Sunderkotter et al., 2004) and with previous findings (Geissmann et al., 2003). However, when inflammation was induced, donor-derived cells were detected (Fig. 5.2c). Donor derived cells in blood, BM and peritoneum did not express high levels of CD11c nor MHC II (data not shown) and so remained as monocytes. However, those that had seeded the spleen had upregulated both these molecules and so could be classed as DC (Fig. 5.2d). These monocyte-derived DC were uniquely CD11c^{int} CD11b^{hi} MAC-3^{+}, as well as being low for CD8 and CD4. They were thus a DC subtype that could be distinguished from the resident steady-state CD11c^{hi} CD11b^{int} MAC-3^{-} splenic DC (Fig. 5.2c,d). To ensure that these monocyte-derived cells were DC, they were sorted from the recipient spleens and assessed for their capacity to activate naïve T cells in a mixed leukocyte reaction. Monocyte-derived DC were as effective as steady-state spleen DC at stimulating T

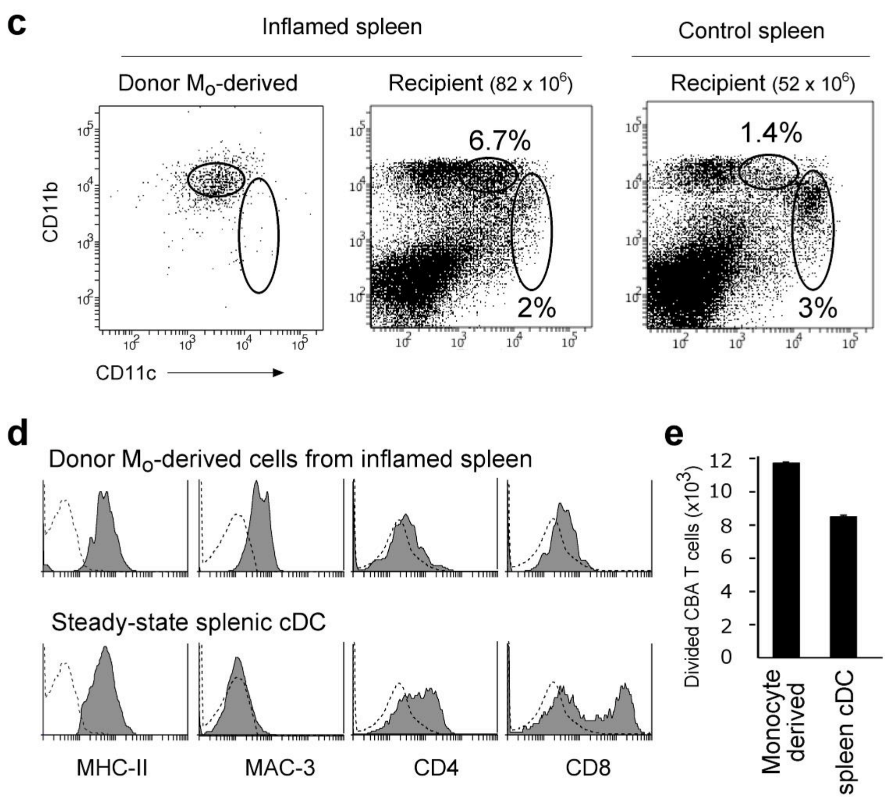
cell proliferation (Fig. 5.2f). It was concluded that under certain conditions of inflammation, monocytes are able to generate a unique form of DC in the spleen.

Figure 5.2 Monocytes produce splenic DC during inflammation.

a) Protocol for monocyte transfer into recipient mice with antigen induced inflammation. To prime Ly5.1 mice for an inflammatory response, mBSA in CFA was injected intradermally at the base of tail, and the mice then boosted with the same injection 14 days later. At least 7 days later, Ly6C^{hi} monocytes from Ly5.2 mice were transferred *i.v.*. Inflammation was induced in some recipients but not control recipients 24 hrs later, and the spleens harvested a further 24 hrs later. **b)** Monocyte recruitment during inflammation. Ly6C^{hi} monocytes were transferred *i.v.* into primed and boosted recipients or untreated control animals. Inflammation was induced by *i.p.* injection of mBSA alone in primed and boosted recipients after 24 hrs. A further 24 hours later bone marrow, peritoneal exudate cells (PEC), blood and spleen were harvested from both inflamed and control recipients, total numbers of recipient cells counted and a fraction stained for Ly5.2. The calculated number of donor-derived cells recovered per 10^6 monocytes transferred is shown for 2 independent experiments. **c)** Analyses of DC-like cells obtained after monocyte transfer. Light density (<1.082g/cm^3) spleen cells were analysed two days after Ly6C^{hi} BM monocytes were transferred into inflamed or control mice. Samples were stained for Ly5.2, CD11c and CD11b, gated for donor-derived (Ly5.2$^+$) or recipient (Ly5.2$^-$) cells, and their phenotype determined. Note the lower CD11c expression of the monocyte-derived cells. No donor-derived cells were obtained in the control recipient spleens. **d)** The surface phenotype of donor monocyte-derived cells from inflamed recipient mice were compared with normal cDC from steady-state mice. **e)** Donor monocyte-derived DC from inflamed recipients or control splenic cDC were sorted and cultured at 200 cells per well with 2 x 10^4 CFSE-labelled CBA T cells. Five days later, the total number of divided T cells was quantitated.


a
Ly5.1
14 d
> 7 d
24 h
24 h
Harvest organs
mBSA/CFA intradermal
mBSA/CFA intradermal
Ly6C^{hi} Mo (Ly5.2)
i.v.
± mBSA
i.p.


b
Inflammation
Control
Cells recovered per 10^6 Ly6C^{hi} monocytes transferred i.v.
12,000
10,000
8,000
6,000
4,000
2,000
0
Bone Marrow
PEC
Blood
Spleen


c
Inflamed spleen
Control spleen
Donor M$_o$-derived
Recipient (82 x 10^6)
Recipient (52 x 10^6)
6.7%
2%
1.4%
3%
CD11b
CD11c
d
Donor M$_o$-derived cells from inflamed spleen
Steady-state splenic cDC
MHC-II
MAC-3
CD4
CD8
e
Divided CBA T cells (x10^3)
12
10
8
6
4
2
0
Monocyte derived
spleen cDC

Discussion

In contrast to reports implicating a role for monocytes in steady-state spleen DC development (Leon et al., 2004), the present study provides evidence that monocytes make only a minor contribution to the steady-state situation in this organ. The Ly6C^{lo} monocyte subset was able to generate some splenic DC in the steady-state. However, none of the progeny were CD8^{+} and the efficiency was 50-fold lower than that of the intrasplenic pre-cDC reported in Chapter 4. Ly6C^{hi} monocytes were even less efficient and did not yield detectable progeny after transfer into non-irradiated hosts, and only a low number after transfer into 'empty' irradiated hosts.

It is perhaps not surprising that monocytes could not generate significant numbers of DC in the steady-state considering GM-CSF is crucial for monocyte to DC conversion in vitro, and that levels of this cytokine are low to undetectable in the steady-state. Indeed, when a GM-CSF-dependent inflammatory stimulus was provided, the situation changed. An overall increase in total splenocyte numbers was observed in recipient mice, which was largely contributed by a 7-fold increase in CD11c^{int} CD11b^{hi} cells of an unknown function or origin. Of note, there was no change in CD11c^{hi} CD11bint conventional DC numbers with inflammation. When the fate of donor Ly6C^{hi} monocytes in these inflamed recipients was assessed they could now be recovered in spleen. These monocytes, which were CD11c^{-} CD11b^{hi} MHC II- when freshly isolated, had also acquired a CD11c^{int} CD11b^{hi} MHC II^{+} phenotype in vivo. Additionally, they were CD4^{-}, CD8^{-} and MAC-3^{+}. Thus, Ly6C^{hi} monocytes could produce a unique inflammatory DC population that did not overlap in phenotype with CD11c^{hi} CD11blo MAC-3^{-} cDC.

These monocyte-derived inflammatory DC closely resembled the CD11c^{int} CD11b^{hi} MAC-3^{+} phenotype of TNF-α/iNOS-producing (Tip) DC that appear after infection with Listeria monocytogenes (Serbina et al., 2003). In that study, Tip DC were presumed to derive from Ly6Chi (CCR2^{+}) monocytes due to the absence Tip DC in CCR2$^{-/-}$ mice (Serbina and Pamer, 2006; Serbina et al., 2003). Infection with Listeria is known to induce high levels of GM-CSF (Cheers et al., 1988), so it fits that monocyte-derived DC emerge in such situations. Other studies also point to the generation of a CD11c^{int} CD11b^{hi} DC population after infection or immunisation with

Streptococcus pneumoniae (Balazs et al., 2002), Mycobacterium tuberculosis (Gonzalez-Juarrero et al., 2003; Peters et al., 2004), Salmonella typhimurium (Sundquist and Wick, 2005) and LCMV (Homann et al., 2004), or during autoimmune sequelae such as psoriasis (Lowes et al., 2005) and diabetes (Turley et al., 2003). Perhaps these are other examples of inflammatory monocyte-derived DC.

While monocytes were not precursors of steady-state DC in spleen, this does not exclude them as precursors for steay-state DC in other tissues, particularly to the migratory DC of lymph nodes. Initial studies had indicated that Langerhans cell reconstitution after UV-irradiation was dependent on CCR2, again implicating a Ly6Chi ($CCR2^+$) monocyte precursor (Merad et al., 2002). More direct testing of transferred Ly6Chi monocytes have since shown that they can act as Langerhans cell precursors (Ginhoux et al., 2006). The caveat of this study was that UV-irradiation was obligatory to clear resident LC for subsequent re-constitution, which may reflect a kind of inflammation. Whether skin-resident monocytes contribute to LC development in the absence of UV-irradiation is not clear.

Another current topic of debate is whether $Ly6C^{hi}$ monocytes give rise to $Ly6C^{lo}$ monocytes (Geissmann et al., 2003; Gordon and Taylor, 2005; Sunderkotter et al., 2004). The use of an in vivo monocyte depletion method and assessment of the reconstitution kinetics indicated $Ly6C^{hi}$ monocytes were the upstream precursor of the Ly6Clo subset (Sunderkotter et al., 2004). In vitro data in that study also corroborated such a conversion. However, attempts at the transfer of purified $Ly6C^{hi}$ monocytes in vivo did not yield any progeny (Geissmann et al., 2003; Sunderkotter et al., 2004), with identical results in this study. That the isolation procedure for monocytes affects their survival has been offered as one explanation for this observation. However, as $Ly6C^{hi}$ monocytes could be recovered in recipients that had inflammation in this study, it suggests monocyte survival is not absolutely affected by their isolation. It is still possible that $Ly6C^{hi}$ to Ly6Clo monocyte differentiation in the steady-state is a rare event and so not able to be measured with traditional transfer approaches.

From the results of this study and other evidence, it is proposed here that the *in vitro*-derived GM-DC culture method mimics the *in vivo* process of monocyte to DC conversion with a GM-CSF-dependent inflammation. Further evidence for such a

correlation is that $Ly6C^{hi}$ monocytes are the last precursor stage en route to DC development when early BM precursors are cultured with GM-CSF (Nikolic et al., 2003), and that *in vivo* Tip DC produce TNF-α and iNOS like GM-DC, but unlike freshly isolated DC (Lu et al., 1996; Powell et al., 2003). Thus studies on GM-DC probably best represent an inflammatory situation.

The model put forward here is that only $Ly6C^{lo}$ monocytes can make a minor contribution to "$CD8^{-}$" DC in the steady-state. However, when there is a microbial or inflammatory stimulus, additional forms of DC may be generated from monocytes, including a unique $CD11c^{int}$ $CD11b^{hi}$ Mac-3^{+} subset.

Chapter 6:
Generation of $CD8^+$ and $CD8^-$ cDC equivalents *in vitro*.

Abstract:

A culture system able to generate the subsets of steady-state conventional dendritic cells (cDC) has not been characterised. We demonstrate that DC generated from bone marrow cultured with flt3 ligand (FL) comprised three distinct $CD11c^+$ subtypes (FL-DC). In addition to $CD45RA^{hi}$ plasmacytoid DC, the cDC from FL cultures could be segregated into two distinct $CD24^{hi}$ and $CD11b^{hi}$ subsets that were equivalents of splenic $CD8^+$ cDC and $CD8^-$ cDC, respectively. The FL-DC subsets resembled their spleen DC counterparts in: 1) surface expression of CD45RA, CD11b, CD24 and Sirp-α; 2) developmental dependence on interferon regulatory factor (IRF)-8; 3) mRNA expression of IRF-4, IRF-8, of CD4 and CD8, of toll-like receptors (TLR) 3, 4, 7 and 9, and chemokine receptors CCR9, CXCR3, CCR6 and CX3CR1; 4) production of IL-12, IL-6, IFN-α, MIP-1α and RANTES in response to appropriate TLR ligands; 5) naïve T cell activation and cross-presentation of exogenous antigen to CD8 T cells; and 6) expression of the protease inhibitor cystatin C. Furthermore, despite the absence of surface CD4 and CD8 in FL-DC, the $CD24^{hi}$ subset expressed CD8α when transferred into mice. This culture system should allow access to greater numbers of the DC subtypes, especially to equivalents of the $CD8^+$ cDC.

Introduction:

Three functionally distinct subsets can be defined in steady-state mouse spleen. These include the plasmacytoid pre-DC (pDC), CD8$^+$ conventional DC (cDC) and CD8$^-$ cDC subsets, which appear to be distinct and not precursor-product related in the steady-state ((Kamath et al., 2000; O'Keeffe et al., 2002a) and Chapter 3). However, the diminutive numbers of DC in the mouse, and difficulty in their isolation, has often precluded study of splenic DC development and function. For example, at most 1 x 10^6 CD8$^+$ cDC can be recovered with high purity from one mouse spleen after an elaborate purification protocol (Vremec et al., 2000). A culture method for generating higher yields of these subtypes would make them more accessible.

There are several well-established procedures for generating DC in culture from BM precursors or from blood monocytes using GM-CSF with or without IL-4 (GM-DC) (Inaba et al., 1992a; Inaba et al., 1992b; Lu et al., 1995; Peters et al., 1993; Sallusto and Lanzavecchia, 1994; Scheicher et al., 1992). However, GM-DC do not seem to show the heterogeneity in DC phenotype and function found with splenic DC. In fact, it is not clear whether GM-DC have any counterparts amongst steady-state DC *in vivo*. In relation to this, a recent study found the development of steady-state DC was Stat3-dependent whereas GM-DC development from monocytes was Stat3-independent, suggesting these two groups of DC have distinct developmental origins (Laouar et al., 2003).

Culture of BM with fms-like tyrosine kinase 3 ligand (FL) is a more recent method that allows the generation of both cDC and pDC in large numbers (now referred to as FL-DC) (Brasel et al., 2000; Brawand et al., 2002; Gilliet et al., 2002). The dependency on FL for DC generation in this culture system correlates with the observations that FL$^{-/-}$ mice have reduced DC numbers (McKenna et al., 2000), that injection of mice with FL greatly increase DC numbers (Maraskovsky et al., 1996; O'Keeffe et al., 2002b) and that only early BM progenitors that express Flt3 are effective precursors of DC (D'Amico and Wu, 2003). Some initial observations suggested that FL-cDC may further divide into subsets, but their relationship to splenic DC subsets was not further investigated (Brasel et al., 2000; Suzuki et al., 2004).

