A novel screen to understand preDC migration and improve recruitment to the tumour microenvironment

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"I am on the verge of mysteries, and the veil which covers them is getting thinner and thinner."

Louis Pasteur

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Author Declaration

I declare that this thesis is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution. Work completed by other people is acknowledged here:
Dr Marieke Pingen carried out CCL2 ELISAs on provided supernatant. Dr Craig Norse prepared sorted preDCs for scRNAseq analysis. Tom Gilbey, Dr Yi-Hsia Liu and Jennifer Cassels carried out cell sorting. Alex Young contributed to all chemokine overexpression optimisations and experiments in Chapter 2. Dr Robert Wiesheu prepared samples for liver analysis. Animal Unit staff maintained animal husbandry and carried out the majority of experimental procedures other than IP injections which were carried out by myself or Dr Chiara Pirillo. Dr Anna Andrusaite prepared samples for small intestine analysis.
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SUMMARY

Classical dendritic cells (cDCs) are a rare, but vital, component of the tumour microenvironment (TME). Their presence improves prognosis and response to current immunotherapies. Although therapeutics have been developed to increase cDC development, and thus their abundance in the tumour, we aimed to understand if cDCs could be induced to specifically infiltrate the TME instead of increasing their numbers systemically. Thus, we set out to investigate if tissue specific signals were involved in the recruitment of cDC precursors.

Precursors of cDC (preDCs) are a heterogeneous group that differentiate in the bone marrow. In the periphery, pre-cDC1s develop into cDC1s and pre-cDC2 into cDC2s, respectively. To infiltrate these tissues, preDCs must migrate through the blood to peripheral tissues but the signals controlling tissue recruitment are unknown, including whether both subsets respond to common factors and if there exist tissue-specific controls. Understanding preDC recruitment will allow us to understand cDC development as well as exploit means to manipulate their seeding in the periphery. This manipulation has the potential to improve cDC infiltration in the tumour microenvironment with potential therapeutic benefit.

Here we present evidence that preDC subsets express distinct chemokine receptors and respond to different chemokine signals in the TME. Additionally, we provide a universal assay that can be used to measure the migration of all immune cells that arise from haematopoiesis to all peripheral tissues and during multiple contexts. With this assay we have unpicked which chemokine receptors are involved in the tissue specific seeding of preDCs peripherally, including subcutaneous tumours.

Thus, this project has contributed to the knowledge of how cDC infiltration is controlled and identified means to exploit their migration patterns.

CONTENTS

ABBREVIATIONS

BM	Bone marrow
CCL/R	C-C chemokine ligand / receptor
ACKR	atypical chemokine receptors
ACT	adoptive cell therapy
APC	antigen presenting cell
CBD	collagen binding domain
cCKR	conventional chemokine receptors
cDC	classical/conventional dendritic cell
CDP	Common DC progenitors
CLP	common lymphoid progenitors
CLR	C-type lectin receptor
cMOP	common monocyte progenitor
CMP	Common myeloid progenitors
CMP	common myeloid progenitors
DAMP	Damage-associated molecular pattern
FACS	Fluorescence-activated cell sorting
Flt3L	FMS-like tyrosine kinase 3 ligand
FRC	fibrobastic reticular cells
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	granulocyte-monocyte progenitor
HBSS	Hanks' Balanced Salt Solution
HES	H. Polygyrus Excretory-secretory material
HLA	human leukocyte antigen
HSC	Hematopoietic stem cells
HSP	heat shock protein
ICB	immune checkpoint blockade
INS	Infectious-nonself
KO	knockout
LC	Langerhan cells
LPS	lipopolysaccharide
LT-HSC	long term HSC
MC	monocytic cells
mDC	migratory dendritic cell
MDP	macrophage and dendritic cell progenitors
MFI	Mean fluorescent intensity
MHC	major histocompatibility complex
MLN	Mesenteric lymph node
MMPS	multipotent progenitors
MPC	mononuclear phagocytic cells
NK	Natural killer
NLR	Nod-like receptor
PAMP	Pathogen-associated molecular pattern
PC	Plasmacytoid cell
pDC	Plasmacytoid dendritic cells
pre-cDC	cDC precursor
pre-preDC	non-committed cDC precursor
PRR	pattern recognition receptors

RA	Retinoic acid
RAMP	resolution associated molecular patterns
rDC	resident dendritic cell
RLR	RIG-I–like receptor
Sca-1	stem cell antigen-1
SNS	self-non-self
ST-HSC	short term HSC
TAA	tumour associated antigen
TCR	T-cell receptor
tdLN	Tumour draining lymph node
TF	Transcription factor
Th	T helper cell
TLR	Toll-like receptor
TME	Tumour microenvironment
Treg	Regulatory T cell
VWF	von Willebrand factor
WT	Wild type

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1 INTRODUCTION

1.1 Dendritic cells

Dendritic cells were first identified by Ralph M. Steinman and Zanvil A. Cohn in the 1970s (Steinman and Cohn 1973) and named as such due to the dendrites which extended from the cell in a stellar fashion. Since then, our knowledge of dendritic cellsand their importance in orchestrating the immune response has grown rapidly. Now we know that conventional dendritic cells (cDCs) are a highly diverse group of cells which originate from the bone marrow, seed multiple tissues, and differentiate into various subtypes with diverse functions (Cabeza-Cabrerizo, Cardoso, et al. 2021a; Merad et al. 2013a; Sichien et al. 2017a). This introduction will describe the current understanding of cDC phenotype, ontogeny, and function with a focus on the role of cDCs in cancer.

1.1.1 Defining dendritic cells

In the past decades, cDCs have been extensively studied, however, characterisation of cDC subsets remains a point of contention. This contention is due to multiple subsets of heterogeneous cells with overlapping functions and phenotypes. Currently, the criteria suggested to define a cell as cDC is based on cell ontogeny (discussed in depth in section 1.1.2) (Guilliams et al. 2014). This has allowed the characterisation of mononuclear phagocytic cells (MPCs) into distinct subsets based on their distinct differentiation. Historically, all MPCs were considered members of the same family descending from tissue infiltrating monocytes (Guilliams et al. 2014). With the advent of phenotypic, ontological, and functional analysis, distinctions between MPCs have been appreciated.

It is now accepted that monocytes arise separately from cDCs from a common monocyte progenitor (cMoP) (Hettinger et al. 2013; Z. Liu et al. 2019; Meredith, Liu, Darrasse-Jeze, et al. 2012) in a colony-stimulating factor-1 receptor (CSF1R) dependent manner. Monocytes circulate in the blood, infiltrate tissues, and receive environmental signals that induce maturation. The expansion of a state of monocytes during inflammation, with characteristics associated with cDC cells, resulted in their definition as monocyte-derived-DCs (mo-DCs) (Cabeza-Cabrerizo, Cardoso, et al. 2021a). However, it is unclear whether these cells are able to exert comparable functions to *bona fide* cDCs. Ontogenically, mo-DCs arise independently from cDCs (discussed in section 1.1.2), however, their definition as DC-like cells arose from their ability to act as antigen presenting cells in certain contexts (Cabeza-Cabrerizo et al. 2021). Nonetheless, when adoptively transferred for analysis in

murine respiratory viral infection models, monocytes lacked the ability to migrate to the draining lymph node and were not able to present antigen and prime T-cell responses (Cedric Bosteels et al. 2020). It is likely that the consideration of monocytes acting as DC-like cells is due to observations from artificial *in vitro* models (Guilliams and Malissen 2015) and non-rigorous separation of cDCs and monocyte cells during analysis.

Macrophages arise in tissues during foetal development and are maintained predominately by self-renewal within the tissue (Wu and Hirschi 2021). Similarly, based on ontogeny and Flt3L-independence, Langerhans cells (LCs) within the skin have also been appreciated as a type of macrophage instead of cDC (Cabeza-Cabrerizo, Cardoso, et al. 2021a). While previously regarded as a type of dendritic cell (Guilliams et al. 2014), new evidence has led to the suggestion that plasmacytoid dendritic cells (pDC) be named plasmacytoid cells (PCs), a term recently coined (Cabeza-Cabrerizo, Cardoso, et al. 2021a). This is due to their differing development and function to cDCs. Original suggestions that PCs could function as antigen presenting cells with cDC functions is likely due to the contamination of cDC precursors in PC studies (Cabeza-Cabrerizo, Cardoso, et al. 2021a; Dress et al. 2019a; Z. Liu et al. 2023; Reizis 2019). However, controversy remains in the field and further ontological and functional analysis with stringent cell separation is needed to confirm if these cells are a type of dendritic cell or not.

cDC ontogeny will be discussed more extensively in section 1.1.2, however, cDCs are an ontogenically distinct population of cells with both specific phenotype and functions as well as features which overlap with other MPCs. As such, the remainder of this thesis will focus on the defined cell type of cDC. cDCs in general are identified, in mice, phenotypically by the expression of CD45, major histocompatibility complex class II (MHCII), CD11c and lack of expression of other immune cell lineage specific markers. Additionally, expression of a CD45 isoform, CD45RA, was expressed on cDCs but downregulated on monocytes and granulocytes (Z. Liu et al. 2023) and thus may be used to identify cDC lineage. Within cDCs, cDC1 and cDC2 subsets have been defined. Although they share a common role in professional antigen presentation to T-cells and instruction of immune responses, the subsets vary in development, phenotype, functional capabilities, and anatomical localisation (discussed in depth in the following sections). cDC location also contributes to their characterisation where lymphoid restricted cDCs are classed here as resident cDCs (rDC) and peripheral tissue cDCs, with the potential to migrate to the lymph nodes in a CCR7-dependent manner, are classed here as migratory cDCs (mDC) (Randolph, Angeli, and

Swartz 2005). It remains difficult to distinguish mDC and rDC in lymph nodes due to the overlapping expression of surface markers. Despite this, during homeostasis, mDCs express a higher level of MHCII and lower CD11c compared to rDC, however, this is less distinct during inflammation (Merad et al. 2013b). Within these subsets, cDCs are considered as rDC1, rDC2, mDC1 and mDC2. It is likely that different functional capabilities are associated with cDCs in distinct locations based on tissue-specific factors. For example, cDCs in mucosal sites will be subject to an abundance of environmental stimuli which may influence their functions. Additionally, lymph node resident cDCs which are distally located from the draining tissue may have distinct functions associated with how they "survey" the tissue. Further analysis of tissue-specific cDCs is needed to understand how location contributes to the cells characteristics.

cDC1 rely on the transcription factors (TFs) *Batf3* and *Irf8* and express XCR1 and Clec9a (DNGR-1) with mDC1 being $CD103^+$ and rDC $CD8\alpha^+$ (Bosteels and Scott 2020; Cabeza-Cabrerizo, Cardoso, et al. 2021b; Sichien et al. 2017b). *Irf8* is crucial to the initial differentiation of cDC1 precursors and the maintenance of cDC subset specificity (Bosteels and Scott 2020). Additionally, cDC1s express a higher level of CD135 (Flt3) to cDC2 (Ginhoux et al. 2009; Waskow et al. 2008a). On the other hand, cDC2s consist of a more heterogeneous group of cells that have less well-defined characteristics. In general, cDC2s rely on *Irf4* TF not during precursor development, but at the maturation stage. CD11c specific knockout of *Irf4* did not lead to an ablation of the cDC2 population but functional and phenotypic abnormalities, particularly their ability to migrate from the peripheral tissues to the draining lymph nodes (Bosteels and Scott 2020; Murphy et al. 2016). cDC2s express CD11b and Sirpα and lack CD103, XCR1, Clec9α and CD8α (Bosteels and Scott 2020; Cabeza-Cabrerizo, Cardoso, et al. 2021b; Sichien et al. 2017b). However, in the small intestinea subset of double positive $CD11b^+$ $CD103^+$ cDCs exist as the prominent cDC subset ((Cerovic et al. 2014; Bain et al. 2017; Scott et al. 2015c). This cDC subset can also be identified in the small intestine's draining lymph nodes and as a less abundant population in the colonic lamina propria.

In mice, cDC2s are more abundant in the majority of tissues compared to cDC1, however, this may be due to multiple cell subsets/cell states being characterised as cDC2s. A consequence of more in-depth analysis defining MPCs is the understanding of the vastness of the cDC2 population. Whether or not all cDC2s are true cDC is a source of confusion and debate (Cabeza-Cabrerizo, Cardoso, et al. 2021a; Guilliams et al. 2014; Merad et al. 2013a).

Further characterisation of the cDC2 populations are needed to confirm if indeed there exist multiple cDC subsets or these differences are a consequence of various cell states. It is also likely that the same cells are being analysed by different researchers, while being classed as different cell types based on the individual's characterisation of cDCs and other MPCs.

Furthermore, a recent fate mapping and scRNAseq study (Z. Liu et al. 2023) proposed a new subset of murine dendritic cell, the DC3, which shares features with cDCs and monocytes. The development of the DC3 was independent of the monocyte lineage and the conventional dendritic cell lineage and will be discussed in section 1.1.2. This study elucidates that currently, we are unable to completely separate cDCs, monocytes and other DC/monocyte-like lineages from our analysis with the array of markers we have identified for each, and this is a factor that must be considered when identifying cDC specific attributes.

It is likely that functional states do not represent separate subsets of cDCs but fluid states that reflect the context of the tissue in which the cell resides. Therefore, it is worth being cautious when defining new subsets of cDCs which may reflect cDC1 and cDC2 in different states. For example, the use of the Flt3L receptor CD135 as a marker of cDC lineage can be problematic since, not only do other lineage specific cells express it in the bone marrow, but also the marker downregulates following exposure to Flt3L (Williams et al. 2017; Ugur et al. 2023a). In this case, cDC precursors could contaminate CD135-negative cells and explain the overlap with cDC attributes in analysis of monocytic cells. Additionally, Zbtb46, which is often used as a specific marker of dendritic cells, is downregulated in cDC upon activation, upregulated on activated monocytes, and expressed in non-immune cells, therefore, Zbtb46 expression alone shouldn't define a cDC (Meredith, Liu, Kamphorst, et al. 2012; Satpathy et al. 2012). Furthermore, both cDCs and monocytes can adapt in a context dependent manner, for instance cDCs upregulate markers CCR7, PD-L1, CD80 and CD86 post-activation (Cabeza-Cabrerizo, Cardoso, et al. 2021b; Merad et al. 2013b). Additionally, a state of cDC2s were identified during infection which took on aspects of cDC1s and monocytes; a cell state classed as inf-cDC2 (Bosteels et al. 2020). These inf-cDC2s were shown to derive from cDC precursors, require Flt3L and differentiate from cDC2s in response to type 1 interferon. Inf-cDC2s shared genes associated with monocytes and all cDCs but ,unlike monocytes, they were able to transport antigen to the draining lymph node and activate T cells. It is likely that these cells are not a new subset of cDCs but rather an inflammatory state that cDC2s take on during infection. Such states will also likely be

identified in cDC1s. In terms of humans, similar subsets have been identified (Cabeza-Cabrerizo et al. 2021). For example, scRNAseq of human blood identified six populations of DC-like cells (Villani et al. 2017): DC1 were associated with CLEC9A^{+} cross-presenting cDC1; DC2 and DC3 were cDC2 subclusters; DC4 shared characteristics with both cDCs and monocytes; DC5 shared features of both cDC2 and pDC; and DC6 were identified as pDC. While confirming the existence of cDC subsets in humans and highlighting that cDC heterogeneity exits in mice and man, the study lacks ontological analysis and analysis of cDCs in distinct peripheral tissues. Moreover,subsets similar to the murine DC3 which arise independently of cDC1 and cDC2 (Bourdely et al. 2020; Cytlak et al. 2020) and other monocyte-derived dendritic cell like subsets (Villar and Segura 2020) have been identified in human blood. Interestingly, it was GM-CSF and not the cDC dependent growth factor, Flt3L, that induced a human DC3 phenotype *in vitro* (Bourdely et al. 2020). The consensus is that human pDCs derive from cDC precursors in a Flt3L dependent manner (Villar and Segura 2020). However, it is possible that, like mice, human pDCs are actually PCs which develop independently from cDCs, and this overlap can be explained by contamination of cDC precursors in PC analysis. Overall, it is certain that cDC biology is a highly complex field of which we are only beginning to understand. It is important that the future of cDC research develops a means to classify states of cDCs to reduce the confusion in the literature that new subsets have been identified. This should involve identifying if the feature of cDC is present in multiple tissues during different contexts. If the feature is distinct to an inflammatory challenge, this would suggest that the feature represents a state of cDC. However, if present in multiple locations during steady-state and inflammation; the feature may represent a cDC subset. Thus, a consensus is required in the cDC field to prevent premature classifications that novel subsets have been identified.

Based on the consensus that cDC1 and the heterogeneous cDC2 exist and well-defined markers are known for each, this research for this thesis aimed to focus on cDC1 and cDC2 only, but it is worth recognising that it is likely these subsets contain contamination of other cell subsets/states (such as the inf-cDC2 and DC3) that have not yet been identified or characterised.

1.1.2 preDC development

cDCs develop from precursor cells within the bone marrow during haematopoiesis when self-renewing multipotent hematopoietic stem cells (HSCs) give rise to lineage specific

blood and immune cells (Sichien et al. 2017a). The development of cDC precursors has been coined “DC-poiesis” (Cabeza-Cabrerizo, Cardoso, et al. 2021a). Haematopoiesis occurs primarily in the bone marrow of adults; an area which provides an ideal niche for cell expansion (Fröbel et al. 2021). The bone marrow niche is a dense vascularised area which provides access to oxygen rich blood, nutrients, routes for egress of mature cells as well as stromal production of growth factors essential for cell development. Progenitors can be separated into multiple cell types based on their surface proteins and capacity to self-renew and differentiate. HSCs themselves are defined by the absence of lineage specific markers and the expression of stem cell antigen-1 (Sca-1) and stem cell factor receptor (c-Kit) which can be subdivided into long-term $CD150^{+}$ (LT-HSCs) and short-term $CD150^{lo/-}$ HSCs (ST-HSCs) which have differing abilities to reconstitute irradiated bone marrow (Kondo et al. 2003). LT-HSCs are characterised by their ability to maintain the HSC pool for the life-time of the organism compared to ST-HSCs which have a reduced capacity for self-renewal (Kondo et al. 2003). In the classical model of haematopoiesis (Naik 2020), the differentiation of HSCs into multipotent progenitors (MMPs) marks the point on the haematopoietic tree when progenitors lose their ability to self-renew but retain multipotency; the ability to differentiate into multiple lineage-restricted cell types. Here, MMPs give rise to common myeloid (CMPs) and lymphoid (CLPs) progenitors. The development of dendritic cell precursors comes from the further differentiation of CMPs into macrophage and dendritic cell progenitors (MDPs) which go on to give rise to common/conventional dendritic progenitors (CDPs) and, subsequently, non-committed preDCs (pre-preDCs) which maintain the potential to develop into precursors of both cDC subsets (K. Liu et al. 2009). This classical model suggests that all cells within each step of differentiation act equally in terms of their differentiation bias. Although out of the scope of this thesis discussion, it is worth noting that other models have been proposed (Naik 2020). In the ‘clonal trajectory’ model, each cell has individual differentiation bias which can occur at any point of haematopoiesis. In this case, cDC commitment may occur early during haematopoiesis.

In general, cDC development relies on the TF *Pu.1* while specific regulation of cDC1 involves *Irf8, Batf3, Nfil3*, and *Id2* and cDC2 involves *Irf4*, *Irf2*, T-*bet*, *Rorδt*, and the repressor of *Irf8*, *Zeb2* (Bosteels and Scott 2020; Brown et al. 2019; Cabeza-Cabrerizo, Cardoso, et al. 2021a; Merad et al. 2013a; Schraml and Reis e Sousa 2015). However, as with all aspects of characterising dendritic cells, transcriptional regulation is complicated. For instance, the expression of *Irf4* and *Irf8* alone may not determine the differentiation of a preDC subset

but rather the combinational dose of both TFs (Ma et al. 2019). At which point cDC subset commitment occurs and which factors control this differentiation remains unclear. However, using scRNAseq analysis, Schlitzer and colleagues identified that cDC gene signatures were identified as early as MDP and increased through CDPs to preDCs. Interestingly, subset specific signatures for cDC2 could be seen transcriptionally at the CDP stage while cDC1 signatures could only be identified at the preDC stage (Grajales-Reyes et al. 2015; Schlitzer et al. 2015). This could suggest that CDPs are programmed to develop into pre-cDC2s which then require additional signals to diverge into pre-cDC1s. This additional signal could be the downregulation of Zeb2, the repressor of Irf8, allowing the crucial TF for pre-cDC1 development to be accessed (Bagadia et al. 2019). In this case, pre-cDC2s would be a developmental stage of pre-cDC1s rather than subsets which develop independently. However, a separate study analysing the development of *in vitro* CDPs, suggested that the preDCs subsets develop directly from CDPs (Grajales-Reyes et al. 2015). Additionally, two populations of CDP were identified based on *Zeb2* expression levels (Bagadia et al. 2019), suggesting the bias towards the individual preDC subsets occurs at the CDP stage based on availability of *Irf8*. In fact, while both preDC subsets rely on Flt3L to develop (Ginhoux et al. 2009; Karsunky et al. 2003b; McKenna et al. 2000a; Waskow et al. 2008b, 2008a), pre-cDC1s have a greater reliance on the growth factor (Lin et al. 2021a). Potentially, the level of available Flt3L controls the differentiation of pre-cDC1s.

What we understand about cDC development was further complicated by a recent study (Z. Liu et al. 2023). The group identified a cell with features of both cDCs and monocytes, which they coined DC3. This DC3 was shown to develop independently from monocytes, using Ms4a3^{Cre}-Rosa^{TdT} monocyte fate mapping mice, and belonging to cDC lineage, using the Zbtb46^{Cre}-Rosa^{TdT} cDC fate mapping mice. While analysing developmental trajectory, the group suggested that cDCs separated into three branches, suggesting the existence of a third cDC subset. However, DC3 arose from Ly6C^{+} MDPs, independent from the conventional DC subsets which develop from CDPs to preDCs. By adoptive transfers, the group identified that while Ly6C^{-} MDPs had dendritic and monocytic potential, Ly6C^{+} MDPs developed into pro-DC3 (precursors of DC3) but not CDPs. Most interestingly, CDPs seemed to be bias to cDC1 development while pro-DC3 gave rise to cDC2s and DC3. When separating pro-DC3 by expression of Lyz2 and CD34, the group identified that Lyz2^{-} CD34^{+} cells developed into cDC2 while Lyz2^{+} CD34^{-} cells became DC3Additionally, CDPs gave rise to some pre-cDC2s while others came from Lyz2^{-} CD34^{+} cells that originated from MDPs not

CDPs.Previous work (Grajales-Reyes et al. 2015) suggested that both preDC subsets arose directly from CDPs. Together this could suggsest that our classification of pre-cDC2s accounts for a heterogeneous group of ontogenically distinct cells. This further supports the notion that our conception of cDC2s encompasses a heterogeneous group comprising multiple types of cDC cellS. It was previously proposed by Guilliams and colleagues (Guilliams et al. 2014) that cDCs should be defined by the ontogeny of the cell. However, these criteria may have to be adjusted since some pre-cDC2s and DC3s can arise from MDPs, without the CDP step of development, and hence do not share the same ontogeny as pre-cDC1s and other pre-cDC2s. Since we are currently unable to separate these subsets, our research focuses on pre-cDC1s and the heterogeneous pre-cDC2s. Figure 1.1 displays how we believe preDCs develop based on the current literature. Determining what controls preDC subset divergence may prove useful in the treatment of viral infections, cancer, and vaccine development where cDC1 presence is beneficial (Hildner et al. 2008a; Ng et al. 2018; Roth et al. 2021).

In any case, differentiated pre-cDC1 and pre-cDC2 exit the bone marrow, a process associated with loss of CXCR4 expression and promoted by gain of CCR2, which is induced more readily during infection (Pereira da Costa et al. 2023a). CXCR4 is vital to the retention of HSCs and precursors within the bone marrow (Nakano et al. 2017). It has been suggested that interactions with haematopoietic cells and Flt3L producing stroma, via CXCR4, supports the survival of developing cells and once the cell number has exceeded the niche capacity, cells are able to leave the bone marrow to migrate to other peripheral niches (Williams et al. 2017a). Supporting this, flt3L administration led to increased CXCR4 on HSCs which contributed to their ability to interact with stroma. Within the blood, preDCs have a short half-life of less than two hours (Kang Liu et al. 2007) suggesting that replenishment of peripheral tissues and lymphoid organs with preDCs occurs rapidly. Within the tissues, preDCs complete their final developmental stage and become cDC, a process controlled by unknown mechanisms but may involve Flt3L signalling for cDC1 and niche specific cues (Ugur et al. 2023b). Interestingly, pro-DC3s can fully develop into DC3 both in the bone marrow and the periphery (Z. Liu et al. 2023). Despite the basics of cDC development being accepted, there remains many unanswered questions: which signals induce their differentiation into the preDC subsets? Is preDC migration to the peripheral tissues and lymph nodes tissue specific? and which signals within the tissues induce preDC maturation

into cDC? Therefore, we sought to elucidate how preDCs infiltrate peripheral tissues and lymph nodes by understanding their use of chemokine receptors.

1.1.3 Identifying preDCs

Before analysing the mechanisms controlling preDC biology, reliable characterisation of the cells is needed. Fortunately, flow cytometric and transcriptomic analysis have allowed the identification of distinct preDCs surface makers that allow individual preDC subsets to be analysed. The majority of analysis has been carried out on bone marrow precursors, however, preDCs have also been identified in murine blood, lymph nodes, peripheral tissues, and tumours (Cabeza-Cabrerizo, Minutti, et al. 2021a; Diao et al. 2010a; Pereira da Costa et al. 2023a; Rivera et al. 2022; Scott et al. 2015d; Ugur et al. 2023b). In general, preDCs are phenotypically identified by their expression of surface markers: lineage$^-$ (B220, NK1.1, Ter119, SiglecF (in lung samples to negate alveolar macrophages)) CD11c$^+$ MHCII$^-$ CD43$^+$ CD135$^+$ Sirpα$^-$ CX$_3$CR1$^+$ (K. Liu et al. 2009; Schlitzer et al. 2015a). PreDC commitments can be phenotypically identified at the preDC stage: non-committed preDC (SiglecH$^+$ Ly6C$^+$), cDC1 preDC (SiglecH$^-$ Ly6C$^-$) and cDC2 preDC (SiglecH$^-$ Ly6C$^+$) definitions which have been previously defined ((Cabeza-Cabrerizo, Minutti, et al. 2021a;Schlitzer et al. 2015a). However, it is important to note that different researchers use alternative strategies to identify preDC subsets. For example Pre-cDC1s have been identified as Lin$^-$ CD117^{int} CD135$^+$ Zbtb46-GFP$^+$ CD226$^+$ and pre-cDC2s were identified as Lin$^-$ CD117^{lo} CD135$^+$ CD115$^+$ (Durai et al. 2019). . It is now clear that excluding Ly6D$^+$ cells can remove contaminating PCs (Dress et al. 2019a). More recently, CD11b has been removed from the lineage$^-$ gates for preDC identification as intermediate levels of expression were identified on preDCs (Cabeza-Cabrerizo, Minutti, et al. 2021a). It is likely that the future of preDC identification will include Lyz2 and CD34 to include pre-cDC2s which develop from MDPs as well as pro-DC3 (Z. Liu et al. 2023). Similar

to murine preDCs, defined subsets can also be identified in human blood (Breton et al. 2016; Z. Liu et al. 2023; See et al. 2017; Villani et al. 2017).

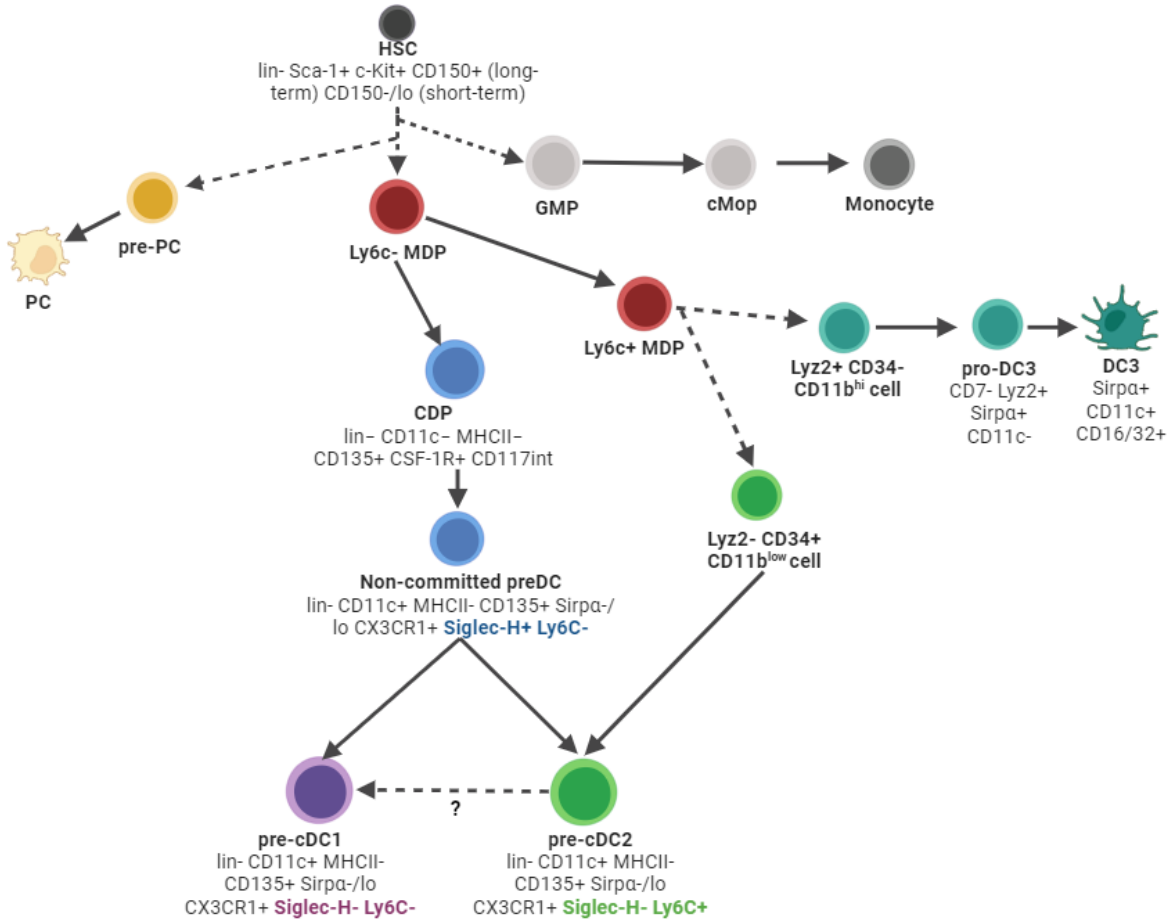


Figure 1-1 - cDC-poesis occurs in the bone marrow. *HSC, haematopoietic stem cell; MDP, monocyte-dendritic progenitors; cMoP, common monocyte progenitors; CDP, common dendritic progenitor; pDC, plasmacytoid dendritic cell; preDC, dendritic cell precursor; LN, lymph node. Following preDC development in the bone marrow, preDC exit into the blood circulation where they then migrate to lymph node and peripheral tissues and differentiate into separate cDC subsets. Solid arrows represent developmental pathways with supporting evidence while dashed arrows represent pathways needing further elucidation. Question mark represents potential theories of preDC development.*

1.1.4 The importance of Flt3L in preDC development

FMS-like tyrosine kinase 3 ligand (Flt3L) is a growth factor essential for cDC development (Cabeza-Cabrerizo, Cardoso, et al. 2021a; Karsunky et al. 2003b; Merad et al. 2013a) while GM-CSF is dispensable for the differentiation of cDC precursors but may promote cDC homeostasis (Greter et al. 2012; Vremec et al. 1997). Mice deficient in Flt3L (McKenna et al. 2000b) or the receptor Flt3 (Waskow et al. 2008b), also known as CD135 (which will be used throughout this thesis), had reduced cDC numbers while administration of Flt3L increased cDC numbers *in vivo* (Karsunky et al. 2003b; Manfra et al. 2003; Maraskovsky et

al. 1997; Tu et al. 2014). Within the bone marrow, Flt3L is produced by bone marrow stromal and immune cells (Gilliland and Griffin 2002) and is involved in the survival and renewal of HSCs (Rasko et al. 1995; Williams et al. 2017a). Interestingly, mice with NK cells unable to produce Flt3L have reduced cDC1s, but not cDC2, within tumours, tumour draining and non-draining lymph nodes (Barry et al. 2018a). Although only tumour and lymph nodes were examined here, it suggests that NK-Flt3L production is vital for cDC1 development. In this study, NK production of Flt3L was not essential for cDC2 development, supporting a differential reliance on Flt3L. Others have also shown that Flt3L preferentially expands pre-cDC1s (Van De Laar, Coffer, and Woltman 2012a; Lin et al. 2021b).

Flt3L receptor CD135 is expressed on many cells in the haematopoietic tree, particularly those involved in DC-poeisis (K. Liu et al. 2009; Merad et al. 2013a; Schlitzer et al. 2015b; Waskow et al. 2008b). Flt3L engagement with CD135 induces a cascade of downstream signalling involving STAT3 and PI3K activation of the mTOR pathway (Laouar et al. 2003; Sathaliyawala et al. 2010; Sichien et al. 2017b). Interestingly, CD135 is maintained on tissue cDC, suggesting a maintenance or functional role for FLt3L throughout the later stages of cDC survival (Karsunky et al. 2003b). In fact, it is likely that a Flt3L feedback loop controls tissue cDC numbers during homeostasis, as suggested in the lymph node where reduction of developing cDC1 within the medulla, as they migrate to the paracortex in a CCR7-dependent manner, increased Flt3L availability and increased cDC1 differentiation from newly arrived pre-cDC1s (Ugur et al. 2023a).

The finding that Flt3L increases cDC numbers has allowed the development of protocols to generate *in vitro* cells which more closely resemble *in vivo* cDC (Grajales-Reyes et al. 2015; Kirkling et al. 2018; Van De Laar, Coffer, and Woltman 2012b) as well as a means to increase cDC numbers *in vivo*. Overall, it seems that flt3L has roles in the generation of cDC precursors but also the complete development of cDCs within the periphery (Ugur et al. 2023b), however, understanding the extent of these roles requires future research.

1.1.5 Dendritic cell sensing, activation, and function

1.1.5.1 Decision making within the immune system

We exist in an environment in which we are constantly being challenged by pathogens, tissue damage and cell intrinsic changes. As such, our immune system has evolved to maintain homeostasis while also attempting to prevent overt immune responses that could induce immunopathology. Therefore, vital decisions must be made on whether a response, and which type of response, must be mounted against a particular threat. Below, a brief explanation of the history and current theories describing the immune system's decision making will be described, however, an in-depth analysis of these theories is outwith the scope of this thesis.

Traditionally, the immune system was perceived to sense "self" from "non-self" subsequently mounting a response to antigen from the "non-self" source based on Burnet and Medawar's work (Pradeu and Vitanza 2012). However, this self-non-self (SNS) model is too simplistic to explain the complex decision making that the immune system makes to mount responses to antigen from "self" sources, such as mutated and cancerous cells and healthy tissue during autoimmunity. Similarly, tolerance to "non-self" antigen, such as that originating from the commensal microbiome, food, and foetal cells, is evidence that the SNS is not sufficient to explain the induction of the immune response. Then, Janeway offered an explanation suggesting that antigen presenting cells (APCs) had their own discriminatory machinery which allowed them to decide whether a response should be mounted towards the source of the antigen or not. This was named the Infectious-nonself (INS) model (Janeway 1992) and describes the ability of the immune system to identify infectious-non-self from non-infectious-self. In this case, pattern recognition receptors (PRRs) expressed by APCs are signalled by pathogen associated molecular patterns (PAMPs) that allow the cell to define the foreign nature of the source of the antigen. However, the INS model remains unable to explain all aspects of decisions made by the immune system, such as responses against non-infectious-self during autoimmunity and tumour development as well as tolerance to infectious-non-self-microbiome and food. The Danger Model (Matzinger 2002) enhanced the INS model and more truly reflects the situation whereby the immune response is mounted not by infection-specific cues but by those that result from self-tissue stress/damage known as "danger". In this case, harmless bacteria, food, and foetal cells can be tolerated while responses can be mounted against self and non-self in the context of danger. Here, Matzinger proposed danger associated molecular

patterns (DAMPs) as the key control signals that induce APC activation. However, this theory also lacks complete clarity on the immune system's decision making. For example, why are responses mounted towards allergens or food which do not exert danger signals or in some cases, rejection of healthy tissues? Matzinger later suggested that allergens are by default immunogenic but whether allergies occur or not is due to person-specific characteristics (Matzinger 2012). Additionally, it could be that bystander antigen presentation in the context of danger can induce these unnecessary responses. Furthermore, definition of what these danger signals are and experimental evidence proving their existence is weak. It has been suggested that the Danger model be renamed the Damage model as cell damage/stress can be experimentally tested compared to the vague and undefined "danger" (Pradeu and Cooper 2012). DAMPs have been suggested to include host molecules released from damaged or stressed cells, such as heat shock proteins (HSPs), however, researchers have suggested that evidence of HSP activating APCs were due to the contamination of PAMPs, specifically bacterial lipopolysaccharide (LPS), and therefore evidence supporting the theory is lacking (Pradeu and Cooper 2012). Additionally, certain HSPs have been suggested to act as resolution associated molecular patterns (RAMPs) which induce tolerogenic responses Shields, Panayi, and Corrigall 2011). On the contrary, the type of cell death has been shown to be able to induce or prevent APC activation. For instance, necrotic cell death and the release of HSPs activated dendritic cells compared to apoptotic cell death (Basu et al. 2000). With this, a new model, the Hidden-self model, was proposed to suggest that intracellular DAMPs that are released during inflammatory cell death are inducers of the immune decision to mount a response (Kono and Rock 2008). The same researchers proposed criteria that a signal must meet to be considered a DAMP, including evidence that LPS or other PAMP contamination is avoided, and inactivation of the DAMP inhibits its ability to induce a response. Although multiple DAMPs have been identified, most have been unable to meet these criteria (Pradeu and Cooper 2012). Despite this, the focus of DAMP research has been on uric acid, High Mobility Group Box 1 (HMGB1) protein and the NLRP3 inflammasome (Pradeu and Cooper 2012). Furthermore, Matzinger's original Danger theory proposed that no immune response is mounted against growing tumours as "tumors are healthy, growing cells that do not normally die necrotically or send out alarm signals". However, she did recognise the possibility for tumours to be "spontaneously rejected" and later reassessed the model (Matzinger 2012). Later, it was shown that the immune system can, and does, respond to tumour (Dunn, Old, and

Schreiber 2004b, 2004a; Hanahan and Weinberg 2011a). Overall, it is clear that multiple aspects of the immune system's decision-making are unknown and an area of debate. A greater understanding will allow us to exploit, and control, immune responses to improve anti-tumour and vaccine responses while inhibit autoimmunity and allergy. As with many processes within the immune system, such as the activation of T-cells, the balance of multiple signals is key and provide protective mechanisms against overt immune responses. Therefore, potentially, induction of the immune response requires multiple activator signals combined, as well as lack of inhibitory signals. In fact, sensing a balance of microbe and damage associated signals was identified in invertebrates (Lazzaro and Rolff 2011). Maybe the presence of DAMPs and PAMPs together, or certain PAMPs alone, can induce responses against non-self-antigen while PAMPs alone with additional tolerogenic signals keep healthy microbiome safe. Additionally, multiple DAMPs together may be able to induce a break in tolerance which allows responses to self-antigen and harmless allergens and food. In this case, the context in which the antigen is presented in is what defines the response. Furthermore, it is likely that both genetic and environmental factors play a vital role in the heterogeneous decision making that we see from person to person. For example, why do some people develop allergies and autoimmunity while others don't?