Herein, we show that despite the presence of some CD11b on all cDC from FL cultures, and the absence of surface CD4 and CD8 expression, FL-DC could be clearly segregated by surface markers into the equivalents of steady-state splenic pDC, $CD8^+$ cDC and $CD8^-$ cDC. The shared properties between the FL-DC subsets and the splenic DC subset counterparts included surface marker expression, transcription factor expression and dependence for development, ability to cross-present cellular antigen to CD8 T cells, expression of toll-like receptors (TLR) and chemokine receptors, and production of cytokines and chemokines in response to TLR stimulation. This system should allow access to large numbers of the DC subsets for further study. In particular, up to 25 x 10^6 of the $CD8^+$ cDC equivalents can be generated from culturing the BM of one mouse with FL.

Results:

Phenotypic characterisation of FL-DC and splenic DC subsets

Dendritic cells derived from FL-stimulated BM cultures (FL-DC) were compared in surface antigen expression to freshly isolated splenic DC. Both FL culture-derived and splenic $CD11c^+$ DC contained distinct $CD45RA^-$ cDC and $CD45RA^+$ pDC populations (Figure 5.1A, 1st column), as others have noted previously (Brawand et al., 2002; Gilliet et al., 2002; Hochrein et al., 2004). However, as the cDC component had been shown to express both surface CD11b and CD205 (Brawand et al., 2002), but not CD4 or CD8, the markers normally used to segregate the splenic $CD8^+$ and $CD8^-$ cDC subtypes were not applicable for FL-cDC subset discrimination (Shortman and Liu, 2002; Vremec and Shortman, 1997) (Figure 5.1).

To assess whether FL-cDC nevertheless contained subsets, we analysed for other surface markers differentially expressed by spleen cDC. These included CD24, a surface molecule selectively expressed by $CD8^+$ cDC (O'Keeffe et al., 2002a; Vremec et al., 2000), and SIRP-α, recently identified by microarrays as being selectively expressed by $CD8^-$ cDC (unpublished data). FL-cDC expressed these markers, as well

as CD11b, at different levels, which allowed their further division into two subtypes: CD24^{hi}SIRP-α^{lo}CD11b^{lo} and CD24^{lo}SIRP-α^{hi}CD11b^{hi} (Figure 5.1a, top panels). We found that four-colour staining for CD11c, CD45RA, CD11b and CD24 was the ideal combination for discrimination of the three FL-DC subtypes.

An almost identical separation of subsets using the same combination of markers was found with splenic DC, but with differences in the relative proportions of each (Figure 5.1a, lower panels). In particular there was a larger proportion of CD24^{hi} FL-DC, the putative CD8^{+} cDC equivalent. Also, while the splenic cDC subsets are selective in their surface expression of CD11b and CD205 in the steady-state (Vremec et al., 2000), all FL-cDC expressed some levels of each (Fig 6.1a) (Brawand et al., 2002; Gilliet et al., 2002). This is not surprising considering all splenic cDC upregulate CD11b and CD205 upon *in vitro* culture (Vremec and Shortman, 1997). In view of these similarities in aspects of surface phenotype we tested whether the FL-DC and splenic DC populations were developmentally and functionally equivalent. The terminology used hereafter for the three subsets is pDC, CD24^{hi} DC (CD8^{+} cDC equivalents) and CD11b^{hi} DC (CD8^{-} cDC equivalents).

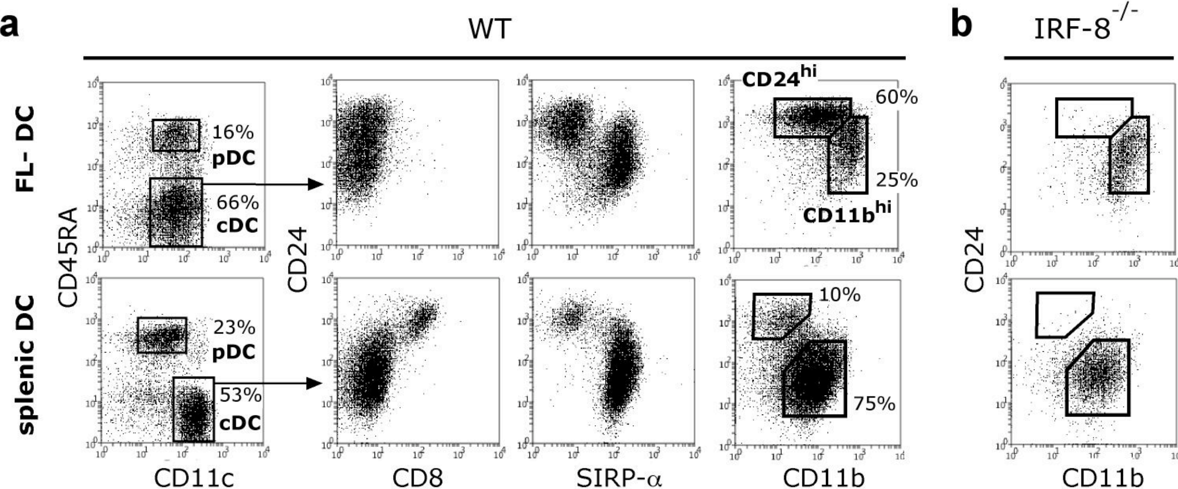


Figure 6.1 **Surface marker discrimination of FL and spleen DC subsets in WT and IRF8$^{-/-}$ mice.**

a) Both FL-DC and splenic DC from WT mice were stained with indicated combinations of surface markers and analysed by flow cytometry. **b)** IRF8$^{-/-}$ FL-DC and splenic DC were stained for similar surface markers. Analysis is representative of 2-3 individual experiments.

DC subset differences in IRF-8$^{-/-}$ mice

Mice genetically deficient for the transcription factor IRF-8 retain splenic CD8$^-$ cDC but have reduced numbers of pDC and CD8$^+$ cDC (Fig 6.1b, lower panel and (Schiavoni et al., 2002)). To assess whether a related defect occurred within FL-DC, we cultured BM from IRF-8$^{-/-}$ and WT mice with FL and compared the DC produced. Amongst the IRF8$^{-/-}$ FL-DC, there was comparable production of CD11b^{hi} DC but greatly reduced numbers of pDC and CD24^{hi} DC compared to WT FL-DC (Figure 6.1b, top panel and unpublished data). The presence of WT BM "feeder cells" in transwell cultures did not affect IRF-8$^{-/-}$ FL-DC subset differentiation, suggesting the defect was intrinsic and not due to a deficiency in a soluble factor (unpublished data). Interestingly, a converse deficit in DC subset production has been reported using IRF-4$^{-/-}$ mice, which have an absence of CD4$^+$ DC *in vivo* and an absence of CD11b^{hi} DC in FL BM cultures (Suzuki et al., 2004).

IRF expression

Not only do the different spleen DC subsets require particular IRFs for their normal development, they also vary in their expression of these transcription factors in the steady-state: the CD8$^+$ DC mainly express IRF-8, CD8$^-$ DC mainly express IRF-4, while pDC express both (Suzuki et al., 2004; Tsujimura et al., 2003). The FL-DC subsets were examined for IRF-4 and IRF-8 mRNA. Indeed, the expression pattern of IRFs in FL-DC subsets correlated with those in splenic DC (Figure 6.2a), in line with previous studies (Suzuki et al., 2004).

Expression of CD4 and CD8

Even though unstimulated FL-DC do not express surface CD4 or CD8α, unlike their putative *in vivo* counterparts, mRNA expression of these molecules was tested. CD24^{hi} DC and pDC did express some CD8 transcript, whereas CD11b^{hi} DC did not. Only pDC expressed CD4 transcript (Figure 6.2a). To see whether surface CD4 or CD8 expression might be dependent on the normal *in vivo* DC environment, we transferred each purified FL-DC subset into non-irradiated mice differing in Ly5

allotype. After 3 days we compared host and donor-derived splenic DC for surface CD4, CD8, CD45RA and MHC II expression (Figure 6.2b). Almost 75% of the recovered $CD24^{hi}$ DC had upregulated CD8 but very few expressed CD4. Transferred FL-pDC upregulated both CD4 and CD8, so they then resembled their *in vivo* counterparts (O'Keeffe et al., 2002a). However, transferred $CD11b^{hi}$ DC upregulated very little surface CD4 or CD8 in this time. The interpretation that $CD11b^{hi}$ FL-DC fail to upregulate CD4 *in vivo* because they are counterparts of splenic $CD4^-CD8^-$ cDC is unlikely considering IRF-$4^{-/-}$ mice, which fail to develop $CD11b^{hi}$ FL-DC, have reduced numbers of splenic $CD4^+$ cDC, but normal numbers of $CD4^-CD8^-$ cDC (Suzuki et al., 2004). More likely is that an *in vivo* factor is required for CD4 expression at an earlier stage of DC development.

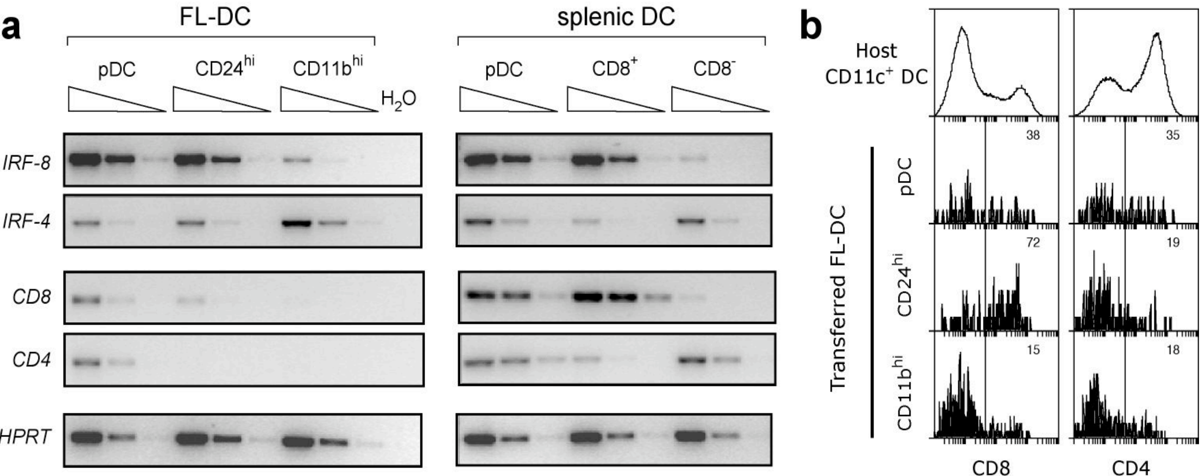


Figure 6.2 **IRF, CD4 and CD8 expression.**

a) IRF-4 and IRF-8, CD4 and CD8 mRNA expression levels were detected by semi-quantitative PCR amongst the FL and spleen DC subsets. **b)** Ly5.2 pDC, $CD24^{hi}$ and $CD11b^{hi}$ FL-DC were sorted to high purity and injected *i.v.* into non-irradiated Ly5.1 recipients. Three days later, recipient splenic DC were enriched and assessed for indicated markers on both host and donor-derived DC. Results are representative of 2-3 independent experiments.

Antigen presentation and cross-presentation

To assess the capacity of the cDC subsets to activate naïve T cells, DC were coated with synthetic OVA peptides for MHC I or II then incubated with OT-I CD8 T cells or OT-II CD4 T cells, respectively. FL-DC showed comparable efficiency to their splenic DC counterparts in stimulating the proliferation of naïve CD8 (Figure 6.3a) and CD4 T cells (Fig 6.3a). Cross-presentation, a process whereby exogenous antigens are taken up and presented via MHC I, is carried out efficiently by splenic $CD8^+$ cDC but not $CD8^-$ DC nor pDC (Heath et al., 2004; Pooley et al., 2001). To examine whether any of the FL-cDC populations were able to cross-present, we incubated each subset with cellular antigen and OT-I cells. In this instance, antigen was provided in the form of OVA protein-coated γ-irradiated splenocytes from bm1 mice (unable to present OVA to OT-I cells) (Smith et al., 2004). Of the FL-DC, only the $CD24^{hi}$ subset was able to cross-present with efficiency similar to splenic $CD8^+$ cDC (Figure 6.cC).

Cystatin C expression

In the steady-state, the splenic $CD8^+$ cDC are unique amongst the DC subsets in their expression of the cysteine protease inhibitor cystatin C (CyC) (El-Sukkari et al., 2003). Once believed to be important in MHC II presentation (Pierre and Mellman, 1998), the precise role of CyC in $CD8^+$ cDC biology is still unknown (El-Sukkari et al., 2003; Villadangos et al., 2001). We found that only $CD24^{hi}$ FL-DC expressed significant levels of CyC (Figure 5.3d).

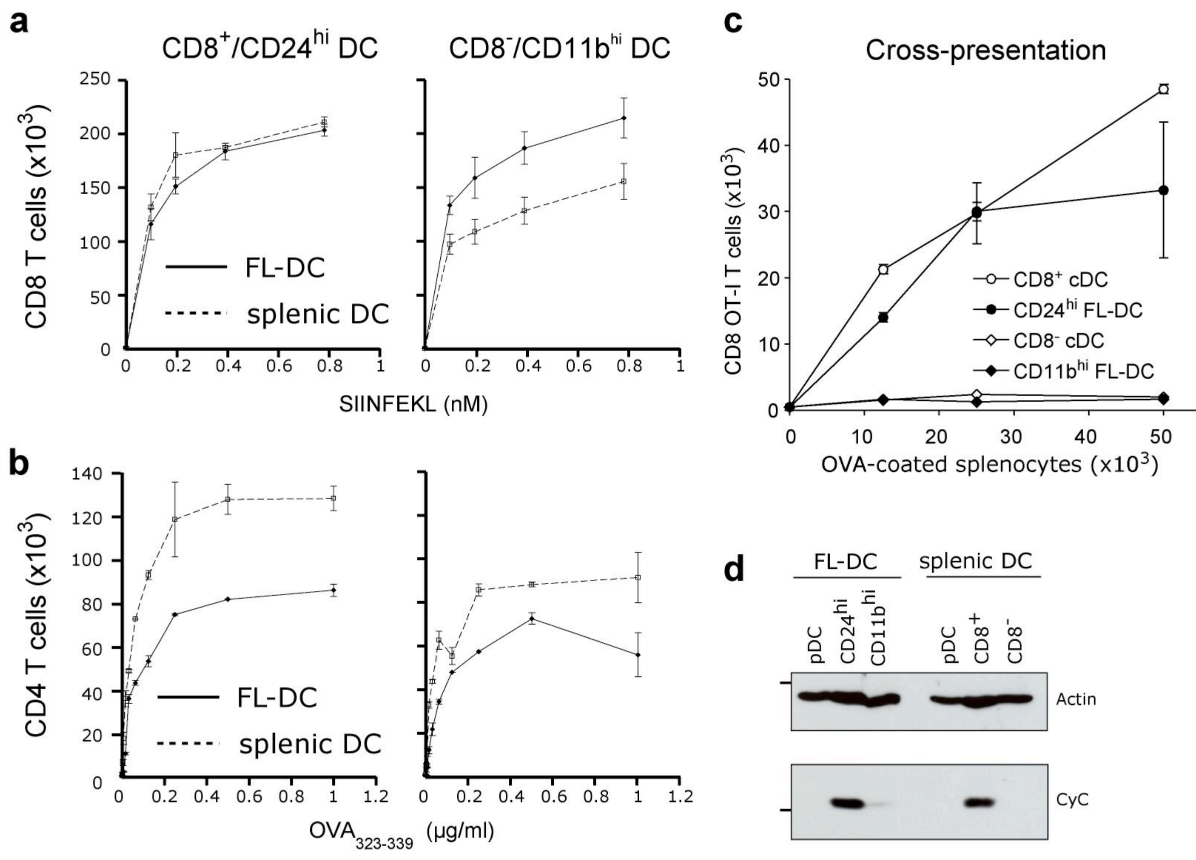


Figure 6.3 T cell activation, cross-presentation and CyC expression.