In any case, it is clear that a significant step in the decision-making process of the immune system lies within the activation of key sentinels within tissues; cDCs.

1.1.5.2 Dendritic cell activation

cDC activation is vital to the orchestration of the adaptive immune response towards the source of antigen. In fact, presentation of antigen by immature, non-activated cDCs to T-cells results in T-cell anergy (Mescher et al. 2006a); a state of tolerance towards the antigen in non-inflamed contexts and protects against immunopathology. Immaturity in cDCs is defined by their lack of activation markers, also known as costimulatory makers; CD80, CD86, and CD40 (Cabeza-Cabrerizo, Cardoso, et al. 2021a). It is the lack of costimulation provided by immature cDCs which prevents the full activation of T-cells (Mescher et al. 2006b). Despite this, immature cDCs are continually surveying tissues, processing and presenting antigen and homeostatically migrating to the draining lymph nodes. This feature of immature cDCs is vital to the maintenance of tolerance by presenting antigen in the context of the homeostatic tissue environment (Scheinecker et al. 2002). However, to obtain the full activation of T-cells, cDCs must provide costimulation. As discussed in the previous section, PRRs expressed on and within, cDCs allow them to sense, and become

activated in response to, contextual cues. These cues have been termed PAMPs and DAMPs/RAMPs. In mice and humans, four main groups of PRRs have been identified: Toll-like-receptors (TLRs); RIG-I–like receptor (RLRs); Nod-like receptor (NLRs); and C-type lectin receptor (CLRs) (Kumagai and Akira 2010). cDC subsets have distinct levels of PRR expression allowing for their differing capabilities in sensing environmental cues (Cabeza-Cabrerizo, Cardoso, et al. 2021b; Cheng et al. 2023). cDC1 express: TLR3,4,9,11 and 13; and CLEC12A and 9A. While cDC2s have a larger range of PRR expression, potentially due to the heterogeneity of the group: TLR2, 4, 5 and 7; RLRs; NLRs; and CLEC4A, 6A and 7A. Additionally, the location of PRRs in, and on, cDCs allows for optimal sensing, for instance, TLR4, which binds LPS, is expressed on the surface of cDC2s while TLR7 and TLR9 is expressed in endosomes of cDC1 and cDC2, respectively, and allows TLR7 to sense internal ssRNA and TLR9 to sense CpG-DNA (Cheng et al. 2023; Kumagai and Akira 2010). Therefore, cues which are external (e.g. bacterial cell wall components such as LPS) or internal (e.g. nucleic acids from viruses of bacteria) to sampling cDCs can be identified. Furthermore, cDCs are positioned at strategic sites to survey tissues, sensing environmental changes and responding to contextual cues: mDC survey the peripheral tissues where cells are exposed to environmental factors or cell intrinsic changes/damage; rDC remain resident in the lymph node (Ugur et al. 2023c) where drainage of peripheral tissues enters the node; and splenic cDCs are located ideally for sensing the circulating blood (Merad et al. 2013a). Tissues have a constant replenishment of new cDCs, from precursor infiltration, and the lifespan of cDCs is short lived at around 7-10 days (Patel, Ginhoux, and Yona 2021). Here, cDCs survey the environment, sampling cell debris and responding to contextual signals. When PRRs sense P/D/RAMPS, a cascade of signalling within the cDC induces the cell to take on a mature state. Although there is controversy regarding the properties of RAMPs and MAMPs and their ability to activate cDCs, the PAMP LPS is well-defined and provides a model for cDC activation. Briefly, LPS is recognised by TLR4 which induces downstream signalling via Myd88 and ultimately the activation of the transcription factor NF-κB (Cheng et al. 2023). This leads to adaptations in the immature cDC which induces their maturation, defined phenotypically by increased MHC:antigen expression and upregulation of costimulatory molecules CD80, CD86, and CD40 (Cabeza-Cabrerizo, Cardoso, et al. 2021a) and at this stage, cDCs are classed as being at full maturation. Activated cDCs halt their sampling of the tissue environment (L. J. Young et al. 2007), upregulate the chemokine receptor CCR7 which allows their enhanced migration to the tissue draining lymph node (Förster, Davalos-

Misslitz, and Rot 2008; Ohl et al. 2004; Roberts et al. 2016). Here, mDCs have a vital role in transferring antigen and contextual information to rDC and inducing the activation of antigen-specific T-cells. The activation of peripherally located cDC is better defined compared to lymph node rDC. How distally located rDC became activated in a specific manner has been underappreciated and understudied, due to a lack of experimental models to unpick the role of rDC. However, it was previously considered that drainage of inflammatory cytokines, such as IFNγ, or $CD4^+$ T-cell help can induce rDC maturation. In this case antigen presenting rDCs were thought to be "licenced" by $CD4^+$ T-cells via CD40:CD40L interactions on antigen-specific $CD4^+$ T-cells (Laidlaw, Craft, and Kaech 2016). These assumptions describe a scenario whereby rDC activation is somewhat uncontrolled by tissue-specific cues which could lead to miscommunication between the site of inflammation and the draining lymph node and subsequently result in the improper activation of rDC, and thus the immune response. Instead, our group recently identified that rDC activation occurred independently of these and instead following the direct transfer of antigen along with contextual information, in the form of PRR ligands, from mDC (Pirillo et al. 2023a). Thus, activation of cDCs local to the peripheral inflammation and rDC located distally are specifically activated in a context-dependent manner. Subsequently, mDC and rDC initiate the activation of antigen-specific T-cells. In the absence of activation signals, homeostatic maturation of cDCs can still occur. This state is associated with a lack of activation genes but the ability for the cells to migrate to the draining lymph nodes. In this case, mDCs continue to transfer antigen in a context-dependent manner; the context being homeostasis. This is vital to the maintenance of tolerance to antigen presented in a non-inflammatory context (Probst et al. 2003; Spörri and Reis e Sousa 2005).

1.1.5.3 Dendritic cell functions and ability to activate T-cells

The induction of an adaptive immune response is reliant on an efficient interaction between cDCs and T-cells, key players of the adaptive immune response. T-cells have specialised T-cell receptors (TCRs) which develop at random allowing a repertoire of T-cells expressing TCRs with varying specificities (Baker et al. 2017). This repertoire increases the chance that T-cells will recognise most antigen the host comes into contact with. These naïve T-cells are located within the T-cell zone of lymph nodes surrounded by a network of fibroblastic reticular cells (FRCs) which provide the framework for cDCs within the lymph node to migrate and interact with naïve T-cells (Acton et al. 2014). Here, cDCs may also induce the migration of T-cells by generating a gradient of CCL19 which T-cells respond to (Alanko et

al. 2023). Thus, cDCs may directly contribute to induction of their interaction with naïve T-cells within the lymph node. This, along with the structural organisation of the lymph node, increase the likelihood that antigen-presenting cDCs will interact with rare antigen specific-T-cells. When antigen-specific T-cells and antigen-presenting cDCs interact, the response of the T-cell is induced. However, antigen recognition by a T-cell is not sufficient to induce full T-cell activation. For a T-cell to become fully activated, and an effective adaptive immune response be initiated, the T-cell must receive three stimulatory signals (Banchereau and Steinman 1998; Mescher et al. 2006a): firstly, the TCR must recognise the antigen displayed by the cDC; secondly, cDCs must provide costimulatory signals (e.g. CD80/86 on cDCs binds to CD28 on T-cells); and finally additional signals mould the type of T-cell response. The current dogma suggests that these final signals are in the form of cytokines provided from the local environment or the cDCs themselves (Mogensen 2009a). However, this may be too simplistic to explain the specific responses induced by T-cells (reviewed elsewhere (J. Zhu, Yamane, and Paul 2010)). Potentially, contextual information from the site of inflammation is also transferred from mDC to induce context-specific T-cell activation. In any case, maintenance of a long and stable interaction is important for the induction of activated T-cells (Pittet et al. 2023). On the other hand, in the absence of other inflammatory factors, immature cDCs express low levels of costimulatory molecules which can induce the anergy of antigen-specific T-cells (Mogensen 2009b). This is associated with short and unstable interactions measured between cDCs and T-cells (Pittet et al. 2023). This feature of cDCs is a vital component of maintaining tolerance (Probst et al. 2003; Spörri and Reis e Sousa 2005). Importantly, immature cDC can spontaneously activate leading to a continuous stream migrating to the draining lymph nodes (J. Liu et al. 2021). This homeostatic migration contributes to the maintenance of tolerance to antigen that coincides without cDC maturation (Probst et al. 2003; Spörri and Reis e Sousa 2005). In mice with epidermal cells fluorescently labelled with ZsGreen, mDCs in the draining lymph node were ZsGreen$^+$ but not rDC (Ruhland et al. 2020a). This suggests that antigen presentation by mDCs is contextually controlled as in homeostasis epidermal-bound antigen was not passed to rDC and, potentially, tolerance is maintained by the lack of rDC activation.

Within this area of research, the current dogma characterises cDC1s as being specialised in the polarisation of CD4$^+$ T-cells to the Th1 subset upon activation and antigen cross-presentation to activate CD8$^+$ T-cells (Merad et al. 2013a). On cDC1s, expression of CD36 allows them to recognise and bind dead cells while Clec9A allows them to sense necrotic

bodies (J. G. Zhang et al. 2012); making them ideal cells to cross-present endogenous antigen. Cross-presentation describes the mechanism used by cDCs to present internally sourced antigens (e.g. virus or mutations in stressed/cancerous cells), that they have retrieved and processed from external sources, on their own MHC class I (Nierkens et al. 2013), a process reliant on the gene Wdfy4 (Theisen et al. 2018) and Perforin-2 (Rodríguez-Silvestre et al. 2023). This allows $CD8^+$ T-cells to become activated in response to MHCI-restricted antigen such as those from viral infections or cancerous cells. cDC2s are known for their role in presenting antigen on MHC class II and activating $CD4^+$ T-cells, including regulatory T-cells (T-regs).

Despite the dogma, it is becoming clear that these roles for each subset are not as strictly defined as once thought. It is likely that there exists both complementary, and compensatory functions, by all cDC subsets (Cabeza-Cabrerizo, Cardoso, et al. 2021b). There is evidence of both cDC1 and cDC2 populations activating $CD8^+$ T-cells (Roberts et al. 2019). Additionally, cDC2s have been shown to carry out "cDC1 specific functions" such as upregulation of IRF8 and IL-12 production (Cedric Bosteels et al. 2020) and activating $CD8^+$ T-cells (Ruhland et al. 2020). Both subsets have been shown to be essential for $CD4^+$ and $CD8^+$ T-cell activation (Eickhoff et al. 2015; Ferris et al. 2020a). Another area of confusion is the roles of mDC and rDC within the cDC1 and cDC2 subsets. Previously, it was identified that mDC migration to the draining lymph node allowed them to transfer antigen (Broz et al. 2014; Gurevich et al. 2017; Roberts et al. 2016). However, evidence of how rDCs were activated context-dependently was lacking until our group identified that mDCs were able to transfer not only antigen but also contextual information, in the form of PRR ligands (Pirillo et al. 2023b), to rDCs in draining lymph nodes. Importantly, this work has elucidated a vital process which ensures rDCs, located distant to the site of inflammation, can be activated in a context-dependent manner allowing them to respond appropriately to the source of antigen. Whether the main role of mDC is only to transfer information to rDC, which have the responsibility of activating antigen specific T-cells, is unknown. However, it has been suggested that mDC1s are the main activators of $CD8^+$ T-cells (Roberts et al. 2016; Salmon et al. 2016a) but both antigen presenting mDC and rDCs have been shown to have potential to activate T-cells *in vitro* (Pirillo et al. 2023b). To understand if mDC and rDC have specific roles, it is important to develop experimental models to test this. Preventing mDCs from migrating to draining lymph nodes can be achieved by blocking CCR7 mediated migration (Roberts et al. 2016). However, in this case, antigen and contextual information

are not transferred and thus rDCs cannot be activated and the importance of rDCs cannot be investigated. Understanding the signals that induce the infiltration of rDCs to lymph nodes may allow the development of techniques to inhibit rDC infiltration and understand their importance.

The conflicting evidence of the roles of migratory and resident cDCs, as well as between the cDC1 and cDC2 subsets, in T-cell activation may be explained by the form and dosage of the antigen examined as well as the spatial organisation of cDCs (Cabeza-Cabrerizo, Cardoso, et al. 2021b; Pittet et al. 2023; Snapper 2018). Antigen must be delivered to the lymph node for T-cell activation to occur, however, how this delivery occurs can vary depending on the source and location of the antigen (Cabeza-Cabrerizo, Cardoso, et al. 2021b). In brief, small molecules, such as certain viruses, can pass through conduit filters and enter the lymph node; lymph borne particles can be sampled directly by rDCs without the requirement of mDC transport; and antigen that remains localised in the periphery can be transported by mDC to the lymph node. Thus, coordination of the immune response may also be dictated by the nature of the antigen being presented. Supporting this, in one study free antigen draining to the lymph node was acquired by rDC2s in the lymphatic sinus while rDC1 in the paracortex required a much higher dose of free antigen to induce their antigen presentation and efficient $CD8^+$ T-cell priming (Gerner et al. 2017). Similarly, cDC1s were suggested to be irrelevant for the activation of $CD4^+$ T-cells to soluble antigen (Binnewies et al. 2019a) while $CD4^+$ T-cell activation to membrane-bound antigen was impacted in cDC1-deficient mice due to reduced antigen transport and antigen presentation (Ferris et al. 2020b). Overall, the nature of the antigen is likely to also influence the orchestration of which roles cDCs play in T-cell activation.

In any case, within the inflamed tissue, where activated T-cells infiltrate, cDCs which do not migrate to the lymph node, but remain in the tissue (Lee et al. 2023), have a vital role in: recruiting T-cells, via CXCL9 and CXCL10 expression (Pfirschke et al. 2017a; Spranger et al. 2017a); restimulating and maintaining the functionality of infiltrating T-cells (Ruffell et al. 2014); and providing final activation signals (Dähling et al. 2022a). For instance, it was seen in a model of chronic lymphocytic choriomeningitis virus (LCMV) infection that T-cells located within niches of cDC1 were protected from exhaustion (Dähling et al. 2022a) potentially due to continued CD28 signalling (Humblin et al. 2023). Overall, despite the specific roles of cDCs needing to be exposed, it is clear that cDCs are vital conductors of the

immune system with their roles in connecting the site of inflammation to the immune hubs of the draining lymph node.

1.2 Dendritic cells in Cancer

1.2.1 The tumour microenvironment (TME)

The immune system plays a complex role in tumour development: anti-tumour immune responses are key to destroying cancerous cells, yet this very response can also contribute to the development of an established tumour. Cancerous cells arise when the accumulation of mutations allow cells to overcome the regulation of controlled cellular processes, particularly those involved in proliferation and survival (Hanahan and Weinberg 2011b). The immune system can recognise these changes in host cells and, in most cases, mount an immune response against the malignant cells, preventing the growth of a tumour; a theory coined immunosurveillance. Therefore, in order to grow and survive, cancer cells also need to evolve the ability to avoid immune destruction. This means that by the time a tumour is of clinical relevance the cancer cells have developed this ability. The escape of tumours from the immune system occurs when cancer cells acquire multiple mutations which can exploit mechanisms that avoid immune destruction and additionally through a process known as cancer immunoediting. Cancer immunoediting describes a theory whereby the immune system moulds tumour development and results in the outgrowth of cancerous cells with the ability to avoid immune destruction. The process involves three phases (the 3E's of cancer immunoediting) and describes a Darwinian process driven by random mutations and the subsequent selection of cells with a survival advantage (Dunn, Old, and Schreiber 2004c; Mittal et al. 2014). Elimination involves immune cells detecting and clearing cancerous cells. During this phase, random mutations in the cancer cells can allow the survival of cells which are able to avoid this immune elimination. This leads to the growing pre-tumour entering the equilibrium phase in which certain malignant cells are being recognised and destroyed by the immune system whereas others avoid destruction and outgrow. Survival of cancerous cells during the equilibrium phase allows them to enter the escape phase which consists of heterogenous and immuno-edited cancerous cells in the growing tumour. Here, the outgrowing cells can exploit multiple mechanisms to evade destruction by suppressing or controlling immune processes.

There are multiple mechanisms known to be exploited by tumours to avoid immune destruction (Drake, Jaffee, and Pardoll 2006). In particular, inhibition of the activation

and/or function of anti-tumour $CD8^+$ T-cells can protect tumour cells against destruction (Anderson, Stromnes, and Greenberg 2017). $CD8^+$ T-cells are key players in the elimination of cancerous cells due to their role in specific killing (Hadrup, Donia, and Thor Straten 2013) and they have been one of the main focus areas of research on the anti-tumour immune response. Their importance has been shown in multiple studies which highlight the positive prognostic factor with $CD8^+$ T-cell abundance in the tumour microenvironment (TME) and patient survival and response to treatments (Hiraoka et al. 2006; Mahmoud et al. 2011). This knowledge has allowed the advent of interventions to improve the anti-tumour immune response with T-cell based immunotherapies (discussed in section 1.4).

The Cancer Immunity Cycle provides a model to explain the steps required for tumour specific T-cells to become activated and exude their cytotoxic functions (Chen and Mellman 2013). It is a model which describes the ideal setting in which T-cells are activated in the context of inflammation and not tolerance. Briefly, tumour cell death releases tumour associated antigens (TAAs), several of which have been identified (Haen et al. 2020) and are most often molecules not expressed by healthy cells rather than mutated proteins themselves (Iheagwara et al. 2014). These include molecules that are: expressed unusually high; expressed within tissues where or when they shouldn't normally; or post-translationally modified (Iheagwara et al. 2014). Interestingly, many of these antigens are not specific to tumours as they have been identified during infection to induce protective immunity and therefore, this may be one explanation for why reduced risk of cancer can be seen in people with history of multiple infections (Hoption Cann, Van Netten, and Van Netten 2006; Iheagwara et al. 2014;Albonico, Bräker, and Hüsler 1998; Hoption Cann, Van Netten, and Van Netten 2006) Additionally, overlap of antigens, such as HSP90 and Annexin2 (Iheagwara et al. 2014), expressed by tumour cells and in previous infections may explain why early tumour development elicits immune responses. This was shown in mice who exhibited increased tumour control following infection with influenza and similar antigens were found in the tumour lysate and infected lung compared to healthy lung tissue (Iheagwara et al. 2014). Furthermore, the release of DAMPs from cancer cells is described in the Cancer Immunity Cycle as a key stage in induction of an inflammatory response. While many DAMPs have been identified to be released from tumour cells, the ability of DAMPs to induce APC activation is an area of confusion, as previously described. Based on the Danger model (discussed in section 1.1.5.1), these should induce the activation of APCs, and hence promote the anti-tumour immune response. While some have been suggested

to induce anti-tumour responses, others have been shown to promote tumour growth and survival (Hernandez, Huebener, and Schwabe 2016). Therefore, the role of DAMPs in tumour immunology, and in immune system processes in general, are unclear. In any case,within the TME, cDC play a vital role in surveying the environment, processing TAAs and contextual cues which can lead to the activation of cDCs (as discussed in detail in section 7.1.5). As such, these activated cDCs migrate to the tumour draining lymph node (tdLN) in a CCR7 dependent manner (Roberts et al. 2016) where they enter the T-cell zone, interacting with naïve T-cells via T-cell receptor (TCR) and pMHC:antigen contact. Upon recognition of antigen specific T-cells, cDCs provide costimulatory and cytokine signals to the T-cells. Activated $CD8^+$ T-cells traffic to, and infiltrate the TME, and, here, tumour cDC niches provide T-cells with signals to gain and maintain their full effector function (Prokhnevska et al. 2023). $CD8^+$ induced cell death is antigen-specific and involves the release of cytotoxic molecules such as granzyme B and IFNγ (Cassioli and Baldari 2022). Tumour cell death continues the cycle, releasing new TAAs and DAMPs inducing priming of *de novo* anti-tumour T-cells (A. L. Young et al. 2023). However, this paradigm explains the ideal situation leading to tumour rejection which, by the time a tumour has reached clinical relevance, this has been avoided. A key mechanism that tumours use to avoid tumour rejection is by inhibiting the Cancer Immunity Cycle, for example, preventing the vital role of cDCs.

1.2.2 The role of tumoural dendritic cells

cDCs are a rare population in most tumours (Broz et al. 2014), a feature often associated with tumour immune microenvironments (TIMEs) (Binnewies et al. 2018) classed as “cold” or “immune-excluded”. Identifying mechanisms to convert these tumours into “hot” or “immune-infiltrated” environments by promoting cDC accumulation poses as a promising target for immunotherapy due to their role in activating and restimulating anti-tumour T-cells in the tdLN then in the TME, respectively. It is now understood that TMEs rich in gene signatures associated with cDC1s show a correlation with increased survival (Böttcher and Reis e Sousa 2018; Broz et al. 2014; Y. Kim, Shin, and Kang 2019; Salmon et al. 2016b) and increased response to immunotherapy (Barry et al. 2018b; Salmon et al. 2016a; Sánchez-Paulete et al. 2016; Spranger et al. 2017b). This is most likely due to the role of cDCs in inducing anti-tumour $CD8^+$ T-cell activation (Roberts et al. 2016) and maintaining T-cell function in the TME (Broz et al. 2014; Dähling et al. 2022b). In mice, cDC1s were shown to be vital to the rejection of tumours (Hildner et al. 2008b). Other than induction of the

adaptive immune system, cDC1s have various important roles within the TME such as promoting T-cell infiltration, via CXCL9 and CXCL10 expression, (Pfirschke et al. 2017b; Spranger et al. 2017b) and restimulating and maintaining the functionality of TME infiltrating T-cells and preventing exhaustion (Dähling et al. 2022b; Ruffell et al. 2014). Hence, promoting cDC1 accumulation within the TME should not only promote T-cell activation to *de novo* TAAs but also promote the maintenance of T-cells to current tumour specific T-cells. Much less is known about the role of cDC2s in cancer. This is due to the increased heterogeneity of cDC2s compared to cDC1s. It is known that mDC2 can activate $CD4^+$ anti-tumour T-cells (Binnewies et al. 2019c) and, although it has been suggested that cDC1s are the only efficient antigen transporters (Salmon et al. 2016a), we have shown that both cDC1 and cDC2 can transport TAAs to tdLNs (Pirillo et al. 2023b). Their role in activation of $CD4^+$ T-cells (Merad et al. 2013a) may play an important role in shaping the cytokine-producing T-helper cell population of the TME. Additionally, patients with tumours rich in cDC2s only had an increased progression free survival if high cDC2 accumulation was accompanied with low regulatory Treg numbers (Binnewies et al. 2019c). Therefore, cDCs are rare within TMEs and those present are often unable to inhibit the growth of the tumour. In fact, activation of cDCs, and hence T-cell priming, is less evident in tumour associated cDCs compared to those in influenza infection (Pirillo et al. 2023a). This may be due to tumour intrinsic factors which prevent the anti-tumour functions of cDCs while promoting a regulatory phenotype of cDCs; such as the homeostatic maturation of cDCs.

1.2.3 Tumour inhibition of dendritic cells

Tumours are notorious for mutating and evolving to escape immune destruction. It is therefore fair to assume that tumours can develop means to inhibit cDC function due to the importance of cDCs in the initiating and maintaining anti-tumour immune responses (Pittet et al. 2023). Supporting this, Maier and colleagues described the contextual state of TME infiltrated cDCs as mregDC1 and mregDC2s (Maier et al. 2020), which are likely cDCs which have undergone homeostatic maturation and not inflammatory activation. As with these classically activated cDCs, mregDCs expressed costimulatory markers CD80, CD86 and CD40 highlighting their maturity and potential to activate T-cells. However, mregDCs also expressed a variety of genes associated with immunoregulation and were identified in draining lymph nodes, a presence reliant on CCR7 as with classically activated cDCs.Compared to naïve lung, tumours were more abundant in mregDCs suggesting that this state of cDCs is context dependent. Subsequently, mregDCs have been identified in

multiple human tumours and murine tumour models and have been identified as lysosomal-associated membrane protein 3 (LAMP3)$^+$ CCR7$^+$ PD-L1$^+$ (Li et al. 2023). Within the tdLN, mregDCs (Movassagh et al. 2004) are enriched; suggesting their involvement in the activation of naive anti-tumour T-cells. Activation from cDCs in this state may induce T-cell anergy or Treg differentiation. Interestingly, mregDCs have been identified in non-tumour sites and as having the function of maintaining tolerance (Li et al. 2023). This highlights the context specific induction of this regulatory activation state of cDCs which TMEs may exploit to protect from immune destruction. It is unknown which factors within the TME bias cDC activation towards this state and whether presence of additional activation signals are able to convert this state to a more classical activation. It is important to note that presence of mregDCs has been associated with positive prognosis (Li et al. 2023). This can potentially be accounted for by the dual anti- and pro-tumour functions that mregDCs may exert or their presence coinciding with the presence of cDCs activated to a less regulatory state. To understand this, it is important to study if mregDC states exist during non-cancer immune challenges to confirm if they are a feature of inflammation in general or a state specific to cancer and potentially steady-state tissues. As such, it is likely that the term “mregDCs” describes the homeostatic maturation state of cDC which is identified in the absence of inflammatory signals. Therefore, the term can be misleading as it suggests that these cells are a subset specific to inducing tolerance, and potentially specific to the context of a tumour challenge. As discussed earlier, a consistent approach to the nomenclature of cDC states and subsets is required to avoid confusion in the field.

Nonetheless, the TME can inhibit the general anti-tumour function of cDCs. Functions associated with activating anti-tumour T-cells such as cDC phagocytosis (Caronni et al., 2021) or cross presentation (Cao et al., 2014; Giampazolias et al., 2021), and cDC survival in general (Min et al. 2013), can be inhibited by factors within the TME such as immunomodulatory molecules (Pittet et al. 2023) and Tregs (Binnewies et al. 2019c). Importantly, and the focus of this thesis, cDCs can be excluded from infiltrating the TME, for example, Meyer and colleagues identified that tumours can systemically reduce cDC numbers and specifically interrupt cDC1 precursor development by production of the growth factor G-CSF which can induce a bias of bone marrow myelopoiesis to granulocytes while inhibiting the cDC1 essential TF, IRF8 (Meyer et al. 2018). Interestingly, this highlights the ability of tumours to distally influence myelopoiesis by the release of growth factors. Similarly, tumour-derived IL-6 interfered with CDP development through enhancing Zeb2, a

suppressor of *Irf8*, thus preventingan essential step in pre-cDC1 differentiation (S. Kim et al. 2023). In another study, it was shown that there was reduced infiltration of cDCs in the TME via tumour inhibition of CCL4 (Spranger, Bao, and Gajewski 2015a). Similarly, tumour production of Prostaglandin E2 impacted NK cell viability and chemokine production which in turn reduced cDC1 recruitment to the TME in mouse models of melanoma, colorectal and breast cancer (Böttcher et al. 2018a). Importantly, within the tumour, NK cells are the predominant producers of cDC growth factor Flt3L (Barry et al. 2018b), therefore, inhibition of NK cells can impact the local production of this vital cDC factor. Additionally, tumour production of vascular endothelial growth factor (VEGF) can interfere with the role of FLt3L in cDC development (Shurin et al. 1997). Thus, multiple mechanisms can be exploited by tumours to inhibit cDC efficiency. With this, it is important to recognise that each tumour and patient is heterogeneous and different mechanisms of inhibition may be at play in individual tumours and in different niches within tumours.

Furthermore, when analysing cDC status in models of cancer, it is important to compare to cDC in more robust immune challenges and regulatory contexts to confirm if the status is a feature of inflammation/tolerance in general or is cancer specific. Misinterpreting a particular feature as cancer-specific may skew our understanding of the normal processes that the immune system undergoes in an inflammatory or regulatory setting. When comparing cDC activation during influenza infection and lung metastasis of a melanoma model, we previously identified that in antigen presenting cDCs, both lung and draining LN cDCs express significantly less costimulatory molecules during tumour challenge (Pirillo et al. 2023b). These costimulatory molecules were significantly increased when mice were treated with Poly (I:C) (a synthetic, double-stranded RNA TLR3 agonist) suggesting that changing the context of the TME with immunogenic molecules can change the activation status of cDC to match the more robust cDCs seen in influenza infections. In this study, mDC transported antigen and contextual information which was vital for the activation of distal rDC. Thus, lack of information transfer to rDC could prevent robust immune activation in tumour settings. As in the case of a model of homeostasis/tolerance, epithelial antigen, and potentially activation signals (Pirillo et al. 2023a), were not transferred to rDC from mDC. Based on this, it is probable that tumours may exploit inefficient mDC transport and, thus, rDC inactivation to maintain tolerance against tumour antigen (Ruhland et al. 2020b). Supporting this hypothesis, a recent study identified that in the tdLN, using cell-to-cell contact analysis, T-cells interacted with mDC, and not rDC, (Chudnovskiy et al. 2022),

suggesting that lack of rDC involvement in a tumour setting may prevent a robust response. To confirm this, comparison of cell-to-cell contact in the draining lymph node during tolerance, cancer and infection is needed.

Overall, there are multiple approaches that are available to promote the anti-tumour functions of cDCs by targeting the pathways of inhibition; one of which being to increase preDC recruitment to the TME. Therefore, we set out to understand the signals involved in the infiltration of cDCs to the TME.

1.3 Dendritic cell recruitment

1.3.1 A brief introduction to chemotaxis

The locomotion of immune cells is fundamental to immune and developmental processes during both homeostasis and inflammation. Chemotaxis is an umbrella term to define the movement of cells in response to a chemical signal (Rumianek and Greaves 2020a). Briefly, chemokinesis refers to the non-directional, random movement of cells; a process seen during cell scanning of tissue microenvironments. Unlike the soluble signals seen in many chemotaxis models, haptotaxis involves the movement of cells to signals which are surface bound. Necrotaxis describes chemotaxis signals that are released from dead or dying cells, such as HMGB1 (Yang et al. 2013). The major inducers of chemotaxis are chemokines. Chemokines are a family of secreted or surface-bound cytokines which can induce and/or direct the chemotaxis of responding cells. In terms of chemokine denotations, CC, CXC, CX_3C and XC describe the subfamilies that are separated by the location of the first two conserved cysteine residues in their protein sequence. ‘L’ after the subfamily name describes the chemokine “ligand” while ‘R’ describes receptors. Lastly, the number is used to list when the gene was first identified. Chemokine receptors are seven transmembrane-spanning G protein-coupled receptors (GPCRs) which can be further subdivided into conventional chemokine receptors (cCKRs) and atypical chemokine receptors (ACKRs). While ACKRs structurally resemble cCKRs, they cannot induce cell migration directly. ACKRs scavenge and degrade chemokines to promote or inhibit cCKR-directed migration. An in-depth review of chemokine and chemokine receptor biology can be found elsewhere (Hughes and Nibbs 2018; Nagarsheth, Wicha, and Zou 2017a; Nibbs and Graham 2013). Chemokines and their receptors have been traditionally classed as either ‘homeostatic’ or ‘inflammatory’ depending on the context in which they induce chemotaxis. However, it is likely that this is an oversimplification where the different classes are involved in either context (Nibbs and Graham 2013). Previous work investigating the functions of individual chemokines have

been complicated by three characteristics of chemokines: promiscuity, redundancy and pleiotropism. Redundancy refers to the view that different chemokines perform similar functions; promiscuous receptors respond to multiple chemokines; and pleiotropism referring to one chemokine inducing multiple distinct outcomes. However, it is becoming clear that this is an oversimplification and the situation is much more specific than the 'promiscuity, redundancy and pleiotropism' model suggests. Recent evidence has suggested that each chemokine and receptor pair have specific roles in the chemotaxis of immune cells, and this may include tissue and context specificity (Dyer 2020; Proudfoot, Bonvin, and Power 2015; Schall and Proudfoot 2011). Despite this, our understanding of the specific pathways controlling chemotaxis are still vague. Thus, although the system may not be promiscuous, redundant, or pleiotropic, our understanding of it is. Therefore, the current understanding of the chemokine system requires more in-depth analysis of immune cells and tissues during homeostasis and inflammation to define the specific pathways. Another oversimplification regarding chemotaxis includes the dynamics involved in cell sensing and response. The current dogma of chemokine induced cell migration involves a source cell which secretes the chemokine; a sink cell that internalizes the protein creating a gradient of signal; and a target cell that migrates towards the source by following this gradient. This model describes an imposed gradient of chemokine signal which is highest at the source cell and lowest at the sink cell location whereby target cells can sense, but not influence, the concentration of chemokine gradient. However, this process relies on target cells, potentially a large distance from the source cells, being able to follow these weak signals and migrate in a directed way through the complex networks of blood vessels and lymphatics time efficiently. The model depends on these gradients extending over large distances in the body and the target cells being able to sense very low concentrations of the chemokine and follow a shallow gradient for a long distance. However, a recent publication has mathematically modelled such chemokine gradients and predicted that not only are weak signals of chemokine not enough to induce cell migration, but cells require very steep gradients to migrate (Dowdell et al., 2023). Therefore, the imposed gradient model fails to explain the directionality of leukocyte chemotaxis that allows the immune response to function specifically and rapidly. A recent study identified that cDCs expressing CCR7, sense CCL19 signals as well as internalizing the protein, thus creating a self-generated gradient. A self-generated gradient model describes target cells sensing uniform chemokine signals and subsequently breaking down these signals creating a steep gradient where the

low concentration exists where the cell is located, and the high concentration exists in front of the cell; inducing their migration forward (Alanko et al. 2023). Hence, chemotaxis is a complex system in which the specificity of the processes needs to be elucidated.

1.3.2 preDC chemotaxis from the bone marrow to the periphery

Migration is key to the majority of cDC functions (Figure 1.2) (Alvarez, Vollmann, and von Andrian 2008), the most well-known being the CCR7:CCL19/21 mediated migration of mDCs to the lymph node. Less well studied, but particularly important is the initial seeding of cDCs in tissues. Following development, preDCs exit the bone marrow and migrate via the blood where they remain in a steady state until they reach the peripheral tissues and lymph nodes and differentiate into cDCs (K. Liu et al. 2009). Within tissues, cDCs are replaced with preDCs every 10-14 days, as shown in parabiotic experiments (Ginhoux et al. 2009). Currently there are some indications of how preDC migration to the peripheral tissues and lymph nodes is controlled. In terms of chemotactic signals controlling preDC migration, Daio and colleagues have seen that CCL3 induced preDC migration *in vitro* (Diao et al. 2006). PreDCs in general have been shown to express CXCR4, CCR2 and CX_3CR1 (Nakano et al. 2016a). Later, subset specific expression of CXCR3 and CCR5 was identified on pre-cDC1s while CX_3CR1 was expressed by pre-cDC2s (Cook et al. 2018a). XCR1 has also been reported to be expressed on pre-cDC1s and not pre-cDC2s (Lança et al. 2022) and high CCR2 was associated with pre-cDC1 (Schlitzer et al. 2015b). In homeostasis, loss of CXCR4 and gain of CCR2 was shown to be required for bone marrow egress of differentiated preDCs (Pereira da Costa et al. 2023b). Furthermore, the upregulation of CCR2 to egress from the bone marrow was more readily acquired when influenza infection was present distally. Interestingly, CXCR3 KO mice had reduced cDC1 in melanoma tissue and the tdLN but not in non-inflamed skin or spleen while cDC2 numbers remained unchanged in both settings (Cook et al. 2018b). This suggests that preDC migration may be context specific. Supporting this context specific migration, CCR2 was shown to be unnecessary for recruitment to the homeostatic lung but required during inflammation (Cabeza-Cabrerizo, Minutti, et al. 2021a; Pereira da Costa et al. 2023a). Interestingly, CCL2 was mostly produced by lung epithelial cells in homeostasis compared to monocytic production of CCL2 in influenza infected areas if the lung (Cabeza-Cabrerizo, Minutti, et al. 2021b). This suggests that the source of the chemokine may also influence the chemotaxis of preDCs. In contrast, CCR2 was found to be important in seeding lung tissue during homeostasis with both cDC1 and cDC2 whereas knocking out CX_3CR1 in $CD11c^+$ cells only reduced cDC2 in lung tissue (Nakano et al. 2017). Additionally, modelling

lung inflammation with inhaled LPS significantly increased the expression of CCR2 ligands (CCL2 and CCL7) in whole lung tissue and recombinant CCL2 was shown to induce pre-DC migration *in vitro.* Furthermore, it was shown in CCR2 KO mice and mixed bone marrow chimeras that CCR2 was important for the infiltration of $CD103^{-}CD11b^{+}$ cDC2s in the small intestine and the mesenteric draining lymph node (MLN/mesLN) (Scott et al. 2015a). In a model of lung allergic inflammation, eosinophils were shown to produce chemokines which recruited $CCR1^{+}$ cDC2s (Rose et al. 2010) and blocking CCR1 reduced cDC1s in ischemic brain lesions (Gallizioli et al. 2020). Together, these publications suggest that preDC migration to the peripheral tissues is subset, tissue and context specific.

In terms of lymph node infiltration, much less is understood. This is likely due to the lack of tools to specifically target lymph node rDC, however, some advances have been made. Liu and colleagues identified that antibody blockage of CD62L prevented preDC seeding of lymph nodes (K. Liu et al. 2009). Additionally, seeding occurred in less than 24 hours after adoptive transfer and preDCs were phenotypically cDCs at day 6 (K. Liu et al. 2009), however, when this maturation occurs and what induces it needs to be clarified. It is well characterised that mDC require CCR7 to migrate to the draining lymph nodes (Förster, Davalos-Misslitz, and Rot 2008; Ohl et al. 2004; Roberts et al. 2016) and this reliance led to investigations of CCR7 in preDC infiltration (Ugur et al. 2023a). In this study, the researchers descried that pre-cDC1s initially enter the medulla of the lymph node, from the blood, and then differentiate into cDC1 as they migrate towards the paracortex. With the use of mixed bone marrow chimeras, it was demonstrated that the spatiotemporal control of lymph node rDC1 within the paracortex required CCR7. rDC1 were suggested to be trapped in the medulla, close to the blood vessels, and unable to migrate within the tissue. This accumulation within the paracortex was significantly increased in response to inflammation. Thus, as with mDC, lymph node infiltration of developing rDC appears to require CCR7.

Furthermore, whether tissue location is predetermined during preDC development is unknown. Tissue specificity is an accepted feature of activated T-cells allowing them to home to the site of infection and exert their effector functions (Brinkman, Peske, and Engelhard 2013). In addition, specific migration of T-cell precursors from the bone marrow to the thymus is required for final stages of T-cell development (Germain 2002). It is likely that specific tissue homing is involved in more aspects of immune regulation. In fact, one study has provided an exciting suggestion that preDCs in the bone marrow are primed to

home to the gut via retinoic acid induction of α4β7 expression on precursors allowing intestinal entry via interaction with mucosal vascular addressin MAdCAM1 (Zeng et al. 2013a). The group named these tissue specific precursors pre-mucosal DCs (pre-μDCs) and were identified as lineage- (CD3, CD19, NK1.1) $CD11c^{int}$ $B220^{+}$ $\alpha4\beta7^{+}$ $CCR9^{-}$. Unfortunately, specific preDC markers were missing from this sorting of cells for adoptive transfer and thus it cannot be certain that they are true preDCs. Later however, the researchers confirmed the expression preDC markers (SiglecH, Sirpα, Ly6c, CD135 and lack of MHCII) on pre-μDCs, confirming their preDC phenotype. These pre-μDCs were isolated from lymphoid tissues of donor mice and adoptively transferred into recipient mice and recipient tissues were analysed 3 days post injection. $\alpha4\beta7^{+}$ pre-μDCs preferentially homed to the small intestine lamina propria (LP) compared to spleen, blood, mesenteric lymph node (MLN), peripheral lymph nodes (PLN) and surrounding intraepithelial lymphocytes (IEL) and *in vitro* gave rise to $CD103^{+}$ DCs and $CCR9^{+}$ pDCs. *In vivo*, the cells developed preferentially into CD103 single positive LP cDCs and MLN cDCs; small intestine $CCR9^{+}$ pDCs;$CD8\alpha^{+}$ splenic cDCs ;and $CD103^{+}$ lung cDCs. It is interesting that these precursors were bias to cDC1 development and not equal differentiation of both subsets. This could suggest that pre-cDC2s with a bias to migrate to these locations make use of a different homing marker. Importantly, pre-μDCs were significantly better at gut homing than non-$\alpha4\beta7^{+}$ preDCs. As non-$\alpha4\beta7^{+}$ preDCs did also migrate to the gut, albeit to a lesser extent, this could suggest that the expression of the homing marker improves specific migration but is not the sole dictator of location. cDCs were significantly decreased in the LP when $\alpha4\beta7^{+}$ was inhibited with antibody treatment, however, it was not clarified if this reduction was specifically cDC1s or both cDC subsets.Despite some interesting insights, it will be important to confirm these findings by improving the experimental design. In this study, sorting cells from lymph nodes and not the BM may have enriched preDCs further down the developmental pathway than BM preDCs, which was confirmed by the increased expression of CD11c and MHCII on small intestine $\alpha4\beta7^{+}$ preDCs compared to spleen and BM. Additionally, without utilising previously defined preDC markers to sort these cells, the true identity of the precursor cannot be confirmed. Finally, *In vitro* treatment of retinoic acid induced $\alpha4\beta7^{+}$ pre-μDC development in BM cells. Therefore, tissue specific environmental cues may induce the differentiation of cDC precursors with homing markers allowing them to be bias to migrate to a particular location before bone marrow egress. It will be interesting to understand if

these signals are produced by niches within the bone marrow or if the cues are retrieved from external sources, such as the target tissue.

Together, these studies have provided useful information about preDC seeding and suggest that committed precursors utilise different receptors to migrate and that these mechanisms may vary depending on tissue and condition. However, there are gaps in our knowledge of the complexity of preDC migration and there is no consensus of which chemokine receptors are important for preDC migration and how tissue location is controlled. The development of a universal assay to measure preDC migration systemically and during multiple tissue contexts will help unpick these complex pathways and provide opportunities to promote or intervene with preDC migration.

1.3.3 preDC recruitment to the TME

In the initial stages of tumour development, tissue resident cDCs are likely to be involved in the early anti-tumour response. However, as a tumour develops into its own tissue, new preDC infiltration can occur (Diao et al.2010a). Whether the mechanisms of recruitment to the TME are comparable to the source tissue the tumour develops from is unknown. Based on research on preDC recruitment in a viral setting, chemokine receptor dependency changes from homeostasis to inflammation (Cabeza-Cabrerizo, Minutti, et al. 2021a). Therefore, it is essential to include tumours as separate tissues to their healthy tissue counterparts when measuring preDC infiltration. Within the literature, there are some indications of the signals involved in cDC TME infiltration. Overexpression of CCL3 in B16 tumour cell lines induced increased numbers of TME preDCs *in vivo* (Diao et al. 2010a). In *in vitro* assays, CCL4 in tumour supernatants recruited mature cDC1 and β-catenin signalling in melanoma tumour cells inhibited CCL4 production and recruitment of cDC1 *in vivo* (Spranger, Bao, and Gajewski 2015b). Supporting the role of CCL4 in augmenting cDC1 within the TME, Williford and colleagues localised CCL4 to the TME using collagen-binding-domains which significantly increased the numbers of cDC1 per mg of tumour (Williford et al. 2019a). On the other hand, cDC1 accumulation in melanoma tumours was dependent on NK-derived CCL5 and XCL1 (Böttcher et al. 2018a). This process was inhibited by tumour production of Prostaglandin E2 (PGE_2) by reducing chemokine production by NK cells and chemokine responsiveness of cDC1s. In B16F10 melanoma cell lines overexpressing CCL21, qualitative immunofluorescence analysis of tumour sections suggested that CCL21 tumours had increased CD11c expression compared to WT tumours (Novak et al. 2007). In a separate

study, melanoma cell lines which expressed high levels of CCL21 had increased $CD11c^+$ cells, a feature not seen in CCR7 knockout mice that are unable to respond to CCL21 (J. D. Shields et al. 2010a). Interestingly, these tumours also developed lymphoid-like structures, tolerogenic environments and had increased invasiveness. In terms of which chemokine receptors are involved, total body CXCR3 KO mice had reduced cDC1 in melanoma tissue and the tdLN but not in non-inflamed skin or spleen while cDC2 numbers remained unchanged in both settings (Cook et al. 2018b). Overall, despite some evidence, there remains no consensus of which chemokine receptors are involved in the recruitment of preDC subsets to the TME. Whether preDC subsets use differing signals to migrate to the TME and if there exists tumour type specificity of these signals needs to be elucidated. By understanding the signals involved, we may be able to exploit preDC migration to increase cDCs within the TME and augment anti-tumour responses and the efficiency of immunotherapies.

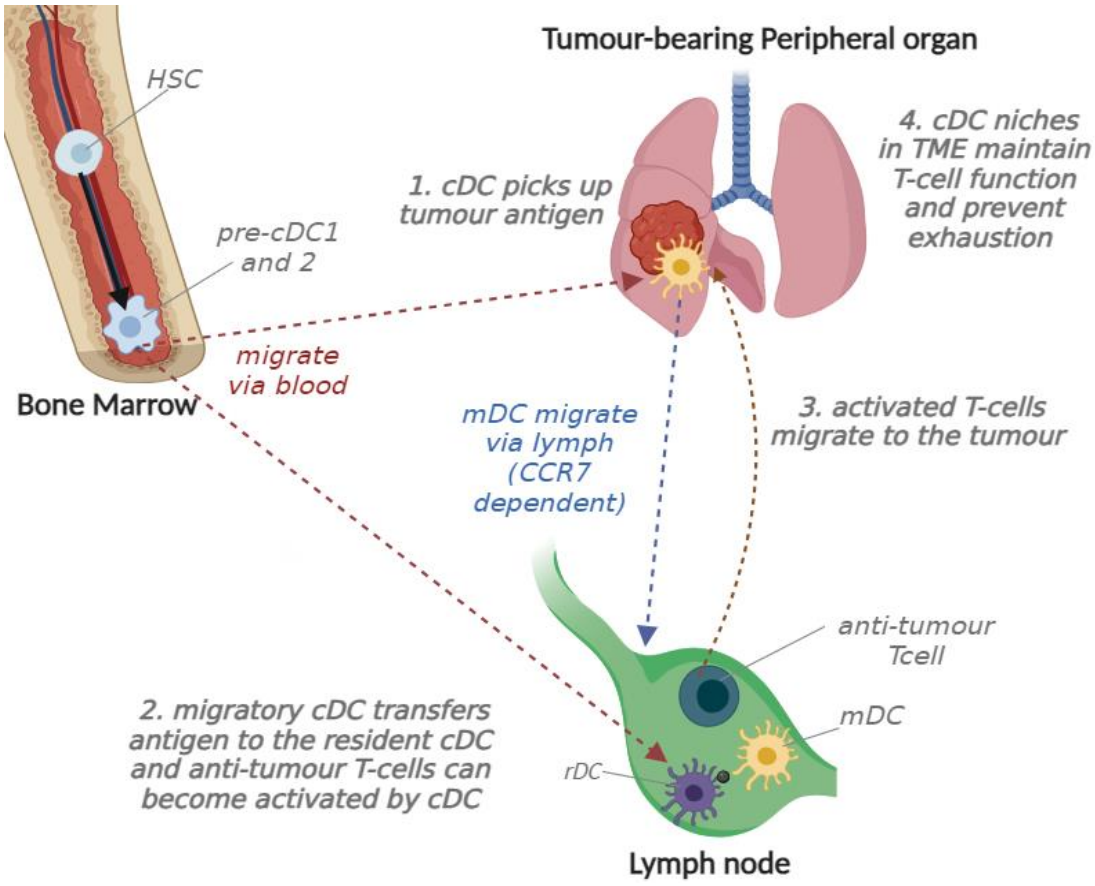


Figure 1-2 – Migration is key to cDC function. *preDCs migrate via the blood to the peripheral tissue and lymph nodes where they mature into cDCs. cDCs within the tumour pick up antigen and become migratory (mDC) when they migrate in a CCR7-dependent manner to the draining lymph node. Here, mDC interact with resident cDCs (rDC) and pass on antigen and contextual information. cDCs are then able to activate tumour specific T-cells. Within the tumour, cDC niches maintain the function of anti-tumour T-cells.*

1.4 Utilising dendritic cells to improve immunotherapy

1.4.1 A brief introduction to immunotherapy

In the last decade, the landscape of cancer treatment has changed with the advent of immunotherapies, however, their applications date back to ancient texts and early medical experiments (Dobosz and Dzieciątkowski 2019). The origin of modern immunotherapies is accredited to "The Father of Immunotherapy" William B. Coley and his infectious "Coley's Toxins" which were used in the late 1800s to induce tumour rejection. However, lack of a clear mechanism and risk of infections prevented its acceptance for many decades. Following advances in immunology and the advent of immunoncology, interest in immunotherapies resurged and the development of multiple immunotherapies have revolutionised the treatment of cancer.

Immunotherapies exploit intrinsic immune mechanisms to stimulate the immune system's ability to target cancer cells (Kruger et al. 2019). T-cells have been the central focus of immunotherapies: Immune checkpoint blockade (ICB; e.g anti-PD-1 and anti-CTLA4) and chimeric antigen receptor (CAR) T-cells promote T-cell activation and function (Seidel, Otsuka, and Kabashima 2018) and tumour-infiltrating lymphocyte (TIL)-based adoptive cell therapies (ACT) aim to boost anti-tumour T-cell numbers and infiltration to the TME (June et al. 2018; Rosenberg and Restifo 2015). An in-depth review of the multiple immunotherapies for cancer treatment can be found elsewhere (Kruger et al. 2019; Y. Zhang and Zhang 2020). Of these ICBs, PD-1 and CTLA-4 blockades are most well studied. They inhibit signals induced by receptors known as checkpoints. These checkpoint signals, by-in-large, provide inhibitory signals that prevent overt immune responses (Greenwald, Freeman, and Sharpe 2005; Sharpe and Pauken 2017). The immune system is precisely balanced to allow responses to be mounted to antigen in the context of inflammation while preventing immunopathology. To do this, the immune system has evolved to involve activator and inhibitory signals, the balance of which controls the dynamics of the immune response. Checkpoints are an example of these inhibitory signals which downregulate activator signals. A consequence of these protective mechanisms in preventing immunopathology is their availability to tumours and, as such, their contribution to tumour progression. Tumours are able to exploit these tolerogenic mechanisms, for instance, PD-L1 and PD-L2 (the ligands to PD-1 checkpoint) are upregulated in human tumours (Okazaki and Honjo 2007) and correlate with poor prognosis (Gandini, Massi, and Mandalà 2016). Multiple additional checkpoints have been uncovered and are under investigation for their

ability to treat cancer (Marin-Acevedo, Kimbrough, and Lou 2021). While the majority of patients gain partial prolonged survival benefits from traditional cancer therapies, PD-1 and CTLA-4 ICBs see some patients experiencing complete regression of tumours as well as increased survival time. For example, complete responses to anti-PD-1/PD-L1 immunotherapeutics varied from 15-32% in multiple studies (Ansell et al. 2015; Eroglu et al. 2018; P. Nghiem et al. 2019; P. T. Nghiem et al. 2016). In one trial, the one-year survival rate of patients with metastatic melanoma was 72.9% when patients were treated with nivolumab (anti-PD-1) compared to only 42.1% in patients treated with traditional chemotherapy (dacarbazine chemotherapeutic) (Robert et al. 2015). In another study, the 5-year overall survival (OS) was 19% in patients with metastatic Non-small cell lung cancer (NSCLC) treated with nivolumab plus ipilimumab (anti-CTLA-4) while the OS was 10% in patients treated with nivolumab alone and compared to 7% with treatment of platinum-doublet chemotherapy alone. Most strikingly, the duration of response (DOR) after 5 years was 28%, 24%, and 3% in the combination treatment, nivolumab alone, and chemotherapy alone groups, respectively (Brahmer et al. 2023), highlighting the long-term benefits of ICB therapy compared to traditional treatments. Furthermore, the adverse effects of traditional therapies such as chemo- and radiotherapy can be life altering (Prieto-Callejero et al. 2020; Vaz et al. 2011) while adverse effects occur less often during ICB treatment in comparison (Herbst et al. 2016; Rittmeyer et al. 2017; F. Tang et al. 2018). Despite the benefits provided by ICB, most patients do not respond, or go on to acquire resistance to treatment (Shah and Fry 2019; Shergold, Millar, and Nibbs 2019; Tan, Li, and Zhu 2020). If these mechanisms of resistance are understood and prevented, immunotherapies, including ICB, have the potential to provide more patients with complete and durable responses instead of treatments focused on increased survival and slowing disease progression (Figure 1.3).

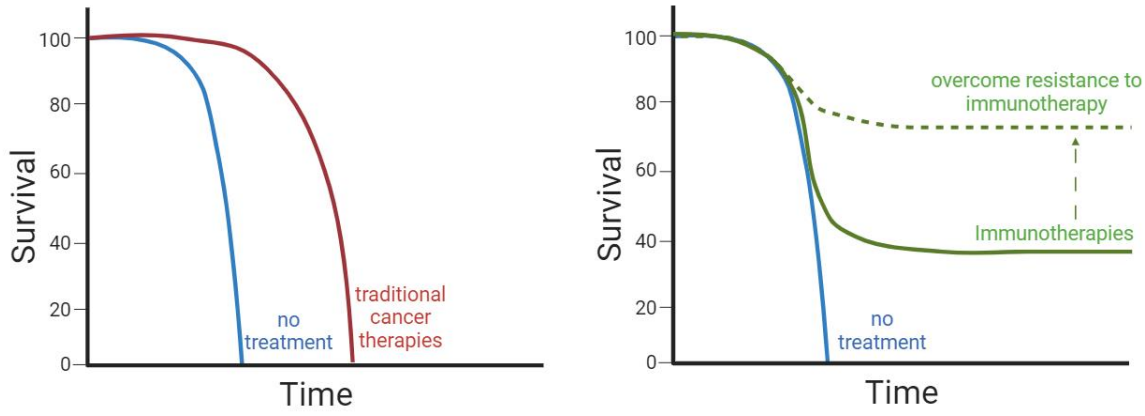


Figure 1-3 – Overcoming resistance to immunotherapy treatments have the potential to provide more patients with complete responses. *Traditional cancer therapies, such as chemo- and radiotherapy, have largely increased the survival time of patients with cancer and improvements in these treatments can continue to slow disease progression and increase survival time. Meanwhile, immunotherapies, particularly immune checkpoint blockade, has allowed a small proportion of patients to receive complete and durable responses. However, the majority of patients are initially resistant, or go on to acquire resistance to treatment. By understanding these mechanisms of resistance, we may be able to overcome resistance and increase the number of patients experiencing complete and durable responses.*

1.4.2 Overcoming resistance to immunotherapy

Resistance to T-cell based immunotherapies occurs when tumours are, or become, no longer vulnerable to cytotoxic T-cell killing. This can happen when the TME exploits mechanisms to inhibit T-cell function, either by direct inhibition or via the inhibition, or promotion, of other immune cells (Shergold, Millar, and Nibbs 2019; Tan, Li, and Zhu 2020). The Cancer Immunity Cycle highlights the key steps that tumours can interrupt to prevent T-cell killing and, thus, resist T-cell focused immunotherapy treatments. Briefly, inhibition of: cDC infiltration and function; T-cell priming, infiltration and killing; the presence of DAMPs; the release and presentation of TAAs; tumour sensitivity to T-cell killing; and exploitation of tolerogenic mechanisms can all contribute to an environment that allows tumours to resist T-cell based immunotherapies (Bai et al. 2020; Shergold, Millar, and Nibbs 2019). Patients that are initially resistant to therapymay be potential responders if these mechanisms of resistance are inhibited. Furthermore, preventing resistance could avert, or treat, acquired resistance in initial responders. Evidence of improving immunotherapy responses by inhibiting resistance can be seen when ICB is augmented by combination therapies. Combination of two ICB treatments, anti-PD-1 and anti-CTLA-4 increased survival rates compared to single therapies: survival rates were 58% to combination therapy, 52% to anti-PD-1 alone and 34% to anti-CTLA-4 alone (Wolchok et al. 2017). Thus, targeting two

inhibitory mechanisms on T-cell function, or inhibiting Tregs (Du et al. 2018), augments responses to single treatment. Additionally, combinations with traditional therapies, chemo- or radiotherapy, can improve responses to ICB by inducing immunogenic cancer cell death which releases TAAs and DAMPs (Pfirschke et al. 2016). Moreover, combinations with small molecule and hormone inhibitors, and surgery are under clinical investigation or have been FDA approved to augment ICB therapies Patel and Minn 2018; Shergold, Millar, and Nibbs 2019). Given the heterogeneity between patients, tumour types and within areas of the TME itself, individual resistance mechanisms will be involved in the escape of tumours from responding to immunotherapy and thus, multiple approaches need to be developed to overcome resistance in more patients. An understanding of the variations of tumours, patients and tumour immune response has allowed us to begin to identify the mechanisms of resistance. Given their vital role in priming and restimulating anti-tumour T-cells in the tdLN and TME, respectively, as well as correlating with increased survival and response to immunotherapy, cDCs are ideal candidates for targeting to eradicate resistance to T-cell based immunotherapies.

1.4.3 Dendritic cells as immunotherapy targets

Notably, despite the focus of immunotherapies being T-cell centred, cDCs have proven to be vital to their efficacy; highlighting their importance to the anti-tumour immune response and their potential to be ideal candidates to prevent resistance to T-cell immunotherapies. For instance, anti-CTLA-4-induced depletion of Tregs, in a model of melanoma, improved the activation status of tumour infiltrating cDC2, characterised by an increase in $ICOS^+$, $PD\text{-}1^+$ and $IFNy^+$ on cDC2s in the tdLN and TME (Binnewies et al. 2019c). This, in turn, improved and the activation of $CD4^+$ T-cells and contributed to tumour regression. This regression relied on cDC2 as *Irf4* KO mice which lack cDC2 were unable to induce activation of $CD4^+$ T-cells (Binnewies et al. 2019c). Additionally, cDC1 were suggested to be vital to the efficacy of anti-PD-L1 as responses were lost in *Batf3* KO mice which lack cDC1 (Salmon et al. 2016a). However, as *Batf3* is expressed in more than just cDC1s and their precursors, specific deletion of cDC1s is needed to confirm the importance of cDC1s in this immunotherapy response. Importantly, deletion of PD-L1 in $Clec9a^+$ cDCs induced controlled tumour growth to the same extent as total body PD-L1 knockout mice (Oh et al. 2020) confirming the importance of cDC1 here. Strikingly, in this study, tumour control was not improved any further in mice with cDCs lacking PD-L1 when treated with anti-PD-L1. Additionally, it was shown that PD-1 blockade therapy acted directly in the tdLN by blocking PD-1:PD-L

interactions between T-cells and cDCs (Dammeijer et al. 2020). These studies suggest that efficacy of PD-1/PD-L blockade functions directly through PD-L1 expressing cDCs in the tdLN during T-cell priming. This was further supported by efficacy of anti-PD-1 being reliant on the presence of tdLNs (Fransen et al. 2018). Furthermore, given cDCs role in sensing immunogenic signals and processing TAAs, it is likely that the synergistic effect of immunogenic cell death on ICB therapy relies on cDCs (Lamberti et al. 2020; Salah et al. 2021a; S. Zhu et al. 2022). Thus, cDCs play a pivotal role in the effectiveness of ICB therapy and therefore are ideal candidates to target as means to prevent resistance to therapy.

Multiple mechanisms are under investigation as potential means to create cDC-focused immunotherapies. Approaches include stimulants which directly influence cDC development and state, and cell/peptide vaccinations. In terms of cDC stimulants, treatment with Flt3L has been extensively investigated with the aim to increase cDC TME infiltration. Flt3L can be used to both enrich preDC development in the bone marrow and tissue development of precursors into cDCs (Ugur et al. 2023). In a murine model of pancreatic cancer, FLt3L treatment significantly increased both cDC1 and cDC2 tumour numbers and increased mDC numbers in the tdLN (Hegde et al. 2020). However, treatment alone did not increase cytotoxic T-cell tumour infiltration but did promote Treg expansion and, consequently, did not control tumour growth. Thus, recruitment of cDCs alone is not enough to promote the anti-tumour immune response. The researchers then combined Flt3L treatment with anti-CD40, to promote cDC maturation, and observed reduced Tregs, increased effector T-cells and promotion of tumour control. Importantly, as with Flt3L treatment alone, anti-CD40 alone did not induce these anti-tumour effects. This highlights the benefits of targeting multiple inhibitory pathways by combination treatments. In another study (Salmon et al. 2016a), Flt3L was combined with PolyI:C which acts as a ligand for endosomal TLR3 and other RNA sensors within cDCs and induces the activation of cDCs (Azuma et al. 2016). As expected, increasing cDC development and activation synergistically improved responses to PD-L1 blockade in mice with melanoma (Salmon et al. 2016a). This response was associated with increased activated cDCs and effector $CD8^+$ T-cells within the TME (Salmon et al. 2016a) and was synergised by anti-GCSF which prevented neutrophilia (Meyer et al. 2018). Similar approaches to activate cDCs include additional PRR agonists that induce local inflammation (Adams et al. 2012; Cance et al. 2019; Galluzzi et al. 2017; Krawczyk et al. 2020; Torres et al. 2007). Excitingly, Flt3L treatment to increase cDCs is being tested clinically in several tumour types. For instance, Flt3L in combination with radiation

and PolyI:C in patients with Non-Hodgkin's Lymphoma, metastatic breast cancer and head and neck squamous cell carcinoma (NCT03789097) and in combination with PolyI:C in Low-Grade B-cell Lymphoma (NCT01976585) (Krawczyk et al. 2020). It is important to note that these approaches are systemic treatments that do not specifically target cDC infiltration to the TME or their function within, therefore, their efficacy may induce off-target effects. To tackle this, researchers have adaptive T-cells, for adoptive cell therapy (ACT), which release Flt3L, with the aim of targeting delivery to the TME (Lai et al. 2020). Tumour growth was controlled, especially when combined with PolyI:C treatment. However, ACT have been suboptimal in the treatment of solid tumours often due to their exclusion from the TME (Kankeu Fonkoua et al. 2022). In this study, the ACT with Flt3L induced endogenous cDC and T-cell TME infiltration but evidence of transferred cells within the TME was not presented, suggesting that this Flt3L treatment remained peripheral in this study.Thus, peripheral Flt3L distal to the BM may influence haematopoesis. Some other tactics aim to narrow their focus to the TME. For instance, prostaglandin E2 (PGE_2) tumour production was shown to prevent cDC recruitment by preventing chemokine production by NK cells and chemokine responsiveness of cDC1s (Böttcher et al. 2018a). PGE_2 tumour production was induced by cyclooxygenase (COX) enzymes and COX inhibition prevented cDC inhibition and improved the frequency of tumour infiltrating activated cDCs (Zelenay et al. 2015). However, production of COX is not unique to the TME and thus blocking the enzyme may have adverse effects by interrupting endogenous pathways (FA 2004).

An additional TME-focused approach is dendritic cell vaccinations. These strategies aim to load cDCs/DC-like cells with personalised antigens, acquired from patient tumour samples or universal antigens associated with cancer type, aiming to induce priming of tumour-specific T-cells. Dendritic cell vaccinations have a long history since initial trials. Professor Ralph Steinman is credited with the discovery of dendritic cells as well as his involvement in the preliminary vaccination approaches. As treatment for his own pancreatic cancer, Steinman and colleagues harvested his peripheral blood mononuclear cells and loaded DC-like cells with TAAs acquired from his tumour biopsy and with prostate cancer specific antigen. Unfortunately, Steinman died due to complications associated with his cancer, however, his legacy continues. These early experiments were likely to not truly reflect the functions of cDCs as peripheral blood is a location where cDCs and preDCs are not as abundant compared to monocyte-DC cells which do not possess the same potential as cDC proper. Despite this, Sipuleucel-t (Provenge), was the first, and is the only, FDA approved

"DC" vaccine. It was approved in 2010 for the treatment of hormone-refractory prostate cancer (Anassi and Ndefo 2011) and induces a slight improvement in survival of an average of 4 months (Wculek, Cueto, et al. 2019). Unfortunately, these cell therapies have not been overall clinically successful, with responses rarely exceeding 15% in patients treated (Garg et al. 2017), likely due to vaccinations not being purely cDC proper as well as intrinsic tumour suppressive mechanisms (Wculek, Cueto, et al. 2019). Nevertheless, these DC-like vaccination strategies are continuing to be investigated as treatments (Wculek, Cueto, et al. 2019). cDC exert greater antigen presentation and T-cell activation capabilities that DC-like cells in these vaccinations likely do not possess. The potential of specific cDC vaccinations was demonstrated in a study in which murine cDC1s were isolated from the spleen, treated *ex vivo* with PolyI:C and loaded with dead tumour cell lysate containing TAAs (Wculek, Amores-Iniesta, et al. 2019). Subsequently, these activated, antigen bearing cDC1s were intravenously injected into donor mice. Following vaccination, mice were protected from tumour growth. This scenario demonstrates an ideal situation in which treatment is personalised to the patient's tumour and vaccination occurs pre-tumour growth, a scenario which is currently unlikely to be clinically relevant. Additionally, these cDC1s were sorted from murine spleen and not precursors from the bone marrow. Clinically, human DC-like cells are harvested from blood and are not cDC. Strategies to harvest human cDC for "next generation" vaccinations are being investigated (Garg et al. 2017; Saxena and Bhardwaj 2018). In one trial (NCT01690377), intra-nodal administration of human cDCs, magnetically isolated from patient blood, and loaded with tumour-associated antigens tyrosinase and gp100, was investigated for the treatment of metastatic melanoma (Schreibelt et al. 2016). A proportion of patients (~28%) experienced increased progression-free survival which correlated with increased $CD8^+$ T-cell responses. Despite this, realistically, such personalised approaches are unlikely to be available. Personalised approaches are time-consuming and expensive which reduces their accessibility. Semi-personalised approaches such as the harvest of HSCs from human leukocyte antigen (HLA) donor umbilical blood, which can be expanded and differentiated *ex vivo* into cDCs, are under investigation (Kumar, Kale, and Limaye 2015; Saxena and Bhardwaj 2018). Another universal approach is the use DC-like immortalised cell lines such as the MUTZ-3 cell line, derived from human leukemic myeloid cells (Santegoets et al. 2008). Yet, these are not ideal as they are also not endogenous cDC proper and therefore may not exert cDC functions to the same extent. Therefore, cDC vaccinations and cell therapies have the potential to revolutionise cancer therapy, but, at

this time, such personalised medicines remain inaccessible to the majority of patients. Universal treatments to improve cDC infiltration to the TME and activation within the TME have shown success in murine models and are being investigated clinically. However, they lack tumour specificity. Furthermore, it is likely that cDC1 and cDC2 have complementary roles in the anti-tumour immune response and is unclear the role of development in priming cDCs to infiltrate the TME. A means to overcome this would be utilizing patient endogenous cDCs. Chemo- and radiotherapies and oncolytic viral treatments which induce immunogenic cell death are one such approach to do this. Another, and potentially less toxic, approach is the use of universal tumour antigens as vaccinations that endogenous cDCs could process and present. Several universal tumour antigens have been identified (Haen et al. 2020), including ones used in these DC-like vaccination trials. In one study, RNA lipoplexes encoding TAAs were shown to be taken up by cDCs, induce cDC activation by TLR7 stimulation, induce expansion of antigen specific T-cells in mice and promote tumour control (Kranz et al. 2016). Additionally, in a small clinical experiment (NCT02410733), results from the first three patients with advanced malignant melanoma, RNA lipoplexes induced novel antigen-specific T-cell responses (Kranz et al. 2016). However, the true benefits of such approaches will likely be unsuccessful if tumour inhibition of cDC recruitment and function are at play. Thus, another exciting approach would be to combine these TME-specific strategies with the recruitment of cDCs precursors specifically to the TME. Although Flt3L improves cDC development, we aimed to investigate the role of tumour specific signals in the migration of preDCs to the TME to also enhance their TME infiltration in a more tissue-specific manner.

1.4.4 Harnessing the chemokine system as cDC immunotherapy

Since chemokines are vital to the recruitment of preDCs and chemotaxis is potentially tissue specific (discussed in section 1.3), utilising the chemokine system as cDC immunotherapy targets is an area of interest (Karin 2021a; Poeta et al. 2019a). However, traditionally, such attempts have not been extremely successful. At present, only two drugs have been approved as chemokine-based therapeutics: plerixafor and maraviroc antagonists of CXCR4 and CCR5, respectively. Maraviroc is used clinically as a therapy option for patients with HIV to prevent viral entry via CCR5 (Hütter et al. 2015). Meanwhile, plerixafor induces the mobilisation of bone marrow cells by interfering with CXCR4-dependent bone marrow localisation (Uy, Rettig, and Cashen 2008), a treatment particularly useful during cell harvesting for bone marrow transplants. In terms of cancer, exploitation of the chemokine

system has seen most attempts focusing on inhibition of chemokine ligands and receptors (Bernardini et al. 2019; Karin 2021b; Nagarsheth, Wicha, and Zou 2017b; Poeta et al. 2019b; Vela et al. 2015). Of note, inhibition of CXCR4:CXCL12 ligation is under clinical investigation for prevention of tumour cell metastasis (Reinholdt et al. 2016). Similarly, CCR2:CCL2 inhibition aims to prevent the infiltration of monocyte cells which can develop into pro-tumour tumour associated macrophage-like cells. Additionally, CCR7 antagonism has been investigated to prevent lymph node metastasis of tumour cells (Vela et al. 2015). Multiple other chemokine pathways have been the focus of clinical research, however the aim has remained to inhibit tumour cell metastasis directly or to prevent the infiltration of certain immune cells. While some focus on the recruitment of T-cells via CXCL9 and CXCL10 administration (Karin 2021b), murine studies suggest that exploitation of the chemokine system to promote the influx of cDCs is a promising route. For example, one group have made use of fusion of CCL4 to collagen-binding domain (CBD) (specifically domain A3 of the of von Willebrand factor (VWF)) (Ishihara et al. 2019a; Williford et al. 2019b). The group demonstrated that the CCL4:CBD accumulated in orthotopic EMT6 breast cancer tumours likely due to leaky tumour vasculature and abundance of collagen, allowing the influx and localisation, respectively. Compared to systemic treatment with CCL4 alone, CBD:CCL4 increased the concentration of intratumoral CCL4 significantly in B16F10 melanoma tumours. Here, cDC1s, $CD8^+$ and $CD4^+$ T-cells and NK cells were significantly increased upon CBD:CCL4 treatment compared to no treatment or WT CCL4. Excitingly, this resulted in an increased response to anti-PD-L1 treatment in B16F10 melanoma tumours and EMT6 immune-excluded breast cancer and spontaneous MMTV-PyMT breast tumours. When analysing the lung, liver and kidney, the group reported no adverse effects of CBD:CCL4 treatment based on tissue morphology and immune infiltration histological analysis. Thus, tumour specific treatment with chemokines may be an effective way to increase cDCs within the TME and, in turn, promote T-cell accumulation and activation and response to immunotherapy. In another study, a CCL21 nanoparticle treatment increased intratumoural IL-12 (suggested to be produced by cDCs) and slowed Lewis lung carcinoma growth in mice (Kar et al. 2011). Further approaches to exploit chemokines for cancer therapy have been investigated (Bobanga, Petrosiute, and Huang 2013), for example, murine bone marrow derived DCs were transduced to express CCL21 and intratumorally injecting these DCs induced increased infiltration of cDCs, T-cells and decreased Tregs (Yang et al. 2004). Additionally, a chemotactic-antigen plasmid DNA vaccine was created by fusion of the gene

sequences for a melanoma specific TAA, CCL21 and the Fc region of IgG (to induce internalisation by FcγR expressing cDCs). Treatment with this fusion vaccine induced tumour regression compared to vaccinations without CCL21 in a model of lung cancer (R. Liu et al. 2006). This therapeutic was later shown to augment anti-PD-1 therapy (Moeini et al. 2017). Thus, there is potential for chemokine therapeutics to augment cDC TME infiltration and responses to immunotherapy.

Despite the potential of chemotaxis-based therapeutics, the benefits of targeted delivery of chemokines to improve cDC infiltration remains elusive. For instance, chemokines have been implicated in tumour development (Poeta et al. 2019a): certain chemokines are associated with the promotion of tumour angiogenesis and endothelial cell survival (CCL2, CCL11, CCL16, CCL18, and CXCL8) and cancer metastasis (CCR7 and CXCR4 on tumour cells involved in their metastasis to lymph node and bone marrow, respectively). Therefore, there is a valid concern that interfering with the chemokine system could induce the recruitment of pro-tumour immune cells or promote the survival and evasiveness of tumour cells. Thus, at this stage, it is probable that targeting chemokines and receptors will remain a non-ideal treatment plan due to the redundancy, promiscuity and pleiotropism of how we understand the specificity of the ligand and receptor interactions. Once we are able to better characterize the tissue and cell specifics of chemotaxis, we may be able to tailor treatments to specifically target tumour type and specific immune cells. As such, we believe it is important to better understand the signals involved in preDC migration and hence cDC infiltration within the TME.

1.5 Aims and hypothesis

Based on the literature, we hypothesised that preDC recruitment may be controlled by distinct chemokine signals which induce tissue-specific infiltration. Therefore, we set out to understand the chemokine receptor expression patterns of preDC subsets (Chapter 1). We next screened chemokines overexpressed within tumours as candidates to increase cDCs within the TME. (Chapter 2). Lacking from the literature was a universal means to measure preDC migration. Therefore, we next aimed to optimise an assay which provided this universal measurement (Chapter 3). After attempting adoptive transfers and knockout mice, we decided that the most accurate and sensitive way to measure preDC migration was with the use of mixed bone marrow chimeras (Chapter 4). As such, we aimed to identify the key chemokine receptors involved in preDC subset migration peripherally.

2 MATERIALS AND METHODS

2.1 Mice

C57BL/6 and Ly5.1 (CD45.1; Strain Code 494) (purchased from Charles River, UK; C57BL/6 background); CCR7-KO (Strain Code006621; purchased from The Jackson Laboratory); and iCCR-KO, CCR2-KO, CCR5-KO and iCCReporter mice (gifted from Professor Gerard Graham) were housed and bred at the Beatson Research Unit in accordance with the UK Animal (Scientific Procedures) Act 1986 and the EU Directive 86/609, under UK Home Office Project License P72BA642F. All mice used to acquire the data presented in this thesis were male and specific pathogen free (SPF).

2.2 Flow Cytometry

Single cell suspensions were acquired from digested murine tissues (Table 8.1). Cells were incubated for 20 minutes at 4°C with Zombie NIR (BioLegend) before incubating for 30 minutes at 4°C with surface protein antibodies (Table 8.2). Cells were fixed at room temperature for 10 minutes with 4% PFA. Fixed cells were resuspended in FACS Buffer (FB, made in house; 1X Dulbecco's phosphate-buffered saline (DPBS) + 2% Foetal Bovine Serum (FBS), + 1% 5000U/ ml Penicillin-Streptomycin + 1% 1M HEPES + 0.2% 500nM EDTA (all from ThermoFisher Scientific, UK)) and CountBright™ Absolute Counting Beads (ThermoFisher). Cells were analysed on a BD LSR Fortessa II™. Important to note, CD11b and CD103 were used to identify cDC1 and cDC2 but others have found that XCR1 and Sirpα may be superior at separating the populations by flow cytometry (Cabeza-Cabrerizo, Cardoso, et al. 2021a).

Table 2-1 - Tissue digestion buffers and methods

Tissue	Buffers	Digestion Method
Lung	RPMI 1640 (Gibco™) + 500U/ml Collagenase IV (Worthington Biochemical, USA) + 100U/ml Collagenase I (Worthington Biochemical, USA) + 0.2mg/ml DNAse1 (Roche, UK)	Lungs were roughly chopped using tissue dissection scissors and placed into a 50ml falcon tube with 10ml digestion mix per set of lungs. Lungs were shaken at 37°C for 60 minutes. Tissue was then pipetted into a 50ml falcon tube through a 70uM filter, centrifuged for 5 minutes at 4°C at 400g and then incubated in Red Blood Cell lysis buffer (eBioscience, UK) for 5 minutes at room temperature. Cells were washed in FB, centrifuged, as before, and resuspended in FB and kept on ice before viability and antibody staining.
Lymph node	RPMI 1640 + 500U/ml Collagenase IV (Worthington Biochemical, USA) + 100U/ml Collagenase I (Worthington Biochemical, USA) + 0.2mg/ml DNASE1 (Roche, UK)	Individual LNs were pierced and torn with sharp forceps to open the LN. LNs were placed into wells of a 24-well plate (3 LNs per well) and covered with 1ml digestion mix. Plate was incubated for 30 minutes and then triturated with a 1ml pipette. Cells were incubated for a further 30 minutes and then pipetted into an Eppendorf through a 70uM filter. Cells were washed in FB, centrifuged for 5 minutes at 4°C at 400g and resuspended in FB and kept on ice before viability and antibody staining.
Bone marrow	N/A	Bones were crushed in PBS using a pestle and mortar to release BM. BM was pipetted into a falcon tube through a 70μM filter. BM was washed with PBS and incubated in Red Blood Cell lysis buffer (eBioscience, UK) for 5 minutes at room temperature. Cells were washed in FB, centrifuged for 5 minutes at 4°C at 400g and resuspended in FB and kept on ice before viability and antibody staining.
Spleen	N/A	Spleen was crushed into a 50ml falcon tube through a 70uM filter using a syringe. Cells were washed with PBS and incubated in Red Blood Cell lysis buffer for 5 minutes at room temperature. Cells were washed in FB, centrifuged for 5 minutes at 4°C at 400g and resuspended in FB and kept on ice before viability and antibody staining.
Blood	N/A	Blood was collected EDTA coated collection tubes by cardiac puncture. Cells were washed with PBS and incubated in Red Blood Cell lysis buffer for 5 minutes at room temperature. Lysis step was repeated for a total of 3 times to ensure all red blood cells were lysed. Cells were washed in FB, centrifuged for 5 minutes at 4°C at 400g and resuspended in FB and kept on ice before viability and antibody staining.
Small intestine	Washing buffer: warm calcium/magnesium free HBSS containing 2mM EDTA Digestion buffer: RPMI1640 (Gibco™ no glutamine) supplemented with 10% FCS, 10% FCS, 100 U/ml Penicillin, 100μg/ml Streptomycin, 2 mM L-glutamine (Life Technologies 15,140,122) and 62.5 CDU/ml Collagenase VIII (CDU, collagenase digestion units) Complete medium: RPMI1640 + 10% heat inactivated FCS, 2mM L-glutamine, 100μm/mL penicillin/streptomycin and 50μm β-mercaptoethanol (all Gibco™).	Small intestine was cut longitudinally and rinsed in cold PBS and Peyer's patches were removed using tissue dissection scissors. Tissue was digested according to Webster, Andrusaite et al., 2019. In brief, the epithelium was removed by incubating with 30ml washing buffer for 15 minutes at 37°C at 250rpm and repeating this step 2 additional times. Small intestines were then incubated with 15ml digestion buffer for 15-20 minutes at 37°C at 250rpm. Digestion was terminated by adding 35ml cold complete medium. Samples were then filtered through a 100μm followed by a 40μm mesh EASYstrainer (Greiner), then washed twice with cold complete medium and centrifuging for 10 minutes in 4°C at 400g. Cells were resuspended in FB and kept on ice before viability and antibody staining.
Liver	4.7ml DMEM (Gibco™) plus 200ml enzyme D, 100ml enzyme R, 20ul enzyme A (as per manufacturer's instruction; Miltenyi Biotec Liver Dissociation Kit, mouse) per liver.	Livers were roughly chopped using scalpels and transferred into C-tubes (Miltenyi Biotec) containing digestion mix. Livers were dissociated using pre-installed program '37C_m_LIDK_1' of gentleMACS Octo Dissociator. After termination of the program, enzymatic activity was stopped by addition of 2ml FCS and the sample was strained through a 70um filter and washed with FB, centrifuged at 4°C for 5min at 400g. Cells were resuspended in Red Blood Cell lysis buffer (eBioscience, UK) and incubated for 5min at room temperature then centrifuged at 4°C for 5min at 400g. This lysis step was repeated, and the final cell suspension was resuspended in FB and kept on ice before viability and antibody staining.