5 x10^3 sorted FL-DC or splenic DC subsets were incubated in duplicate with varying concentrations of peptide, **a)** SIINFEKL for 45 minutes, then washed or **b)** $OVA_{323-339}$ peptide, prior to the addition of 5 x10^4 CFSE-labeled OT-I or OT-II T cells. After 60 hrs, divided (CFSE^{lo}) OT cells were quantitated. **c)** Varying numbers of OVA protein-coated bm1 splenocytes were incubated with 2.5 x10^4 sorted DC subsets and 5 x 10^4 OT-I. Divided OT-I cells were quantitated after 60 hrs. **d)** Equal numbers of each DC subset were sorted to high purity and a western blot performed against CyC and actin. Results are representatives of 2-3 individual experiments.

TLR expression

The splenic DC subsets have unique TLR expression patterns, which enable them to directly respond to particular TLR ligands (Edwards et al., 2003b; Proietto et al., 2004). Splenic pDC express TLR 7 and 9 but not TLR 3 or 4; $CD8^+$ cDC express TLRs 3, 4 and 9 but not TLR7; $CD8^-$ cDC express TLRs 4, 7 and 9 but not TLR 3. Using real-time PCR, an almost identical pattern of TLR mRNA expression was found between the FL-DC and splenic DC subset equivalents (Figure 6.4a).

Chemokine receptor expression

The spleen DC subsets show quantitative differences in chemokine receptor expression, with pDC being the highest expressors of CXCR3 and CCR9, $CD8^-$ cDC the highest expressors of CCR6 and CX3CR1, while $CD8^+$ cDC express some CXCR3 and CX3CR1 (Anna Proietto, personal communication). We found comparable mRNA expression of most of these receptors between the FL-DC and splenic DC (Figure 5.4B). One striking difference was the lack of CCR6 expression in $CD11b^{hi}$ FL-DC as compared to splenic $CD8^-$ cDC. This suggests that a factor present *in vivo* but not in FL cultures is required for normal expression of CCR6.

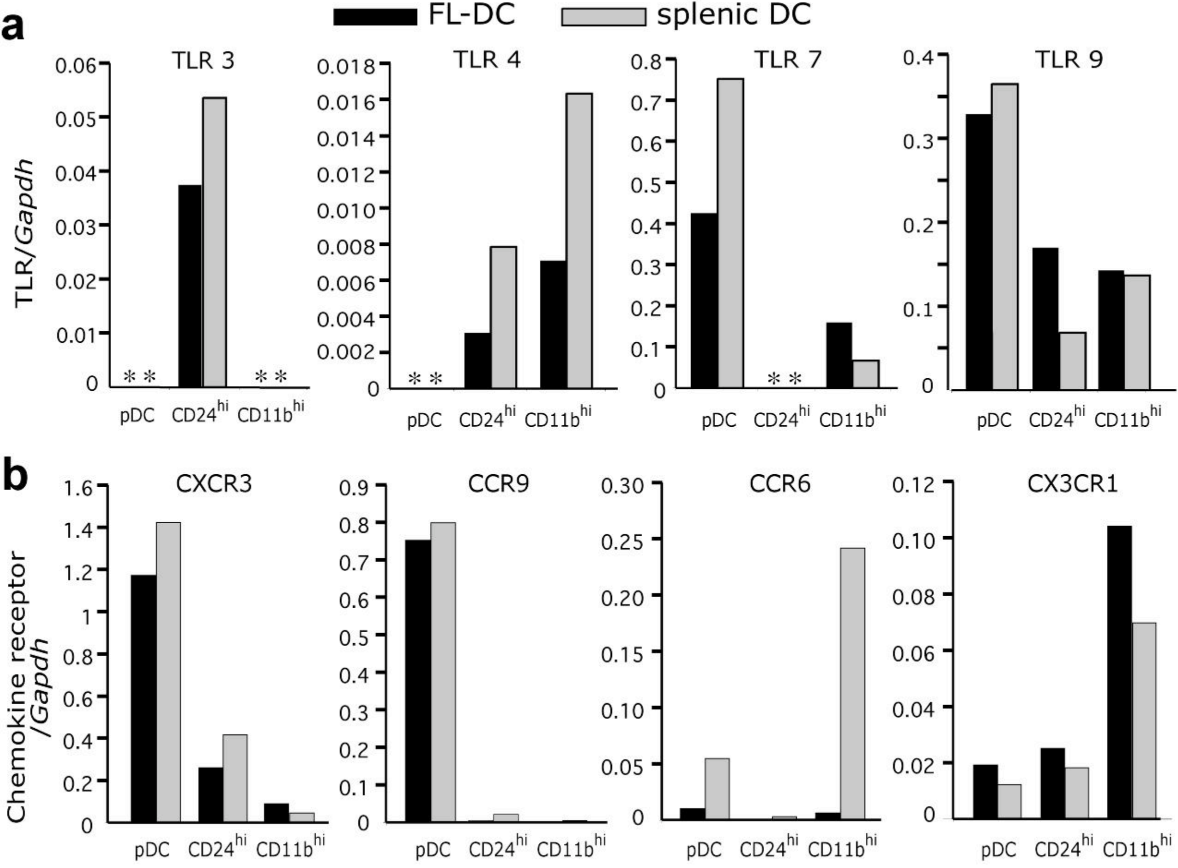


Figure 6.4 TLR and chemokine receptor expression.

Real-time PCR was performed on sorted FL and splenic DC subsets for **a)** TLR 3, 4, 7 & 9, and **b)** CXCR3, CCR9, CCR6 and CX3CR1. All values are expressed relative to GAPDH with (*) indicating levels below detection. Data is representative of 2 independent experiments.

Cytokine and chemokine production

A major functional difference between the splenic DC subsets is their capacity to produce particular cytokines. Cytokine production often requires TLR engagement, so production will depend on the TLR expression pattern of the DC subset, the TLR ligand, and on other factors such as the cytokine environment. Moreover, the splenic DC subsets differ in the cytokines they produce to the same TLR ligand (Hochrein et al., 2001; Proietto et al., 2004). For example, only pDC and $CD8^-$ cDC express TLR7 and produce cytokines in response to the TLR7 ligand, R848. On the other hand, all splenic DC express TLR9, but when stimulated with the TLR9 ligand, CpG DNA, pDC produce the highest levels of IFN-α while $CD8^+$ cDC produce the highest levels of IL-12 p70.

To compare the FL-DC subset to their splenic DC counterparts, purified DC were stimulated with R848, CpG 2216 or LPS, in conditions known to induce cytokine production, and the supernatants analysed by ELISA. The patterns of cytokine production were similar between the FL-DC and splenic DC equivalents (Figure 6.5a). Of note, only the pDC produced high levels of IFN-α in response to CpG, only the $CD24^{hi}$ DC produced high levels of IL-12 p70 in response to CpG, and the $CD11b^{hi}$ DC produced some IL-12 p40 to TLR stimuli. IL-6 production was also similar between the FL-DC and splenic DC subsets (unpublished data).

Chemokine production in response to the TLR7 ligand R848 was comparable between FL-DC and splenic DC (Figure 6.5b). In particular, $CD11b^{hi}$ DC were the major producer of RANTES, while pDC were the major producers of MIP1-α. The $CD24^{hi}$ DC subsets showed negligible chemokine production in response to R848, which correlates with their lack of TLR7 expression.

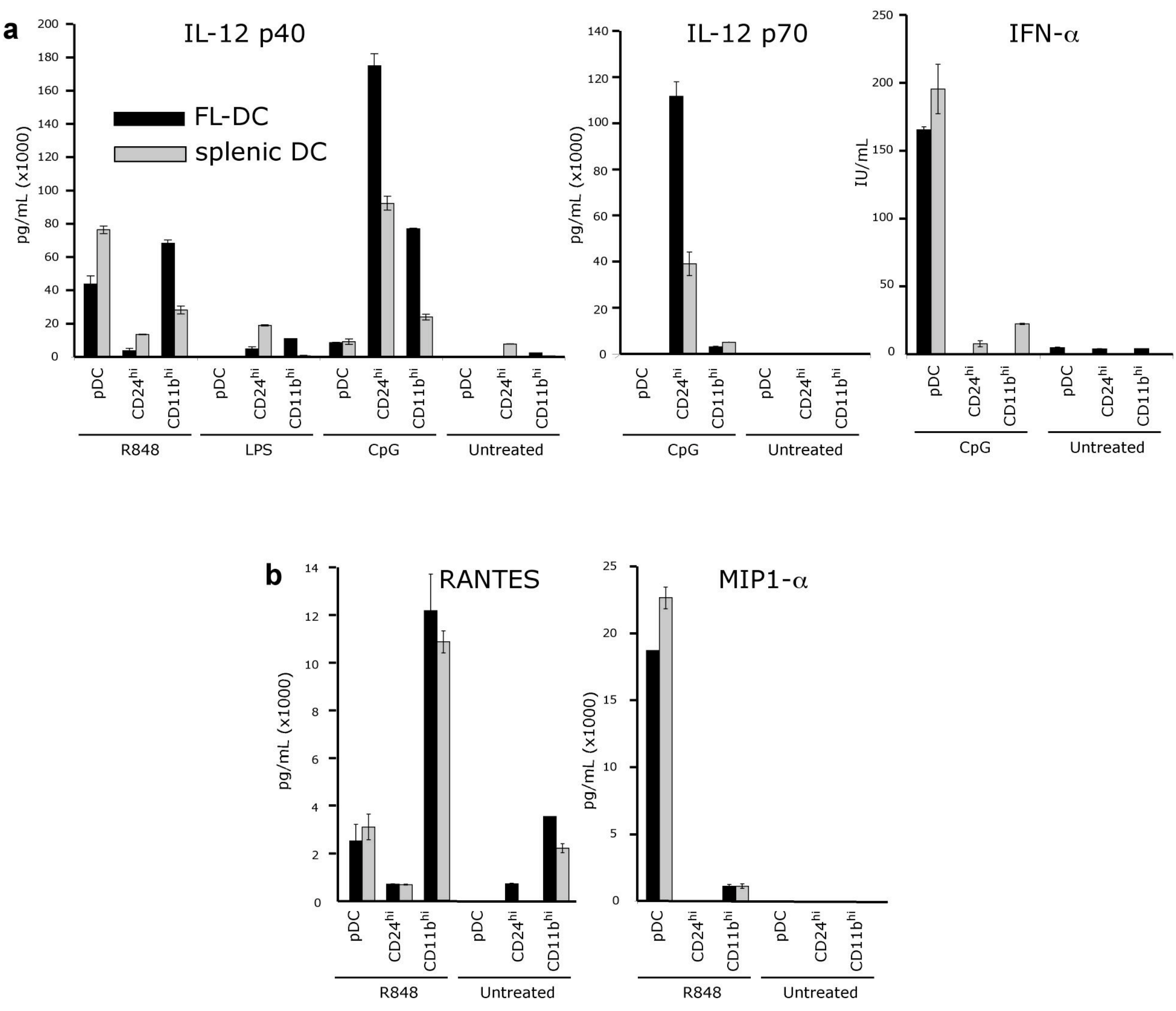


Figure 6.5 Cytokine and chemokine production.

Sorted DC (1 x10^5) were cultured in duplicate in either culture medium alone or with the indicated TLR agonists for 24 hrs, except for IFN-α and IL12 p40/70 where DC stimulation was for 48 hrs in medium containing GM-CSF, IL-4 and IFN-γ. Supernatants were analysed by ELISA for the indicated **a)** cytokines and **b)** chemokines. Data is representative of 2-5 independent experiments with duplicates from one experiment shown.

Discussion

This Chapter reports on the findings that FL BM cultures produce DC subsets that, despite differences in CD4 and CD8 expression, are close equivalents of the steady-state splenic pDC, $CD8^+$ cDC and $CD8^-$ cDC subtypes. The FL-DC subtypes shared many properties with their *in vivo* counterparts, including expression of surface markers, IRFs, TLRs and chemokine receptors. Importantly, key functional differences were also conserved between the FL-DC and spleen DC equivalents including in antigen presentation, particularly cross-presentation, and their production of cytokines and chemokines. The major novel finding in this study is the generation of a functionally distinct spleen $CD8^+$ cDC equivalent, and a strategy for its isolation in high yield. It is thus proposed that, in contrast to GM-DC, FL-DC better facilitate the study of steady state DC subset ontogeny and function. Finally, FL-stimulated cultures may be useful for identifying the precursors involved in their generation, and producing human DC counterparts to the murine DC subsets.

Chapter 7:
Identification of pro-DC and pre-DC as precursor stages

Abstract:

In Chapter 6 I demonstrated that FL-supplemented BM cultures produce DC subtypes that are equivalent to splenic plasmacytoid DC, $CD8^+$ conventional DC (cDC) and $CD8^-$ cDC by many functional parameters and most phenotypic markers. Therefore, this culture system provides a valuable model for the study of steady-state DC development from unknown BM precursors. In this Chapter, I report on the identification of two distinct but sequential dividing DC precursor populations that gave rise to pDC, $CD8^+$ and $CD8^-$ DC. A dividing 'pro-DC' stage ($CD11c^-$ MHC II^-) gives rise to an intermediate 'pre-DC' ($CD11c^+$ MHC II^-) *en route* to generation of all $CD11c^+$ MHC II^+ splenic DC populations. Thus, I have established a robust method to obtain and track large numbers of intermediate DC precursors and a system to study their clonal development. I have described two novel developmental steps in the transition between haematopoietic stem cells to the end product steady-state DC subtypes.

Introduction:

Downstream of the BM haematopoietic stem cell (HSC), it appears that both common myeloid (CMP) and common lymphoid progenitors (CLP) have the potential to give rise to pDC, $CD8^+$ cDC and $CD8^-$ cDC, with peak generation after 14-21 days (Manz et al., 2001a; Manz et al., 2001b; Wu et al., 2001). DC production occurs only from progenitors within the populations that express the fms-like tyrosine kinase receptor 3 (flt3) (D'Amico and Wu, 2003; Karsunky et al., 2003). Some later precursor stages that are immediately prior to generation of the DC subtypes (2-5 d prior) have also been identified. These include the intrasplenic pre-cDC that generate $CD8^+$ and $CD8^-$ cDC (Chapter 4), a $Ly49Q^-$ $B220^+$ BM precursor of pDC (Kamogawa-Schifter et al., 2005; Omatsu et al., 2005), monocyte precursors of Langerhans cells (Ginhoux et al., 2006; Merad et al., 2002), and monocyte precursors of inflammatory DC (Chapter 5 and (Geissmann et al., 2003; Randolph et al., 1999; Serbina et al., 2003)). However, apart from one description of a $CX3CR1^+$ macrophage/DC precursor (MDP), (Fogg et al., 2006), the DC developmental stages in the intervening gap between the early and late DC precursors is still poorly resolved.