Table 2-2 - antibodies used for flow cytometry and cell sorting

Target	Fluorochrome	Reactivity	Host and isotype	Clone	Manufacturer
CD16/32 (FC block)	N/A	Mouse	Rat IgG2a,λ	93	BioLegend, UK
CD90.2 (Thy1.2)	AF700	Mouse	Rat IgG2b,k	30-H12	BioLegend, UK
MHCII	AF700	Mouse	Rat IgG2b,k	M5/114.15.2	BioLegend, UK
CD103	APC	Mouse	Armenian Hamster IgG	2.00E+07	BioLegend, UK
cKIT (CD117)	APC	Mouse	Rat IgG2b,k	2B8	BioLegend, UK
CD103	APC	Mouse	Armenian Hamster IgG	2.00E+07	BioLegend, UK
CD135	APC	Mouse	Rat IgG2a,k	A2F10	BioLegend, UK
CD150	APC	Mouse	Rat IgG2a, λ	W19132B	BioLegend, UK
CD90.1	APC	Mouse/Rat	Mouse IgG1, κ	OX-7	BioLegend, UK
cKit	APC-cy7	Mouse	Rat IgG2b, κ	2B8	BioLegend, UK
CD117 (c-kit)	Biotin	Mouse	Rat IgG2b, κ	2B8	BioLegend, UK
B220	BV421	Mouse	Rat IgG2a,k	RA3-6B2	BioLegend, UK
CSF1R (CD115)	BV421	Mouse	Rat IgG2a,k	AFS98	BioLegend, UK
F4/80	BV510	Mouse	Rat IgG2a,k	BM8	BioLegend, UK
CD45	BV510	Mouse	Rat IgG2b, κ	30-F11	BioLegend, UK
NK1.1	BV605	Mouse	Mouse IgG2a,k	PK136	BioLegend, UK
B220	BV605	Mouse	Rat IgG2a,k	RA3-6B2	BioLegend, UK
CD3ε	BV605	Mouse	Armenian Hamster IgG	145-2C11	BioLegend, UK
CD19	BV605	Mouse	Rat IgG2a,k	6D5	BioLegend, UK
CD11b	BV605	Mouse	Rat IgG2b, k	M1/70	BioLegend, UK
CD11c	BV650	Mouse	Armenian Hamster IgG	N418	BioLegend, UK
Ly6C	BV711	Mouse	Rat IgG2c,k	HK1.4	BioLegend, UK
CD3e	BV785	Mouse	Armenian Hamster IgG	145-2C11	BioLegend, UK
B220	BV785	Mouse	Rat IgG2a,k	RA3-6B2	BioLegend, UK
Ly6G	BV785	Mouse	Rat IgG2a, κ	1A8	BioLegend, UK
CD8α	FITC	Mouse	Rat IgG2a,k	53-6.7	BioLegend, UK
CD45.1	FITC	Mouse	Mouse IgG2a,k	A20	BioLegend, UK
TruStain FcX™	N/A	Mouse	Rat IgG2a, λ	93	BioLegend, UK
HSC lineage dump (anti-mCD3, anti-mLy-6G(Ly-6C), anti-mCD11b, anti-mCD45R(B220), anti-mTer-119)	Pacific Blue™	Mouse	Rat IgG2a/Rat IgG2b	17A2; RB6-8C5; RA3-6B2; Ter-119; M1/70;	BioLegend, UK
CD135	PE	Mouse	Rat IgG2a,k	A2F10	BioLegend, UK
CD170 (Siglec-F)	PE	Mouse	Rat IgG2a,k	S17007L	BioLegend, UK
CD48	PE	Mouse	Armenian Hamster IgG	HM48-1	BioLegend, UK
CD45.2	PE	Mouse	Rat IgG1, κ	QA18A15	BioLegend, UK
CD24	PeCy7	Mouse	Rat IgG2b,k	M1/69	BioLegend, UK
Sirpα (CD127α)	PE-Cy7	Mouse	Rat IgG1,k	P84	BioLegend, UK
Sca-1	PE-Cy7	Mouse	Rat IgG2a, κ	D7	BioLegend, UK
CX_3CR1	PerCpCy5.5	Mouse	Mouse IgG2a,k	SA011F11	BioLegend, UK
Siglec-H	PerCpCy5.5	Mouse	Rat IgG1	551	BioLegend, UK
CD8α	PerCpCy5.5	Mouse	Rat IgG2a,k	53-6.7	BioLegend, UK
CD103	PerCpCy5.5	Mouse	Armenian Hamster IgG	2 E 7	BioLegend, UK

2.3 Flt3L production and use

The codon optimised human FLT3L gene (cross-reactive with mouse) sequence was synthesised with a Kozak sequence, IgK chain leader sequence upstream of the gene, *Eco-RI-HF* and *XhoI-HF* (New England Biolabs) cut sites, CCG overhangs and the stop codon removed (Integrated DNA Technologies, Inc.; Table 8.3). pcDNA™3.1/His (ThermoFisher Scientific) plasmid was digested with *EcoRI-HF* and *XhoI-HF* and the Flt3L sequence was ligated into the plasmid using T4 ligase (New England Biolabs; according to manufactures protocol). The pcDNA™3.1/His:Flt3L plasmid (Figure 2.2) was then used to transfect Freestyle HEK 293FT cells. On the day of transfection, cells were seeded at ~1-1.5 x 10^6/ml in 250ml in 1L flasks. Transfection reagent was made up in 40ml OptiPro (Invitrogen). 0.8ml of 200mM L-glutamine stock was added, giving a final concentration of 4mM. 1.25mg of the pcDNA™3.1/His:Flt3L was added and 1.875mg polyethylenimine (PEI). The reagent was mixed 5-10 times by inverting. After 10 minutes, 10ml of the reagent was added to the cells and the flasks were incubated for 3-4 days in a shaking incubator. Supernatant was collected from the cells and the protein purified using a HisTrap™ column (Cytiva) on an ÄKTA™ (Cytiva). Flt3L was resuspended at a concentration of 100µg/ml in PBS and stored at -80°C.

For *in vivo* use, 100µl (10µg) of Flt3L was injected intraperitoneally into mice daily for 4 days.

Table 2-3 - Flt3L insert components.

Flt3L insert	Key
CCGGAATTCGCCACCATG GAGACAGACACACTCCTG CTATGGGTACTGCTGCTCT GGGTTCCAGGTTCCACTG GTGACACTCAAGATTGCTC CTTTCAGCACAGTCCAATA AGCTCAGACTTTGCTGTGA AAATCCGAGAACTTTCCGA TTACCTGTTGCAAGATTAC CCCGTCACTGTTGCCTCAA ATTTGCAAGACGAGGAACT GTGTGGCGGGCTCTGGCG ACTTGTATTGGCACAGAGA TGGATGGAACGACTGAAG ACAGTTGCAGGATCTAAGA TGCAGGGCTTGTTGGAGC GAGTTAATACGGAAATACA TTTTGTTACGAAATGCGCA TTTCAGCCTCCACCCTCAT GTTTGCGATTTGTGCAAAC CAACATTTCCCGCTTGCTG CAGGAAACTTCTGAGCAA CTTGTGGCTCTCAAACCTT GGATCACGCGACAAAATTT TAGCAGATGCTTGGAACTT CAATGCCAACCTGACTCTA GTACCCTTCCTCCGCCCTG GTCCCCGAGACCACTGGA GGCTACAGCTCCTACCGCA CCTCAACCCCTCGAGCGG	• **EcoRI restriction site** CCGGAATTC • **XhoI restriction site** CTCGAGCGG • **Kozak sequence** GCCACC • **IgK chain leader sequence** ATGGAGACAGACACACTCCTGCTATGGGTACTGCTGCTCTGGGTTCC AGGTTCCACTGGTGAC • **Codon optimised human Flt3L gene sequence** ACTCAAGATTGCTCCTTTCAGCACAGTCCAATAAGCTCAGACTTTGCT GTGAAAATCCGAGAACTTTCCGATTACCTGTTGCAAGATTACCCCGTC ACTGTTGCCTCAAATTTGCAAGACGAGGAACTGTGTGGCGGGCTCT GGCGACTTGTATTGGCACAGAGATGGATGGAACGACTGAAGACAGTT GCAGGATCTAAGATGCAGGGCTTGTTGGAGCGAGTTAATACGGAAAT ACATTTTGTTACGAAATGCGCATTTCAGCCTCCACCCTCATGTTTGCG ATTTGTGCAAACCAACATTTCCCGCTTGCTGCAGGAAACTTCTGAGC AACTTGTGGCTCTCAAACCTTGGATCACGCGACAAAATTTTAGCAGAT GCTTGGAACTTCAATGCCAACCTGACTCTAGTACCCTTCCTCCGCCC TGGTCCCCGAGACCACTGGAGGCTACAGCTCCTACCGCACCTCAAC CC

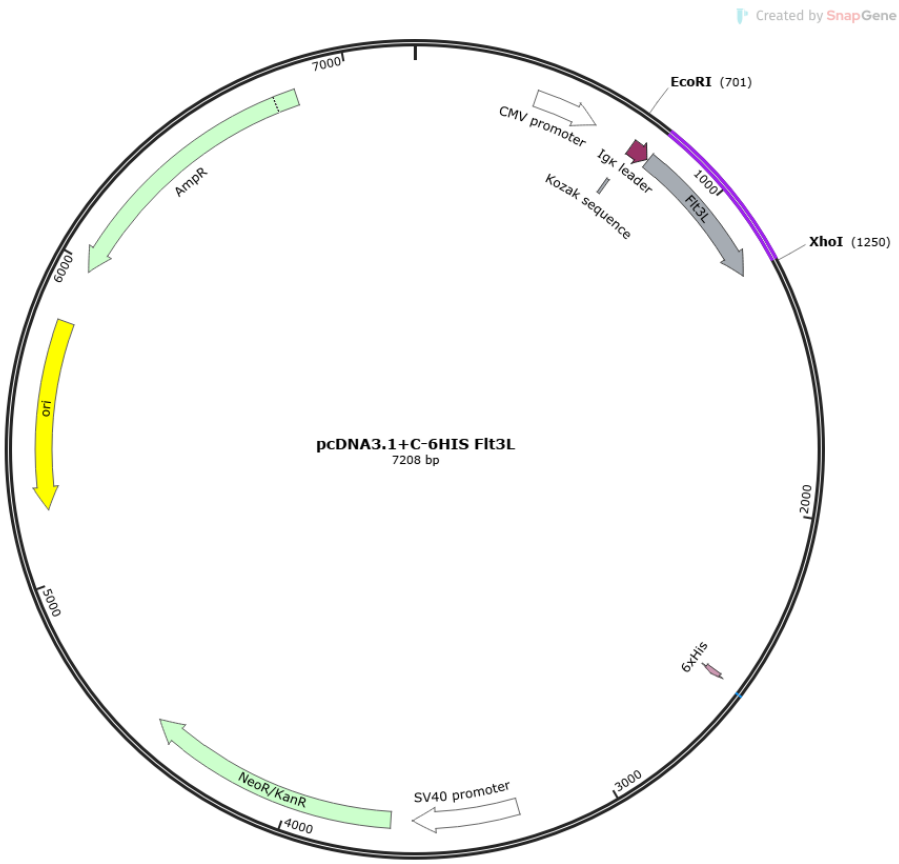


Figure 2-1 - pcDNA3.1+C-6HIS flt3L plasmid map

2.4 Bone marrow derived dendritic cell cultures

Bone marrow (BM) was harvested from murine hind legs and hips by crushing bones in a pestle mortar (Table 1). $1x10^5$ BM cells were plated in each well of a 6-well plate in 2ml R10 (RPMI 1640 + 10% Foetal Bovine Serum (FBS), + 1% 5000U/ml Penicillin-Streptomycin + 1% 1M HEPES + 1% MEM Non-Essential Amino Acids Solution (100X) + 1% 100X GlutaMAX™ Supplement + 0.2% 500nM EDTA (all from ThermoFisher Scientific, UK)) + 200ng/ml Flt3L and 10 ng/ml GM-CSF (Peprotech, UK) at 37°C + 5% CO_2. On day 3 of culture additional 200ng/ml Flt3L and 10 ng/ml GM-CSF was added and cells were cultured until day 7. Cells were analysed at various days of culture. Both adherent cells and suspension cells were collected for analysis (adherent cells were removed with ice cold PBS and a cell scraper).

2.5 Adoptive transfers

Bone marrow cells were sorted on a BD FACSAria™ III sorter with an 80µM nozzle at a flow rate of 1 into FB. Single, live, lineage negative (NK1.1, B220, CD3, CD19, CD11b) cells were sorted. MDPs ($cKit^{hi}$ $CD135^+$ $CX3CR1^+$); CDPs ($cKit^{lo}$ $CD115^+$); and preDCs ($CD11c^+$ $MHCII^-$ $CD135^+$ $Sirpa^-$) were separated sorted. Cells were injected intravenously into recipient mice

(control mice were injected with 100µl PBS only). Tissues were harvested 6 days post-transfer, digested, and stained with antibodies for flow cytometry analysis.

2.6 Haematopoietic stem cell cultures

Bone marrow from C57BL/6 mice was harvested and enriched for HSCs using positive magnetic selection. In brief, single, non-RBC lysed, cells were resuspended in PBS and incubated for 15 minutes on ice with anti-mouse Biotinylated cKit (1:200; Biolegend, UK). Following incubation, cells were washed in PBS and centrifuged for 5 minutes at 400xg. 100µl of MojoSort™ Nanobeads were added to cells in 1ml PBS and incubated for 15 minutes on ice. Cells were washed in PBS as before and resuspended in 2.5ml PBS. Cells were added to FACS tubes and the tube was placed in cell isolation magnet for 5 minutes. The fraction of cells bound to the tube walls was collected by pouring the non-HSC cells into waste while the tube remained in the magnet. The bound fraction was resuspended in 2.5ml PBS and this isolation step was repeated to improve the purity. $cKit^+$ cells were then resuspended in HSC enrichment media (made fresh; 958µl Ham's F12 + 10µl 100X PenStrep + 10µl 1M Hepes + 10µl 100mg/ml PVA + 1µl 100µg/ml TPO + 1µl 10µg/ml SCF) as described previously (Ochi et al. 2021). $1x10^6$ cells were added per well in a 24 well plate. Every few days media was gently removed from cells and fresh, pre-warmed media was replaced. Cells were moved into larger plates as they expanded.

2.7 Mixed bone marrow chimeras

Recipient mice were injected intraperitoneally with Busulfan (Merck)(15mg/kg) for 2 days. The following day, donor bone marrow was harvested, red blood cell lysed and reconstituted in PBS. 1×10^6 bone marrow cells were transplanted intravenously into recipient mice. Peripheral blood re-constitution was assessed by flow cytometry 8 weeks after transplantation at which point chimeras were used for further experiments. Chimeras made from *in vitro* HSCs were created in the same way with 1×10^6, 1×10^5, 1×10^4, 1×10^3 cultured cells or PBS as a control.

2.8 Transfections and Transductions

$2\text{-}2.5x10^6$ human embryonic kidney (HEK) cells were seeded in 10cm plates in D10 (DMEM, + 10% Foetal Bovine Serum (FBS), + 50U/ml Penicillin-Streptomycin + 1X MEM Non-Essential Amino Acids Solution + 1X GlutaMAX™ Supplement) and the following day were transfected as follows. D10 on the HEK cells was replaced with 7ml D10 + 25uM chloroquine. Transfection mix was made in a 15ml falcon: 16µg DNA, 6.5µg amphotrophic

helper virus, 415µl dH20, 62.5µl 250mM CaCl2, 500µl 2x HBSS (added last dropwise while creating bubbles in the transfection mix with a pipette gun). The 1ml of transfection mix was then added dropwise across the surface of the HEK cells and the plate was left unmoved for at least 2 minutes before being transferred to 37°C incubator. The following day, the media was changed on the transfected cells and target cells were seeded at $2.5x10^5$/well in a 6-well plate with 2ml of D10. The next day, the viral supernatant was collected from the HEK cells and centrifuged for 5 minutes at 1,500 rpm. 1/1000x Polybrene (Merck) was added to the viral supernatant. Media was removed from target cells and replaced with 1ml viral supernatant, 0.5ml D10 and 0.5ml 2x HBSS. Transduction was repeated the next day and two days later the cells were then sorted for transduced cells using a selection marker encoded in the plasmid. For suspension cells (EL4), transfection was performed as described, however, to transduce, viral supernatant was added to the cells and the plates were centrifuged for 60-90 minutes at 33°C at 2,000rcf. HSCs were transduced by adding 100,000 cells, in 500µL HSC media, to each well rectronectin coated 24 well plates. 100µL concentrated virus (concentrated with Lenti-X Concentrator (Tarkara)) was added to the cells and the plates were centrifuged for 60-90 minutes at 33°C at 2,000rcf. Cells were left in incubator for 2 days following transduction before analysing.

2.9 Chemokine overexpression

Chemokine protein coding sequences were identified from chemokine mRNA sequences (NCBI) with the ExPASy translation tool (Table 8.4). Sequences were synthesised (Integrated DNA Technologies, Inc) and ligated into the MSCV-IRES-Thy1.1 (addgene Plasmid #17442 Figure 2.3), digested with *BstBI-HF* and *SalI-HF*, with T4 ligase according to manufacturer's protcol. The modified plasmid was used to transduce EL4 cells as described in section 8.8. Control cells were transduced with an empty MSCV-IRES-Thy1.1 control. Transduced cells were sorted by expression of the selection marker Thy1.1. Sorted cells were re-sorted after one week in culture to improve purity. To confirm cell expression of CCL2, a CCL2 ELISA was carried out according to manufacturer's protocol (Mouse CCL2/JE/MCP-1 DuoSet ELISA; biotechne).

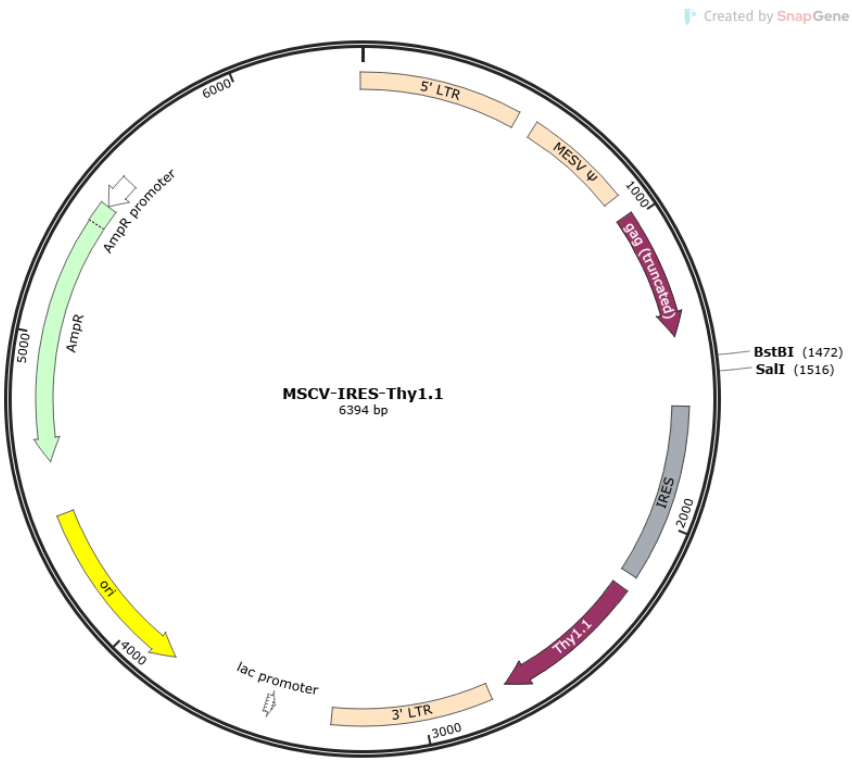


Figure 2-2 - MSCV-IRES-Thy1.1 plasmid map

Table 2-4 - Chemokine protein coding sequences

Chemokine	Protein Coding sequence	NCBI reference sequence
CCL2	ATGCAGGTCCCTGTCATGCTTCTGGGCCTGCTGTTCACAGTTGCCGGCTGGAGCATCCACGTGTTGGCTCAGCCAGATGCAGTTAACGCCCCACTCAC CTGCTGCTACTCATTCACCAGCAAGATGATCCCAATGAGTAGGCTGGAGAGCTACAAGAGGATCACCAGCAGCAGGTGTCCCAAAGAAGCTGTAGTT TTTGTCACCAAGCTCAAGAGAGAGGTCTGTGCTGACCCCAAGAAGGAATGGGTCCAGACATACATTAAAAACCTGGATCGGAACCAAATGAGATCAG AACCTACAAACTTTATTTAAAACTGCATCTGCCCTAAGGTCTTCAGCACCTTTGAATGTGAAGTTGACCCGTAAATCTGAAGCTAATGCATCCACTACCTT TTCCACAACCACCTCAAGCACTTCTGTAGGAGTGACCAGTGTGACAGTGAACTAG	NM_011333.3 Mus musculus chemokine (C-C motif) ligand 2 (Ccl2), mRNA
CCL7	AGGATCTCTGCCACGCTTCTGTGCCTGCTGCTCATAGCCGCTGCTTTCAGCATCCAAGTGTGGGCCCAACCAGATGGGCCCAATGCATCCACATGCTG CTATGTCAAGAAACAAAAGATCCCCAAGAGGAATCTCAAGAGCTACAGAAGGATCACCAGTAGTCGGTGTCCCTGGGAAGCTGTTATCTTCAAGACA AAGAAGGGCATGGAAGTCTGCGCTGAAGCCCATCAGAAGTGGGTCGAGGAGGCTATAGCATACTTAGACATGAAAACCCCAACTCCAAAGCCTTGA	NM_013654.3 Mus musculus chemokine (C-C motif) ligand 7 (Ccl7), mRNA
CXCL13	ATGAGGCTCAGCACAGCAACGCTGCTTCTCCTCCTGGCCAGCTGCCTCTCTCCAGGCCACGGTATTCTGGAAGCCCATTACACAAACTTAAAATGTAG GTGTTCTGGAGTGATTTCAACTGTTGTCGGTCTAAACATCATAGATCGGATTCAAGTTACGCCCCCTGGGAATGGCTGCCCCAAAACTGAAGTTGTGA TCTGGACCAAGATGAAGAAAGTTATATGTGTGAATCCTCGTGCCAAATGGTTACAAAGATTATTAAGACATGTCCAAAGCAAAAGTCTGTCTTCAACT CCCCAAGCTCCAGTGAGTAAGAGAAGAGCTGCCTGA	NM_018866.2 Mus musculus chemokine (C-X-C motif) ligand 13 (Cxcl13), mRNA
CX3CL1	ATGGCTCCCTCGCCGCTCGCGTGGCTGCTGCGCCTGGCCGCGTTCTTCCATTTGTGTACTCTGCTGCCGGGTCAGCACCTCGGCATGACGAAATGCGA AATCATGTGCGACAAGATGACCTCACGAATCCCAGTGGCTTTGCTCATCCGCTATCAGCTAAACCAGGAGTCCTGCGGCAAGCGTGCCATTGTCCTGG AGACGACACAGCACAGACGCTTCTGTGCTGACCCGAAGGAGAAATGGGTCCAAGACGCCATGAAGCATCTGGATCACCAGGCTGCTGCCCTCACTAA AAATGGTGGCAAGTTTGAGAAGCGGGTGGACAATGTGACACCTGGGATCACCTTGGCCACTAGGGGACTGTCCCCATCTGCCCTGACAAAGCCTGAA TCCGCCACATTGGAAGACCTTGCTTTGGAACTGACTACTATTTCCCAGGAGGCCAGGGGGACCATGGGGACTTCCCAAGAGCCACCGGCAGCAGTGA CCGGATCATCTCTCTCAACTTCCGAGGCACAGGATGCAGGGCTTACGGCTAAGCCTCAGAGCATTGGAAGTTTTGAGGCGGCTGACATCTCCACCACC GTTTGGCCGAGTCCTGCTGTCTACCAATCTGGATCTAGCTCCTGGGCTGAGGAAAAAGCTACTGAGTCCCCCTCCACTACAGCCCCATCTCCTCAGGT GTCCACTACTTCACCTTCAACCCCAGAGGAAAATGTTGGGTCCGAAGGCCAACCCCCATGGGTCCAGGGACAGGACCTCAGTCCAGAGAAGTCTCTA GGGTCTGAGGAGATAAACCCAGTTCATACTGATAATTTCCAGGAGAGGGGGCCTGGCAACACAGTCCACCCCTCAGTGGCTCCCATCTCCTCTGAAG AGACCCCCAGCCCAGAGCTGGTGGCCTCGGGCAGCCAGGCTCCTAAGATAGAGGAACCCATCCATGCCACTCGGCAGAAACTGAGTGTGCT TATCACTCCTGTCCCCGACACCCAGGCAGCCACAAGGAGGCAGGCAGTGGGGCTACTGGCTTTCCTTGGTCTTCTTTTCTGCCTAGGGGTGGCCATGT TTGCTTACCAGAGCCTTCAGGGCTGTCCCCGCAAAATGGCGGGGGAAATGGTAGAAGGCCTCCGCTACGTCCCCCGTAGCTGTGGCAGTAACTCATA CGTCCTGGTGCCAGTGTGA	NM_009142.3 Mus musculus C-X3-C motif chemokine ligand 1 (Cx3cl1), mRNA
CCL3	ATGAAGGTCTCCACCACTGCCCTTGCTGTTCTTCTCTGTACCATGACACTCTGCAACCAAGTCTTCTCAGCGCCATATGGAGCTGACACCCCGACTGCC TGCTGCTTCTCCTACAGCCGGAAGATTCCACGCCAATTCATCGTTGACTATTTTGAAACCAGCAGCCTTTGCTCCCAGCCAGGTGTCATTTTCCTGACTA AGAGAAACCGGCAGATCTGCGCTGACTCCAAAGAGACCTGGGTCCAAGAATACATCACTGACCTGGAACTGAATGCCTGA	NM_011337.2 Mus musculus chemokine (C-C motif) ligand 3 (Ccl3), mRNA
CCL4	ATGAAGCTCTGCGTGTCTGCCCTCTCTCTCCTCTTGCTCGTGGCTGCCTTCTGTGCTCCAGGGTTCTCAGCACCAATGGGCTCTGACCCTCCCACTTCCT GCTGTTTCTCTTACACCTCCCGGCAGCTTCACAGAAGCTTTGTGATGGATTACTATGAGACCAGCAGTCTTTGCTCCAAGCCAGCTGTGGTATTCCTGA CCAAAAGAGGCAGACAGATCTGTGCTAACCCCAGTGAGCCCTGGGTCACTGAGTACATGAGTGACTTGGAGTTGAACTGA	NM_013652.2 Mus musculus chemokine (C-C motif) ligand 4 (Ccl4), mRNA
CXCL16	ATGAGGCGGGGCTTTGGACCCTTGTCTCTTGCGTTCTTCCTTTTCTTGTTGGCGCTGCTGACCCTGCCAGGCGATGGCAACCAGGGCAGTGTCGCTGG AAGTTGTTCTTGTGATCGTACCATTTCTTCTGGCACCCAGATACCGCAGGGTACTTTGGATCACATCCGAAAATACCTGAAAGCATTTCATCGTTGTCC ATTCTTTATCAGGTTCCAGTTGCAGTCCAAAAGCGTGTGTGGGGGAAGCCAAGACCAGTGGGTCCGTGAACTAGTGGACTGCTTTGAGCGCAAAGAG TGTGGAACTGGTCATGGGAAGAGTTTTCACCACCAAAAACATTTGCCTCAAGCCAGTACCCAGACCCCTGAGGCCGCAGAGGGGACACCTTCGGACA CGAGCACCCCTGCACATAGTCAGAGCACTCAGCACTCCACTCTTCCATCAGGAGCACTGTCCTTAAACAAAGAGCACACCCAACCCTGGGAGATGACC ACTCTCCCTTCAGGCTATGGTCTGGAAGCTAGGCCTGAGGCTGAGGCAAATGAGAAACAGCAAGATGACAGACAGCAAGAAGCACCAGGAGCTGGA GCTAGCACACCAGCTTGGGTACCGGTGCTGTCCCTCCTGGCCATTGTCTTCTTCCTCACTGCAGCCATGGCCTATGTGCTGTGCAACAGGAGAGCGAC ACAGCAGAACTCTGCAGGTTTGCAGCTCTGGTACACTCCTGTTGAACCAAGACCCTAG	NM_023158.7 Mus musculus chemokine (C-X-C motif) ligand 16 (Cxcl16), transcript variant 1, mRNA
CCL19	ATGGCCCCCCGTGTGACCCCACTCCTGGCCTTCAGCCTGCTGGTTCTCTGGACCTTCCCAGCCCCAACTCTGGGGGGTGCTAATGATGCGGAAGACTG CTGCCTGTCTGTGACCCAGCGCCCCATCCCTGGGAACATCGTGAAAGCCTTCCGCTACCTTCTTAATGAAGATGGCTGCAGGGTGCCTGCTGTTGTGT TCACCACACTAAGGGGCTATCAGCTCTGTGCACCTCCAGACCAGCCCTGGGTGGATCGCATCATCCGAAGACTGAAGAAGTCTTCTGCCAAGAACAA AGGCAACAGCACCAGAAGGAGCCCTGTGTCTTGA	NM_011888.4 Mus musculus C-C motif chemokine ligand 19 (Ccl19), mRNA
CCL21	ATGGCTCAGATGATGACTCTGAGCCTCCTTAGCCTGGTCCTGGCTCTCTGCATCCCCTGGACCCAAGGCAGTGATGGAGGGGGTCAGGACTGCTGCC TTAAGTACAGCCAGAAGAAAATTCCCTACAGTATTGTCCGAGGCTATAGGAAGCAAGAACCAAGTTTAGGCTGTCCCATCCGGCAATCCTGTTCTCA CCCCGGAAGCACTCTAAGCCTGAGCTATGTGCAAACCCTGAGGAAGGCTGGGTGCAGAACCTGATGCGCCGCCTGGACCAGCCTCCAGCCCCAGGG AAACAAAGCCCCGGCTGCAGGAAGAACCGGGGAACCTCTAAGTCTGGAAAGAAAGGAAAGGGCTCCAAGGGCTGCAAGAGAACTGAACAGACACA GCCCTCAAGAGGATAG	NM_011124.4 Mus musculus chemokine (C-C motif) ligand 21a (serine) (Ccl21a), mRNA
CCL5	ATGAAGATCTCTGCAGCTGCCCTCACCATCATCCTCACTGCAGCCGCCCTCTGCACCCCCGCACCTGCCTCACCATATGGCTCGGACACCACTCCCTGC TGCTTTGCCTACCTCTCCCTCGCGCTGCCTCGTGCCCACGTCAAGGAGTATTTCTACACCAGCAGCAAGTGCTCCAATCTTGCAGTCGTGTTTGTCACTCC GAAGGAACCGCCAAGTGTGTGCCAACCCAGAGAAGAAGTGGGTTCAAGAATACATCAACTATTTGGAGATGAGCTAG	NM_013653.3 Mus musculus chemokine (C-C motif) ligand 5 (Ccl5), mRNA
XCL1	ATGAGACTTCTCCTCCTGACTTTCCTGGGAGTCTGCTGCCTCACCCCATGGGTTGTGGAAGGTGTGGGGACTGAAGTCCTAGAAGAGAGTAGCTGTG TGAACTTACAAACCCAGCGGCTGCCAGTTCAAAAAATCAAGACCTATATCATCTGGGAGGGGGCCATGAGAGCTGTAATTTTTGTCACCAAACGAGG ACTAAAAATTTGTGCTGATCCAGAAGCCAAATGGGTGAAAGCAGCGATCAAGACTGTGGATGGCAGGGCCAGTACCAGAAAGAACATGGCTGAAAC TGTTCCCACAGGAGCCCAGAGGTCCACCAGCACAGCGATAACCCTGACTGGGTAA	NM_008510.3 Mus musculus chemokine (C motif) ligand 1 (Xcl1), mRNA
CXCL9	ATGAAGTCCGCTGTTCTTTTCCTCTTGGGCATCATCTTCCTGGAGCAGTGTGGAGTTCGAGGAAACCCTAGTGATAAGGAATGCACGATGCTCCTGCAT CAGCACCAGCCGAGGCACGATCCACTACAAATCCCTCAAAGACCTCAAACAGTTTGCCCCAAGCCCCAATTGCAACAAAACTGAAATCATTGCTACAC TGAAGAACGGAGATCAAACCTGCCTAGATCCGGACTCGGCAAATGTGAAGAAGCTGATGAAAGAATGGGAAAAGAAGATCAGCCAAAAGAAAAAG CAAAAGAGGGGGAAAAAACATCAAAAGAACATGAAAAACAGAAAACCCAAAACACCCCAAAGTCGTCGTCGTTCAAGGAAGACTACATAAGAGACC ATTACTTTACCAACAAGCACCCTGAATCTTAATGGGTTTTAGATTGTACTGAAAAGCCTTCCCTGGCAGAGCAGCCTTTAATACATAG	NM_008599.4 Mus musculus chemokine (C-X-C motif) ligand 9 (Cxcl9), mRNA
CXCL10	ATGAACCCAAGTGCTGCCGTCATTTTCTGCCTCATCCTGCTGGGTCTGAGTGGGACTCAAGGGATCCCTCTCGCAGGACGGTCCGCTGCAACTGCATC CATATCGATGACGGGCCAGTGAGAATGAGGGCCATAGGGAAGCTTGAAATCATCCCTGCGAGCCTATCCTGCCCACGTGTTGAGATCATTGCCACGA TGAAAAAGAATGATGAGCAGAGATGTCTGAATCCGGAATCTAAGACCATCAAGAATTTAATGAAAGCGTTTAGCCAAAAAAGGTCTAAAAGGGCTCC TTAA	NM_021274.2 Mus musculus chemokine (C-X-C motif) ligand 10 (Cxcl10), mRNA
CXCL11	ATGAACAGGAAGGTCACAGCCATAGCCCTGGCTGCGATCATCTGGGCCACAGCTGCTCAAGGCTTCCTTATGTTCAAACAGGGGCGCTGTCTTTGCAT CGGCCCCGGGATGAAAGCCGTCAAAATGGCAGAGATCGAGAAAGCTTCTGTAATTTACCCGAGTAACGGCTGCGACAAAGTTGAAGTGATTGTTACT ATGAAGGCTCATAAACGACAAAGGTGCCTGGACCCCAGATCCAAGCAAGCTCGCCTCATAATGCAGGCAATAGAAAAAAAGAATTTTTTAAGGCGTC AAAACATGTGA	NM_019494.1 Mus musculus chemokine (C-X-C motif) ligand 11 (Cxcl11), transcript variant 1, coding, mRNA
CCL1	ATGAAACCCACTGCCATGGCACTGATGTGCCTGCTGCTGGCTGCCGTGTGGATACAGGATGTTGACAGCAAGAGCATGCTTACGGTCTCCAATAGCT GCTGCTTGAACACCTTGAAGAAAGAGCTTCCCCTGAAGTTTATCCAGTGTTACAGAAAGATGGGCTCCTCCTGTCCTGATCCCCCAGCTGTGGTATTCA GGCTGAACAAAGGTAGAGAAAGCTGCGCCTCAACTAACAAAACGTGGGTTCAAAATCACCTGAAGAAGGTGAACCCCTGCTAACCGGGGTGAAGAT GAGCCACCTTCCCATCCCAGCACCAGAGACTGCAGTTTCCCTCAGTCCTCCACCCTCCCA	NM_011329.3 Mus musculus chemokine (C-C motif) ligand 1 (Ccl1), mRNA
CCL8	ATGAAGATCTACGCAGTGCTTCTTTGCCTGCTGCTCATAGCTGTCCCTGTCAGCCCAGAGAAGCTGACTGGGCCAGATAAGGCTCCAGTCACCTGCTG CTTTCATGTACTAAAGCTGAAGATCCCCCTTCGGGTGCTGAAAAGCTACGAGAGAATCAACAATATCCAGTGCCCCATGGAAGCTGTGGTTTTCCAGA CCAAGCAGGGTATGTCTCTCTGTGTAGACCCCACACAGAAGTGGGTCAGTGAGTACATGGAGATCCTTGACCAGAAGTCTCAAATTCTGCAGCCTTG A	NM_021443.3 Mus musculus chemokine (C-C motif) ligand 8 (Ccl8), mRNA
CCL20	ATGGCCTGCGGTGGCAAGCGTCTGCTCTTCCTTGCTTTGGCATGGGTACTGCTGGCTCACCTCTGCAGCCAGGCAGAAGCAGCAAGCAACTACGACT GTTGCCTCTCGTACATACAGACGCCTCTTCCTTCCAGAGCTATTGTGGGTTTCACAAGACAGATGGCCGATGAAGCTTGTGACATTAATGCTATCATCT TTCACACGAAGAAAAGAAAATCTGTGTGCGCTGATCCAAAGCAGAACTGGGTGAAAAGGGCTGTGAACCTCCTCAGCCTAAGAGTCAAGAAGATGT AA	NM_016960.2 Mus musculus chemokine (C-C motif) ligand 20 (Ccl20), transcript variant 1, mRNA
CCL17	ATGAAGACCTTCACCTCAGCTTTTGGTACCATGAGGTCACTTCAGATGCTGCTCCTGGCTGCTCTGCTTCTGGGGACTTTTCTGCAGCATGCCAGAGCT GCTCGAGCCACCAATGTAGGCCGAGAGTGCTGCCTGGATTACTTCAAAGGGGCCATTCCTATCAGGAAGTTGGTGAGCTGGTATAAGACCTCAGTGG AGTGTTCCAGGGATGCCATCGTGTTTCTGACTGTCCAGGGCAAGCTCATCTGTGCAGACCCCAAAGACAAACATGTGAAGAAGGCCATCAGATTGGT GAAAAACCCAAGGCCATGA	NM_011332.3 Mus musculus chemokine (C-C motif) ligand 17 (Ccl17), mRNA
CCL22	ATGGCTACCCTGCGTGTCCCACTCCTGGTGGCTCTCGTCCTTCTTGCTGTGGCAATTCAGACCTCTGATGCAGGTCCCTATGGTGCCAATGTGGAAGAC AGTATCTGCTGCCAGGACTACATCCGTCACCCTCTGCCATCACGTTTAGTGAAGGAGTTCTTCTGGACCTCAAAATCCTGCCGCAAGCCTGGCGTTGTT TTGATAACCGTCAAGAACCGAGATATCTGTGCCGATCCCAGGCAGGTCTGGGTGAAGAAGCTACTCCATAAACTGTCCTAG	NM_009137.2 Mus musculus chemokine (C-C motif) ligand 22 (Ccl22), mRNA
CXCL12	ATGGACGCCAAGGTCGTCGCCGTGCTGGCCCTGGTGCTGGCCGCGCTCTGCATCAGTGACGGTAAACCAGTCAGCCTGAGCTACCGATGCCCCTGCC GGTTCTTCGAGAGCCACATCGCCAGAGCCAACGTCAAGCATCTGAAAATCCTCAACACTCCAAACTGTGCCCTTCAGATTGTTGCACGGCTGAAGAAC AACAACAGACAAGTGTGCATTGACCCGAAATTAAAGTGGATCCAAGAGTACCTGGAGAAAGCTTTAAACAAGTAA	NM_021704.3 Mus musculus chemokine (C-X-C motif) ligand 12 (Cxcl12), transcript variant 1, mRNA
RARRES2	ATGAAGTGCTTGCTGATCTCCCTAGCCCTATGGCTGGGCACAGTGGGCACACGTGGGACAGAGCCCGAACTCAGCGAGACCCAGCGCAGGAGCCTA CAGGTGGCTCTGGAGGAGTTCCACAAACACCCACCTGTGCAGTTGGCCTTCCAAGAGATCGGTGTGGACAGAGCTGAAGAAGTGCTCTTCTCAGCTG GCACCTTTGTGAGGTTGGAATTTAAGCTCCAGCAGACCAACTGCCCCAAGAAGGACTGGAAAAAGCCGGAGTGCACAATCAAACCAAACGGGAGAA GGCGGAAATGCCTGGCCTGCATTAAAATGGACCCCAAGGGTAAAATTCTAGGCCGGATAGTCCACTGCCCAATTCTGAAGCAAGGGCCTCAGGATCC TCAGGAGTTGCAATGCATTAAGATAGCACAGGCTGGCGAAGACCCCCACGGCTACTTCCTACCTGGACAGTTTGCCTTCTCCAGGGCCCTGAGAACCA AATAA	NM_027852.3 Mus musculus retinoic acid receptor responder (tazarotene induced) 2 (Rarres2), transcript variant 2, mRNA
CCL21	ATGGCTCAGATGATGACTCTGAGCCTCCTTAGCCTGGTCCTGGCTCTCTGCATCCCCTGGACCCAAGGCAGTGATGGAGGGGGTCAGGACTGCTGCC TTAAGTACAGCCAGAAGAAAATTCCCTACAGTATTGTCCGAGGCTATAGGAAGCAAGAACCAAGTTTAGGCTGTCCCATCCGGCAATCCTGTTCTCA CCCCGGAAGCACTCTAAGCCTGAGCTATGTGCAAACCCTGAGGAAGGCTGGGTGCAGAACCTGATGCGCCGCCTGGACCAGCCTCCAGCCCCAGGG AAACAAAGCCCCGGCTGCAGGAAGAACCGGGGAACCTCTAAGTCTGGAAAGAAAGGAAAGGGCTCCAAGGGCTGCAAGAGAACTGAACAGACACA GCCCTCAAGAGGATAG	NM_011124.4 Mus musculus chemokine (C-C motif) ligand 21a (serine) (Ccl21a), mRNA

2.10 Tumour injections

Tumour cells were cultured at 37°C and 5% CO_2 in D10. For subcutaneous tumour injections, $2x10^6$ EL4 cells, $5x10^5$ B16ZsGreen-minOVA or $1x10^6$ B16-F10 cells were resuspended in 25µl of Matrigel (Corning Life Science) and 25µl of RPMI and injected subcutaneously. Once endpoint, defined as $15mm^2$ x $15mm^2$, was reached tumours and tdLN were harvested and processed for flow cytometry.

2.11 Chemotaxis associated gene expression analysis

Murine microarray (GSE60782) and scRNAseq (GDE60781) data were acquired from Schlitzer and colleagues (Schlitzer et al. 2015b). Bulk RNAseq was acquired from Lutz and colleagues (GSE189780) and Cabeza-Cabrerizo and colleagues (GSE18383) (Cabeza-Cabrerizo, Minutti, et al. 2021a; Lutz et al. 2022). Analysis was carried out on R-studio (version 1.2.5033). For analysis of microarray data, genes with ontology classified as involved in chemotaxis (GO:0016493) and possessing receptor activity (GO:0004950) were selected, filtered for the top 11 receptors expressed on preDCs and Z-scores for each gene were calculated across the replicates of each population (pre-cDC1 and pre-cDC2). Genes were arranged by Z-score in the pre-cDC1 population highlighting the genes most overexpressed in that population. scRNAseq data of sorted CDP, MDP and preDCs was analysed using Seurat (Butler et al. 2018) and expression of chemokine receptors were analysed. Chemokine receptor expression of sorted preDCs was isolated from bulk RNAseq data.

For our own scRNAseq of preDCs, we harvested BM from three 4-week-old C57BL/6 male mice. Cells were enriched for preDCs using negative magnetic selection. In brief, cells were resuspended in PBS, anti-mouse Biotin Ter119, CD3, CD19 (Biolegend, UK) was added to cell suspension and incubated for 15 minutes on ice. Following incubation, cells were washed in PBS and centrifuged for 5 minutes at 400g. 100µl of MojoSort™ Nanobeads were added to cells in 1ml PBS and incubated for 15 minutes on ice. Cells were washed in PBS as before and resuspended in 2.5ml PBS. Cells were added to FACS tubes and tube was placed in cell isolation magnet for 5 minutes. The fraction of cells not bound to the tube walls was collected by pouring the cells into a falcon tube while the tube remained in the magnet. This isolation step was repeated on the collected fraction to improve the purity. Cells were then stained using flow cytometry antibodies as described in section 8.2. PreDCs were sorted as single live $CD45^+$, lineage negative (B220, NK1.1, CD90.1, F4/80, ly6D) $CD11c^+$ $MHCII^-$ $CD11b^{-/lo}$ $CD135^+$ $Sirp\alpha^{-/int}$ $CD43^+$ on a BD FACSAria™ III Cell Sorter into FACS buffer.

~30,000 preDCs were sorted from each bone marrow and 10,000 from each spleen. The next steps were carried out by the omics facility at the Beatson Institute for Cancer Research. Briefly, cell suspensions were centrifuged for 5 minutes at 1000xg at 4°C and as much supernatant removed as possible without disturbing the cell pellet. The cells were then resuspended in 50µL of cell staining buffer (CSB) and blocked by the addition of 0.5µL of TruStain FcX™ PLUS (anti-mouse CD16/32) antibody (BioLegend) for 10 min at 4°C. 4µL each Hashtag TotalSeq™ antibodies (#1,#2,#3,#4,#5 and #6) in a total volume of 50µL of CSB was then added to the 50µL of blocked cell suspension and incubated for a further 30 minutes at 4°C. A total of three washes using 1mL of CSB was then performed, with the final wash being passed through a 40µm nylon cell strainer (BD Falcon) and the cells resuspended in 50µL of CSB. Cell number and viability was assessed using a haemocytometer after staining with Trypan Blue. Cells were then pooled at equal numbers in a final volume of 43.1µL and a total of 10,000 cells. This cell suspension was then processed through 10X Genomics Chromium controller using the Single Cell Gene Expression 3' v3.1 kit (10X Genomics, Chromium Next GEM Single Cell 3' Kit v3.1) to generate an emulsion which was first reverse transcribed and then PCR amplified to generate cDNA. Sequencing libraries were then generated using 10µl of cDNA as outlined in the 10X Genomics protocol (CG000315 Rev C), with the only change being the addition of 1µL of 0.2µM of HTO Additive Primer v2 (BioLegend) to cDNA Amplification step. Briefly, cDNA was first fragmented, end repaired and adaptors ligated, followed by PCR amplification and size selection to generate final libraries which were sequenced on a NovaSeq S4 flowcell (Illumina) to a depth of 25,000 reads per cell. Cell surface Hashtag libraries were generated from the supernatant containing the HTO index resulting from the bead clean up step post cDNA amplification. This supernatant underwent a SPRI select clean up and PCR amplified using the QuantaBio PCR mix utilizing HTO i5 and i7 primers as set out in the table below with 15 cycles of amplification.

Correlation analysis of human cDC and chemokine genes was carried out on the GEPIA interactive software (Z. Tang et al. 2017). Human cDC1s were identified by expression of Xcr1, Clec91 and Clnk while cDC2s were identified by expression of Cd1c, Fcer1a and Cd1d.

2.12 Statistical Analysis

Statistical analysis was performed using GraphPad Prism 8.3.0 software. In general, statistical significance between two groups was determined by a paired t-test and one-way

ANOVAs with multiple comparisons were used for more than two groups, as specified for each data set. *P < 0.05, **P < 0.01, ***P < 0.001. Specific statistical tests are listed in figure legends.

2.13 Illustrations

All illustrations were created using BioRender, SnapGene or Adobe Illustrator with academic subscriptions.

3 Chapter 1: preDC migration heterogeneity

3.1 Introduction

cDCs develop from a process known as DC-poesis in which self-renewing multipotent hematopoietic stem cells (HSCs) give rise to lineage specific blood and immune cells in the bone marrow (Cabeza-Cabrerizo, Cardoso, et al. 2021a). To understand the mechanisms used by preDCs to develop and migrate from the bone marrow to the peripheral tissues, insight can be gained from their transcriptional profile and expression of surface markers. PreDCs, in general, are phenotypically identified by their lack of expression of other lineage specific markers and CD11c^{+} MHCII^{-} CD135^{+} Sirpα^{-} CX$_3$CR1^{+} CD43^{+} (Cabeza-Cabrerizo, Minutti, et al. 2021a; Pereira da Costa et al. 2023a; Schlitzer et al. 2015a). Subsets and developmental states can be further categorised: SiglecH^{+}Ly6C^{+} cells are known as non-committed preDC (pre-preDC) which have the potential to develop into pre-cDC1 (SiglecH^{-} Ly6C^{-}) and pre-cDC2 (SiglecH^{-} Ly6C^{+}), definitions which have been previously defined ((Cabeza-Cabrerizo, Minutti, et al. 2021a;Schlitzer et al. 2015a). However, it is important to note that different researchers use alternative strategies to identify preDC subsets. For example Pre-cDC1s have been identified as Lin^{-} CD117^{int} CD135^{+} Zbtb46-GFP^{+} CD226^{+} and pre-cDC2s were identified as Lin^{-} CD117^{lo} CD135^{+} CD115^{+} (Durai et al. 2019). Similar to murine preDCs, subsets can also be identified in humans (Breton et al. 2016; See et al. 2017; Villani et al. 2017). At which point cDC subset commitment occurs remains unclear. Using scRNAseq analysis, Schlitzer and colleagues identified that gene signatures associated with the cDC general lineage were identified as early as the MDP stage and increased within CDPs to preDCs. Interestingly, subset specific signatures for cDC2 could be seen transcriptionally at the CDP stage while cDC1 signatures could only be identified at the preDC stage (Grajales-Reyes et al. 2015; Schlitzer et al. 2015). Differentiated pre-cDC1 and pre-cDC2 exit the bone marrow, a process associated with loss of CXCR4 expression and by gain of CCR2 (Pereira da Costa et al. 2023a). Within the blood, preDCs have a short half-life of less than two hours (Kang Liu et al. 2007) suggesting that replenishment of the tissues with preDCs occurs rapidly. In terms of chemotaxis, defining the expression of chemokine receptors on preDC subsets will allow us to begin to understand the process. PreDCs in general have been shown to express CXCR4, CCR2 and CX$_3$CR1 (Nakano et al. 2016a). Later, subset specific expression of CXCR3 and CCR5 was identified on pre-cDC1s while CX$_3$CR1 was identifiedon pre-cDC2s (Cook et al. 2018a). XCR1 has also been reported to be expressed on pre-cDC1s and not pre-cDC2s (Lança et al. 2022) and high CCR2 was associated with pre-cDC1 (Schlitzer et al. 2015b). Despite this, we

still do not know the specific signals which control preDC migration and whether these signals are cell and tissue specific. Additionally, there are suggestions that these signals change depending on the context of the tissue, for instance CCR2 was shown to be unnecessary for recruitment to the homeostatic lung but required during inflammation (Cabeza-Cabrerizo, Minutti, et al. 2021a; Pereira da Costa et al. 2023a). Therefore, we aimed to characterise the signalling pathways directing preDC chemotaxis. This chapter describes our process in defining preDC chemokine receptor expression to create a list of candidate receptors and ligands to further study.

3.2 Results

3.2.1 preDCs express chemokine receptors

Initially, we utilised publicly available datasets to analyse the expression of chemokine receptors on preDCs in general. scRNAseq (GDE60781) data were acquired from Schlitzer and colleagues (Schlitzer et al. 2015b) and bulk RNAseq was acquired from Lutz and colleagues (GSE189780) and Cabeza-Cabrerizo and colleagues (GSE18383) (Cabeza-Cabrerizo, Minutti, et al. 2021a; Lutz et al. 2022) (Figure 3.1 A, B and C). scRNAseq data was analysed using the standard Seurat pipeline (Butler et al. 2018) and expression of chemokine receptors were analysed. Chemokine receptor expression of preDCs was isolated from bulk RNAseq data. This analysis allowed us to compile a list of 11 chemokine receptors expressed on preDCs in all three sets: CCR1, 2, 5, 7, 9, CXCR2, 3, 4, XCR1, CX_3CR1 and GpR35 (Figure 3.1 D). These data align with previous reports of preDC chemokine receptor expression (Cook et al. 2018b; Lança et al. 2022; Nakano et al. 2017).

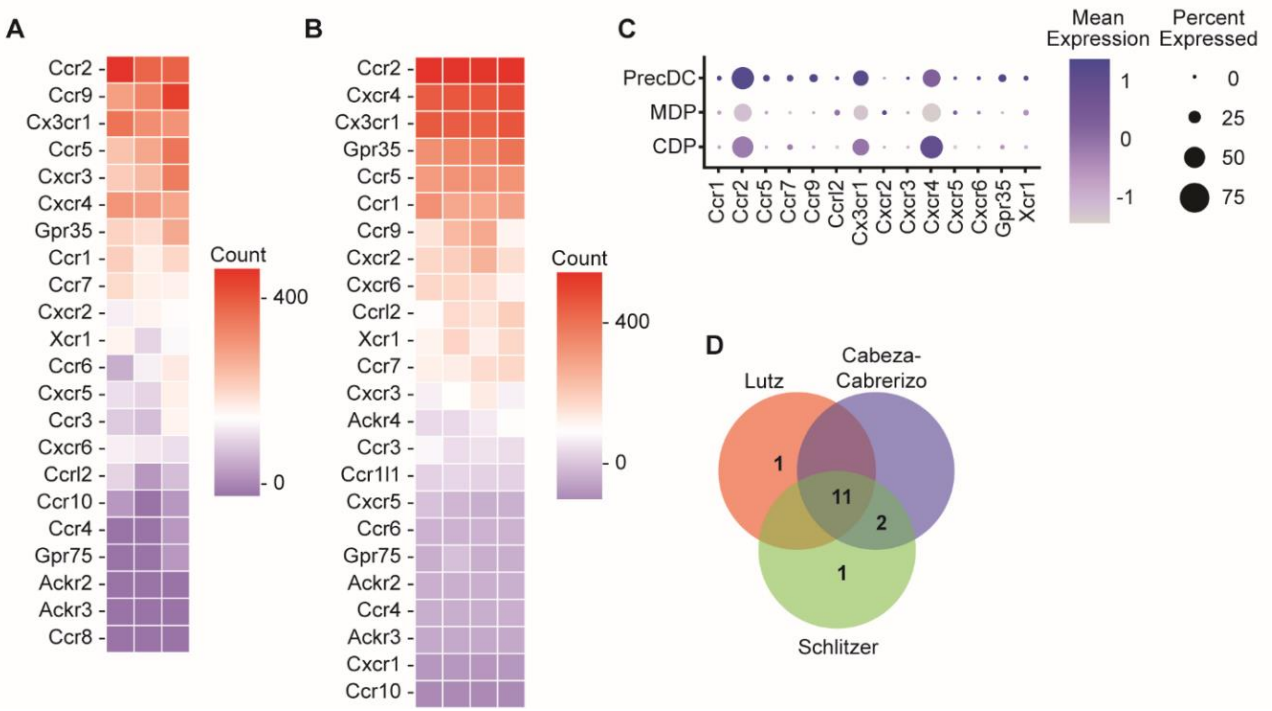


Figure 3-1 – Level of expression of chemokine receptors on bone marrow preDCs. *Bulk RNAseq A) GSE18383; Cabeza-Cabrerizo, Minutti, et al. 2021 and B) GSE189780; Lutz et al. 2022. Columns represent individual biological replicates of sorted preDCs. Data was organised from highest to lowest gene expression. scRNAseq C) GDE60781; Schlitzer et al., 2015 dataset analysis was carried out on R-studio (version 1.2.5033). scRNAseq data of sorted MDP, CDP and preDC was analysed using Seurat (Butler et al. 2018) and expression of chemokine receptors was analysed. Chemokine receptor expression of preDCs was isolated from bulk RNAseq data.*

3.2.2 Chemokine receptor expression is heterogeneous between preDC subsets.

These datasets allowed us to identify chemokine receptor expression on preDCs, however, as cDC subsets differ in terms of phenotype and function (Cabeza-Cabrerizo, Cardoso, et al. 2021a), we wanted to investigate if this heterogeneity also contributed to preDC migration. To do this, we utilised another publicly available dataset, this time microarray data (GSE60782) of sorted preDC subsets (Schlitzer et al. 2015b). Genes with ontology classified as involved in chemotaxis (GO:0016493) and possessing receptor activity (GO:0004950) were selected, filtered for the top 11 receptors expressed on preDCs and Z-scores for each gene were calculated across the replicates of each population (pre-cDC1 and pre-cDC2). Although preDC expression of CXCR2 was picked up by RNAseq analysis, the gene was not identified by microarray analysis. Genes were arranged by Z-score in the pre-cDC1 population highlighting the genes most overexpressed in that population and we were able to detect which receptors were differentially expressed between the two subsets. CXCR3, CCR7, CCR5 and XCR1 were expressed at a higher level on pre-cDC1s compared to pre-

cDC2s while CX_3CR1, CXCR4, CCR9 and CCR2 were higher on pre-cDC2s. Promisingly, these results aligned with previous literature on preDC chemokine receptor heterogeneity whereby CXCR3, XCR1 and CCR5 was shown to be expressed highest on pre-cDC1s while CX3CR1 was on pre-cDC2s (Cook et al. 2018a; Lança et al. 2022). Interestingly, CCR2 was previously associated with pre-cDC1s (Schlitzer et al. 2015b) while here we identified it differentially expressed on pre-cDC2s. As far as we are aware, CCR7 has not been reported to display differential expression on pre-cDC1s, nor CCR9 and CXCR4 on pre-cDC2s.

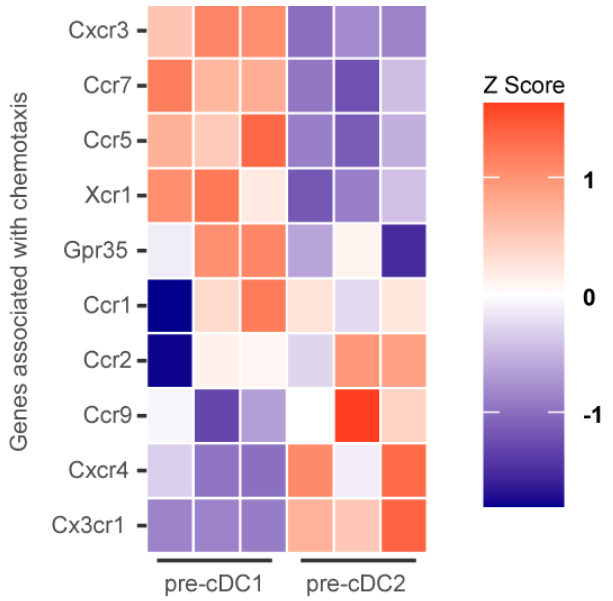


***Figure 3-2 - preDC subsets express distinct chemokine receptors at the RNA level**. Microarray data of bone marrow preDCs (Schlitzer et al., 2015) were analysed. Genes with ontology classified as involved in chemotaxis (GO:0016493) and possessing receptor activity (GO:0004950) were selected, filtered for the top 11 receptors expressed and Z-scores for each gene were calculated across the replicates of each population. Heatmap shows the Z-scores of 3 individual biological replicates of each preDC subsets. Genes were arranged by Z-score in the pre-cDC1 population highlighting the genes most overexpressed in that population. Analysis was carried out using R studio.*

To confirm this heterogeneity at the protein level, CCR1, 2, 3 and 5 were analysed by flow cytometry by the use of transgenic chemokine receptor reporter mice (iCCReporter; gifted from Professor Gerald Graham) (Medina-Ruiz et al. 2022). iCCReporters were created using counterselection recombineering (Wang et al. 2014) to allow the expression of genes along with the iCCR receptors: CCR1 is expressed with Clover, CCR2 with mRuby2, CCR3 with mTagBFP2, and CCR5 with iRFP682 (Figure 3.3 A). However, a limitation of these mice is that they report transcript level and expression may be appear higher due to the half-lives of the flourochromes. However, iCCReporter expression was suggetsed to accurately replicate surface protein expression, as confirmed by flow cytometry analysis (Medina-Ruiz et al. 2022).

By analysing the receptor expression on the non-committed preDCs as well as the dedicated subsets, we were able to identify the changes in the expression of these chemokine receptors throughout the development of preDCs as well as between the committed subsets (Figure 3.3 B). Aligning with our microarray analysis, in the bone marrow CCR2 was more highly expressed on pre-cDC2s compared to pre-cDC1s and expression increased in general from non-committed states to committed. This can be seen by frequency of preDCs expressing CCR2 (Figure 3.3 B). Conversely, CCR5 was higher on non-committed preDCs and decreased upon commitment (Figure 3.3 B). Similar to the microarray dataset, CCR1 was expressed equally between pre-cDC1 and pre-cDC2s. Since CCR3 was identified as highly expressed on preDCs in the bulk RNAseq and not the scRNAseq publicly available datasets (Figure 3.3 A), we had excluded it from further analysis of the microarray data. However, here we identified differential expression on pre-cDC1s. This was confirmed for CCR2 and CCR3 when the MFI of iCCRs were pooled from three separate experiments (Figure 3.3 C). However, no significant difference was identified between subsets of preDCs when the MFI of CCR5 was calculated from the pooled experiments. Further analysis is needed to confirm the differential chemokine receptor expression of preDC subsets.

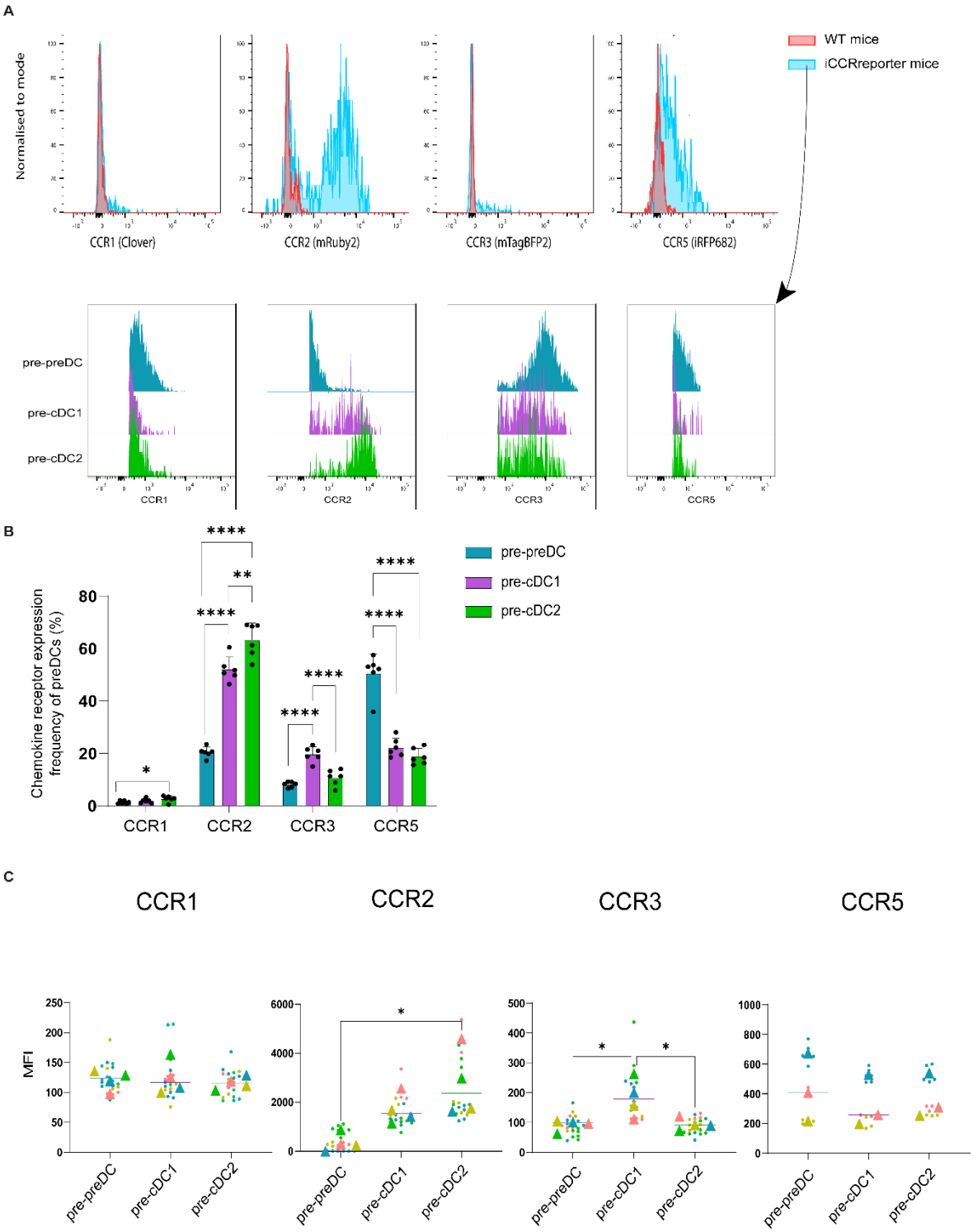


Figure 3-3 - preDC subsets express distinct chemokine receptors at the protein level*. Bone marrow cells of A) iCCReporter mice were analysed by flow cytometry and MFI of CCR1, CCR2, CCR3 and CCR5 was calculated. Histograms show MFI of all preDC subsets and in the bottom panel individual preDC subsets are shown. B) Frequency of pre-preDC, pre-cDC1 and pre-cDC2s expressing each receptor are shown. Data represent percentage ±SD from one independent experiment with n=6 mice and represent data from three separate experiments. One-way ANOVA statistical analysis. C) shows the pooled MFIs of CCR1,2,3 and 5 from three independent experiments. Superplots show individual data points of which colours represent data from separate experiments. Triangle symbols show the average expression from separate experiments and lines*

*display the median of these. One-way ANOVA statistical analysis was carried out between the averages of each replicate experiment. PreDCs in general were single live CD45+ lineage (B220, NK1.1, CD90.1, F4/80)- CD11c+ MHCII- CD135+ Sirpα lo/- and subsets were then separated as pre-preDCs (SiglecH+ Ly6c+), pre-cDC1s (SiglecH- Ly6c-) and pre-cDC2s (SiglecH- Ly6c+). *P<0.01**P<0.005***P<0.0001.*

3.2.3 Chemokine receptor expression is heterogeneous within subsets

Given the heterogeneity we observed in the bone marrow with pre-cDC1 and pre-cDC2 subsets, where only a proportion of the subsets expressed iCCRs, we wanted to see if this was related to a differential state or a feature of a heterogeneous population. Thus, we compared the bone marrow to peripheral tissues with more mature preDCs which have egressed from the bone marrow. Again, using the iCCReporter mice, we analysed the combination of receptors expressed on preDC subsets using FlowJo's Boolean Combination Gate analysis. Here, frequencies of different combinations of receptor expression were calculated within the preDC subsets. Novel findings include the identification of various combinations of iCCR expression within the bone marrow (Figure 3.4 A). Expression of CCR5 alone decreased from non-committed preDC to the committed subsets while the opposite was seen for CCR2. The biggest frequency of receptor combinations was seen in pre-cDC1 subsets expressing both CCR2 and CCR5, and CCR3 and CCR5. Interestingly, combinations were also identified within preDC subsets in peripheral tissues (Figure 3.4 B). Single expression of CCR2 was diminished in lymph nodes compared to bone marrow and peripheral tissues. Differential combination expression between preDC subsets was also identified systemically, for instance, a proportion of pre-cDC2 expressed higher CCR3 alone and a combination of CCR3 and CCR5 in the small intestine, while pre-cDC2s expressed a higher proportion of and CCR2 and CCR5 combination. Interestingly, peyers patches also contained preDCs with combinations of CCR3 and CCR5. It is important to note that in this dataset, CCR2 in general was expressed higher on pre-cDC1 than pre-cDCs. This pattern did not align with our previous experiments. Although this dataset included 8 individual samples, biological repeats are needed to confirm our findings. Since, iCCRreporter mice allow us to analysis only CCR1,2,3 and 5 expression, we were interested to analyse combinations of all chemokine receptors.

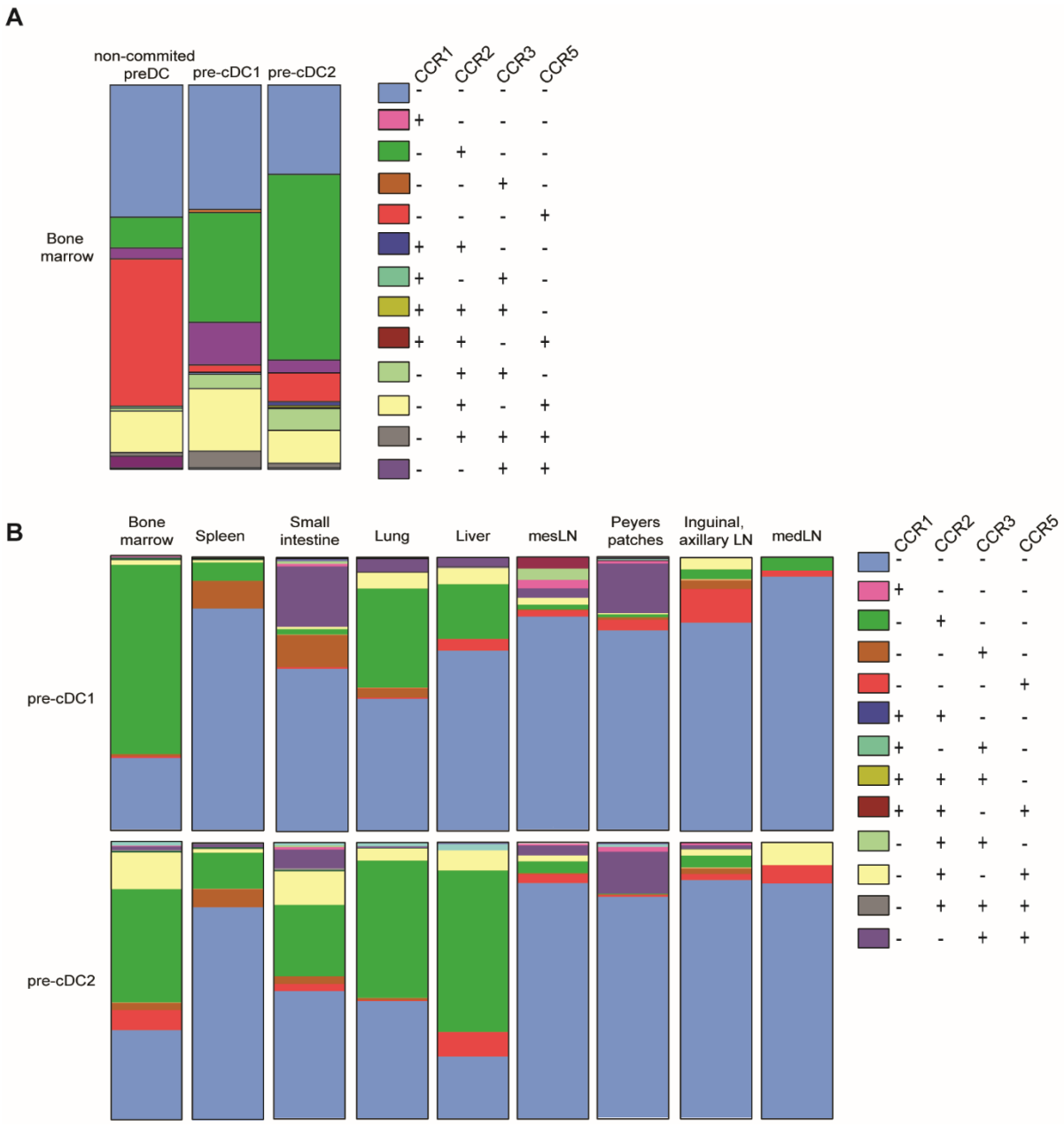


Figure 3-4 - Chemokine receptor expression is heterogeneous within preDC subsets. *Bone marrow cells of iCCReporter mice were analysed by flow cytometry. Expression of chemokine receptors CCR1,2, 3 and 5 on non-committed preDCs, pre-cDC1 and pre-cDC2s are shown. Combination analyse shows the percentage of the cells expressing the chemokine receptors (identified by '+'). PreDCs were gated as lineage- (B220, CD90.2, F4/80), CD11c+, MHCII-, CD135+, Sirpα- and subsets were then separated as pre-preDCs (SiglecH+ Ly6c+), pre-cDC1s (SiglecH- Ly6c-) and pre-cDC2s (SiglecH- Ly6c+). Bone marrow data represents 3 independent experiments and shows experiment with n=6. Systemic tissue data represent one independent experiment with n=8.*

To confirm and expand on this, we sorted preDCs from the bone marrow and spleen of 4-week-old male C57BL/6 mice which have been processed for scRNAseq. Upon return of our datasets, we will analyse chemokine receptor expression. preDCs were identified as single, live, $CD45^{+}$ $lineage^{-}$ (B220, NK1.1, CD90.1, F4/80, ly6D) $CD11c^{+}$ $MHCII^{-}$ $CD11b^{lo/-}$ $CD135^{+}$

Sirpα $^{lo/-}$ CD43^{+} (Figure 3.5). These gates were based on previous publications on preDC identification (Cabeza-Cabrerizo, Cardoso, et al. 2021a; Dress et al. 2019b; Grajales-Reyes et al. 2015; K. Liu et al. 2009; Kang Liu et al. 2007; Schlitzer et al. 2015a). Analysis of bone marrow preDCs will allow us to examine the heterogeneity between and within preDC subsets in depth. Moreover, analysis of heterogeneity within splenic preDCs, which have fully differentiated and left the bone marrow, will allow us to confirm if the heterogeneity is a differential state of preDCs or if there exists inherent heterogeneity that can be identified following migration from the bone marrow.

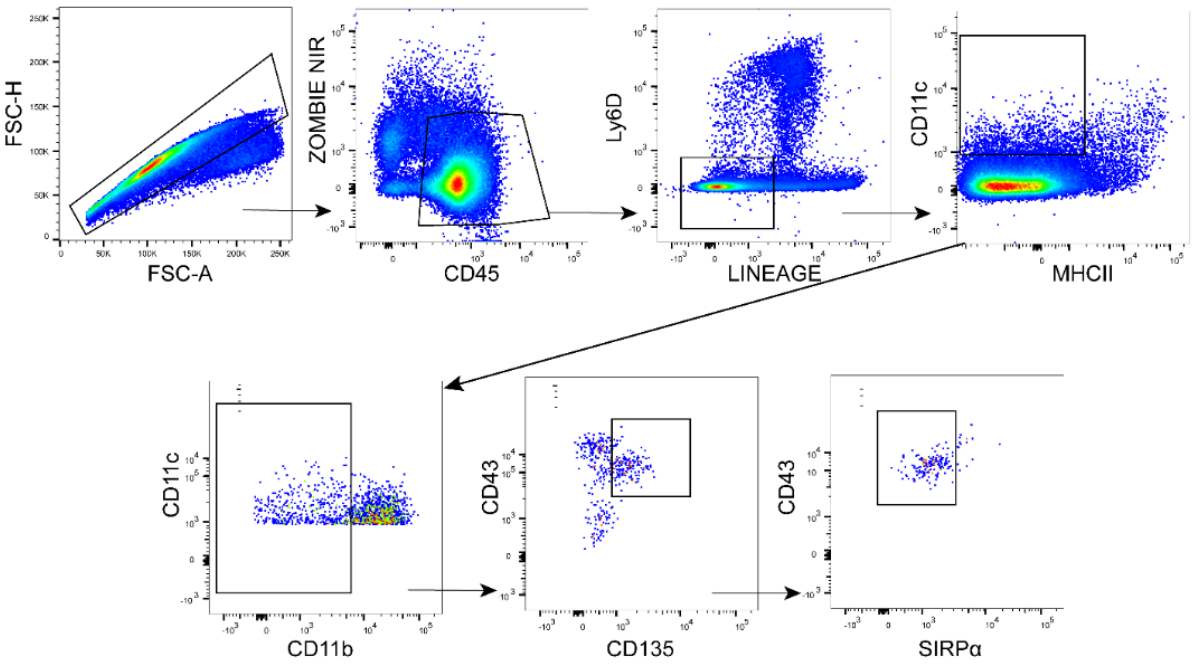


Figure 3-5 Flow cytometric gates used for sorting preDCs. *Hips and hind legs were harvested from 4-week WT C57BL/6 male mice. preDCs were identified as single live CD45+ lineage (B220, NK1.1, CD90.1, F4/80, ly6D)- CD11c+ MHCII- CD11b-/lo CD135+ Sirpα lo/- CD43+. Cells were sorted on a BD FACSAria™ III Cell Sorter into FACS buffer.*

3.3 Discussion

In this chapter, we have demonstrated the differential expression of preDC subsets in terms of chemokine receptor expression suggesting that preDCs utilise different chemokine receptors. These chemokine receptors may be used for bone marrow retention, bone marrow egress or tissue infiltration. . Such heterogeneity has been suggested previously with differential expression of CXCR3, CCR5, CCR2 and XCR1 on pre-cDC1s and CX_3CR1 on pre-cD2 (Cook et al. 2018a; Lança et al. 2022; Schlitzer et al. 2015b). In another study, preDCs in

general have been shown to express CXCR4, CCR2 and CX_3CR1 (Nakano et al. 2016a), however, subsets were not examined independently.