In vitro systems that generate large numbers of DC are useful for the study of DC function and development. One common method is the culture of BM, blood precursors or monocytes with GM-CSF with or without IL-4 (GM-DC) (Inaba et al., 1992a; Inaba et al., 1992b; Lu et al., 1995; Peters et al., 1993; Sallusto and Lanzavecchia, 1994; Scheicher et al., 1992). These “myeloid” DC appear to be homogeneous and have been a useful model for studying many properties of DC. However, they have been difficult to place in the context of DC found *in vivo*. They are often assumed to be the equivalent of splenic $CD8^-$ cDC based on CD11b expression, although this has never been formally shown. In Chapter 5, it was proposed that GM-DC are more representative of ‘inflammatory’ DC rather than steady-state DC.

Nevertheless, the generation in culture of GM-DC provides a useful tool for the study of inflammatory DC development, either from monocytes or BM. In fact, Leenen and colleagues have elegantly identified the steps in development of DC from BM progenitors in the presence of GM-CSF by tracking earlier timepoints in culture

(Nikolic et al., 2003). Results from this study suggested GM-DC development involved a sequential transition: Early Myeloid Precursors ($CD31^{hi}$ $Ly6C^{-}$ $CD58^{hi}$) ⇒ Monoblasts ($CD31^{+}$ $Ly6C^{+}$) ⇒ Monocytes ($CD31^{-}$ $Ly6C^{hi}$) ⇒ DC ($CD31^{-}$ $Ly6C^{-}$). Importantly, this process clearly involved $Ly6C^{hi}$ monocytes as the immediate precursor of DC.

However, for steady-state DC development FL appears to be the crucial cytokine rather than M-CSF or GM-CSF. This correlates with an absence of pDC and cDC in mice genetically deficient for FL and its STAT3 signalling pathway (Laouar et al., 2003; McKenna et al., 2000), an increase in the numbers of splenic DC when FL is injected (Maraskovsky et al., 1996), and the obligatory expression of flt3 on early precursors for splenic DC (D'Amico and Wu, 2003; Karsunky et al., 2003). The importance of FL in DC development also correlates with its use as a single addition cytokine in a different *in vitro* model for DC production (FL-DC). In this system BM is cultured with FL for 9 days, as described in Chapter 6 and previously (Brasel et al., 2000; Brawand et al., 2002; Gilliet et al., 2002). Distinct FL-DC subtypes develop in these cultures that are equivalent to pDC, $CD8^{+}$ cDC and $CD8^{-}$ cDC from the spleen, according to numerous functional and phenotypic parameters (Chaper 6 and (Brasel et al., 2000; Suzuki et al., 2004)). Therefore, unlike GM-DC, the FL-DC culture system is a true mimetic of steady-state DC generation from BM precursors.

Presumably, at earlier time points during FL BM cultures there are putative DC precursor stages *en route* to generation of the DC subtypes. Analogous to the characterisation of DC development in GM-CSF cultures (Nikolic et al., 2003), this Chapter describes the identification and characterisation of two DC precursor populations in FL cultures. The early precursor is a blasting cell that divides several times to give rise to a late-stage precursor. This late precursor can then differentiate with minimal division into all of the DC subsets, but not most other lineages. The identification of these precursor stages thus provides greater resolution of the step-wise development of DC downstream of the early BM progenitors.

Results

Kinetics of DC development in FL BM cultures

In Chapter 6 it was demonstrated that culturing BM with FL for 9 days generates DC subtypes equivalent to splenic pDC, CD8^{+} cDC (CD24^{hi} cDC) and CD8^{-} cDC (CD11b^{hi}), and this data is shown again in Fig. 7.1a. The aims of this study were to isolate, and then characterise, DC precursor populations from earlier timepoints in FL BM cultures. This first required characterisation of the process by which DC developed from BM in this culture system.

There were several possibilities to account for DC derivation from BM in FL cultures. Firstly, pre-existing BM-resident DC may have simply been the only surviving cell type over the culture period, thus accounting for all FL-DC at day 9. Flow cytometric analysis of freshly isolated BM showed that less than 0.5% could be accounted as CD11c^{hi} MHC II^{hi} DC. However, as the number of FL-DC produced in this system was roughly equal to number of BM cells seeded (Chapter 6), DC survival could not account for FL-DC generation. Secondly, as there is evidence that DC can themselves divide (Kabashima et al., 2005; Shortman and Wu, 2004; Zhang et al., 2004), it was possible that this rare population of BM-resident DC could act as precursors for more DC in FL cultures. This would need to be examined**.** Thirdly, and the more likely scenario, was that DC derived from a precursor that was not an already developed BM-resident DC.

It was also important to establish whether DC development involved a simple one-for-one conversion from the starting BM population, or whether it involved precursor division prior to DC formation. To find out which of these took place, BM was first labelled with CFSE, a cytosolic dye that dilutes progressively with division, prior to culture. CFSE-labelling of BM did not affect the numbers and ratio of the resulting FL-DC subtypes after 9 days (data not shown). When assessed for CFSE expression after 1, 3 and 8 days of culture, cells appeared to have divided progressively over time such that most CD11c^{+} FL-DC by the end of the culture period were low for CFSE

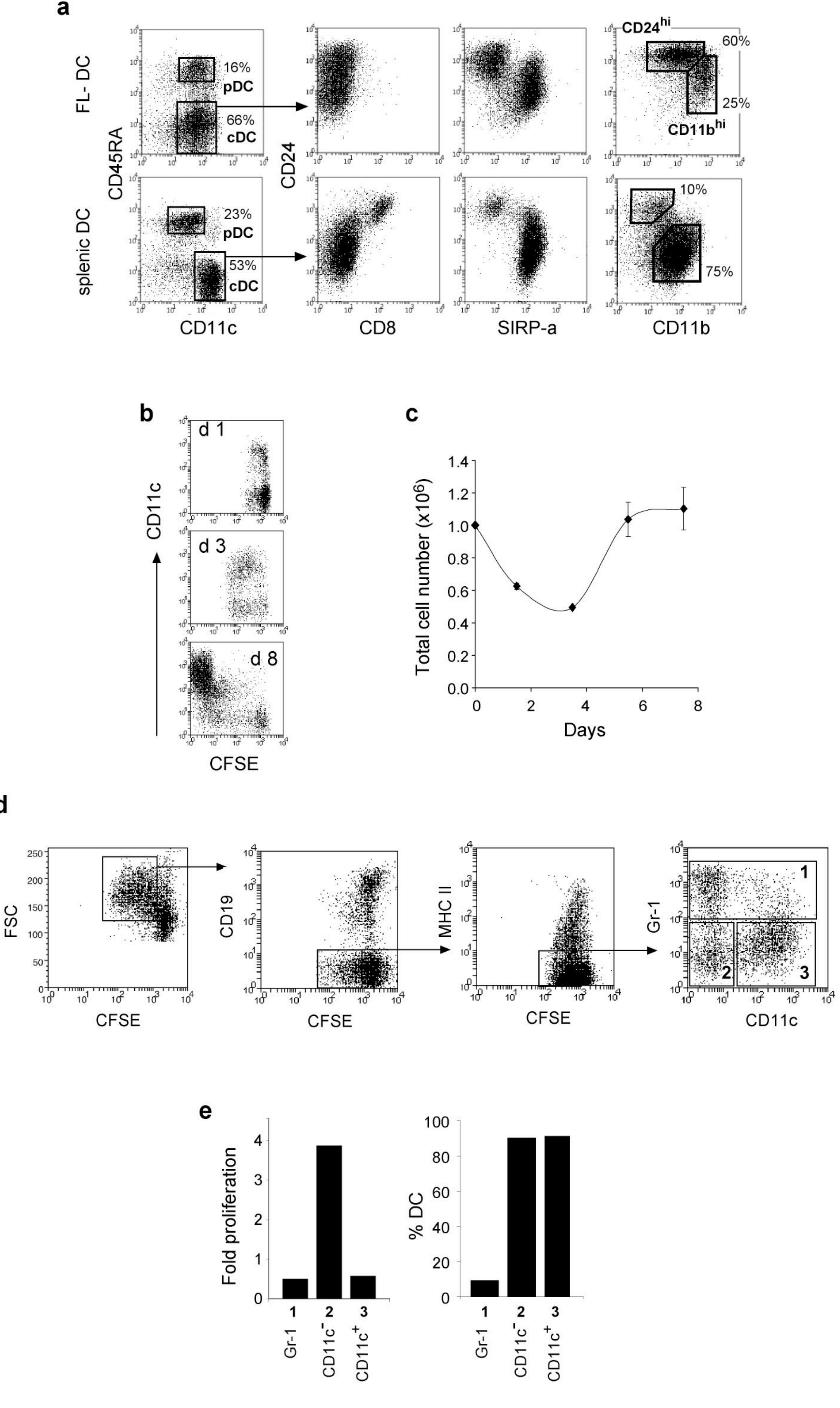
(Fig. 7.1b). This highlighted that a dividing precursor stage was responsible for the generation of FL-DC.

As division was clearly involved in the generation of DC at the culture endpoint, it was reasoned that inspection of earlier timepoints in FL cultures would reveal the putative dividing precursors that gave rise to the DC subtypes. Moreover, it was predicted that the dilution of CFSE within these dividing precursors would provide a useful means not only for their segregation from non-diving non-precursors, but also as a marker useful for their isolation by flow cytometry.

To establish the ideal time for precursor enrichment from cultures, it was necessary to determine when the least number of BM non-precursors was present prior to significant precursor expansion. Thus, the total cell number in FL cultures was tracked over time. From this analysis, the lowest total cell number was found to be at day 3, after which time cell numbers began to rise again (Fig. 7.1c), in line with a previous study (Brasel et al., 2000). Analysis of cultured cells using flow cytometry at this 3-day timepoint resolved a clear population of dividing cells separate from the undivided CFSE^{hi} peak (Fig. 7.1b). Therefore, as day 3 of FL cultures i) yielded an easily identifiable population of dividing cells, ii) retained the lowest number of non-precursors from BM, and iii) was a time prior to the rapid expansion of DC in culture, it was chosen as the timepoint for further isolation of putative DC precursors.

Figure 7.1 DC subsets and fractions with precursor activity in FL-DC BM cultures

a) BM was cultured with 200-300 ng/mL FL for 9 days then the DC harvested. Splenic DC were also freshly isolated. DC from both preparations were stained and assessed for surface expression of CD11c, CD45RA and CD24, in addition to either CD8, SIRP-α or CD11b. b) BM was CFSE-labelled prior to culture with FL. Cultures were harvested at various times and their CFSE versus CD11c expression assessed, as well as c) their total numbers determined. d) FL cultured cells were harvested at day 3, and separated by density centrifugation on 1.086 g/cm^3 Nycodenz to remove dead cells, and some lymphocytes and granulocytes. The light density cells were retained and stained with mAbs against CD19, MHC II, CD11c and Gr-1. Plots show progressive gating for populations until the final gating was for cells that were large and dividing, CD19$^-$, MHC II$^-$ and either 1) Gr-1$^+$, 2) Gr-1$^-$ CD11c$^-$ or 3) Gr-1$^-$ CD11c$^+$. Each of these 3 populations were sorted to high purity and then either transferred in vivo (Table 7.1), or e) 1 x 10^4 cells of each population re-cultured in medium from day 3 FL cultures (conditioned medium) for a further 5 days. For e), cells were then harvested, counted and their surface expression of CD11c and MHC II assessed. Histograms show the fold increase in total numbers, as well as the percentage of cells at the end of the culture period that were CD11c$^+$ MHC II$^+$ DC.


a
FL- DC
splenic DC
CD45RA
CD24
16%
pDC
66%
cDC
23%
pDC
53%
cDC
CD24hi
60%
25%
CD11bhi
10%
75%
CD11c
CD8
SIRP-a
CD11b
b
d 1
d 3
d 8
CD11c
CFSE
c
Total cell number (x10^6)
Days
d
FSC
CFSE
CD19
CFSE
MHC II
CFSE
Gr-1
CD11c
1
2
3
e
Fold proliferation
% DC
Gr-1
CD11c-
CD11c+

Isolation of DC precursors

Both an *in vitro* and *in vivo* assay of DC differentiation was used to gauge the enrichment of DC precursor activity during the course of precursor isolation from day 3 FL cultures. Taking account of the first 3 days in culture, both assays were designed to end after 5 days to reflect a total time of 8 days, correlating to the total time of standard FL BM cultures. The *in vivo* assay was identical to that used in Chapters 3 & 4 whereby Ly5.2$^+$ cells were transferred *i.v.* into non-irradiated Ly5.1$^+$ recipients, with analysis of donor-derived DC 5 days later (see also Materials & Methods) . The per cell DC precursor activity was determined as the number of DC generated per 10^6 cells transferred. By contrast, the *in vitro* assay for DC precursor activity involved the re-culture of cells from different fractions for a further 5 days in the medium that was retained after 3 days of FL BM cultures (now referred to as conditioned medium). The fold increase in total numbers was determined by calculating the number of progeny relative to the number of cells seeded. The percentage of such progeny that had differentiated to CD11c$^+$ MHC II$^+$ DC was also determined by flow cytomtery.

As a first step of enrichment for DC precursors, cells from day 3 of culture were first separated by centrifugation in 1.086 g/cm^3 Nycodenz medium to exclude dead cells as well as contaminating dense BM granulocytes and B cells that survived the culture period. The supernatant of the density separation contained between 60-80% of the total cells, and the pellet contained the remainder. Upon transfer of a sample *in vivo* of both the light and heavy density fractions, it was found that the light density region retained all DC precursor activity while the heavy density cells from the pellet had none (Table 7.1). Therefore, only these lighter density cells were retained for further precursor enrichment.

To discriminate the dividing cells, which should have included the dividing DC precursors, flow cytometry was utilised to sort for cells with a lower intensity of CFSE. Using this criterion, 35-60% of cells were found to have been in division (Fig 7.1d). Of these divided cells, a fraction was MHC II$^+$. Whether these cells were DC that were dividing and thereby acting as precursors of more DC, or else were end-stage cells that had developed from an already divided precursor, needed to be

determined. Thus, $CD11c^+$ MHC II^+ cells from a single division peak, based on CFSE fluorescence, were re-cultured in conditioned medium for 24 hours. While a number of these DC survived this period, there was no evidence of a drop in CFSE fluorescence or an increase in number over this time suggesting that, once developed, DC could not act further as precursors (data not shown). Therefore to exclude already developed DC, dividing cells were gated on MHC II^- cells.

There was also a population of $CD19^+$ B lymphocytes that had undergone division in culture. When transferred *in vivo*, these small dividing $CD19^+$ lymphocytes were unable to generate DC progeny (data not shown). In a separate experiment, the development of such cells was also observed when BM was CFSE-labelled and cultured in medium alone, in the absence of FL, which were conditions that did not produce DC. Therfore, such cells probably represented residual proliferation after extraction from BM. As a result, $CD19^+$ cells were also excluded from precursor purification.