To unpick these chemokine receptor expression profiles of preDC subsets, we initially utilised publicly available data. scRNAseq (GDE60781) (Schlitzer et al. 2015b) and bulk RNAseq (GSE189780 and GSE18383) (Cabeza-Cabrerizo, Minutti, et al. 2021a; Lutz et al. 2022) were analysed allowing us to identify the chemokine receptors most highly expressed on preDCs in general to compile a list of target receptors to investigate. Consistent between all three, we confirmed CCR2, 5, XCR1 and CX_3CR1 expression on preDCs. In addition, we found CCR1,7,9 and CXCR2,3,4 and GpR35 on preDCs.

With this list of receptors expressed on preDCs, we analysed a microarray dataset of sorted preDC subsets (GSE60782) (Schlitzer et al. 2015b). Here we characterized the differential chemokine receptor expression between pre-cDC1 and pre-cDC2. CXCR3, CCR7, CCR5 and XCR1 were expressed at a higher level on pre-cDC1s compared to pre-cDC2s, while CX_3CR1, CXCR4, CCR9 and CCR2 were higher on pre-cDC2s. Promisingly, these results aligned with previous literature on preDC chemokine receptor heterogeneity whereby CXCR3, XCR1 and CCR5 was shown to be expressed highest on pre-cDC1s while CX3CR1 was on pre-cDC2s (Cook et al. 2018a; Lança et al. 2022). However, CCR2 was previously reported as associated with pre-cDC1 subsets (Schlitzer et al. 2015b). Researchers in this study identified bimodal expression and did not present overall expression of CCR2. Therefore, this could suggest that pre-cDC2s exist in different developmental stages identified by low or high CCR2 expression, whereas when pre-cDC1s develop they differentiate with high CCR2 expression. High CCR2 expression, combined with loss CXCR4 (during homeostasis and infection), was associated with preDCs ready to egress from the bone marrow (Jung et al. 2015; Pereira da Costa et al. 2023a) . As such, pre-cDC1s may develop and rapidly egress the bone marrow while pre-cDC2 undergo further differentiation states. Furthermore, previous analysis of pre-cDC2s may not have considered the inclusion of pre-cDC2s from different developmental pathways (as discussed in 1.1). Hence, high and low CCR2 expression on pre-cDC2s may be associated with either population of cells developing directly from CDPs or pro-DC3. Analysis of $Lyz2^+$ in the population of pre-cDC2s here may allow us to see if the differential expression of CCR2 is associated with pro-DC3. Additionally, *ex vivo* cultures of $Lyz2^+$ pro-DC3 and CDPs may allow us to determine which precursor gives rise to CCR2 high and low cDC2s. the differentially high expression of CXCR4 on pre-cDC2 may also be explained by bimodal expression within a heterogeneous population. Unfortunately, in

depth analysis of pre-cDC2 developmental subsets was not carried out for this research. Finally, as far as we are aware, CCR7 has not been reported to display differential expression on pre-cDC1s, nor CCR9 and CXCR4 on pre-cDC2s.

To confirm the differential chemokine receptor expression at the protein level, we made use of transgenic chemokine receptor reporter mice (iCCReporter) (Medina-Ruiz et al. 2022) and analysed CCR1, 2, 3 and 5 by flow cytometry. Aligning with our analysis of the microarray dataset, we report higher expression of CCR2 on pre-cDC2s compared to pre-cDC1s. Interestingly, CCR2 increased in general from non-committed preDC states to committed while CCR5 decreased upon commitment. CCR5 has been previously identified as highly expressed on non-committed preDCs (Cook et al. 2018). Since CCR2 was associated with egress from the bone marrow, it could be suggested here that the balance of higher CCR5 and lower CCR2 expression is associated with early stages of development; a balance that shifts as the cells differentiate. In fact, CCR5 was shown to be important in maintaining macrophage localisation in the bone marrow (Seyfried et al. 2021a). Therefore, CCR5 may induce preDC retention in the bone marrow while upregulation of CCR2 is involved in their egress. Finally, aligning with the similar expression between preDC subsets in the microarray analysis, we report an equal expression of CCR1 at the protein level. CCR3 was very weakly expressed by preDCs in both bulk RNAseq datasets but not the scRNAseq. As such, we had excluded it from further analysis of the microarray data. However, here we identified differential expression on pre-cDC1s.

Next, we wanted to understand if the heterogeneity between preDCs also exists within preDC subsets. If such heterogeneity occurs, it could hint that preDC subsets have tissue specific imprinting before they egress the bone marrow. In this case, cells within the pre-cDC1 and pre-cDC2s subsets are primed to migrate to specific tissues based on their expression of chemokine receptors. So, we carried out combination analysis to assess the bone marrow of iCCReporter mice to identify if combinations of chemokine receptors expression are present within preDC subsets.We have provided evidence that chemokine receptor expression is heterogeneous within pre-cDC1 and pre-cDC2 in the bone marrow. This could suggest that different combinations of chemokine receptor expression induce the tissue specific migration of preDCs to various anatomical locations. However, it may also suggest that we have identified separate states of preDC differentiation that could be characterised by combinations of chemokine receptor expression. In order to define which was the case, we considered that heterogeneity between peripheral preDCs by site would

support our tissue specificity hypothesis. Indeed, we identified differential combination expression within, and between, preDC subsets systemically. This supports previous suggestions of tissue specificity of a subset of preDCs, named pre-mucosal DCs (pre-μDCs), which expressed α4β7 allowing intestinal entry via interaction with mucosal vascular addressin MAdCAM1 (Zeng et al. 2013a). Future analysis could involve sorting preDCs with certain receptor combination, adoptively transferring them and following their migration, as well as characterising their expression of tissue homing markers. Additionally, we have sorted preDCs from WT bone marrow and spleen and, upon return of the dataset, we are eager to identify if clusters exist within preDC subsets. If so, characterising their expression of chemokine receptors, integrins and tissue-specific homing markers may allow us to unpick if tissue imprinting of preDCs occurs within the bone marrow. Additionally, we have identified preDC scRNAseq datasets from peripheral tissues which will allow us to analyse the heterogeneity of preDCs which have infiltrated peripheral tissues.

4 Chapter 2: Can we exploit this therapeutically?

4.1 Introduction

Having identified the chemokine receptors most highly expressed on preDC subsets, we wanted to distinguish which chemokines could most efficiently therapeutically increase the cDC compartment within the TME. cDC1s are a rare population of cells within most tumours (Broz et al. 2014). Despite this, it is now understood that TMEs rich in cDC1s show a correlation with increased survival (Broz et al. 2014; Y. Kim, Shin, and Kang 2019) and increased response to immunotherapy (Salmon et al. 2016a; Sánchez-Paulete et al. 2016; Spranger et al. 2017b). Immunotherapies, particularly T-cell targeting treatments, have changed the landscape of cancer therapy, however, most patients do not develop efficient responses. This is defined as primary, or acquired, resistance to treatment and occurs when tumours are, or go on to be, no longer vulnerable to cytotoxic T-cell killing, respectively. This can happen when the TME exploits mechanisms to inhibit T-cell function, either by direct inhibition or via the inhibition, or promotion, of other immune cells (Shergold, Millar, and Nibbs 2019; Tan, Li, and Zhu 2020). Given the vital role of cDCs in priming and stimulating anti-tumour T-cells in the tdLN and TME, respectively, inhibition of cDC infiltration and/or function can be exploited by tumours to resist immunotherapy (Salah et al. 2021b). The accumulation of cDCs correlates with increased survival and a better response to immunotherapy, making them ideal candidates for targeted intervention to overcome resistance to T-cell-based immunotherapies. Multiple mechanisms are under investigation as potential means to create cDC-focused immunotherapies. Approaches include stimulants that directly influence cDC development and state and cell/peptide vaccinations. Notably, Flt3L treatment to enhance cDC development, along with Poly I:C to induce cDC activation, has been clinically promising (Salah et al. 2021b). However, this treatment systemically increases cDC numbers and is not focused on targeting infiltration of cDCs directly to the TME. While other approaches have been investigated, we were keen to investigate the ability of chemokines to induce tissue specific localisation of preDCs to the TME. Within the literature, there are some indications of the chemokines which can increase cDC in the TME. Overexpression of CCL3 in B16 tumour cell lines induced increased numbers of TME preDCs *in vivo* (Diao et al. 2010a); CCL4 was involved in the recruitment of TME cDC1 *in vivo* (Spranger, Bao, and Gajewski 2015b); Williford and colleagues localised CCL4 to the TME using collagen-binding-domains which significantly increased the numbers of cDC1 per mg of tumour, highlighting the potential for tumour specific recruitment

(Williford et al. 2019a). In another study, in melanoma tumours NK-derived CCL5 and XCL1 induced cDC1 accumulation (Böttcher et al. 2018a). In melanoma models, CCL21 increased CD11c expression compared to WT tumours (Novak et al. 2007; J. D. Shields et al. 2010b). Despite these studies, there is no consensus of which chemokine has the most potential to recruit preDC subsets to the TME. Whether preDC subsets use differing signals to migrate to various tumour types also needs to be elucidated. Accordingly, we developed an assay to screen the ability of chemokines to increase cDCs within the TME by overexpressing chemokines in tumour cells.

4.2 Results

Overexpression of chemokines in tumour cells was used to screen immunotherapy targets aimed at increasing intra-tumoral cDCs Initially, we aimed to use our melanoma model and overexpress chemokines in B16ZsGreen-minOVA cell lines. This would have provided us with the ability to measure antigen transfer (Roberts et al. 2016) as well as OTI and OTII T-cell expansion (Galea-Lauri et al. 2004). However, due to technical difficulties while transducing cell lines, we were only able to use EL4 cell lines. EL4 cell lines were taken from a T-cell lymphoma developed from a carcinogen-induced tumour in C57BL/6 mice (Kramer et al. 2017). While not melanoma models, we chose to persist in investigating the cDC compartment of EL4 tumours subcutaneously injected in mice. This decision stemmed from the notion that it constituted an initial screen to identify chemokines recruiting preDCs in this artificial setting.. We are now optimising the transductions of B78Zsgreen-minOVA cell lines, a model of melanoma derived from the B16 cell line (Gillies et al. 1992; Straten et al. 1998), and MC38-minOVA cells as a model of colorectal cancer (Schrörs et al. 2023). Both cell lines were previously transduced to express Zsgreen-minOVA or minOVA alone in our lab. Using these models with chemokines overexpressed we will be able to investigate preDC recruitment in more physiological models as well as track antigen transfer and T-cell activation. First, we overexpressed chemokines in EL4 cell lines by transducing with a vector expressing the chemokines. Since the vector had the selection marker Thy1.1, we were able to confirm transduction by flow cytometry and enrich the transduced cells by sorting Thy1.1^{+} cells (Figure 4.1 A). As

expected, *in vitro*, EL4 cells released the overexpressed chemokine into culture supernatant, as confirmed by ELISA of CCL2 overexpressed cells (Figure 4.1 B).

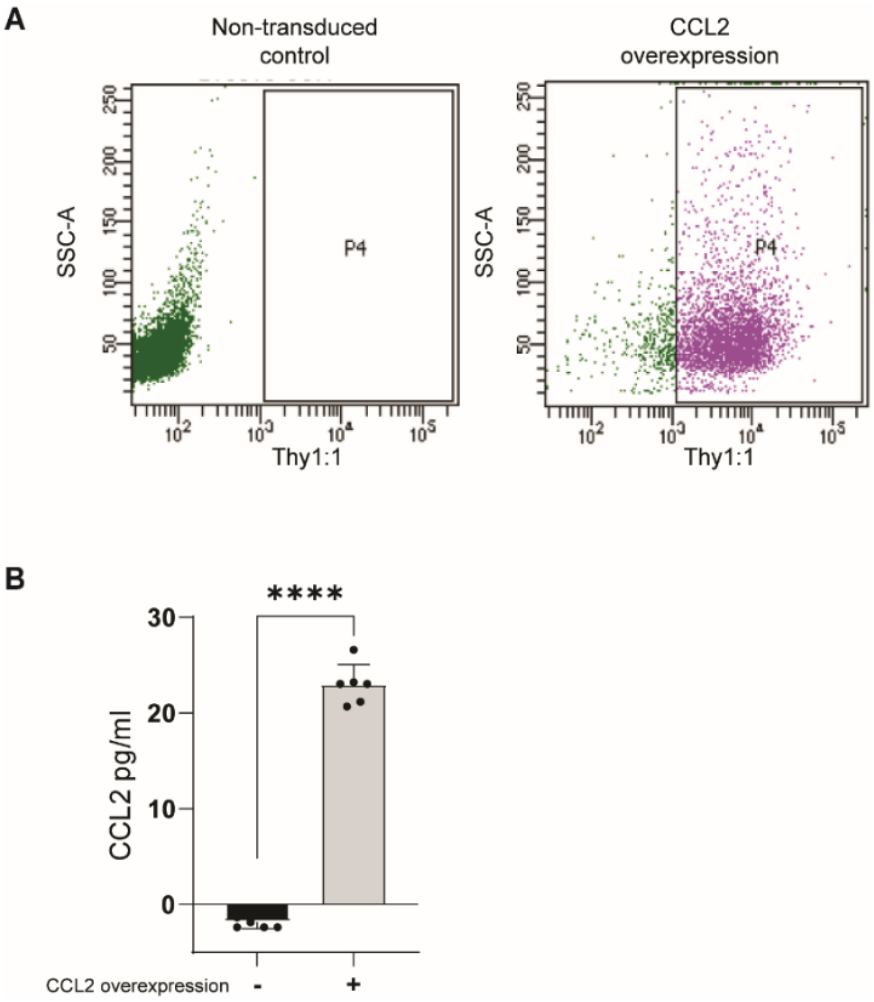


Figure 4-1- EL4 cells can be transduced to overexpress chemokines. *MSCV-IRES-Thy1.1:chemokine plasmids were used to transduce EL4 tumour cell lines. A) Transduced cells were selected by cell sorting by expression of Thy1.1 selection marker. B) In vitro, CCL2 overexpressing cell supernatant was analysed by ELISA for CCL2 expression. P<0.0001**** (unpaired T-test).*

At endpoint (defined by tumour size 15mm*15mm), tumours were harvested and processed for analysis by flow cytometry. Neutrophils, monocytes, macrophages and cDCs were identified by surface expression of cell specific surface proteins (Figure 10.2).

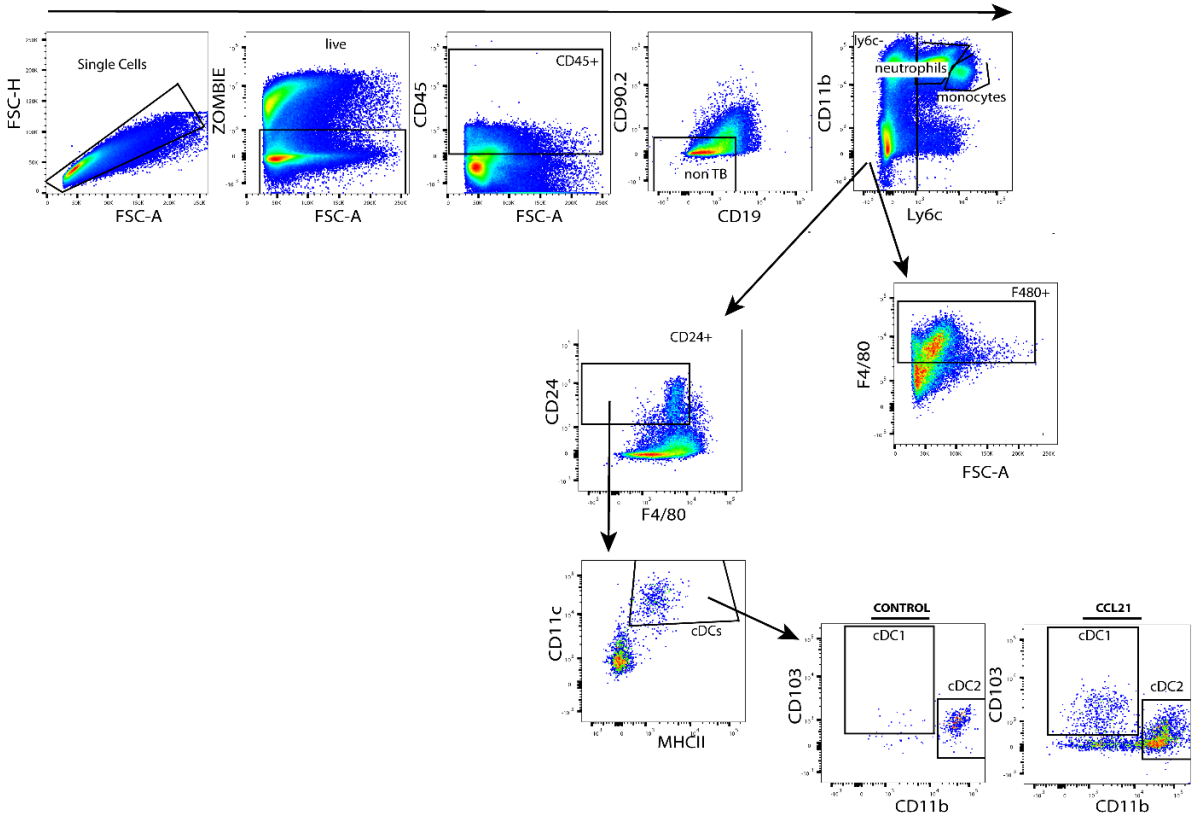


Figure 4-2 - Gating strategy used to immunophenotype EL4 tumours*. Chemokines were overexpressed in EL4 cell lines by transduction a MSCV-IRES-Thy1.1 vector expressing chemokines. 2x10^{6} EL4 cells were subcutaneously injected into WT C57/BL6 mice. At endpoint tumours were harvested and processed for analysis by flow cytometry. cDCs were gated as CD45+, lineage- (B220, CD90.2, F4/80), Ly6c-, CD24+, CD11c+, MHCII+, CD103+ (cDC1s) or CD11b+ (cDC2s). Monocytes as lineage- (B220, CD90.2, F4/80), Ly6c^{Hi}, CD11b+. Neutrophils as lineage- (B220, CD90.2, F4/80), Ly6c^{INT}, CD11b+. Macrophages as lineage- (B220, CD90.2, F4/80), Ly6c-, CD11b+/-, F4/80+.*

Since monocytes are well defined in their ability to use CCR2 to migrate towards CCL2 (Volpe et al. 2012), we confirmed the ability of CCL2 overexpressed EL4 cell lines to recruit monocytes to the TME when they established subcutaneous tumours compared to EL4 tumour cells transduced with an empty vector control (Figure 10.3).

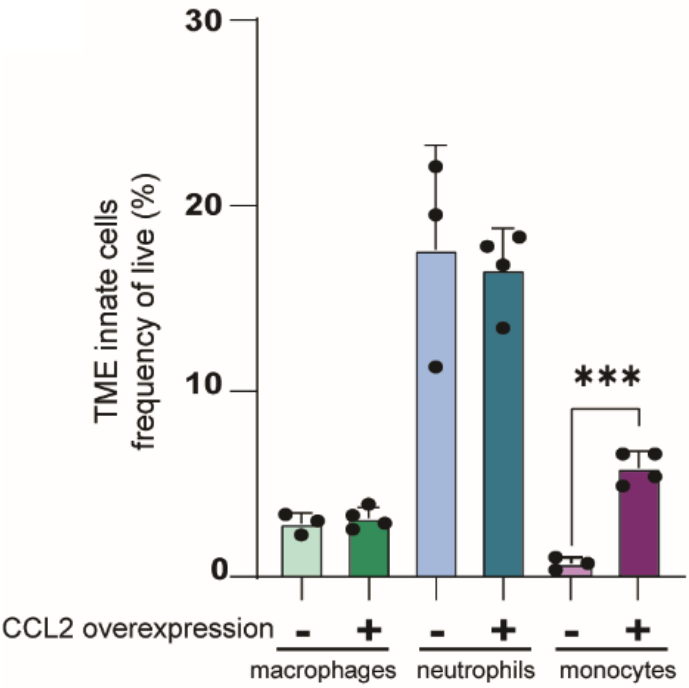


Figure 4-3 – CCL2 overexpressing tumours recruit monocytes. *$2x10^6$ EL4 cells were subcutaneously injected into WT C57/BL6 mice and innate cell frequency within tumours were analysed by flow cytometry at endpoint. Monocytes were gated as lineage- (B220, CD90.2, F4/80), $Ly6c^{Hi}$, CD11b+. Neutrophils as lineage- (B220, CD90.2, F4/80), $Ly6c^{INT}$, CD11b+. Macrophages as lineage- (B220, CD90.2, F4/80), Ly6c-, CD11b+/-, F4/80+. P<0.0005***P<0.0001**** (unpaired T-test).*

Next, we screened chemokines for their association with an increase intratumoural preDCs by measuring the fold change of cDC frequency in TME of the overexpressed tumours compared to WT tumours (transduced with an empty vector) (Figure 10.4). Coinciding with the literature, CCL21 (Novak et al. 2007; J. D. Shields et al. 2010a) significantly increased cDC1 and cDC2. Additionally, XCL1 and RARRES trended to an increase in cDC1 intratumourally, although this did not reach significance. XCL1 was previously shown to increase cDC1 in the TME (Böttcher et al. 2018a). In this same study, CCL5 recruited cDC1 however we did not identify this in our screen. On study has shown that preDCs respond to CCL3 *in vitro* and to TMEs *in vivo* (Diao et al. 2006), and here we have also identified significant increases in both cDC1 and cDC2 in CCL3 overexpressed TMEs. A trend to an increase in cDC1s occurred in CXCL9 overexpressed tumours, although not significant, but not cDC2 which agrees with the evidence that the receptor of CXCR3 is important in cDC1 infiltration to melanoma tumours (Cook et al. 2018a). Importantly, CCL4 has been shown to improve cDC1 tumour infiltration in several studies (Spranger, Bao, and Gajewski 2015a; Williford et al. 2019a), nevertheless we failed to identify this in our model. We identified CCL7 and CX_3CL1 to be

associated with cDC2 tumour infiltration. It is important to note that our screen does not demonstrate direct recruitment of preDCs in response to these chemokines, rather an association with increased chemokines and frequency of the cDC subsets.

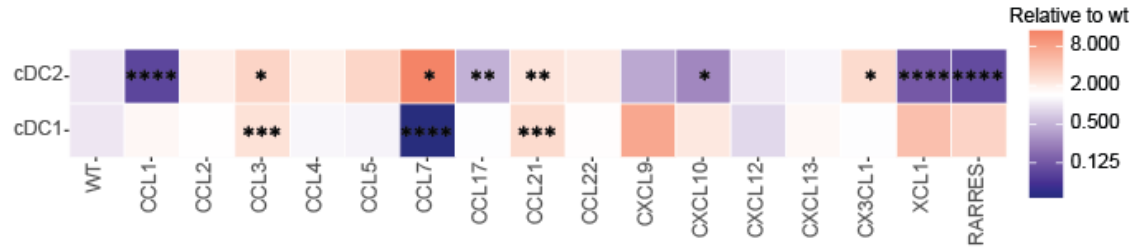


Figure 4-4 – Overexpression of chemokines changes the cDC compartment of the TME. *Chemokine overexpressing EL4s were injected subcutaneously into C57BL/6 mice and immune compartment was analysed by flow cytometry at endpoint (defined by tumour size 15mm*15mm). cDCs were gated as CD45+, lineage- (B220, CD90.2, F4/80), Ly6c-, CD24+, CD11c+, MHCII+, CD103+ (cDC1s) or CD11b+ (cDC2s). Percentage of live cells which are cDCs was calculated and fold change relative to WT control is shown. Data represent three independent experiments *P<0.051**P<0.005 P<0.0005***P<0.0001**** (Unpaired T test compared to WT).*

Since CCL21 overexpression significantly increased both intratumoural cDC1 and cDC2 , we considered that CCR7^{+} activated mDCs were trapped in the TME and were unable to migrate to the tdLN. To investigate this, we harvested the tdLN from tumour bearing mice and measured mDC and rDC frequencies. Compared to control tumours mDCs were slightly reduced in the tdLN of CCL21 overexpressed tumours, however, they were not completely abrogated from the tdLN (Figure 10.5).

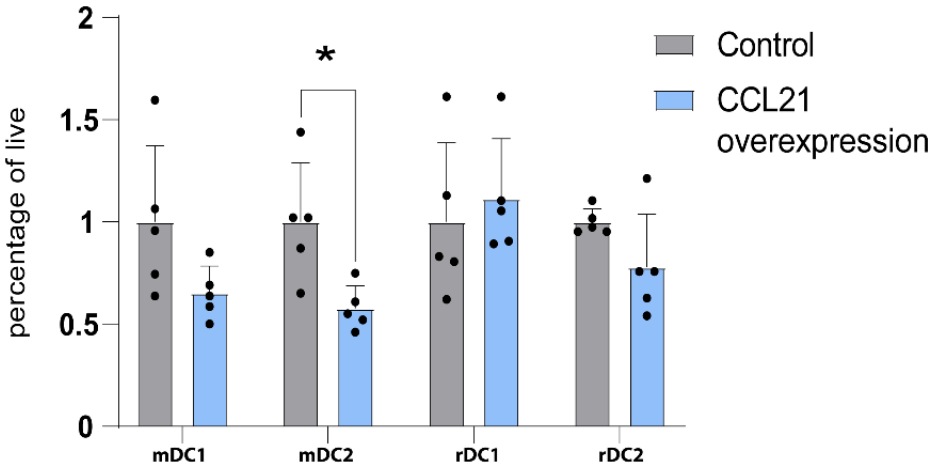


Figure 4-5 - mDC are slightly reduced in the tdLN or TMEs overexpressing CCL21. *Chemokine overexpressing EL4s were injected subcutaneously into C57BL/6 mice and immune compartment of the tdLN was analysed by flow cytometry at endpoint. mDCs were gated as CD45+, lineage- (B220, CD90.2, F4/80), Ly6c-, CD11c+, MHCII^{hi}, CD103+ (cDC1s) or CD11b+ (cDC2s). rDCs were gated as CD45+, lineage- (B220, CD90.2, F4/80), Ly6c-, CD11c+, MHCII^{int}, CD8a+ (cDC1s) or CD11b+ (cDC2s). Percentage of live cells which are cDCs was calculated*

*and fold change relative to WT control is shown. Data represent three independent experiments *P<0.05 (Unpaired T test compared to WT).*

4.2.1 cDC subsets and chemokine correlations in human tumours

Finally, to begin to inspect if certain cDC subsets and chemokines were associated with each other in human tumours, we looked to publicly available human data. The online software, GEPIA (Z. Tang et al. 2017) is an interactive website that allows for the analysis of RNA expression analysis from The Cancer Genome Atlas (TCGA). Using this, we were able to look at the correlation (Correlation coeffecient; R) of genes associated with human cDC1 (Xcr1, Clec91 and Clnk), cDC2s (Cd1c, Fcer1a and Cd1d) (Binnewies et al. 2019b; Cabeza-Cabrerizo, Cardoso, et al. 2021a) and chemokines in multiple human tumour datasets. From these datasets it is clear that correlations between cDC1 and cDC2s and chemokines are heterogeneous between tumour types. For instance, CCL21 correlated with cDC1s in Cholangiocarcinoma (CHOL), Colon adenocarcinoma (COAD), Breast invasive carcinoma (BRCA), Esophageal carcinoma (ESCA) and Liver hepatocellular carcinoma (LHC) but not in Lung adenocarcinoma (LUAD), Adrenocortical carcinoma (ACC), Bladder Urothelial Carcinoma (BLCA), Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), Glioblastoma multiforme (GBM) and Head and Neck squamous cell carcinoma

(HNSCC). Interestingly, certain chemokines displayed little or no correlation with cDCs in human tumour types.

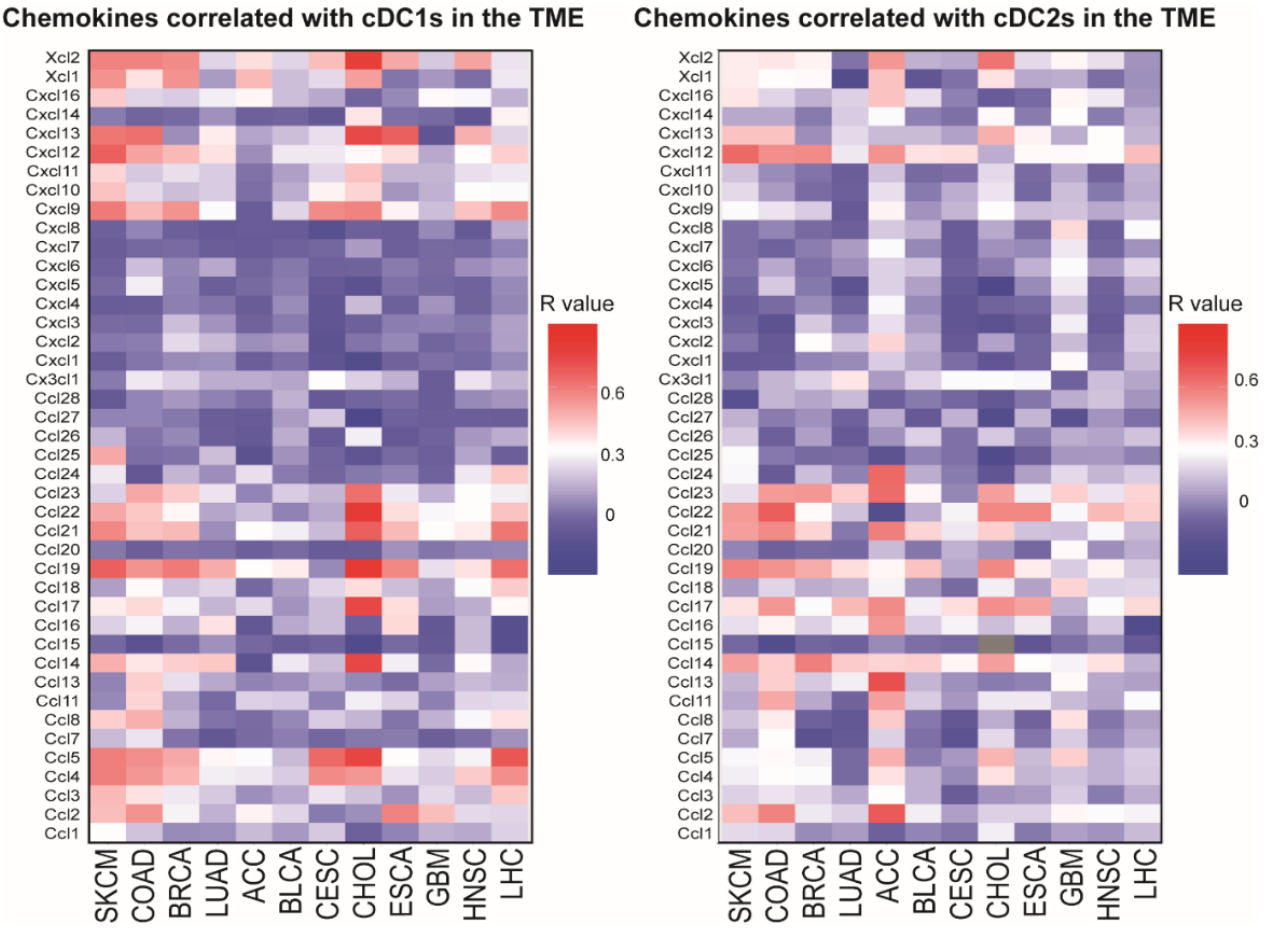


Figure 4-6 - Correlations of cDC and chemokine genes are heterogeneous between cDC subsets and tumour type. *Heatmaps show correlation coefficients (R) of genes associated with human cDCs and chemokines. Data analysed from The Cancer Genome Atlas (TCGA) on GEPIA (Tang, Z. et al. 2017). Human cDC1 gene signatures were defined as XCR1/CLEC91/CLNK and cDC2s were CD1C/FCER1A/CD1D. human skin cutaneous melanoma (SKCM); colon adenocarcinoma (COAD); breast invasive carcinoma (BRCA); lung adenocarcinoma (LUAD); adrenocortical carcinoma (ACC); urothelial bladder carcinoma (BLCA); cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC); cholangiocarcinoma (CHOL); esophageal cancer (ESCA); glioblastoma (GBM); head and neck squamous cell carcinoma (HNSCC); liver hepatocellular carcinoma (LHC).*

4.3 Discussion

Since cDC TME infiltration improves patient prognosis and response to therapy (Broz et al. 2014; Y. Kim, Shin, and Kang 2019; Salmon et al. 2016a; Sánchez-Paulete et al. 2016; Spranger et al. 2017b), we wanted to understand which signals recruited preDC subsets to the TME. Although Flt3L can be used to increase preDC development and cDC numbers within the TME, we considered chemokines could be used to enhance intratumoural cDCs within the TME. The importance of CCL4, CCL5, CCL21 and XCL1 have previously been suggested,

however, we wanted to create a screen to test all of the ligands associated with chemokine receptors expressed on preDCs (Table 10.1).

Table 4-1- Ligands to chemokine receptors expressed preDCs.

Receptor	Ligand(s)
CCR1	CCL3-7,9,10
CCR2	CCL2,7,12
CCR5	CCL3-5
CCR7	CCL19,21
CCR9	CCL25
CXCR2	CXCL1-3,4,7,9,10
CXCR3	CXCL4,9-11
CXCR4	CXCL12
XCR1	XCL1,2
CX3CR1	CX3CL1
Gpr35	

Therefore, we overexpressed these chemokines in EL4 tumour cell lines. We confirmed successful transduction by expression of the selection marker encoded in the vector used to transduce. To confirm that our experimental design resulted in chemokine overexpression, CCL2 production was confirmed in the supernatant of cultured EL4 cells engineered to overexpress CCL2 by ELISA. These overexpressing cells were injected subcutaneously and at tumour growth endpoint, immune cell infiltration was analysed by flow cytometry. As expected, CCL2 overexpression increased recruitment of monocytes, confirming the ability of these tumour cells to act as a screen for increasing intratumoural immune cells..

In terms of preDC recruitment, our data aligned with previous reports that CCL21 improved infiltration of cDC1 and cDC2 (Novak et al. 2007; J. D. Shields et al. 2010a). CCL3 overexpression increased cDC1 and cDC2 in the TME which adds to the *in vitro* and *in vivo* evidence that CCL3 can induce preDC migration (Diao et al. 2006). As with chemokine receptor expression, we identified heterogenous associations with chemokines in the recruitment of preDCs to the TME. CXCL9 overexpression slightly increased cDC1s but not cDC2 which supports reports that the CXCL9 receptor CXCR3 was important in cDC1 infiltration to melanoma tumours (Cook et al. 2018a). Although not significant, in our screen XCL1 overexpression trended to an increase in cDC1s within the TME, a mechanism also previously reported (Böttcher et al. 2018a). Unlike this study, we did not identify an association with CCL5 and

recruitment of cDC1. Additionally, in our screen CCL4 overexpression did not selectively increase cDC1s, despite having been reported by other groups (Spranger, Bao, and Gajewski 2015a; Williford et al. 2019a). These differences could be explained by differences in tumour type and potentially preDC tissue specific migration also applies to tumour type or tissue origin of the tumour. We identified CX_3CL1 to be associated with increasing intratumoural cDC2s, supporting our previous finding that pre-cDC2 differentially express CX_3CR1 compared to pre-cDC1. Additionally, selective recruitment of cDC2s was identified when CCL7 was overexpressed, which has not previously been identified. Thus, we have created a screen that has allowed us to unpick the chemokines associated with preDC recruitment to the TME in this model. However, it is important to note that our screen does not demonstrate direct recruitment of preDCs in response to these chemokines, rather an association with increased chemokines and frequency of the cDC subsets and thus the therapeutic potential of chemokines. Additionally, differences with our data and reports in the literature may be due to differences in tumour type or tumour location. It will be interesting to identify if overexpression of chemokines results in different infiltration patterns in different tumour models. We are optimizing the engineering of chemokine-overexpressing B78 melanoma cells, which will be subcutaneously located, and B16F10 metastatic melanoma cells, which develop within the lung. This may allow us to answer if tumour type or tumour location induces preDC migration by expression of distinct chemokines. Additionally, we are intrigued to identify which cells within these tumours are the main producers of the various chemokines and if this changes between tumour types.

Next, we were concerned that the overexpression of CCL21 within in the TME was only associated with increased cDC1 and cDC2 due to prevention of mDC migration to the tdLN. CCL21 interactions with $CCR7^{hi}$ activated cDCs within the TME could keep these cDCs stuck in the TME and may diminish T-cell activation. To investigate if this was the case, we analysed the cDC subsets in the tdLN and, indeed, we identified a slight reduction in mDCs, particularly mDC2s. This suggests that cDCs within CCL21 overexpressing tumours have a reduced ability to migrate to the tdLN. It is likely that this reduction will impact antigen transfer and T-cell activation thus making CCL21 a non-ideal therapeutic. To investigate this, we aim to engineer OVA expressing tumour cell lines with CCL21 and measure OTI and OTII T-cell activation when increased CCL21 in the TME prevents the normal migration of mDC to the tdLN.

It is important to note the limitations of this approach, artificially increasing chemokine signals within the TME does not allow us to understand the endogenous signals that recruit preDCs to the TME in a normal setting. However, this screen allows us to test the chemokines which could provide potential augmentation to ICB therapy by increasing cDC accumulation. However, based on our previous data and hypothesis that preDC migration may be tissue specific, the tissue of which the tumour develops in may influence which signals recruit preDCs. Additionally, therapeutically increasing certain chemokines may be able to override the endogenous recruitment pathways. Furthermore, the short-term experimental feature of our subcutaneous tumour models prevents our ability to accurately analyse differences in growth patterns when chemokines are used therapeutically. As such, it is certain that we need to screen these signals in multiple, and more relevant, models of cancer. To achieve this, we are optimizing the development of collagen binding domains (CBDs) for the targeted localization of chemokines to the TME.. In this case we do not have to rely on overexpressing tumour cell lines and can investigate our screen in spontaneous/chemically induced cancer models. CBDs can be linked to chemokines and are able to selectively localise to the TME due to their leaky vessels and dense collagen deposition allowing CBD infiltration and binding, respectively (Ishihara et al. 2019b; Williford et al. 2019a). Although this has been previously demonstrated with CCL4 overexpression (Williford et al. 2019c), we ultimately wanted to understand which chemokines increase cDC infiltration, and therefore immunotherapy responses, most efficiently in various tumour types. Despite the association of certain chemokines and cDC infiltration, the pleiotropism of the chemokines may induce the recruitment of additional immune cells, some of which may induce pro-tumour features. Therefore, we aim to utilise these longer-term tumour models to investigate the therapeutic benefits and disadvantages of increasing chemokine expression within the TME. When produced, we will investigate the combination of these chemokine therapies with anti-PD-1 therapy.