When the resulting $CFSE^{low}$ $CD19^-$ MHC II^- cells were co-stained with CD11c and Gr1, three distinct populations could be discerned; (i) $Gr1^+$, (ii) $Gr1^-$ $CD11c^-$ and (iii) $Gr1^-$ $CD11c^+$, referred to as the $Gr1^+$, $CD11c^-$ and $CD11c^+$ fractions, respectively. Each fraction was assessed for their DC precursor activity *in vitro* and *in vivo*. Some $Gr1^+$ cells survived the culture period in conditioned medium after 5 days (Fig. 7.1e), but none of these were $CD11c^+$ DC. Even after transfer *in vivo* no DC could be generated from the $Gr\text{-}1^+$ population (Table 7.1). However, both the $CD11c^-$ and $CD11c^+$ fractions did produce $CD11c^+$ MHC II^+ DC, albeit with differing efficiency. DC production was apparent both *in vitro* and *in vivo*, (Fig. 7.1e and Table 7.1). Moreover, each of the $CD11c^-$ and $CD11c^+$ fractions produced all DC subtypes including pDC, $CD8^+$ cDC and $CD8^-$ cDC *in vivo* and their equivalents *in vitro* (data not shown). Thus both of these fractions contained DC precursors.

Table 7.1. Enrichment of DC precursors

Fraction	Separated populations	DC generated after 5 d (per 10^6 transferred)
Day 3 FL culture	Heavy density	433
	Light density	30,417 ± 9,590
Light density	Undivided	7,117 ± 2,070
	Divided	46,266 ± 15,414
Divided	$Gr1^+$	2,375
	$CD11c^+$	38,158 ± 26,340
	$CD11c^-$	57,920 ± 24,013
Enriched $CD11c^-$	pro-DC	161,046 ± 60,110
Enriched $CD11c^+$	pre-DC	121,859 ± 65,472

Various fractions were recovered during the isolation steps for DC precursors from day 3 FL BM cultures ($Ly5.2^+$ BM), and a known number transferred *in vivo* into at least 2 non-irradiated $Ly5.1^+$ recipients. Five days later, recipient splenic DC were enriched and counted, and the proportion of $CD11c^+$ $Ly5.2^+$ DC determined by flow cytometry. DC precursor activity for each fraction was then calculated as $Ly5.2^+$ DC generated per 10^6 cells of each fraction transferred ± standard deviation where multiple experiments were performed. Results are representative of 3 independent experiments.

Phenotype of DC precursors

To determine if there was any heterogeneity within the $CD11c^-$ and $CD11c^+$ fractions, a panel of over 30 surface markers was assessed. Both DC precursors were homogenous for most markers (Table 7.2). However, heterogeneity was found for several molecules including CD11b, Sca-1, Ly6C, IL-7 receptor-α (IL7R-α), CD45RA and F4/80. To determine co-segregation of surface markers with DC precursor activity, the dividing $CD11c^-$ fraction was first separated by marker expression and then re-cultured in conditioned medium for 5 days. Analysis of the progeny demonstrated that all precursor activity of the $CD11c^-$ fraction was retained within cells low to negative for expression of all these markers, including Ly6C. As cells that were $Ly6C^+$ were also positive for expression of Sca-1, CD11b and F4/80 (data not shown), separation based on Ly6C was sufficient for the final enrichment step for $CD11c^-$ precursors. The $CD11c^+$ fraction contained a majority of cells that were, by contrast, positive for expression of most of these markers, except IL-7Rα (Table 7.2).

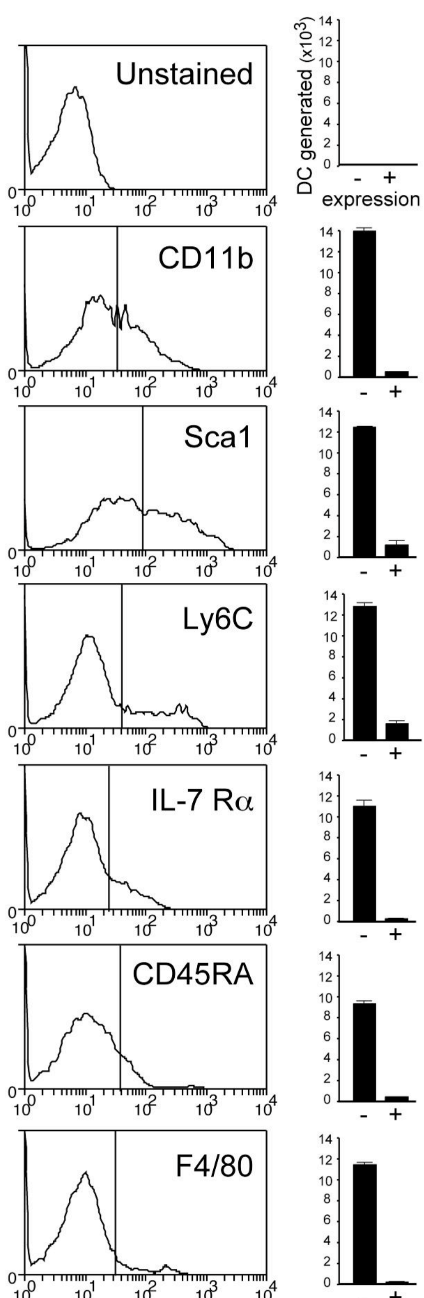


Figure 7.2 Correlation of DC precursor activity with CD11c$^-$ fraction

Gr-1$^-$ CD11c$^-$ cells were sorted as per Fig. 7.1d and then stained for expression of the indicated surface markers. CD11c$^-$ cells were sorted based on either positive or negative expression of these markers, and 2.5 x 10^3 re-cultured in conditioned medium for 5 days. Total numbers and phenotype of DC was determined at this point. The DC generated was calculated by multiplying the percentage of the fraction that were either positive or negative for expression of each of the surface markers prior to separation and re-culture, by the total number of CD11c$^+$ MHC II$^+$ DC generated from that fraction.

Precursor isolation

The final protocol for the isolation of $CD11c^-$ and $CD11c^+$ precursors is described in Fig. 7.3a. CFSE-labelled BM cells were cultured with FL for 3 days, the cells harvested, separated by density centrifugation, and the light density cells retained. Based on surface marker expression by cells that did not contain precursor activity, mAb against IL7R-α, MHC II and CD19 were used in a depletion cocktail for the recovered light density cells, then mAb-bound non-precursor cells depleted using immunomagnetic bead depletion. The enriched precursor fraction was then stained with mAbs against CD11c and Ly6C. $CD11c^+$ precursors were sorted as divided $CD11c^+$ cells, and $CD11c^-$ precursors as divided $CD11c^-$ $Ly6C^-$ cells.

Morphology and surface phenotype

Cytospin preparations of both precursor populations and cDC from the culture endpoint were compared. Pro-DC were characterised as large, monoblast-like in appearance, with a highly active endoplasmic reticulum and golgi. As measured by forward scatter using flow cytometry, pre-DC were generally smaller than pro-DC (data not shown). They also had less cytoplasm, but could not be characterised as monocytes or lymphocytes, although they had some cells that were lymphoid-like in morphology. They resembled somewhat the instrasplenic pre-cDC identified in Chapter 4, except that these pre-cDC did not contain the lymphoid-like cells. The DC products were intermediate in size between pro- and pre-DC and had dendrites characteristic of these cells.

The phenotype of precursors is shown in Table 7.2. $CD11c^-$ precursors were negative for most lineage markers including CD11b, F4/80, CD3, CD19 and CD49b. They were also negative for Sca-1 and IL-7Rα differentiating them from HSC and CMP, respectively. Of note, $CD11c^-$ precursors were $CD43^{int/hi}$ and $Fc\gamma RII/III^+$. No clear set of markers allows their clear delineation from other lineage negative cells from BM and spleen. $CD11c^+$ precursors were uniquely MHC II^-, $DX5^-$, $CD45RA^{low}$, $CD11b^{low}$, $CD43^{int}$. This phenotype was similar to the intrasplenic pre-cDC described in Chapter 4.

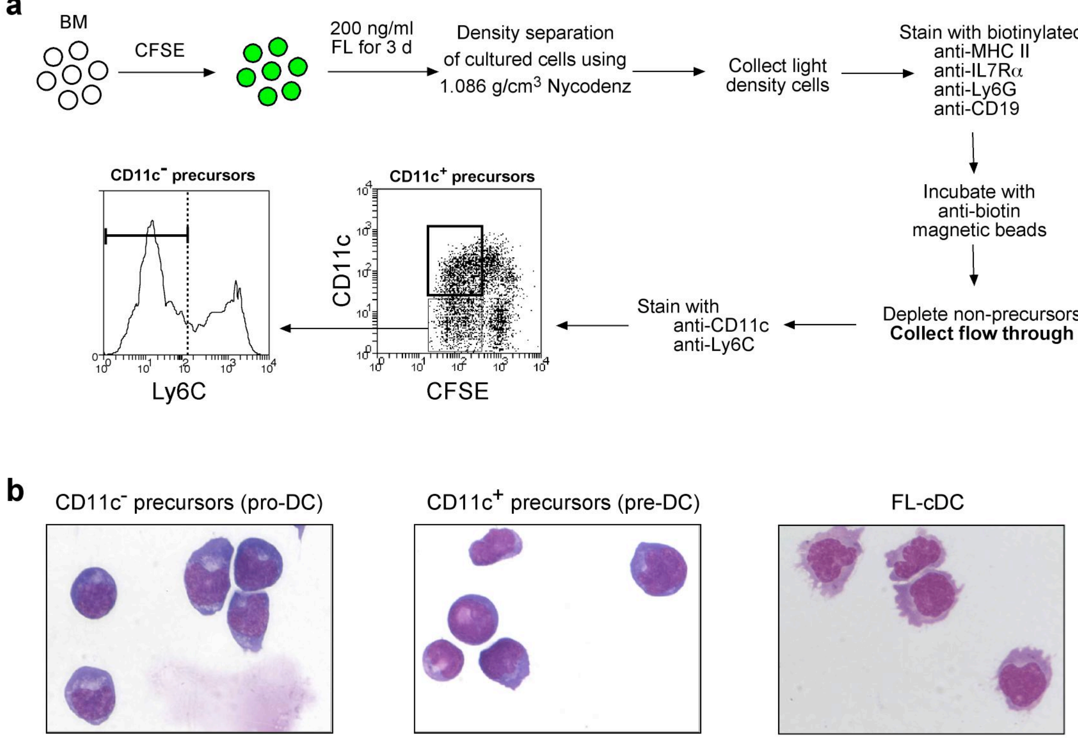


Figure 7.3 Isolation and morphology of CD11c$^-$ and CD11c$^+$ DC precursors

a) To isolate DC precursors, BM was CFSE-labelled and cultured with FL for 3 days. Cells were harvested and separated by density centrifugation in 1.086 g/cm^3 Nycodenz medium. Light density cells were collected, coated with biotinylated mAbs against non-precursor cell types, incubated with anti-biotin beads, and non-precursors depleted using a magnetic column. The flow through containing precursors was then stained for CD11c and Ly6C expression prior to separation by flow cytometry. Large, dividing CFSE^{low} cells were gated as CD11c$^+$ cells (CD11c$^+$ precursors) or CD11c$^-$ Ly6C$^-$ cells (CD11c$^-$ precursors). **b)** Cytospin preparations of both precursor populations, as well as CD11c$^+$ CD45RA$^-$ FL-derived cDC populations were stained with May-Grünwald Giemsa.

Table 7.2 Phenotype of pre-DC and pro-DC

Surface marker	Alternative name	mAb clone	pre-DC	pro-DC
MHC II		M5/114	–	–
CD1d		20H2	+	+
CD3		KT3	–	–
CD4	Ly-4	GK1.5	–	–
CD8	Ly-2	YTS169.4	–	–
CD9	p24	KMC8.8	–/+	–/+
CD11a	LFA-1α	121/7.7	+	+
CD11b	Mac-1	M1/70	+	–/+
CD11c	Integrin-α_X	N418	+	–
CD16/32	FcγRII/III	2.4G2	–	+
CD18	Integrin-β2	M18/2.a.12.7	+	–
CD19	B4	1D3	–	–
CD25	IL-2 Rα	PC61	–	–
CD40	gp39	FGK45.5	–	–
CD43	Leukosialin	S7	+	+/++
CD45R	CD45R	B220	–/+	–
CD45RA	Exon A isoform	14.8	–/+	–
CD49b	Pan NK	DX5	–	–
CD51	Integrin-αV	H9-2B8	–	–
CD54	ICAM-1	YN1/1.7.4	–	–
CD62L	L-selectin	MEL-14	–	–
CD80	B7-1	16-10A1	–	–
CD86	B7-2	GL-1	–	–
CD103	Integrin-α_{IEL}	M2/90	–	–
CD122	IL-2 Rβ	5H4	–	–
CD127	IL-7 Rα	A7R34-2.2	–	–
CD154	CD40L	MR1	–	–
MAC-2	Galectin-3	M3/38	–	–
F4/80	Ly-71	F4/80	–/+	–
Ly6A/E	Sca-1	D7	+	–
Ly6C		5075-3.6	–/+	–
Ly6G		1A8	–	–

–/+ indicates continuous negative to low expression.

+/++ indicates continuous intermediate to high expression.

CFSE-labelled FL BM cultures were harvested at day 3, blocked with rat Ig and anti-FcγRIII for 15 minutes, then stained for CD11c, Ly6C and each of the above surface markers. The pro-DC and pre-DC populations were gated as per Fig. 7.3 and the expression levels of each surface marker determined.

Steps in development

As both the CD11c$^-$ and CD11c$^+$ precursors could generate DC, albeit with differing efficiency, there was a possibility that they were related and represented different stages in a common linear pathway of DC development, both having been captured in day 3 FL cultures. Alternatively, they could have represented separate branches of development from BM precursors, each giving rise to DC independently of the other. In order to assess their relationship, both precursor populations were re-cultured with conditioned medium for a further 5 days to allow DC development, and their phenotype analysed at several timepoints (Fig. 7.4).

When CD11c$^-$ precursors were re-cultured *in vitro* and their CD11c and MHC II phenotype assessed at day 1, 3 and 5 after re-culture, there appeared to be a distinct sequence of events (Fig. 7.4a top panels). The CD11c$^-$ MHC II$^-$ precursors first upregulated CD11c giving rise to a CD11c$^+$ MHC II$^-$ population, which was apparently equivalent to the CD11c$^+$ precursors. This CD11c$^+$ MHC II$^-$ population in turn up-regulated MHC II to give rise to all DC subsets. By contrast, CD11c$^+$ MHCII$^-$ precursors did not down-regulate CD11c at any stage but did acquire surface MHC II expression over time (Fig 7.4a, lower panels). This indicated CD11c$^-$ precursors gave rise to the intermediate CD11c$^+$ precursors, and not vice versa. It was interesting to note that the acquisition of MHC II between the CD11c$^+$ precursor stages and DC did not involve a gradual increase in levels. Rather, the bi-modal expression of MHC II suggested there was a rapid transition between an MHC II$^-$ precursor and MHC II$^+$ product.