Additionally, we are interested to understand the impact of chemokine-induced changes to the cDC compartment on the balance of T-cell subsets within the TME and tdLN. Since both $CD8^+$ and $CD4^+$ T-cells are important to the anti-tumour immune response, the ideal chemokine therapeutic would recruit both subsets. However, $CD4^+$ T-cells are only positively prognostic when the repertoire lacks $CD4^+$ $FOXP3^+$ Tregs (Binnewies et al. 2019a). Thus, analysis of the T-cell compartment will be vital to understand which cDC increasing agent corresponds to increased $CD8^+$ and non-Treg $CD4^+$ T-cells. This will be particularly

important in the case of XCL1 and RARRES which, in our model, trended to increase cDC1s but significantly decreased cDC2s. Whether a decrease in cDC2s induces decreased Tregs and therefore promotes the anti-tumour response, or the decrease in effector $CD4^+$ T-cells abrogates the response, will be essential to understand.

Finally, by analysing the correlation of chemokines and human cDC genes in the The Cancer Genome Atlas (TCGA), we have demonstrated that, within humans, these associations are heterogeneous between tumour types. This highlights the potential for tissue/tumour type specific signals to control preDC recruitment. To further define this, we will investigate the association with chemokine CBD localisation and cDC infiltration in multiple tumour types.

Overall, at this time, it is unlikely that using chemokines to recruit preDCs is the ideal approach for cDC-based immunotherapy.. This is due to our lack of knowledge on the heterogenous signals recruiting preDCs in different tumour types, as well as the off-target effects of chemokines on additional immune cells which may induce pro-tumour functions. Despite this, we are eager to continue to unpick these distinct chemotaxis mechanisms so that, in time, a more personalised approach utilising chemokines may be possible.

5 Chapter 3: Developing an assay to measure preDC migration.

5.1 Introduction

Our initial work allowed us to understand which chemokine receptors are expressed by preDCs, within the bone marrow, as well as gain insight into the heterogeneity of this expression by preDC subsets. We then aimed to investigate the importance of these receptors *in vivo* by developing an assay to measure preDC migration to peripheral tissues and lymph nodes. Previous work investigating the functions of individual chemokines have been complicated by three characteristics of chemokines: promiscuity, redundancy and pleiotropism. However, it is becoming clear that this is an oversimplification. Recent evidence has suggested that each chemokine and receptor pair have specific roles in the chemotaxis of immune cells, and this may include tissue specificity (Dyer 2020; Proudfoot, Bonvin, and Power 2015; Schall and Proudfoot 2011). Therefore, we aimed to create an assay sensitive enough to unpick the specific roles of chemokine receptors in preDC migration. Previous methods have made use of *in vitro* chemokine migration assays; however, these techniques have had limited success investigating preDC migration. Transwell chemokine assays model chemotaxis with chemokine rich media being separated from cells through a porous insert. If the cells respond to the chemokine signal, they will migrate through the insert into the lower section of the assay well (Rumianek and Greaves 2020b). These assays can measure the potential of preDCs to migrate towards specific chemokines but lack physiological relevance of the tissue/context specificity of this migration. Other assays such as under agarose, allow the creation of chemokine gradients by diffusion through the agarose (Rumianek and Greaves 2020b) and, although more relevant, still lack the means to understand the specificity of preDC migration *in vivo.* Other groups have made use of *in vivo* systems such as adoptive transfers and knockout mice which have provided some important insight into the control of preDC migration (Cabeza-Cabrerizo, Minutti, et al. 2021a; Nakano et al. 2017; Pereira da Costa et al. 2023a; Scott et al. 2015a). Adoptive transfers involve the enrichment of cDC precursors from donor mice which are injected intravenously into recipient mice and their migration can be tracked. This system provides the means to investigate the importance of chemokine receptors in preDC migration during various contexts. However, transfers rely on there being a sufficient number of preDCs acquired from donor mice; the assumption that sorted preDCs have completed the developmental stages that allow them to be ready to migrate within the blood to peripheral tissues; and are mostly able to investigate preDC migration in shorter term experimental models.

Knockout mice on the other hand, are useful in understanding the general importance of chemokine receptors in tissue homeostasis, however the whole immune system has developed in an abnormal context which may impact the development, function, and migration of immune cells. Thus, the use of chemokine receptor total body knockout mice should be used with caution when measuring the specific mechanisms of cell migration.

Despite some progress, our understanding of preDC migration in different contexts remains unclear. Additionally, it is unknown whether this migration varies between preDC subsets and if tissue specific controls are at play. We therefore set out to create a universal assay that could measure preDC migration to different tissues during homeostasis, cancer, and infection. In this chapter, we discuss the methods used to optimise a migration assay.

5.2 Results

5.2.1 Systemic chemokine receptor knockout mice are not sensitive enough to model the specificity of preDC migration.

Initially, we investigated if total body knockout mice for chemokine receptors could be used to identify receptors involved in preDC migration. iCCRKO mice lack the inflammatory chemokine (iCCR) receptor locus (encoding for *Ccr1*, *Ccr2*, *Ccr3* and *Ccr5*) (Dyer et al. 2019). Since monocytes use CCR2 to migrate, we measured monocyte infiltration to confirm that iCCRKO mice were deficient in monocytic CCR2 dependent migration. As expected and corresponding with Dyer and colleagues (Dyer et al. 2019), monocytes frequencies were unchanged in the bone marrow of both iCCRKO and WT C567BL/6 mice but were significantly reduced in the lung, spleen and TME (Figure 11.1 A). We then measured cDC frequencies systemically in iCCRKO and WT mice. Interestingly, there was a significant increase in cDC2s in the bone marrow and spleen of iCCRKO mice (Figure 11.1 B). The only significant reduction in cDC infiltration was seen in mDC2s in the inguinal (iLN) and mesenteric (mesLN) lymph node. In line with the evidence that cDCs do not use CCR2 to migrate to the lung during homeostasis (Cabeza-Cabrerizo, Minutti, et al. 2021a), we saw no change in cDC frequency in unchallenged lungs of iCCRKO compared to WT mice. To investigate cDC infiltration in the TME and tdLN, we gave iCCRKO and WT mice subcutaneous B16 tumours and measured the cDC compartment by flow cytometry at tumour endpoint. As discussed, CCL3 has previously been identified to induce preDC tumour infiltration in B16 melanoma models (Diao et al. 2010b). CCL4 (Spranger, Bao, and Gajewski 2015b) and CCL5 (Böttcher et al. 2018a) were also associated with DC migration to

the TME in various melanoma models. Considering this, we expected to see a reduction of all cDCs in the TME of the iCCRKO mice which are deficient in receptors for CCL3, 4 and 5. Interestingly, the data show a significant decrease in cDC2s within the TME but not cDC1s in the iCCRKO mice. Similarly, within the tdLN, rDC2s were significantly reduced and all cDC1s were unchanged. mDC2s within the tdLN show a slight increase in the iCCRKO mice. These data could suggest that iCCRs have less important roles in cDC infiltration, but we also considered that the large number of changes in the development of the immune system and tumour establishment in iCCRKO mice may mask the effect. To increase the sensitivity of our approach we decided to optimise a competitive assay which in other settings has allowed the impact of the gene knockout to be seen (Kwarteng and Heinonen 2016; Mujal et al. 2016). In this way, knockout cells are directly compared to WT cells in WT mice in which the development of the immune system and tumour establishment are not also impacted by the gene knockout.

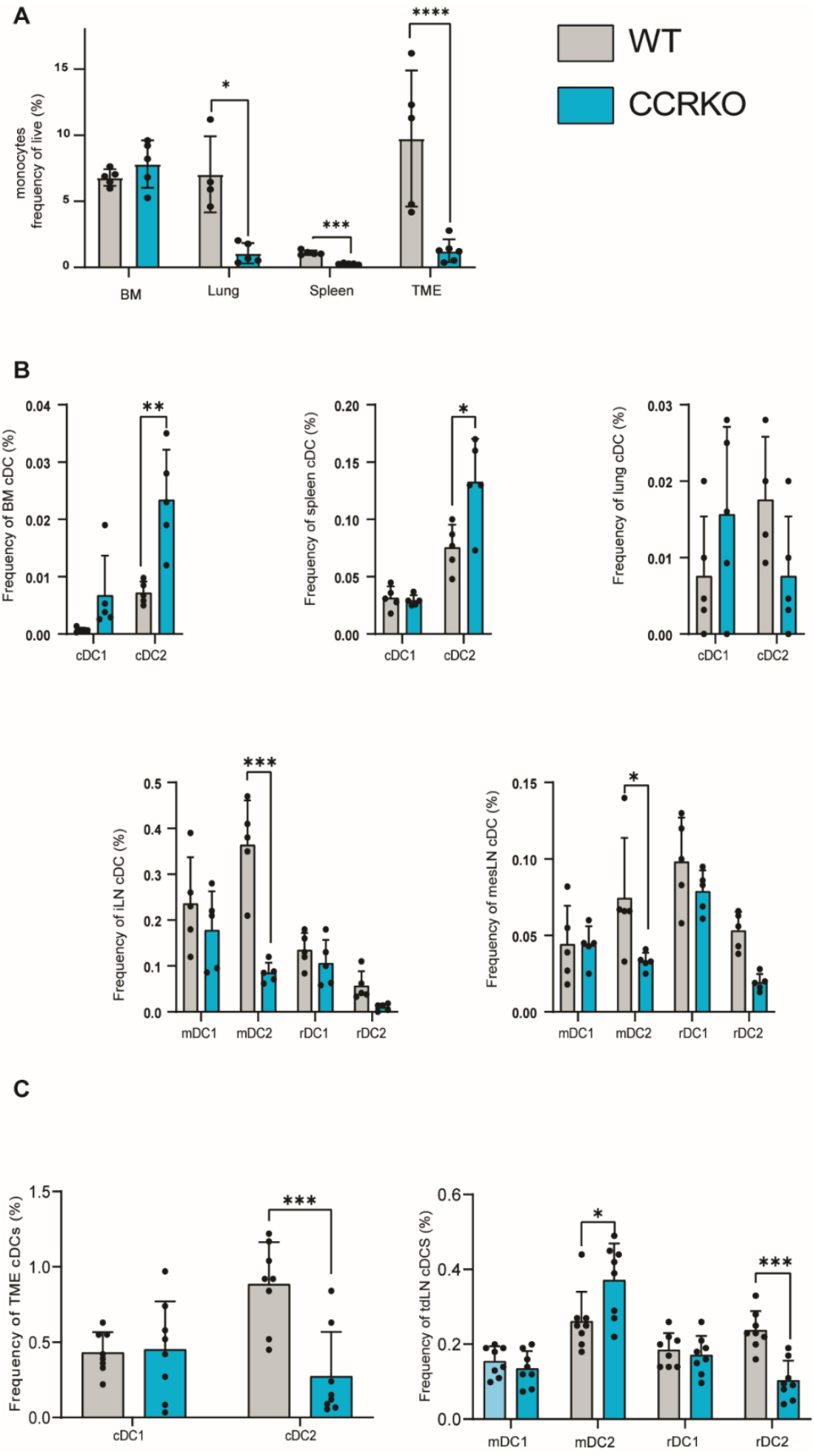

A
monocytes frequency of live (%)
WT
CCRKO
BM
Lung
Spleen
TME
B
Frequency of BM cDC (%)
Frequency of spleen cDC (%)
Frequency of lung cDC (%)
cDC1
cDC2
Frequency of iLN cDC (%)
Frequency of mesLN cDC (%)
mDC1
mDC2
rDC1
rDC2
C
Frequency of TME cDCs (%)
Frequency of tdLN cDCS (%)

Figure 5-1 - cDC frequencies are changed systemically in mice lacking CCR1, 2, 3 and 5. *Bone marrow (BM), spleen, lung, inguinal (iLN) and mesenteric (mesLN) were harvested from homeostatic iCCRKO and WT mice. In a separate experiment, iCCRKO and WT mice were injected with B16 melanoma cells subcutaneously. Tumours and tumour draining lymph nodes (tdLN) were harvested at day 14 and tissues analysed by flow cytometry. cDCs were gated as lineage- (B220, CD90.2, F4/80, CD11c+, MHCII+ and CD103+ (mDC1), CD8a (rDC1) or CD11b (cDC2). Data show A) monocyte frequencies, B) cDC frequencies in the BM, spleen and lung of homeostatic mice and c) cDC frequencies in the tumour microenvironment (TME) and tdLN. Data represent percentage ± SD from two independent experiments. Data points represent biological replicates with n=5-8 per group of mice. Data show frequency of live cells. *P<0.0001 **P<0.005 (paired T test).*

5.2.2 Adoptive transfers are not efficient to model preDC migration during homeostasis.

Our first approach in optimising a competitive assay was to adoptively transfer preDCs from WT mice into WT recipients. The migration of transferred preDCs can be measured by determining the proportion of tissue cDCs which are from donor preDC. Other groups have shown that preDCs and precursors can be sorted from donor mice and adoptively transferred intravenously to recipient mice (Cabeza-Cabrerizo, Minutti, et al. 2021b; Diao et al. 2010a; Scott et al. 2015b) We confirmed that we could successfully sort these precursors using expression of markers reported by other groups (Figure 11.2) (Cabeza-Cabrerizo, Cardoso, et al. 2021; Dress et al. 2019; Grajales-Reyes et al. 2015; K. Liu et al. 2009; Kang Liu et al. 2007; Schlitzer et al. 2015).

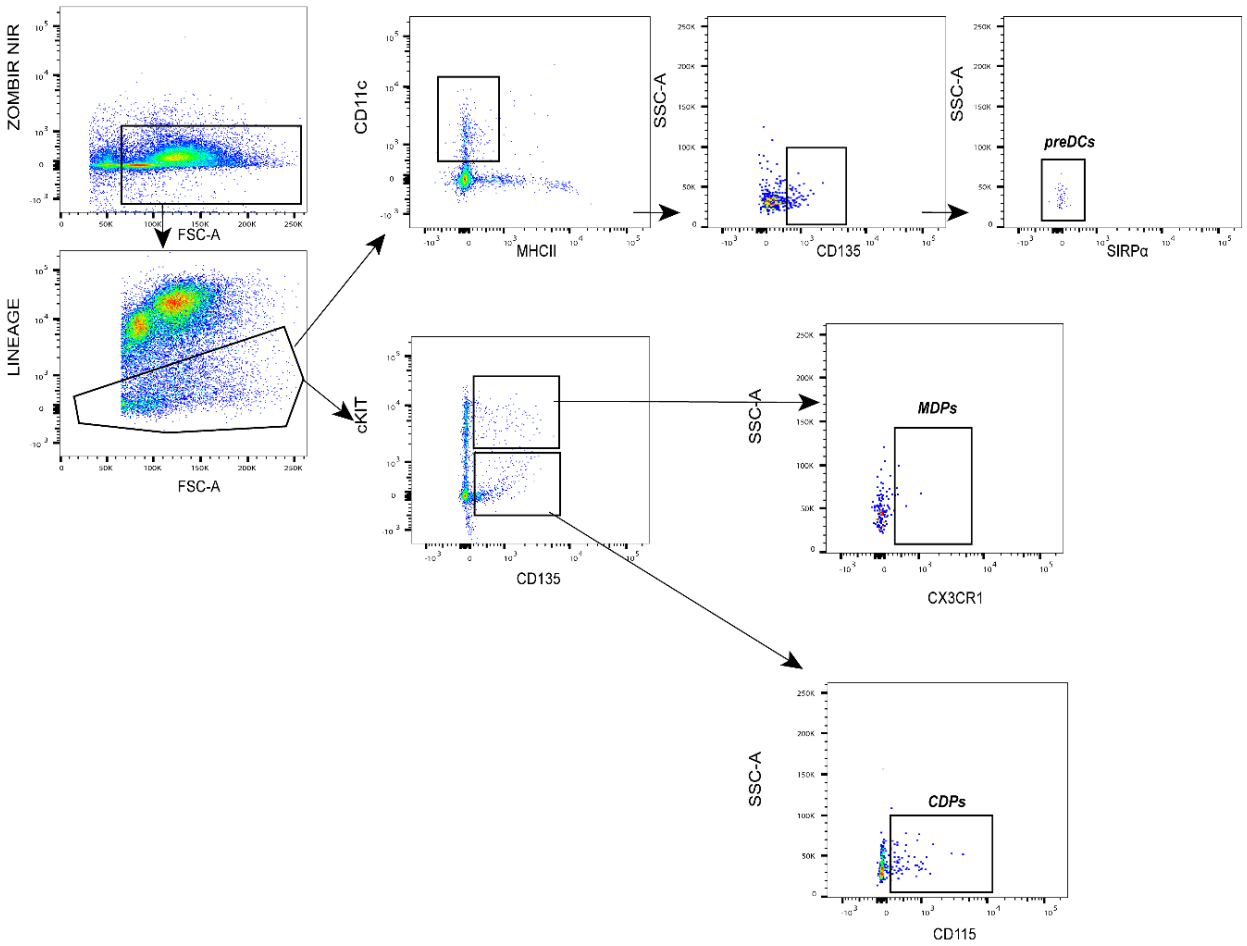


Figure 5-2 - Gating strategy of MDPs, CDPs and preDCs for sorting. *Bone marrow was harvested from CD45.1+ mice. Cells were sorted from single live lineage (NK1.1, B220, CD3, CD19, CD11b) negative cells: MDPs (cKit^{hi} CD135+ CX3CR1+). CDPs (cKit^{lo} CD115+) and preDCs (CD11c+ MHCII- CD135+ Sirpa-).*

With the use of the congenic markers CD45.1 and CD45.1, donor and recipient cells can be separately analysed by flow cytometry allowing us to identify the cDCs that developed from host preDCs and donor preDCs. We aimed to transfer 1x10^6 cells in 100µLPBS however we struggled to acquire adequate and consistent numbers of precursors from each sort. This meant large numbers of donor mice were required per recipient and transfers were inconsistent. When transfers with 33,000 MDPs, 47,000 CDPs or 300,000 preDCs, donor cDCs could be identified in recipient bone marrow (Figure 11.3). However, in this experiment only one recipient mouse was able to be used per transfer due to the small number of donor cells acquired. Therefore, sample numbers were too low to allow robust statistical analysis and would not allow for identification of factors required to migrate. In unchallenged mice, lack of recruitment signals may explain the small numbers of donors identified in tissues, however, this suggested that this approach may be unfeasible. Therefore, we decided to create a means to expand preDC numbers in donor mice.

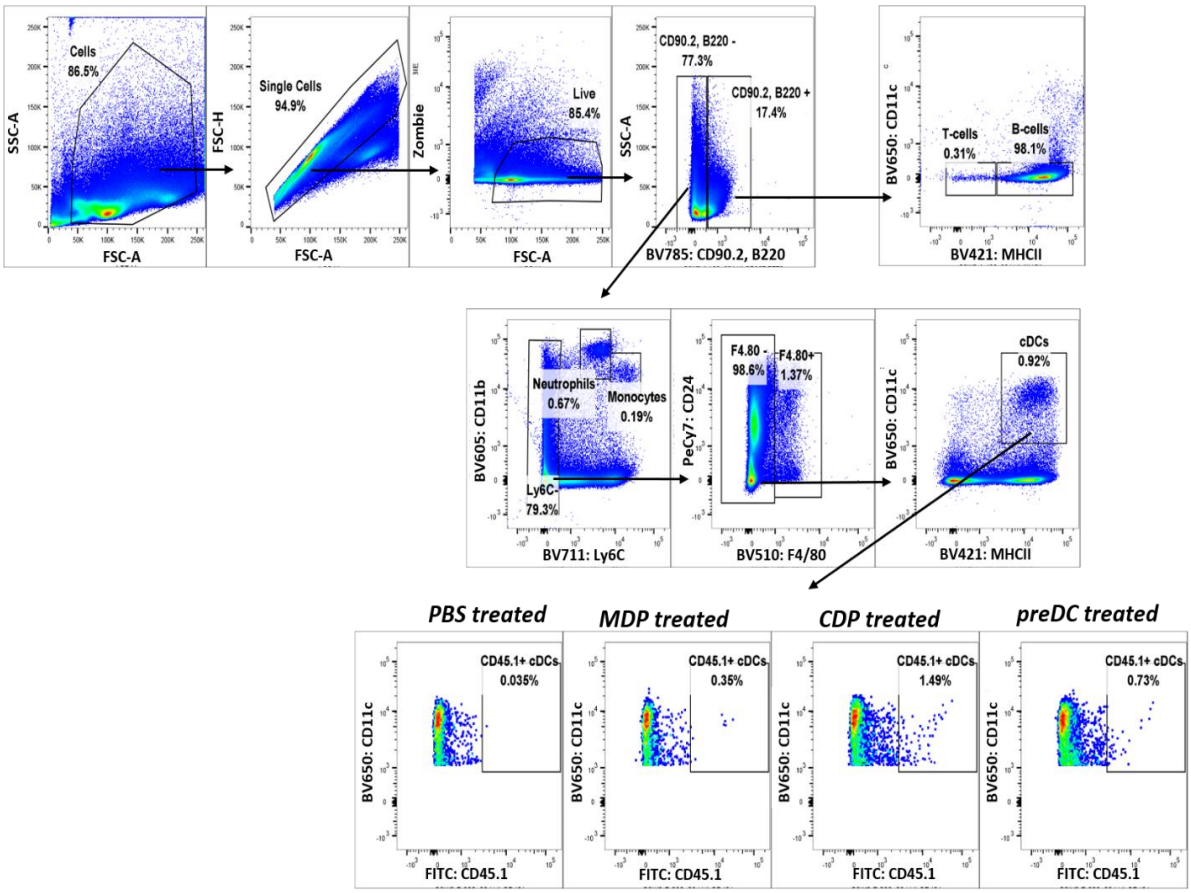


Figure 5-3 - Gating strategy to identify CD45.1+ transferred cells. *WT C57BL/6 mice were transferred with 33,000 MDPs, 47,000 CDPs or 300,000 preDCs. Bone marrow was harvested at Day 6 post-transfer and analysed by Flow Cytometry. Cells were gated on Live, singlets and cDCs (CD90.2-, B220-, Ly6C-, F4/80-, CD11c+, MHCII+), Monocytes (CD90.2-, B220-, CD11b+, Ly6Chi), Neutrophils (CD90.2-, B220-, CD11b+, Ly6Cint), B-cells (B220+, MHCII+, CD11c-) and T-cells (CD90.2+, MHCII-, CD11c-) were analysed for expression of CD45.1.*

5.2.3 Production of Flt3L

To improve the number of cells acquired for adoptive transfers we had to create a means to expand preDCs *in vivo*. Flt3L is a growth factor vital for preDC development (McKenna et al. 2000b; Waskow et al. 2008b) and has been routinely used to expand preDCs in culture as well as systemically in mice (Karsunky et al. 2003b; Maraskovsky et al. 1997; Tu et al. 2014). Previous methods have made use of Flt3L expressing tumour cells (Mach et al. 2000; Scott et al. 2015d), however, we wanted to avoid any impact of tumour growth on preDC development. Due to cost of commercially available Flt3L,, , we decided to produce purified Flt3L in-house (as detailed in Methods section 8.3). To produce these quantities of Flt3l, human Flt3l gene sequence was cloned downstream of a Kozak consensus sequence to optimise translation (Kozak 2002) and an IgK leader sequence to induce efficient protein secretion (Carter et al. 2010). The modified Flt3L sequence was cloned into the pcDNA3.1-6HIS backbone. We transfected this plasmid into FreeStyle™ 293-F cells which released Flt3L into the culture supernatant. The protein was then purified using HisTrap™ column purification on an ÄKTA™. Purification was confirmed by chromatogram (Figure 11.4 A) and SDS-PAGE (Figure 11.4 B). Flt3L protein was reconstituted and stored at 100ug/ml in PBS.

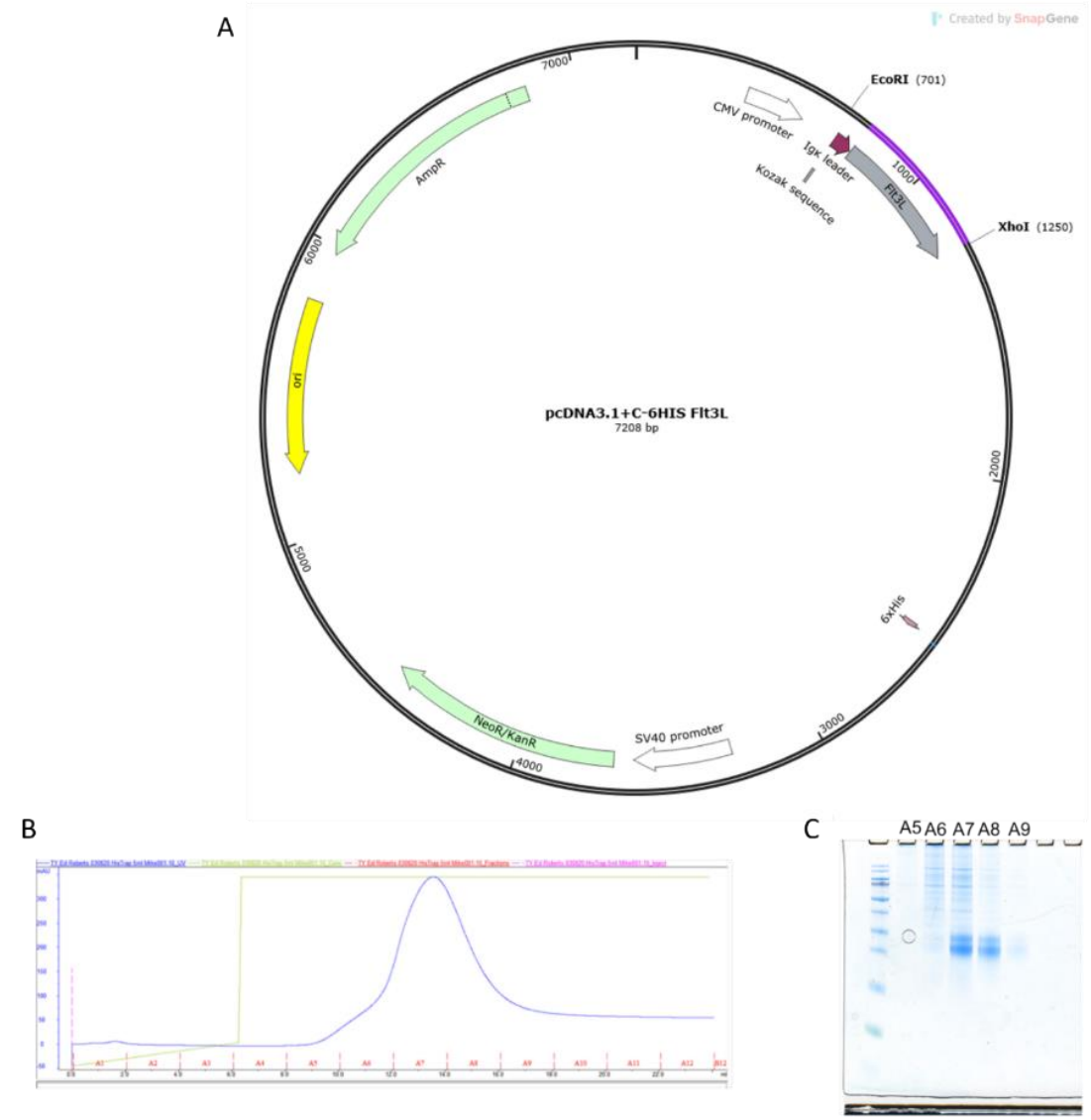


Figure 5-4 - Flt3L protein purification. *Freestyle HEK293FT™ cells were transfected with pcDNA3.1 6HIS:Flt3L. A) pcDNA3.1+6HIS vector (acquired in-house) was digested with EcoRI-HF and XhoI-HF and Flt3L (gblock generated by Integrated DNA technologies, Inc.) was ligated into the plasmid using T4 ligase. The protein was then purified from cell culture supernatant using a HisTrap™ column on an ÄKTA™. B) The chromatogram of the extracted protein showing the UV signal across collected fractions. C) These fractions were run on a SDS PAGE gel showing the Flt3L collected in fractions A7 and A8.*

5.2.4 Our Flt3L expands preDCs *in vitro* and induces pre-cDC1 development

Flt3L is vital to the development of cDCs both *in vitro* and *in vivo* (Karsunky et al. 2003b; McKenna et al. 2000b; Waskow et al. 2008). Updated methods to expand cDC1s in culture, by co-culturing bone marrow cells with OP9 stromal cells which express the Notch ligand Delta-like 1, are being investigated by other groups (Kirkling et al. 2018) and currently optimised in our own hands. Meanwhile, to test the ability of our synthesised Flt3L in

expanding preDCs *in vitro*, we tested it using an established bone marrow dendritic cell (BMDC) culture (Karsunky et al. 2003a). Although BMDC do not truly reflect the phenotype and function of cDCs, these culture conditions produce cDC1 like cells, which rely on Flt3L (Lin et al. 2021a), and so represents an appropriate method to test the Flt3L function. Although GM-CSF isn't vital for the development of cDCs, the growth factor does improve cell expansion both *in vitro* and *in vivo* (Van De Laar, Coffer, and Woltman 2012) and so was included in this assay. Briefly, bone marrow was harvested from C57BL/6 mice and cultured for 7 days before being analysed by flow cytometry (Figure 11.5 A). We cultured the cells with media alone, 10ng/ml GM-CSF, 200ng/ml Flt3L or both. As expected, cultures with both growth factors had the highest expansion of preDCs (Figure 11.5 B), confirming the efficacy of our Flt3L.

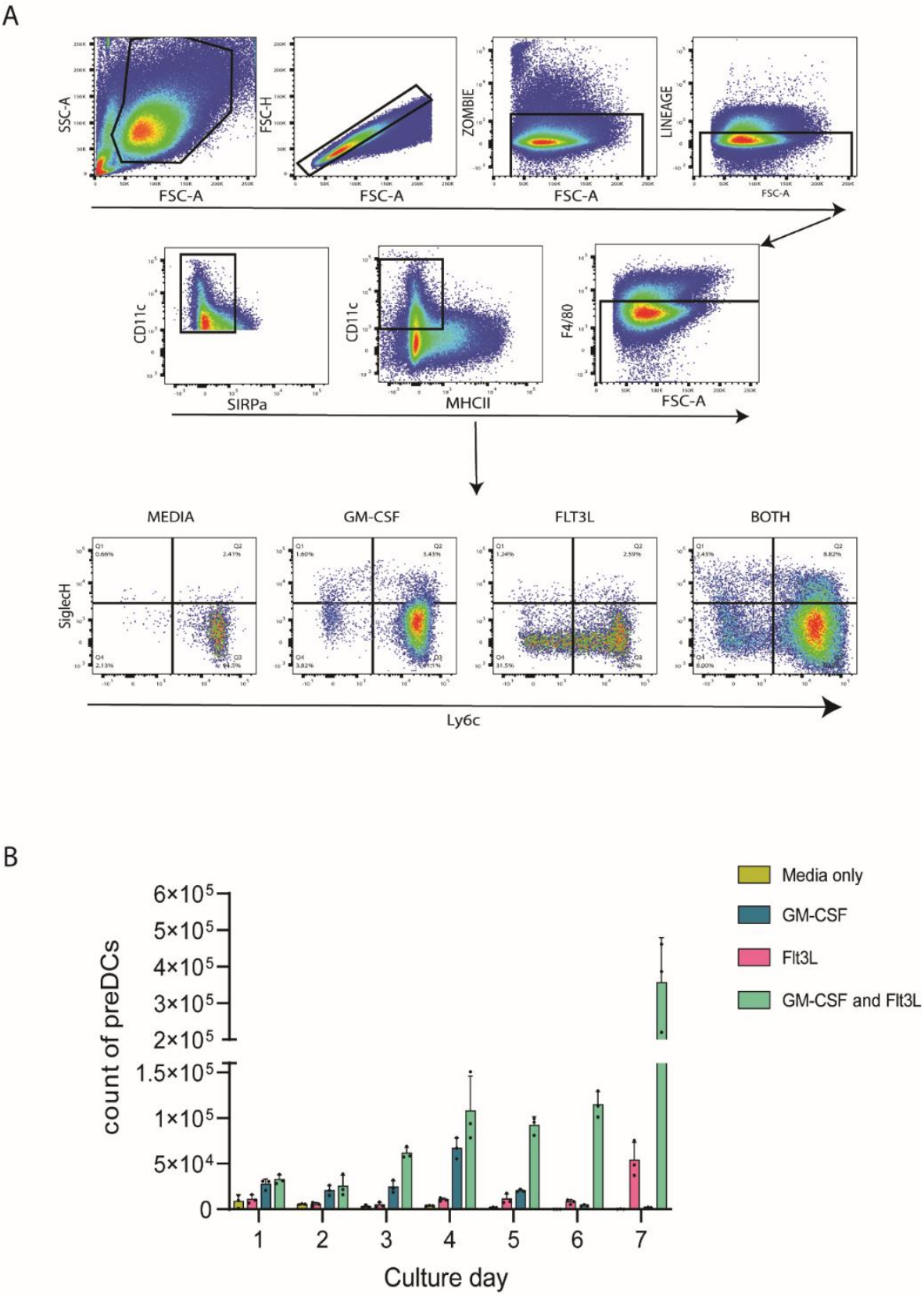


Figure 5-5 - preDCs are expanded in vitro with GM-CSF and FLt3L. *Bone marrow cells were harvested from the hips and hind legs of C57BL/6 mice and cultured in vitro with media, Flt3L, GM-CSF alone or both Flt3L and GM-CSF in 6 well plates. Cells were analysed by flow cytometry. A) PreDCs were gated as lineage- (B220, CD90.2, F4/80), CD11c+, MHCII-, Sirpα-/lo and subsets were then separated as pre-preDCs (SiglecH$^+$ Ly6c$^+$),*

pre-cDC1s (SiglecH$^-$ Ly6c$^-$) and pre-cDC2s (SiglecH$^-$ Ly6c$^+$). Top panel shows gating strategy for preDCs at day 5 of culture with both growth factors. Bottom panel shows cultures with different conditions at day 5 of culture. B) Data represent count of preDCs in bone marrow cultures over 7 days of culture. Data from one experiment and data points represent technical repeats.

It has been suggested that Flt3L selectively expands pre-cDC1s (Barry et al. 2018a; Lin et al. 2021a) and for our assay we would require both pre-cDC1 and pre-cDC2 to be expanded. So, to ensure this, we measured the abundance of the precursor cells in cultures of each condition. We identified, as expected, Flt3L alone selected for pre-cDC1 development (Figure 11.6 C). In comparison, cultures of media or GM-CSF alone were biased to pre-cDC2 development (Figure 11.6 A and B), although GM-CSF cultures had an increased frequency of pre-cDC1s to media alone (Figure 11.6 B). Promisingly, by day 7, cultures treated with both GM-CSF and Flt3L contained equal frequencies of pre-cDC1 and pre-cDC2s (Figure 11.6 D). Neither of the growth factors alone or in combination induced pre-PC expansion (Figure 11.6) which solidifies that PCs develop separately from cDCs (Cabeza-Cabrerizo, Cardoso, et al. 2021a; Dress et al. 2019b). From these data we confirmed the ability of our homemade Flt3L to expand preDCs, in particular improving pre-cDC1 development *in vitro* and in line with previous studies (Van De Laar, Coffer, and Woltman 2012), we confirmed that although GM-CSF is involved in cDC development, Flt3L is required *in vitro* to develop pre-cDC1s. Moving forward, we aimed to utilise Flt3L induced expansion of preDCs *in vivo* to acquire physiologically relevant cDC precursors.

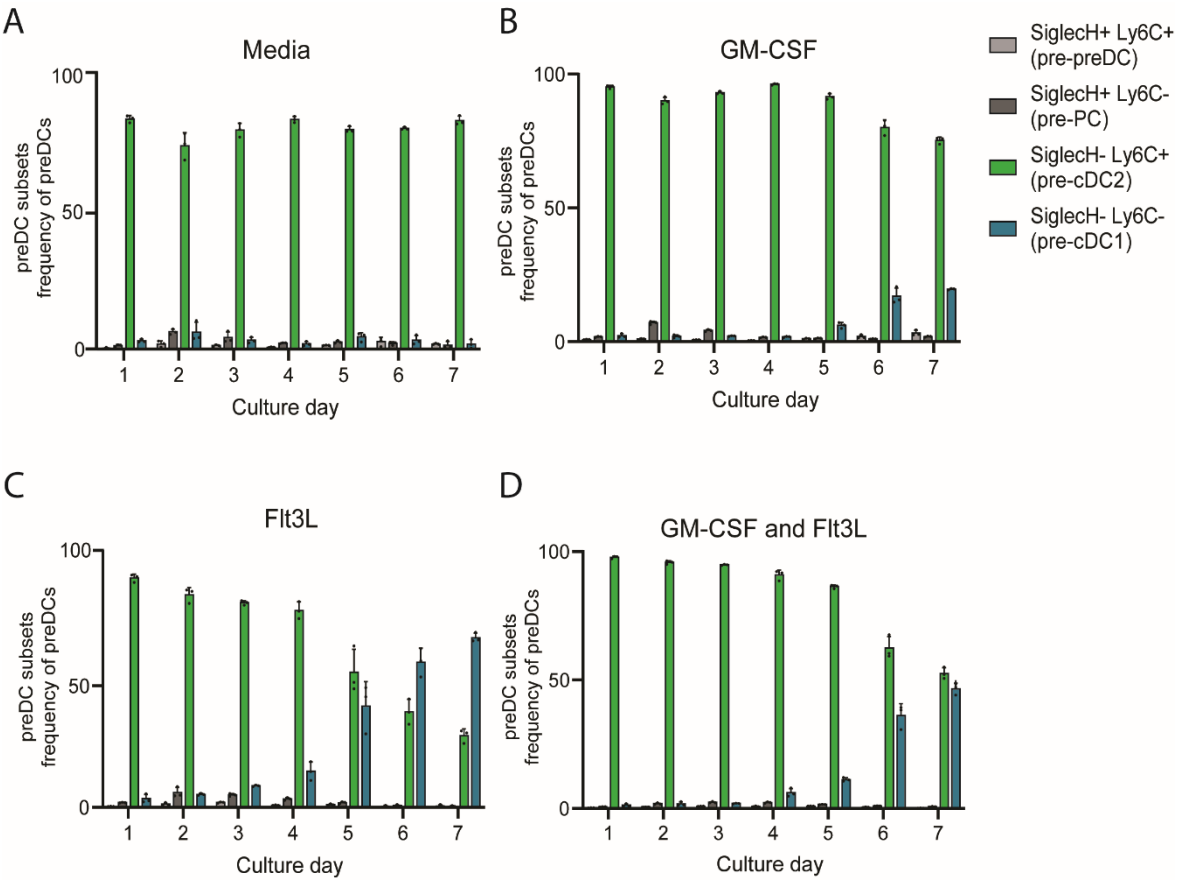


Figure 5-6 - Both Flt3L and GM-CSF are needed to grow preDC subsets in bone marrow cultures. *Bone marrow cells were harvested from the hips and hind legs of C57BL/6 mice and cultured in vitro with A) media, B) GM-CSF, C) Flt3L or both D) Flt3L and GM-CSF. Cells were analysed by flow cytometry. PreDCs were gated as lineage- (B220, CD90.2, F4/80), CD11c+, MHCII-, CD135+, Sirpα-. pre-cDC1s were SiglecH- Ly6C-; pre-cDC1s were SiglecH- Ly6C+; pre-preDCs were SiglecH+ Ly6C+; and pre-PCs were SiglecH+ Ly6C-. Data represent percentage of subsets within the precursor populations. Data from one experiment and data points represent technical repeats.*

5.2.5 Flt3L expands preDCs *in vivo*

Based on previous evidence, we aimed to confirm if ourFlt3L treatment induced cDC expansion (Karsunky et al. 2003b; Tu et al. 2014).We administered 10ug Flt3L to C57BL/6 mice for 4 days before harvesting tissues and measuring preDC and cDC counts by flow cytometry (Figure 11.7 A). The daily dose of 10ug of Flt3l was decided on based on previous publications (Karsunky et al. 2003a). In line with the literature, we reported significant increases in the count of both preDCs and cDCs systemically in mice treated with Flt3L (Figure 11.7). Within the bone marrow, the source of preDCs, both pre-cDC1 and pre-cDC2 were significantly increased and this corresponded to an increased number of BM cDCs (Figure 11.7 B). Although not significant, spleen preDCs and cDCs trended to increase in Flt3L treated mice (Figure 11.7 C). Within the lymph nodes (mesenteric; Figure 11.7 D and

Inguinal, Axillary; Figure 11.7 E), both preDCs and resident cDCs (rDCs) were significantly increased. To confirm that our Flt3L was not inducing an inflammatory response and, consequently, the increase in preDC expansion, we analysed migratory cDC (mDCs) numbers within the pooled inguinal and axillary lymph node and the mesenteric LN. No change in mDCs was identified in these locations (Figure 11.7 F). In summary, our Flt3L expands preDCs and cDC development in mice without inducing inflammation and therefore creates an efficient means to increase donor preDCs for adoptive transfer.