To examine whether DC generation by the two precursors involved expansion or a simple one-for-one differentiation, total cell number after re-culture of both precursors was examined over time. While CD11c$^+$ precursors underwent minimal expansion during this period, CD11c$^-$ precursors increased 7-10 fold over the 5 day re-culture period (Fig. 7.4b). This was confirmed in time course studies where precursors were first sorted on a single division peak based on CFSE prior to culture; CD11c$^-$ precursor progeny reduced CFSE fluorescence rapidly over this time, indicating division, whereas CD11c$^+$ precursors did not, suggesting they differentiated with minimal division (data not shown). Combined, these results implied that

different stages of the same developmental process had been captured in day 3 FL cultures and that CD11c$^-$ precursors gave rise to the CD11c$^+$ precursor intermediate prior to DC generation. In view of these observations that one DC precursor could give rise to the other, and in accordance with the nomenclature for other haematopoietic lineages, the CD11c$^-$ MHC II$^-$ precursors are referred to hereafter as pro-dendritic cells or “pro-DC”, and the CD11c$^+$ MHC II$^-$ precursors as pre-dendritic cells or “pre-DC” (Fig. 7.4c).

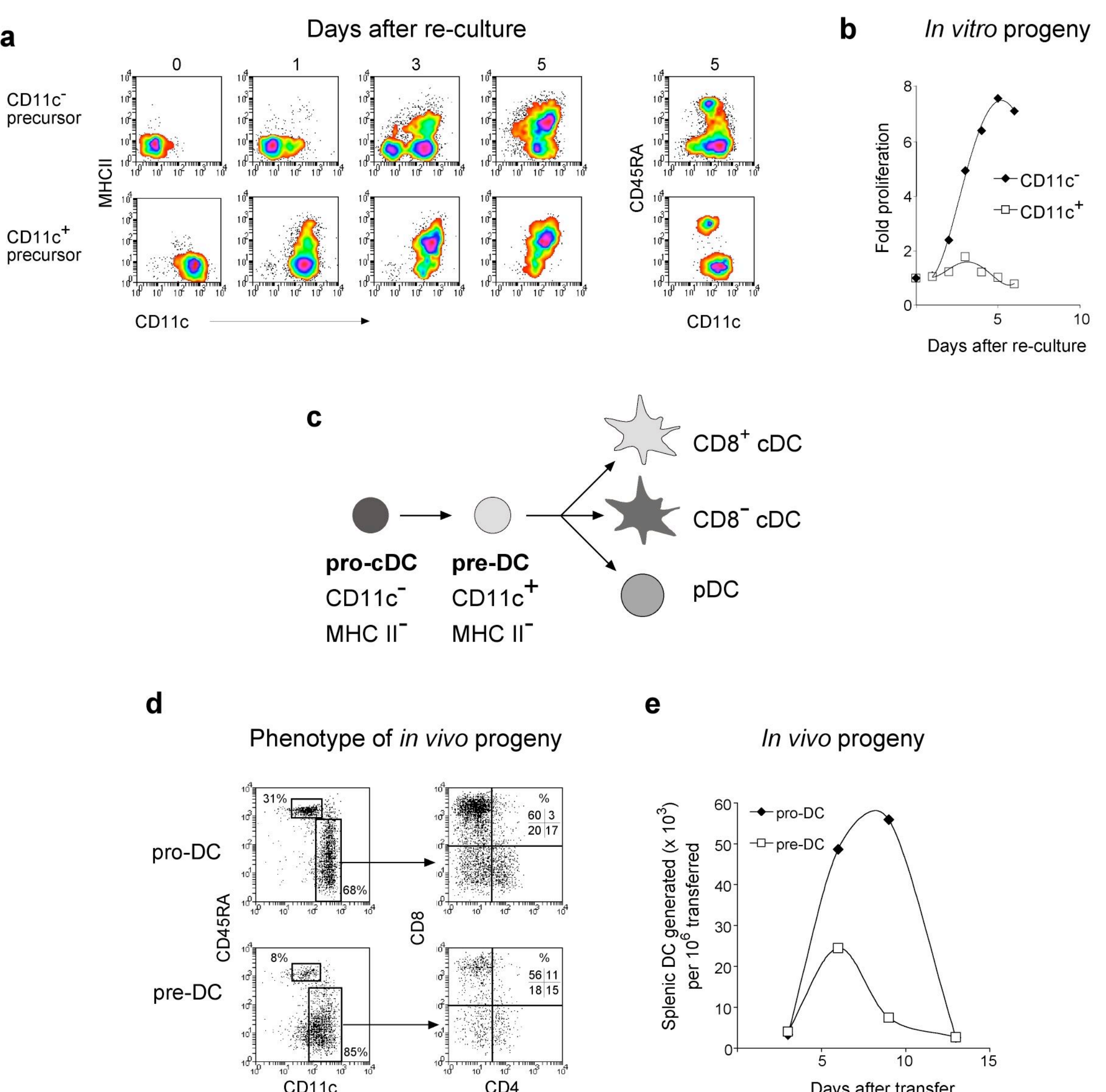
***In vivo* progeny.**

Although the DC subtypes generated in FL BM cultures were demonstrated in Chapter 6 to be equivalent by numerous phenotypic and functional parameters to freshly isolated pDC, CD8$^+$ and CD8$^-$ DC of the spleen, they did not express the CD8 or CD4 molecules *in vitro* (Chapter 6 and Fig. 7.1a). Rather, it was demonstrated that expression of these markers on FL-DC was dependent on the *in vivo* environment (Chapter 6). Therefore, to ensure the *in vitro* precursors identified here were indeed *bona fide* precursors of these subtypes *in vivo*, pro-DC and pre-DC were transferred into non-irradiated hosts and donor-derived splenic DC production measured at later timepoints. At day 5 after transfer, both precursors produced all splenic DC subtypes. Importantly, the DC progeny expressed surface CD4 and CD8 (Fig. 7.4d). Thus the final DC progeny included (i) CD11c$^+$ CD45RA$^+$ plasmacytoid DC, including all subsets based on CD4 and CD8 expression (data not shown), (ii) CD8$^+$ conventional DC, and (iii) both CD4$^+$ and CD4$^-$ subtypes of the CD8$^-$ conventional DC (Fig. 7.4d). Pro-DC could generate 1.6×10^5 DC, and pre-DC 1.2×10^5 DC, per 10^6 precursors transferred after this time (Table 7.1).

To determine whether the difference in DC production of pro- and pre-DC after 5 days was due to a difference in kinetics of peak DC generation *in vivo*, a fixed number of each precursor was transferred into non-irradiated mice and DC numbers tracked at different times (Fig. 7.4e). Pro-DC generated the most DC between 5 and 10 days, but progeny were undetectable by day 14. In contrast, pre-DC generation of DC peaked at 5 days, with low numbers by day 9 and undetectable by day 14. These *in vivo* kinetics and precursor activity data again supported the conclusion that pro-DC were the upstream precursors of pre-DC.

Figure 7.4 The steps in development of DC precursors to DC

CD11c$^-$ and CD11c$^+$ precursors were isolated form FL cultures as per Fig. 7.3 **a)** Each precursor (1×10^4) was cultured in conditioned medium for varying times. At days 1, 3 and 5 after culture, cells were harvested and their expression of CD11c and MHC II analysed. **b)** Total numbers in each well was also determined and expressed as a fold proliferation relative to the number of cells seeded. **c)** Based on the sequence of events in a), pDC, CD8$^+$ cDC and CD8$^-$ cDC are proposed to develop in a sequential process involving CD11c$^-$ MHC II$^-$ precursors (now referred to as pro-DC) that divide and give rise to CD11c$^+$ MHC II$^-$ precursors (now referred to as pre-DC), which subsequently differentiate to the CD11c$^+$ MHC II$^+$ DC subtypes. **d)** A known number of Ly5.2$^+$ pro- and pre-DC were transferred *i.v.* into at least 2 non-irradiated Ly5.1$^+$ recipients for varying times. Five days later, recipient spleens were enriched for DC, and the expression of markers on donor precursor-derived Ly5.2$^+$ DC analysed. **e)** Alternatively, the per cell precursor activity was determined for either pro- or pre-DC either 2, 6, 9 and 14 days after transfer *in vivo* to determine the kinetics of DC generation.


a
Days after re-culture
0
1
3
5
5
CD11c⁻ precursor
CD11c⁺ precursor
MHCII
CD45RA
CD11c
CD11c
b
In vitro progeny
Fold proliferation
CD11c⁻
CD11c⁺
Days after re-culture
c
pro-cDC
CD11c⁻
MHC II⁻
pre-DC
CD11c⁺
MHC II⁻
CD8⁺ cDC
CD8⁻ cDC
pDC
d
Phenotype of in vivo progeny
pro-DC
pre-DC
31%
68%
8%
85%
CD45RA
CD11c
CD8
CD4
%
60 3
20 17
56 11
18 15
e
In vivo progeny
Splenic DC generated (x 10³) per 10⁶ transferred
pro-DC
pre-DC
Days after transfer

Precursor frequency

That the precursor populations could produce DC was not proof in itself that they were a pure population with every cell capable of DC generation. Clonal precursor frequency analysis was required. As pro-DC were the upstream precursor of pre-DC, and as pro-DC had a larger burst size, single cell analysis was examined only with this cell type.

When single pro-DC were cultured in Terasaki wells with conditioned medium for 5 days, in an attempt at clonal analysis, there was no detectable progeny (data not shown). By contrast, culture of 5-10 x 10^3 pro-DC per well had been sufficient for 7-10 fold expansion *in vitro* (Fig. 6.4b). This suggested that cell-cell interaction and a minimum number of precursors was required for DC development. To overcome such issues, a system was developed whereby single pro-DC from mice that expressed eGFP fused to MHC II (MHC II-eGFP) (Boes et al., 2002) was cultured amongst a feeder population of non-GFP pro-DC. In this way, GFP^+ DC progeny from single precursors could be tracked by flow cytometry, yet develop *in vitro* with sufficient cell-cell interaction.

When varying numbers of pro-DC were cultured in conditioned medium, as low as 500 pro-DC per well was sufficient to allow generation of all DC subtypes in a similar ratio and with a relative efficiency to wells containing 1 x 10^4 (data not shown). Accordingly, 500 was selected as the number of feeders to support full differentiation of the single GFP^+ pro-DC.

To generate pro-DC from MHC II-eGFP mice, BM from these animals was first labelled with PKH-26, a membrane dye that halves in fluorescence at each successive division. PKH-26 was used instead of CFSE so as not overlap in fluorescence with GFP. PKH-labelled MHC II-eGFP BM was cultured with FL for 3 days, harvested, and pro-DC isolated as per the standard protocol (Fig 7.3), except that a reduction in PKH-26 fluorescence was used in place of CFSE to gate for dividing cells at day 3.

To gauge the precursor frequency of the pro-DC population, single pro-DC were deposited in wells using flow cytometry amongst 500 sorted wildtype pro-DC and cultured for 5 days. Analysis at this time found at least 44% of wells had GFP^+ progeny. Previous estimations of the efficiency of single cell sorting and spontaneous death of a B cell hybridoma had suggested that 65-80% of wells get one surviving cell (David Vremec, personal communication). Thus, when taking into account any spontaneous death and a less than complete efficiency of single cell deposition, the precursor frequency of pro-DC was demonstrated to be 44% at the very least, but probably closer to 55-75%. In another approach to gauge precursor frequency, 500 wildtype pro-DC were plated in each well and then either 1, 2, 4, 8 or 16 MHC II-eGFP pro-DC seeded in addition. The percentage of wells negative for GFP progeny was plotted against the number of pro-DC seeded. A line of best fit was also determined for all values where the number of pro-DC seeded resulted in wells negative for progeny (Figure 7.5). As few as 4 pro-DC per well was sufficient to yield GFP^+ progeny in all wells, again indicative of a high precursor frequency.

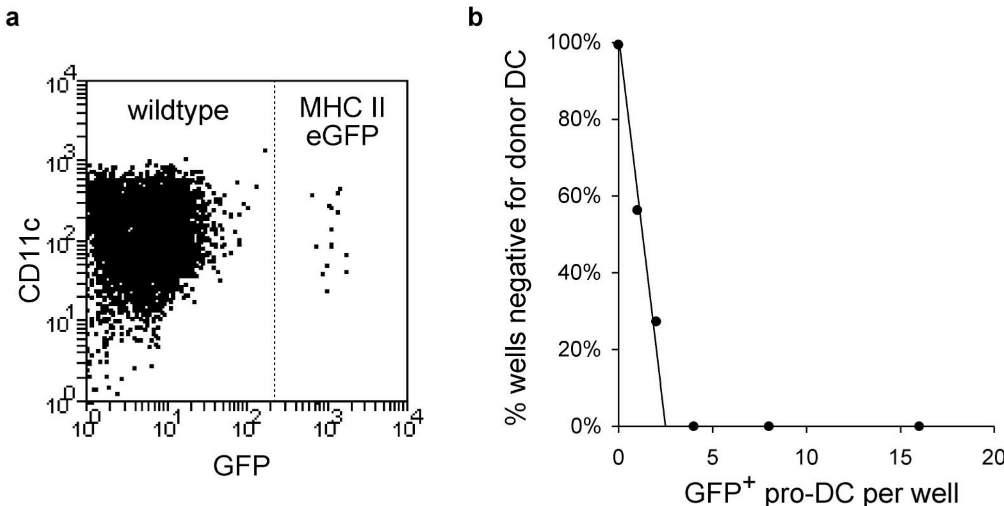


Figure 7.5 Clonal analysis of pro-DC.

Either 1, 2, 4, 8 or 16 pro-DC from MHC II-eGFP mice were sorted, using a flow cytometer, directly into wells containing 500 wildtype pro-DC in conditioned medium. At least 100 wells each containing a single GFP^+ pro-DC, and at least 12 wells for all other values, were cultured as replicates. a) A representative plot from a well in which a single GFP^+ pro-DC was cultured for 5 days is shown. b) Cultures were incubated for 5 days, and then scored for the percentage of wells with GFP^+ $CD11c^+$ progeny. The line of best fit was determined for all points that yielded wells negative for GFP^+ progeny.

In vitro **lineage potential**

It was important to determine at which point the pro- and pre-DC had diverged from other myeloid and lymphoid lineages. To test for the myeloid lineage potential of precursor populations, a fixed number was cultured with various cytokine stimuli in soft-agar colony assays (Table 7.3). Pre-DC did not form any colonies or clusters under any conditions tested, suggesting they were mostly DC-restricted, were relatively late-stage precursors unable to form myeloid colonies in agar cultures and/or were unresponsive to signals for myeloid development. It also demonstrated that they were free of contaminating early precursors. However, pro-DC preparations were able to generate a few colonies of mixed lineage in response to IL-3, GM-CSF, M-CSF or concentrated FL conditioned medium (CM). Most conditions yielded very few colonies. There was some colony formation in response to M-CSF; on average 18% of cells were responsive. Pro-DC were even less responsive to GM-CSF with less than 5% of precursors forming colonies or cluster in response to this cytokine. Of the colonies produced in either condition, most were macrophages (M), rather than granulocytes (G) or mixed granulocyte/macrophage colonies (GM). Whether the cells within the pro-DC preparation with this small degree of myeloid potential were common to those with DC precursor potential, or whether they were contaminating the preparations, is not yet clear.

Table 7.3 Myeloid lineage potential of pro-DC and pre-DC by colony assay

		Mean number of colonies per 1000 cells cultured						Clusters	Number of experiments
		Blast	G	GM	M	Eo	Meg		
Pro-DC	GM-CSF	0	1 ± 2	3 ± 6	39 ± 61	0	0	5	3
	M-CSF	0	0	8 ± 11	127 ± 110	0	0	36 ± 28	3
	IL-3	0	10 ± 10	3 ± 6	21 ± 25	0	0	10 ± 14	3
	SCF+IL-3+Epo	0	3 ± 6	0	29 ± 27	0	0	35 ± 31	3
	FL	0	0	0	0	0	0	0	1
	FL+LIF	0	0	0	0	0	0	10	1
	CM [1x]	0	8	3	72	0	0	133	1
	LPS	0	0	0	0	0	0	0	1
	IL-7	0	0	0	0	0	0	0	1
	Saline	0	0	0	0	0	0	0	1
Pre-DC	GM-CSF	0	0	0	0	0	0	0	1
	M-CSF	0	0	0	0	0	0	0	1
	IL-3	0	0	0	0	0	0	0	1
	SCF+IL-3+Epo	0	0	0	0	0	0	0	1
	FL	0	0	0	0	0	0	0	1
	Saline	0	0	0	0	0	0	0	1
BM	GM-CSF	0	18 ± 1	11.5 ± 2.5	21 ± 1	1.5 ± 1.5	0		2
	M-CSF	0	2.5 ± 0.5	3 ± 0	31.5 ± 2.5	0	0		2
	SCF+IL-3+Epo	6.5 ± 1.5	20 ± 7	13 ± 6	14 ± 0	1 ± 1	21.5 ± 6.5		2
	FL+LIF	4	2	0	0	0	0		1
	Saline	0	0	0	0	0	0		2

Soft agar colony assays were performed on sorted pro-DC, pre-DC or control BM under various conditions. Cells from each population (100-1000) were cultured in 1 ml of Dulbecco's modified Eagle medium containing 0.3% agar and analyzed as described previously (Metcalf et al., 2002). The recombinant cytokines were used at the following final concentrations: GM-CSF, IL-3, M-CSF, LIF (10 ng/ml) and SCF (100 ng/ml), flt3L (500 ng/ml), erythropoietin (EPO) (2I U/ml) or conditioned medium from day 3 FL cultures at a final concentration of 1x (CM). After 7 d of incubation, differential colony counts were performed on fixed whole mount preparations that were stained for acetylcholinesterase, Luxol fast blue, and hematoxylin.

In vivo **lineage potential**

To determine the *in vivo* lineage potential of pro- and pre-DC, especially in view of the low but sill reasonable amount of *in vitro* myeloid potential, precursors were transferred into lethally irradiated recipients to measure haematopoietic reconstitution. Either host Ly5.1$^+$ BM alone, or host BM in combination with each Ly5.2$^+$ DC precursor population, was transferred into lethally irradiated Ly5.1 recipients and their progeny assessed at day 10 and day 21 for both myeloid and lymphoid lineages (Fig. 7.6). These timepoints reflected the peak of development from committed myeloid and lymphoid progenitors, respectively. Spleen and BM were harvested at day 10 after transfer, and spleen and thymus at day 21. Cells that fell in the Ly5.2$^+$ gate in mice reconstituted with Ly5.1$^+$ host BM alone represented the slight background, whereas analysis of mice in which DC precursors were co-transferred with host BM resolved a clear population of Ly5.2$^+$ donor-derived cells (Fig 7.6a). Phenotypic analysis of Ly5.2$^-$ host cells served as an endogenous control.

At day 10 after reconstitution, myeloid progeny of Ly5.2$^-$ host BM had a significant proportion of CD11c$^-$ cells that were CD11b$^+$ or Ly6G$^+$, as expected (Fig 7.6a and 7.6b). When progeny of DC precursor transfers were assessed, most could be accounted for as CD11c^{hi} DC in both BM and spleen. It was unexpected to find CD11c^{hi} DC progeny in BM considering few DC are normally found in this organ. This may have reflected transfer of progenitors committed to DC formation, independent of organ localisation. In any case, few of the precursor progeny of pre-DC could be considered CD11c$^-$ CD11b$^+$ macrophages or CD11c$^-$ Ly6G$^+$ granulocytes in either BM (Fig. 7.6b) or in spleen (Fig. 7.6c). There were some CD11c^{int} CD11b$^+$ cells derived from pro-DC in BM. These were likely to be the pre-DC progeny of pro-DC *en route* to DC formation, although it cannot be discounted that they were CD11c^{lo} macrophages. Nevertheless, the efficiency for myeloid generation from pro-DC was extremely low when compared to the efficiency of half as many unfractionated host BM cells that were co-transferred, gated as Ly5.2$^-$ cells in the analysis. Thus, as pre-DC generated no myeloid progeny, and as pro-DC generated few cells that could be considered classic myeloid cells, both precursors were considered to be relatively DC-committed. The data suggested that both pro- and pre-DC had branched off from the myeloid pathway. Moreover, no CD19$^+$, DX5$^+$ or

$CD3^+$ lymphoid progeny were observed above background at this time (data not shown)

After 21 days, no myeloid cell, T cell, B cell or NK cell progeny of pre-DC could be detected in either spleen or thymus (data not shown). While pro-DC were also unable to generate myeloid cells, T cells or NK cells, some $CD19^+$ $CD45RA^+$ B cell generation was observed from this precursor after 21 d in spleen (Fig. 7.6d), with similar results in thymus (data not shown). Further analysis of the $CD19^+$ progeny for CD21, CD23 and IgM expression demonstrated each of the T1, T2 and marginal zone B cell subsets was present in the expected ratios, suggesting there was no preference (data not shown). The derivation of B cells from the pro-DC population was unexpected but could have implied that either i) they derive from a common DC/B cell committed precursor, or ii) that a small fraction of $CD19^-$ B cell precursors were contaminating in the pro-DC preparation. To determine which was the case, the ability of pro-DC to form B lymphocyte colonies in soft agar cultures was tested. Pro-DC were cultured with LPS, IL-7 or FL for 7 days, conditions known to expand late-stage B lymphocyte progenitors, (Rasko et al., 1995). However, no colonies could be detected under these conditions (Table 7.3). Such an absence of colonies suggested pro-DC did not contain significant late-stage B lymphocyte potential. However, clonal assays for early (Balciunaite et al., 2005) rather than late B cell progenitors are required in the future to truly assess the whether B cell precursors were common to those for DC, or were contaminating within the preparation.

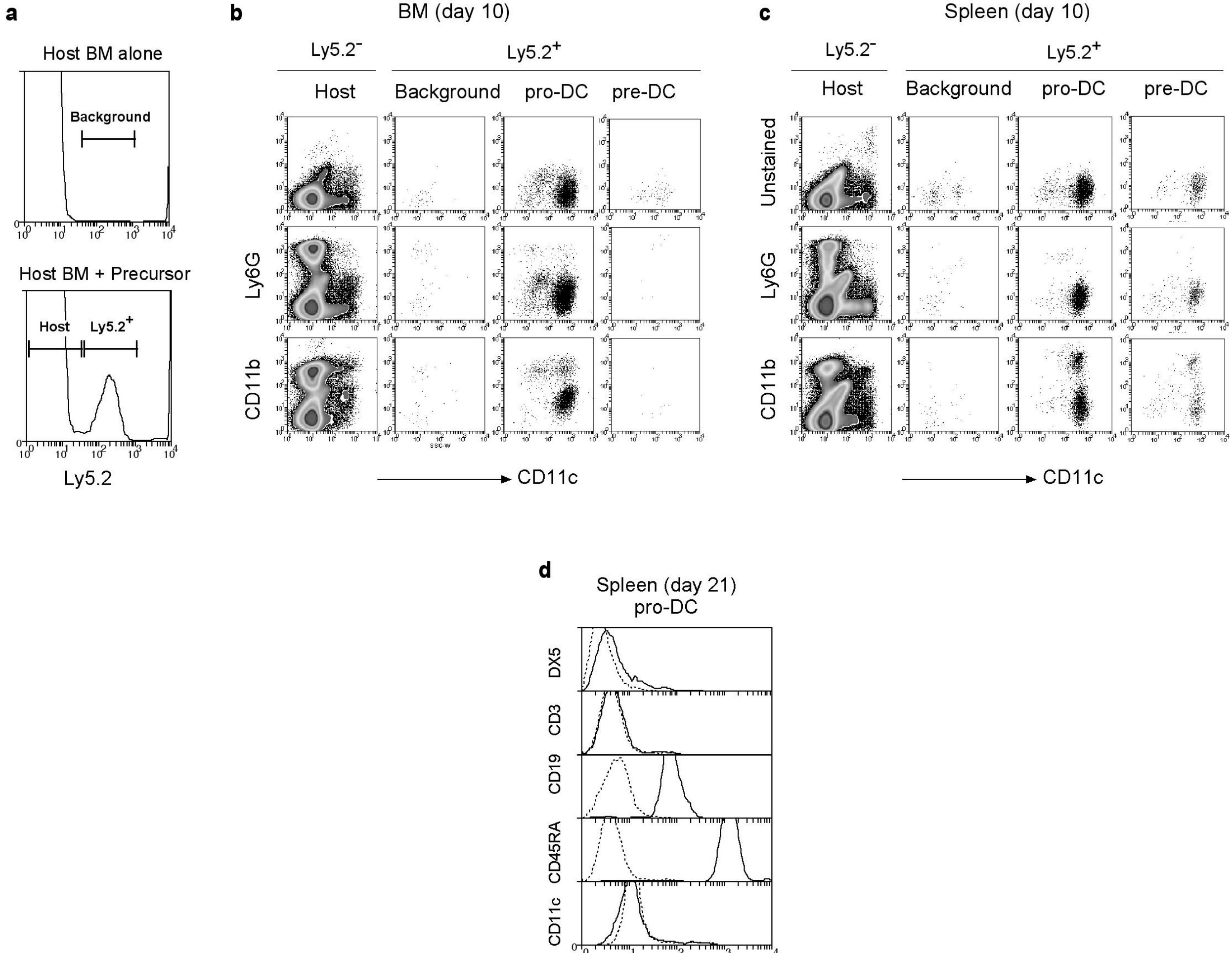


Figure 7.6 Lineage potential *in vivo*

Host Ly5.1$^+$ BM (1 x 10^5 per mouse) was transferred either alone, or with Ly5.2$^+$ pro-DC (2.3 x 10^5) or Ly5.2$^+$ pre-DC (1.5 x 10^5) into at least 2 lethally irradiated Ly5.1 recipient mice. Single cell suspensions were made from tissues at either day 10 or 21 after transfer. **a)** Cells were stained for expression of Ly5.2 to gate for reconstitution by host cells (Ly5.2$^-$), or by donor-derived cells (Ly5.2$^+$). **b)** Phenotype of BM 10 days after reconstitution. Shown is the phenotype of host cells, and Ly5.2$^+$ cells from mice reconstituted with host BM alone (background), with pro-DC, or with pre-DC. **c)** The same analysis as in b) but for spleen. **d)** Spleen was harvested 21 days after transfer of pro-DC, and stained with various markers after gating on Ly5.2$^+$ cells as in a). Broken lines represent the background of unstained controls of Ly5.2$^+$ progeny, and solid lines represent surface marker expression.

Discussion

The culture of BM with FL is an *in vitro* system that mimics steady-state DC development. By examining earlier timepoints of these cultures, two precursor intermediates that give rise to steady-state pDC, $CD8^+$ cDC and $CD8^-$ cDC have been identified. Generation of these DC subtypes occurred both after transfer of precursors *in vivo,* as well as upon re-culture *in vitro*. Careful tracking of their development resolved that the two precursors were related in a linear developmental pathway. A dividing $CD11c^-$ MHC II^- pro-DC stage, gave rise to a $CD11c^+$ MHC II^- pre-DC stage, *en route* to final generation of the DC subtypes.

An important issue in DC development has been to establish the relatedness of the monocyte/macrophage lineage to steady-state splenic DC. There are reports that DC and macrophages can arise from common early precursors such as CMP (Manz et al., 2001b; Traver et al., 2000; Wu et al., 2001), intermediate precursors such as MDP (Fogg et al., 2006), as well as late precursors such as monocytes (Geissmann et al., 2003; Leon et al., 2004; Randolph et al., 1998; Randolph et al., 1999). Certainly, such myeloid precursors can either differentiate into macrophages with M-CSF, or into DC with GM-CSF, suggesting they share a common pathway. Thus, it has generally been assumed that GM-CSF-derived DC are equivalent to those found *in vivo*. However, in Chapters 4, 5 and 6 it was questioned whether monocytes are the actual precursors of steady-state DC. Instead, it was shown that precursors since separated from the myeloid lineage were responsible for steady-state splenic DC via an intrasplenic pre-cDC population. Monocytes were proposed to instead better represent inflammatory DC precursors when GM-CSF levels are high, rather than of the steady-state.

Colony assays were used as a first measure to gauge the myeloid potential of the DC precursors reported in this Chapter. Pre-DC did not generate any myeloid colonies in such assays. For pro-DC, 18% could form colonies in response to M-CSF and less than 5% of pro-DC in response to GM-CSF, which were mostly macrophage-like colonies in both instances. Such properties of pro-DC set them apart from characterised myeloid precursors such as MDP (Fogg et al., 2006) and granulocyte/macrophage progenitors (GMP) (Dakic et al., 2005) from which 50% of

cells form colonies in response to these cytokines. Nevertheless, whether this small degree of macrophage generation *in vitro* by pro-DC comes from the same precursor that gives rise to DC, or whether they represent contaminants has not been determined, but is part of ongoing work.

Both pro- and pre-DC were also poor in myeloid cell generation *in vivo*. Pre-DC did not generate any detectable macrophage or granulocyte progeny in BM. For pro-DC, a maximum of 5% of progeny did not fit the CD11c^{hi} DC profile in BM, and these CD11c^{lo} CD11b^{+} cells were likely to represent the downstream CD11c^{+} pre-DC stage. It is still possible that these few cells represent a small degree of CD11c^{lo} macrophage potential in BM. Even so, the efficiency for macrophage generation by pro-DC was exceedingly low, even below that of unseparated BM. In spleen, the results were even clearer with no macrophage or granulocyte progeny from either precursor above background.

When assessed for lymphoid potential, pre-DC did not generate any T cells, B cells or NK cells. Pro-DC also did not give rise to T cells or NK cells, but did generate B cells at later timepoints. However, these were likely to be contaminating early progenitors rather than representing a common DC/B cell progenitor (Izon et al., 2001), as indicated by their poor B lymphocyte potential in preliminary clonal assays. Further analysis using clonal assays for early B cell precursors will resolve whether precursors with B lymphocyte potential are common to those with DC potential, or instead are contaminating and able to be excluded.

While both pro- and pre-DC are able to generate all pDC and cDC subtypes *in vivo*, they were, nevertheless, derived from *in vitro* cultures. Thus, the question remains: what are their *in vivo* equivalents? Phenotypic examination of spleen and BM is ongoing but, as yet, no putative *in vivo* counterparts of pro-DC have been identified. This is largely due to the overlapping phenotype of pro-DC with many cells negative for lineage markers. Their large size, CD117^{+} Sca-1^{-} phenotype, and peak of DC generation between 9-10 days might suggest they are related to the macrophage/DC progenitor (MDP) recently described by Geissmann and colleagues (Fogg et al., 2006). However, while MDP could generate macrophages, CD8^{+} cDC and CD8^{-} cDC, they were unable to generate pDC. MDP were also efficient in generation of DC

in colony assays with GM-CSF. This is in stark contrast to the pro-DC identified here that has cDC and pDC potential, but generates few macrophages *in vivo*, or colonies in response to GM-CSF. Future studies should focus on the identification of novel restricted markers expressed by pro-DC for their separation *in vivo*, as well as thorough clonal analysis to determine whether they represent a common precursor for all DC subtypes, or separate precursors that overlap in phenotype.

Pre-DC, on the other hand, have a possible identification *in vivo*. The peak DC generation of pre-DC was at 5 days, they had a $CD11c^{int}$ $CD43^{+}$ MHC II^{-} phenotype, and an efficiency of around 1 x 10^5 DC generated per 10^6 transferred. This is very similar to the intrasplenic pre-cDC identified in Chapter 4. However, the one striking difference was that culture-derived pre-DC produced all DC subtypes, whereas intrasplenic pre-cDC generated only cDC and not pDC (Chapter 4). One explanation for this discrepancy could be the differing tissue source used for their isolation. For example, the precursors within total BM that were active in FL cultures may have potential for all DC subtypes, whereas spleen-resident pre-cDC are solely cDC-committed (Chapter 4). There is evidence of such a BM-resident common cDC/pDC precursor (Bruno et al., 2001; Diao et al., 2004) that may be the equivalent of pre-DC identified in this study. However, in those studies, as well as for pre-DC in this Chapter, clonal assays are required to definitively address whether they represent common or separate precursors for the DC subtypes.

If culture-derived pre-DC (this Chapter) and intrasplenic pre-cDC (Chapter 4) are both clonal precursors of their respective lineages, the question remains as to their relationship to each other. There are two possibilities that are not mutually exclusive. Pre-DC may first branch into pDC- and cDC-restricted lineages within BM, the former developing to pDC in the BM and the latter seeding the spleen, where they would then correspond to pre-cDC. Alternatively, the pre-DC identified in this Chapter and analogous BM cell types may be a spatially distinct precursor type that rarely leaves the BM in the steady-state, but can instead act as a reserve that is released during infection or inflammation. This latter hypothesis is supported by the observation that steady-state DC develop from long-term precursors within the spleen but not those from BM via the bloodstream (Kabashima et al., 2005), and also that FL levels are low in the steady-state (Franchini et al., 2004; Lyman et al., 1995) but can

increase with certain viral infections, during which DC numbers also increase (Franchini et al., 2004).

The question also remains as to the identity of the upstream BM progenitors that give rise to pro-DC. These could potentially include pre-existing BM-resident pro-DC, the early flt3$^+$ CMP or CLP progenitors, or other cell types. Preliminary experiments involving the seeding of purified CMP and CLP from BM into FL cultures have demonstrated robust DC generation, suggesting both are upstream precursors of pro- and pre-DC in FL cultures (Li Wu, personal communication). Thorough analysis of dividing cells at times prior to day 3 in FL cultures, or of freshly isolated BM will be required to determine whether additional progenitors are also involved.

The results in this Chapter provide evidence for additional steps in the linear transition between HSC in the BM and DC in the periphery. It is known that HSC give rise to early flt3$^+$ progenitors. Subsequent to this, evidence presented here suggests that these flt3$^+$ progenitors sequentially develop into pro-DC, then pre-DC, and finally all of the steady-state splenic DC subtypes. Additionally, this study describes a simple method for the generation of large numbers of DC precursors and a system to study their development, including clonal development from single marked precursors.

Chapter 8
Final discussion

The results of this thesis are summarised as follows:

1) $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes are not precursors of steady-state spleen DC. However, when there is systemic inflammation, $Ly6C^{hi}$ monocytes produce a unique "inflammatory" DC in spleen, reminiscent of TNF-α iNOS-producing (Tip) DC. These inflammatory DC are distinct from cDC and other steady-state subsets. GM-CSF cultures best represent this subset.
2) A rare precursor population within the spleen itself is responsible for steady-state generation of cDC, including the $CD8^+$ and $CD8^-$ subtypes, but not of pDC or other lineages. This "pre-cDC" population has been purified 600-fold and has the phenotype $CD11c^{int}$ $CD43^{int}$ Sirp-α^{int} MHC II^-. It is not a classical monocyte. Pre-cDC produce cDC within 5 days with only limited division. The population includes some cells already pre-committed to the $CD8^+$ or $CD8^-$ cDC subtypes only. This and direct experiments indicate that $CD8^-$ cDC are not the direct precursors of $CD8^+$ cDC, but are distinct DC lineages.
3) The culture of BM with FL generates three DC subtypes *in vitro* that are equivalent to pDC, $CD8^+$ cDC and $CD8^-$ cDC found *in vivo*. Thus, the FL culture system adequately reflects steady-state DC development. Two unique DC-committed precursor populations within these cultures are responsible for generating all DC subtypes *in vitro*, and when transferred *in vivo*. They include an upstream dividing precursor (pro-DC) that gives rise to a downstream precursor (pre-DC) *en route* to DC formation.

It is evident that DC can no longer be regarded as a homogeneous entity with a common lifecycle. While all DC share some features, the discrete DC subtypes have distinct locations in the body, different functional properties and specialised roles in the immune system. The research presented in this thesis has identified some of the distinct precursors of DC and their paths to differentiation.

Monocytes have been considered the archetypal DC precursor due to their ability to form DC in culture with GM-CSF (GM-DC). However, I eastablished that their *in vivo* contribution to steady-state spleen DC is minimal (Chapter 5), although there are reports that they may be precursors for Langerhans cells. I have demonstrated that monocytes are more likely to be a reserve for the generation of a unique "inflammatory" DC type, as demonstrated in this thesis (Chapter 5) and other studies. Thus, I also propose also that *in vitro*-derived GM-DC are more representative of these *in vivo* "inflammatory" DC, rather than steady-state DC (Fig. 8).

The intrasplenic pre-cDC that I identified, by contrast, represents the immediate precursor stage in the steady-state pathway of cDC generation (Fig. 8) (Chapter 4). Importantly, it has segregated from the pathway of pDC development. What is not clear is whether pre-cDC constantly enter via the blood, or instead are derived from a long-term DC precursor that is spleen-resident. Several lines of evidence from this thesis and other studies support the latter scenario (Chapter 4). Whether these same rules apply for $CD8^+$ and $CD8^-$ cDC of lymph nodes is also not known. Further experiments using parabiotic mice, or the like, should distinguish whether precursors for the different DC subtypes within different lymphoid organs are resident long-term or come in a steady flux from the blood.

While pre-cDC are certainly a major, if not the major, route for cDC generation, the proportion of $CD8^-$ cDC generated is notably lower than the proportion seen within freshly isolated spleen. Homeostatic proliferation of $CD8^-$ cDC might be one mechanism that boosts and maintains $CD8^-$ cDC numbers, after having first differentiated from pre-cDC (Fig. 8) (Kabashima et al., 2005).

Analysis of FL BM cultures, which I found to closely model generation of the steady-state DC subtypes (Chapter 6), reveal that pro-DC and pre-DC are the intermediate

stages between flt3^{+} early BM precursors and DC formation, both *in vitro* and *in vivo* (Fig. 8 and Chapter 7). The *in vivo* counterpart of pro-DC is the subject of ongoing investigation. An *in vivo* counterpart of the culture-derived pre-DC (Chapter 7) appears to be the intrasplenic pre-cDC (Chapter 4), except that they differ in their production of pDC; the former can while the latter cannot. These differences might be due to heterogeneity within precursors, or may be due to intrinsic differences between BM versus spleen as a precursor source.

Related to this last possibility is whether FL BM cultures truly reflect the steady-state situation, and whether BM or spleen is the more representative source of steady-state precursors? As mentioned previously, spleen DC generation may be largely independent of the bloodstream, and therefore independent of the BM as well. This questions the relevance of BM as an active contributor to steady-state spleen DC. Perhaps the answer is that, despite bearing equivalent populations to steady-state DC, FL-DC generation actually reflects an emergency response. It is known that FL levels are low in the steady-state but that serum levels increase after certain viral infections, concomitant with an increase in DC numbers (refer Chapter 7). Perhaps during certain infections, BM-derived pro- and pre-DC are released into the bloodstream to strengthen DC numbers in peripheral organs. Therefore, BM may be a reserve while spleen is the steady-state source of DC.

What are the precursor stages earlier to pro-DC? Both flt3^{+} CMP and flt3^{+} CLP are active precursors within whole BM in the FL-DC system, so these may be the upstream precursor of pro-DC (Chapter 7). There is also a recent report of a BM-resident common macrophage and conventional DC precursor (MDP) that does not generate pDC. How the MDP relates to the pro-DC, which generate pDC and cDC but not macrophages, is not evident. Clonal analysis that is now possible as an extension to the single cell studies of Chapter 7 should help address the DC subtype and macrophage potential of pro-DC and other candidate precursors.

The first major challenge for translation to humans is to identify the human equivalents of CD8^{-} and CD8^{+} cDC of mice. Evidence from several animals demonstrate that equivalent DC subtypes to the mouse occur cross-species (Introduction). In steady-state mice, CD8^{-} and CD8^{+} cDC are virtually absent from

the blood, and their precursors are at very low frequency, if at all (Chapter 5). Perhaps, it is no surprise then that the human equivalents of cDC have not yet been delineated, considering human studies focus mainly on blood DC or blood-derived GM-DC. Few human studies have focused on spleen or lymph node DC from healthy patients, although the few that have done so provide preliminary evidence of DC subtypes (Introduction). Another possibility is to explore FL cultures of human BM, which may harbour equivalents to *in vivo* DC subtypes, analogous to my studies in the mouse system. These experiments are currently in progress.

Considering the importance of individual DC subtypes in mouse models of infection and autoimmunity, the second major challenge will be to apply these discoveries to the putative human DC subtypes. Unlike the injection of already mature DC for immune therapies, as is currently the protocol, DC precursors offer several advantages in that they are more efficient in homing back to lymphoid organs, and have an expansion phase to increase numbers *in situ*.

Many challenges still lie ahead including the identification of the precise steps in DC development, the organ location of these steps, and the regulators of differentiation. Once applied to the human system, DC and their precursors may serve as useful targets or vectors for immune therapies.

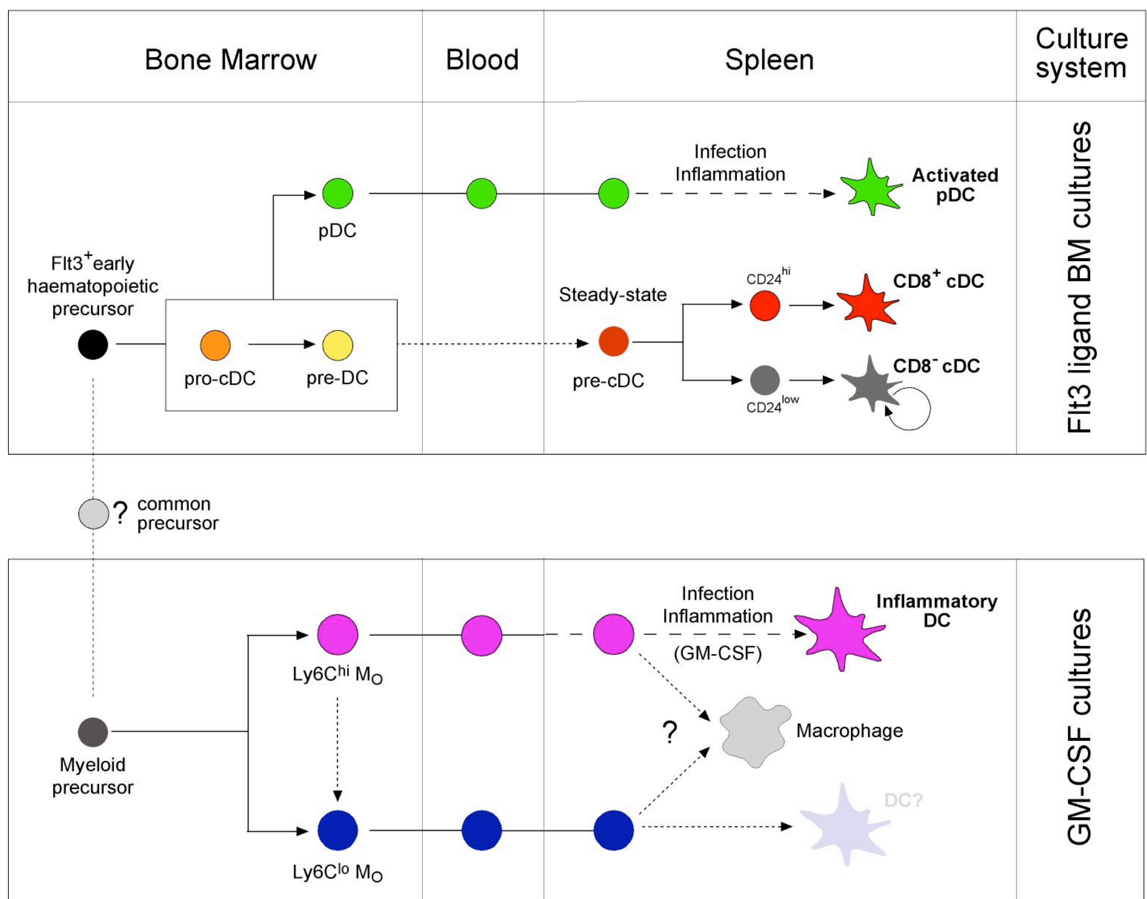


Figure 8 Model of DC development from different precursors

In the steady-state, CD8$^+$ and CD8$^-$ cDC turnover every 3-5 days. The CD8$^+$ cDC are derived from CD24^{hi} pre-cDC, and CD8$^-$ cDC from CD24^{lo} pre-cDC. These, pre-cDC are resident within spleen and may be replenished by an earlier precursor from within spleen or from BM. Of note, pDC are not derived from intrasplenic pre-cDC. Rather, they circulate to the spleen from BM via the blood in the steady-state. The upstream of precursor of both pDC and cDC are BM-resident pro-DC. These precursors can generate all DC subtypes via a pre-DC intermediate. However, the relationship between BM pre-DC and intrasplenic pre-cDC is not yet clear. The earliest flt3$^+$ haematopoietic progenitors in BM generate all steady-state DC subtypes and steady-state DC generation is best reflected by FL BM cultures. Monocytes, by contrast, are derived from myeloid-restricted precursors in BM and circulate in the steady-state as reserves. Only upon infection or inflammation do monocytes become a unique form of “inflammatory” spleen DC. Such DC are best reflected by GM-CSF cultures. The last precursor stage that is downstream of HSC but common for both monocyte and DC generation is not known.

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