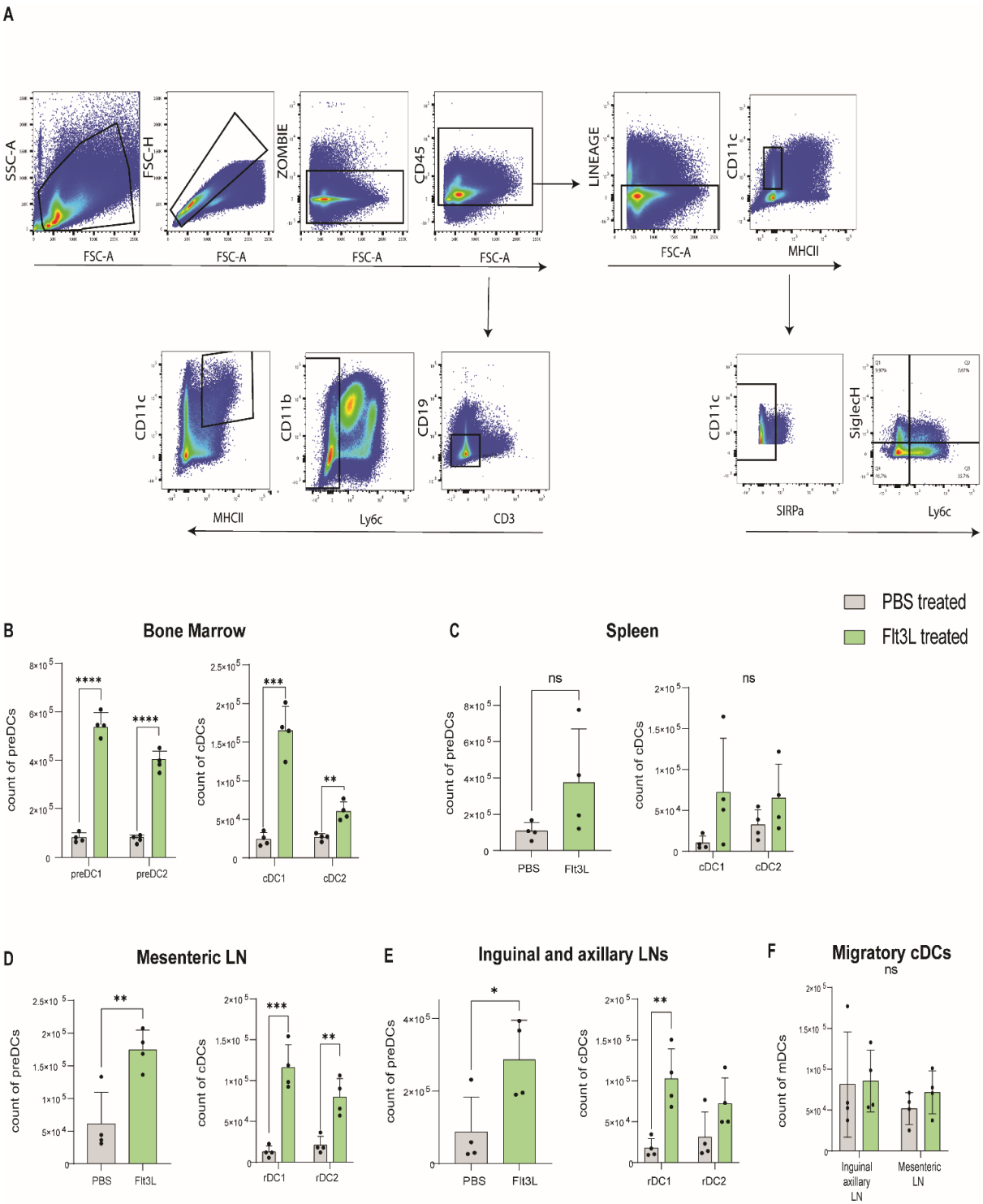


Figure 5-7 - In vivo Flt3L treatment increases preDCs and cDCs systemically. *C57BL/6 mice were treated with 10ug Flt3L for 4 days and preDC and cDC subset numbers were analysed by flow cytometry. A) PreDCs were gated as lineage- (B220, CD90.2, F4/80), CD11c+, MHCII-, CD135+, Sirpα-. pre-cDC1s were SiglecH- Ly6C- and pre-cDC1s were SiglecH- Ly6C+. cDCs were gated as lineage-, CD11c+, MHCII+ and CD103+ (mDC1), CD8a (rDC1) or CD11b (cDC2). Resident and migratory cDCs were defined by low and high MHCII expression, respectively. Data shows preDC numbers in B) bone marrow (BM); C) Spleen; D) mesenteric lymph node (MLN); and E) pooled inguinal and axillary lymph nodes (LN). F) Migratory cDC numbers were analysed for Inguinal and axillary lymph nodes and Mesenteric lymph nodes. Data represent data from three independent experiments and data points represent individual mice. *P<0.0001 **P<0.005 ***P<0.001 (paired T test).*

5.2.6 Flt3L improves adoptive transfer

Since we had successfully produced Flt3L as a means to expand preDCs, we repeated our preDC adoptive transfer experiments. Per donor mouse, Flt3L treatment increased the number of preDCs acquired from sorting (Figure 11.8 A). Recipient bone marrow was harvested as before and cDCs were analysed by flow cytometry (Figure 11.8 B). Donor cDCs could then be identified in recipient bone marrow, spleen and MesLN (Figure 11.8 C and D). Additionally, when treated with Flt3L, one donor mouse provided ~1x10^6 preDCs (Figure 11.8 A) that could be used for the adoptive transfers of one recipient mouse. Although this was a significant improvement from the small numbers of preDCs acquired from non-treated donor mice (Figure 11.8 A), we were eager to develop an assay to measure preDC migration that would be more consistent in terms of acquirement of donor cell numbers and require less donor mice per experimental mouse. Therefore, despite the potential success of adoptively transferring preDCs we looked to the development of a consistent and sensitive assay to answer our questions.

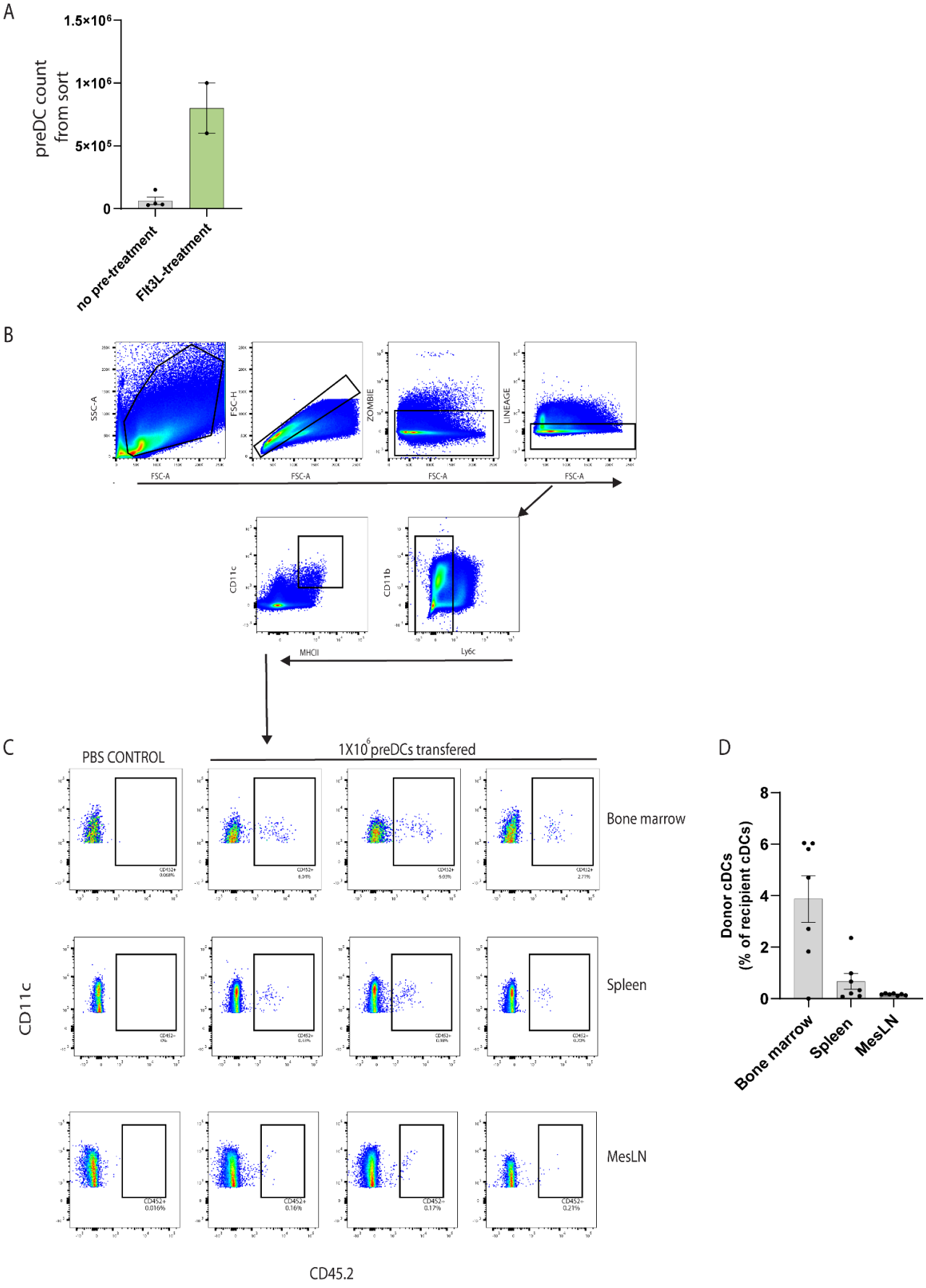


Figure 5-8 - Treatment of Flt3L increases donor preDCs for adoptive transfers. *A) preDC counts acquired from sorting the bone marrow of individual donor mice that had no prior treatment or were treated with 10ug of Flt3L for 4 days prior to bone marrow harvest. The group of mice that had no prior treatment represent 4*

individual experiments of which preDCs were sorted from bone marrow. The group of mice that had Flt3L-treatment represent 2 individual experiments of which preDCs were sorted from bone marrow. B) shows the gating strategy used to identify cDCs in the BM of recipient mice 6 days post transfer of donor preDCs and C) shows the identification of donor cDCs in the BM, spleen and mesLN of one PBS control treated recipient mouse and three individual recipient mice treated with $1x10^6$ preDCs from Flt3L-treated donors. Percentage of donor cDCs are quantified D) in 2 individual experiments in which 3 and 4 donor mice received $1x10^6$ and $6x10^5$ preDCs, respectively.

5.3 Discussion

In order to investigate the chemokine signals involved in the recruitment of preDCs to the periphery, lymph nodes and tumour microenvironment, we aimed to create a sensitive and universal migration assay.

Initially we examined the use of total body chemokine receptor knockout mice to measure which chemokine receptors were involved in preDC migration. We utilised iCCRKO mice which lack Ccr1, Ccr2, Ccr3 and Ccr5 (Dyer et al. 2019). As with our chemokine overexpression experiments (Chapter 2), we used CCR2 dependent migration of monocytes to CCL2 as a control for assay efficiency. As expected and corresponding with Dyer and colleagues (Dyer et al. 2019), monocytes frequencies were unchanged in the bone marrow but were significantly reduced in the lung, spleen and TME of iCCRKO mice, confirming their ability to measure cell migration. Thus, we measured cDC frequencies and interestingly, the only significant reduction in cDC infiltration was seen in mDC2s in the iLN and mesLN. We identified no change in cDC frequency in unchallenged lungs of iCCRKO which supports the evidence that cDCs do not use CCR2 to migrate to the lung during homeostasis (Cabeza-Cabrerizo, Minutti, et al. 2021a). Within the TME, iCCRKO mice had a significant decrease in cDC2s within the TME but not cDC1s. Similarly, within the tdLN, rDC2s were significantly reduced and all cDC1s were unchanged. mDC2s within the tdLN had a slight increase in the iCCRKO mice. These data could suggest that iCCRs have less important roles in cDC infiltration, but we also considered that the large number of changes in the development of the immune system and tumour establishment in iCCRKO mice may mask the effect. We had expected to see a reduction of all cDCs in the TME of the iCCRKO mice since the ligands (CCL3, 4 and 5) to the iCCR receptors had been suggested to induce preDC migration to the TME (Böttcher et al. 2018a; Diao et al. 2010b; Spranger, Bao, and Gajewski 2015b). By blocking monocyte infiltration, and potentially other immune cells, the

immune compartment and TME develops abnormally. This may induce changes to the recruitment of preDCs and hence we decided to optimise a more sensitive approach to measure preDC migration.

Competitive assays have been used in other settings and the impact of the gene knockout can be unveiled (Kwarteng and Heinonen 2016; Mujal et al. 2016). Here, when the majority of the immune system is WT, the importance of the knockout gene can be tested without complimentary mechanisms maintaining the mechanism and, thus, masking the impact of the knocked-out gene. Additionally, the immune system and TME develop in a WT setting without the impact of the knocked-out gene. Hence, we intended to adoptively transfer preDCs from chemokine receptor knockout (KO) mice into WT recipients. Here, we could measure the presence of KO preDCs in recipient tissues to understand their migration. Initially, we began optimising adoptive transfers of preDCs from WT donors into WT recipients. Unfortunately, the number of preDCs we acquired by sorting donor preDCs were inconsistent between experiments and the number of donor cells we identified in recipient tissues was low. Thus, we produced Flt3L as a means to expand the production of preDCs in donor bone marrow. Previous methods have made use of Flt3L expressing tumour cells (Mach et al. 2000; Scott et al. 2015b), however we wanted to avoid any impact of tumour growth on preDC development. Additionally, commercially available Flt3L was ineffective, potentially due to inefficient glycosylation, so we needed to generate Flt3L. We confirmed that our Flt3L induced the development of pre-cDC1 and pre-cDC2 *in vitro* and both precursors and cDC subsets *in vivo.* Although treatment of Flt3L increased the number of preDCs we were able to acquire from sorting donor cells, identification of donor preDCs in recipient tissues remained low. We considered that potentially, the sorted preDCs had not fully developed and migrated to the recipient bone marrow where they needed to complete their differentiation. Therefore, we decided to continue with our competitive approach but develop a more sensitive and consistent assay that more truly modelled the endogenous migration of preDCs. As such we utilised mixed bone marrow chimeras, which will be discussed in the following chapter.

6 Chapter 4: preDC migration chemokine receptor dependence can be measured using bone marrow chimeras

6.1 Introduction

Following development, preDCs exit the bone marrow and migrate via the blood where they remain in a steady state until they reach the peripheral tissues and lymph nodes and differentiate into cDCs (K. Liu et al. 2009). There is currently no consensus regarding the specific mechanisms which control the tissue localisation of preDCs. Despite this, there are some indications, for example, CCL3 (Diao et al. 2006) and CCL4 have both been suggested to induce preDC migration to the TME *in vitro* and *in vivo* (Spranger, Bao, and Gajewski 2015b). Supporting the role of CCL4 in augmenting cDC1 within the TME, Williford and colleagues localised CCL4 to the TME using collagen-binding-domains which significantly increased the numbers of cDC1 per mg of tumour (Williford et al. 2019a). Similarly, in mice, CCL21 expression recruited $CD11c^+$ cells (Novak et al. 2007; J. D. Shields et al. 2010a). On the other hand, cDC1 accumulation in melanoma tumours was dependent on NK-derived CCL5 and XCL1 (Böttcher et al. 2018a). CXCR3 KO mice had reduced cDC1 in melanoma tissue and the tdLN but not in non-inflamed skin or spleen while cDC2 numbers remained unchanged in both settings (Cook et al. 2018b). This suggests that preDC migration may be tissue and context specific. Supporting this context specific migration, CCR2 was shown to be unnecessary for recruitment to the homeostatic lung but required during inflammation (Cabeza-Cabrerizo, Minutti, et al. 2021a; Pereira da Costa et al. 2023a). In contrast, CCR2 was found to be important in seeding lung tissue during homeostasis with both cDC1 and cDC2 whereas knocking out CX_3CR1 in $CD11c^+$ cells only reduced cDC2 in lung tissue (Nakano et al. 2017). Additionally, modelling lung inflammation with inhaled LPS significantly increased the expression of CCR2 ligands (CCL2 and CCL7) in whole lung tissue and recombinant CCL2 was shown to induce preDC migration *in vitro* (Nakano et al. 2017). Furthermore, in a model of lung allergic inflammation, eosinophils were shown to produce chemokines which recruited $CCR1^+$ cDC2s (Rose et al. 2010) and blocking CCR1 reduced cDC1s in ischemic brain lesions (Gallizioli et al. 2020). Overall, despite some evidence, there is no consensus of which chemokines and receptors are involved in the recruitment of preDC subsets to peripheral tissues, including the TME. Our evidence presented in Chapters 1 and 2 suggest that committed precursors may utilise different receptors to migrate and that these mechanisms may vary depending on tissue and tissue condition. The development of a universal assay

to measure preDC migration systemically and during multiple tissue contexts will help these complex pathways and provide opportunities to promote or intervene in preDC migration. Since previous attempts lacked sensitivity and consistency (as discussed in Chapter 3) we developed a mixed bone marrow chimera system and show that it provides a means to accurately measure preDC migration systemically and systematically in different contexts.

6.2 Results

6.2.1 Creating bone marrow chimeric mice to measure preDC migration

Mixed bone marrow chimeras involve the engraftment of donor mouse haematopoietic stem cells (HSCs) into recipient mice creating a model in which the majority of immune cells are wildtype, but a proportion of self-renewing HSCs give rise to donor knockout immune cells (Ferreira et al. 2019). This system provides an alternative approach to transgenic mice in which the importance of genes in immune cell development and function can be investigated without creating total body knockouts which may have a range of unintended effects. It also offers a model, in contrast to adoptive transfers, with a continuous replenishment of donor cells from self-renewing HSCs, enabling the investigation of longer experimental models.. Traditional methods of creating bone marrow chimeras involve reducing the recipient's HSC compartment with the use of total-body irradiation, inducing DNA damage in dividing cells and, subsequently, HSC depletion (Lu et al. 2020). This ablation creates bone marrow niche space for the engraftment of transferred HSCs. However, such approaches can have lasting impacts on the recipient's bone marrow niche, immune cell development and function and require successful engraftment of donor HSCs for the survival of recipients (Cao et al. 2011; Lu et al. 2020; Shao et al. 2014). Therefore, we opted to create chimeras using a myelosuppressive agent, Busulfan, which, while allowing the creation of high percentage chimerism, is generally better tolerated by recipients, maintaining a state closer to homeostasis, and survival is not dependent on donor engraftment as recipient cells are not completely depleted (Andersson et al. 2003; Peake et al. 2015; Youshani et al. 2019). Busulfan is commonly used in preparation for bone marrow transplantations and is believed to induce ablation by DNA alkylation in dividing cells (Sjöö et al. 2006). Therefore, Busulfan treatment will also induce off-target effects by damaging dividing cells other than HSCs, however, it is better tolerated than total-body irradiation. Based on previous work in our lab, we dosed recipient mice intraperitoneally with 15mg/kg for two days prior to cell transfer. To phenotypically identify donor cells from recipient cells,

congenic CD45.1 and CD45.2 mice are often used, like how we identified adoptively transferred cells. Figure 12.1 A describes our experimental process where donor bone marrow cells were transferred at a concentration of $1x10^6/100\mu L$ in PBS intravenously into Busulfan-treated recipients. After 8 weeks, $CD45.1^+$ recipients with WT $CD45.2^+$ donor cells were subcutaneously given B16ZsGreen-minOVA tumours and, at endpoint (defined by tumour size), samples were analysed by flow cytometry for percentage chimerism of cDCs (Figure 12.1 B). Chimerism was defined by percentage of cDCs that expressed the congenic marker of the donor mice. Based on this initial experiment, we were positive that we could successfully create mixed bone marrow chimeras as cDC chimerism was present in most recipient mice analysed (Figure 12.1 C). Using this system, the majority of preDCs develop from WT cells and migrate normally meaning that peripheral tissues and lymph nodes do not lack cell infiltration. Consequently, when the minority of preDCs are donor knockout cells in chimeric mice, the importance of this chemokine receptor can be investigated without the impact of a total body knockout masking the effect and long-term changes due to irradiation.

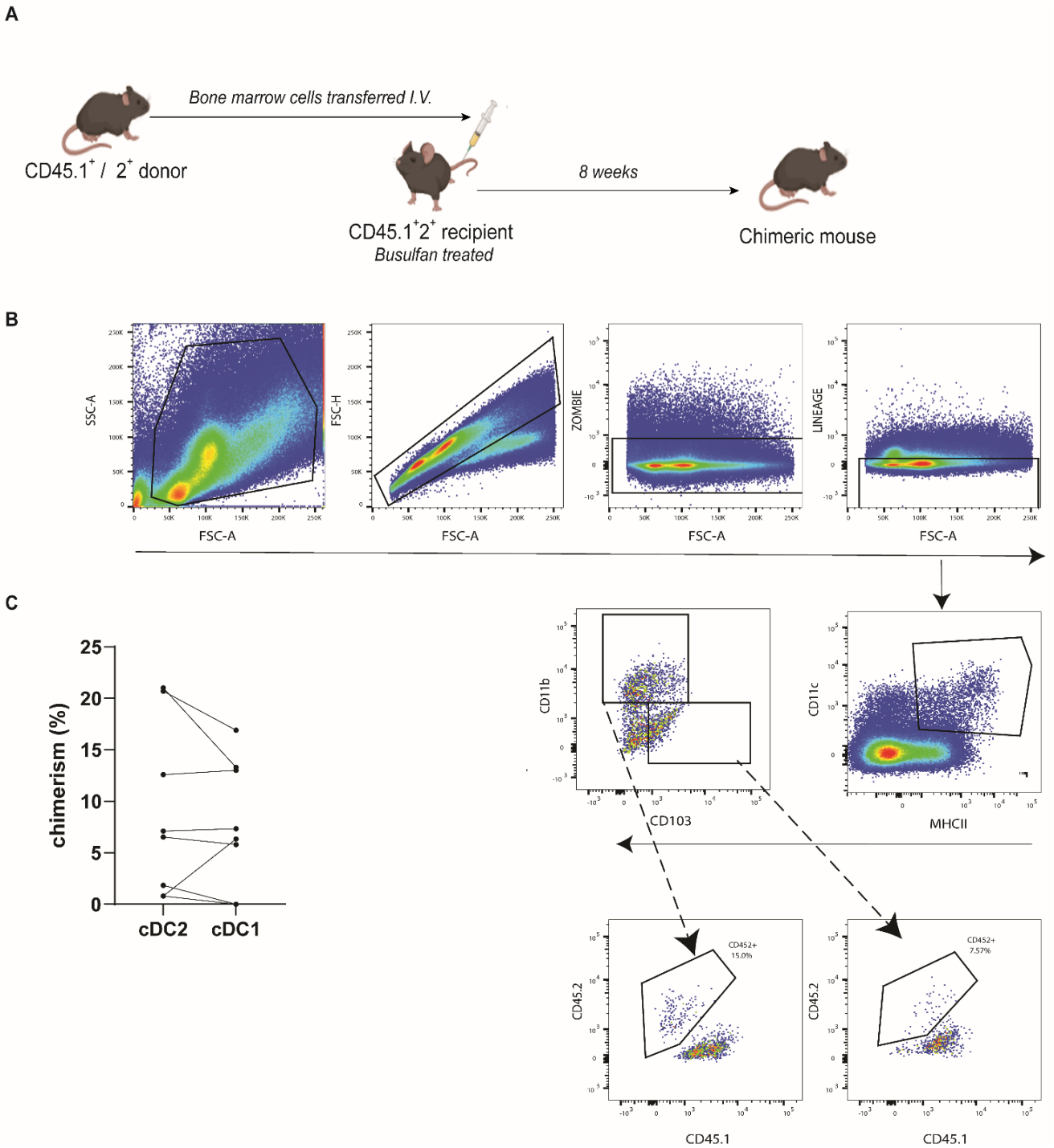


Figure 6-1 - Bone marrow chimeras can be created. *A) Schematic describes experimental process of creating bone marrow chimeras. Donor bone marrow cells were lysed of red blood cells and transferred at a concentration of 1x10^6/100µLin PBS intravenously into Busulfan treated recipients. B) shows flow cytometry gating and C) quantified flow cytometry analysis of bone marrow cDCs of B16ZsGreen-minOVA tumour bearing recipient CD45.1 chimeric mice. Spleen cDCs were gated as CD8a+ or CD11b+. Lymph node mDC were MHCIIhi, CD103+ or CD11b+. Lymph node rDC were MHCIIhi, CD8a+ or CD11b+. Donor cells were acquired from WT CD45.2 mice. Joining lines represent cDC subsets from the same mouse.*

6.2.2 Chimeric mice were used to identify the chemokine receptors involved in preDC migration systemically and to the tumour microenvironment

To confirm that our mixed bone marrow chimera assays measured cell migration as we would expect, we analysed monocytic infiltration. Monocytes utilise CCR2 to migrate, a process which is well defined (Dyer et al. 2019). Individual groups of chimeras were created using donor cells from wildtype (WT), CCR1KO, CCR2KO, CCR5KO, iCCRKO (CCR1,2,3,5KO), CCR7KO and XCR1KO. We calculated percentage chimerism of monocytes in the bone marrow and peripheral tissues. Since each mouse had unique chimerism, to normalise data we calculated the fold change of chimerism in each tissue compared to the bone marrow. We expected to see a significant reduction in chimerism systemically compared to the bone marrow when chimeras were made with donor cells lacking CCR2. Indeed, our assay measured monocyte reliance on CCR2 to migrate since CCR2KO and iCCRKO chimeras had reduced monocyte migration compared to the bone marrow (Figure 12.2). Chimerism in the spleen with WT donor cells was significantly increased compared to the bone marrow, although we did not see this in chimeras with did CCR1, CCR5 and CCR7 KO cells, receptors which monocytes have not been shown to rely on to migrate. Thus, this may be an artefact of analysis and more biological repeats are needed to confirm this. Interestingly and, we identified that monocytes rely on = expression of XCR1. However, whether this reliance is for their ability to develop, egress the bone marrow or infiltrate peripheral tissues is unclear from these data.

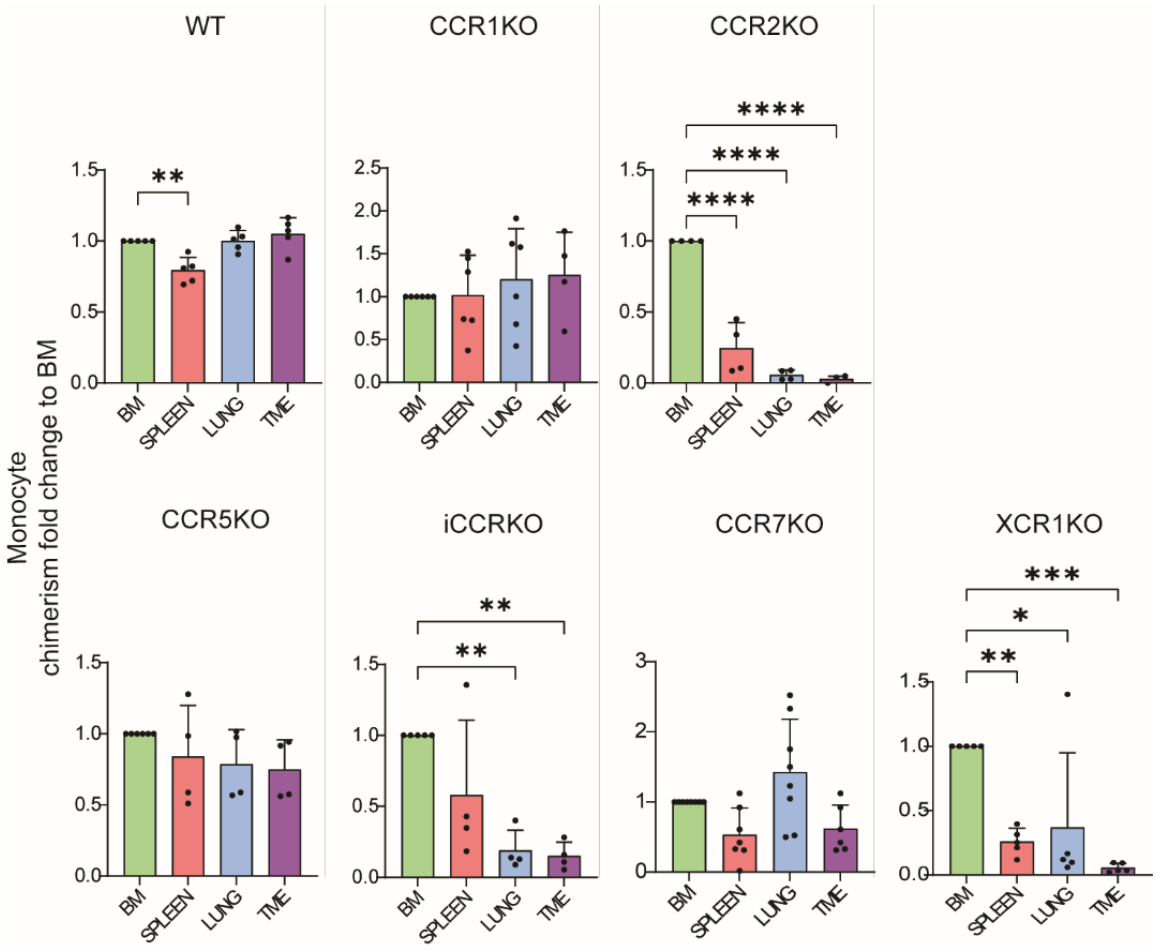


Figure 6-2 – Bone marrow chimeras accurately measure monocyte migration. *Donor bone marrow cells from wildtype (WT), CCR1KO, CCR2KO, CCR5KO, iCCRKO, CCR7KO and XCR1KO CD45.2 mice were lysed of red blood cells and transferred at a concentration of 1x10^6/100µLin PBS intravenously into Busulfan treated CD45.1 recipient chimeric mice. Percentage chimerism of monocytes in the bone marrow and systemic tissues was calculated by CD45.2 expression. Fold change of chimerism in each tissue compared to the bone marrow was calculated and One-way ANOVA with multiple comparisons was used to statistically test the chimerism fold change in each tissue compared to the bone marrow (BM). Monocytes were gated as cDCs were identified by lineage (CD3, B220, NK1.1, SiglecF, F4/80)-, CD11b+ Ly6Chi.*P<0.01**P<0.005***P<0.0005***P<0.0001.*

Next, we analysed preDC migration in our chimeras. Since analysis of cDCs in tissues is well defined by flow cytometry, we analysed chimerism of cDC subsets as an outcome of pre-cDC1 (Figure 12.3) and pre-cDC2 (Figure 12.4) migration. Importantly, due to a technical error, analysis of cDC subsets in spleen and lung samples of CCR1KO chimeras was not possible and this chimerism here represents cDCs as a whole and not individual subsets. Pre-cDC1s from WT mice had chimerism systemically comparable to the bone marrow, as expected. Furthermore, pre-cDC1s did not rely on CCR1 and CCR5 to migrate however they relied on CCR2. Interestingly, pre-cDC1s from iCCRKO donors were able to seed all tissues except from the TME. As expected, CCR7KO pre-cDC1s could migrate to peripheral tissues

but not seed lymph nodes with mDC1. We found that, like mDC, rDC seeding also relied on CCR7. Additionally, supporting evidence that CCL21 in the TME recruits cDC1, CCR7KO pre-cDC1s had reduced TME infiltration. Interestingly, as with monocytes, XCR1 expression was needed for pre-cDC1 seeding systemically. Confirming that preDC migration is heterogeneous between subsets, pre-cDC2 receptor reliance varied from pre-cDC1 (Figure 12.4). As with pre-cDC1, pre-cDC2 migration did not rely on CCR1 but did rely on CCR2 and XCR1. However, iCCRKO pre-cDC2s had reliance on expression of these receptors to migrate to all tissues, particularly the TME. Additionally, there was a significant reduction in mDC2 and rDC2 seeding in the MLN in CCR5KO chimeras. Finally, as expected, mDC2s relied on CCR7 to seed the lymph nodes and, as with rDC1, rDC2 also relied on CCR7 but to a lesser extent in the tdLN compared to the MLN.

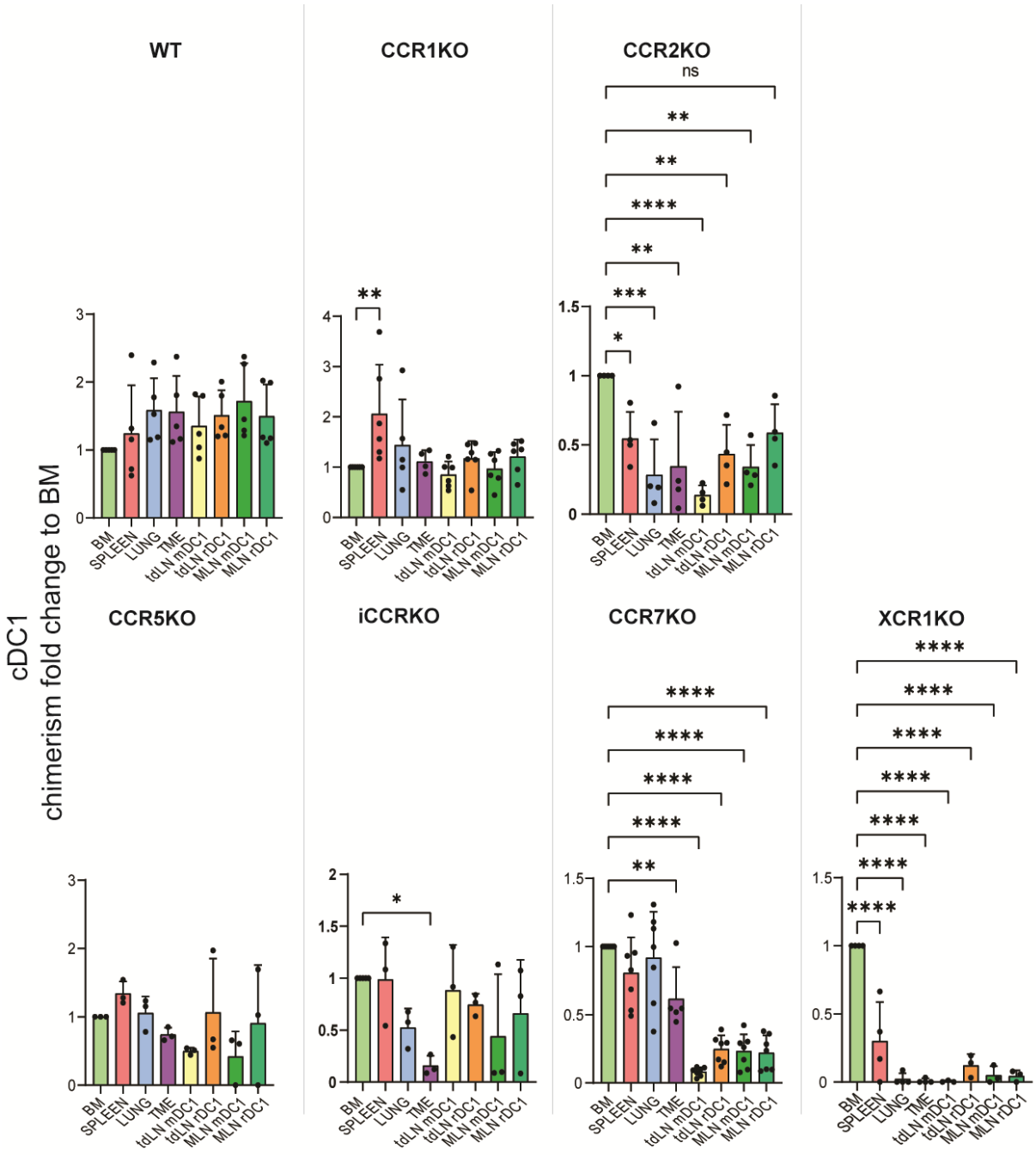


Figure 6-3 – pre-cDC1 subsets have differential reliance on chemokine receptors to egress the BM and migrate systemically. *Donor bone marrow cells from wildtype (WT), CCR1KO, CCR2KO, CCR5KO, iCCRKO, CCR7KO and XCR1KO CD45.2 mice were lysed of red blood cells and transferred at a concentration of 1x10^6/100µLin PBS intravenously into Busulfan treated CD45.1 recipient mice. Percentage chimerism of cDCs in the bone marrow and systemic tissues was calculated by CD45.2 expression. Fold change of chimerism in each tissue compared to the bone marrow was calculated and One-way ANOVA with multiple comparisons was used to statistically test the chimerism fold change in each tissue compared to the bone marrow (BM). cDCs were identified by lineage (CD3, B220, NK1.1, SiglecF, F4/80)-, CD24+ (in the TME) and CD11c+. Then, BM, lung and TME cDCs were gated as CD103+ or CD11b+. Spleen cDCs were gated as CD8a+ or CD11b+. Lymph node mDC were MHCIIhi, CD103+ or CD11b+. Lymph node rDC were MHCIIhi, CD8a+ or CD11b+ *P<0.01**P<0.005***P<0.0005***P<0.0001.*

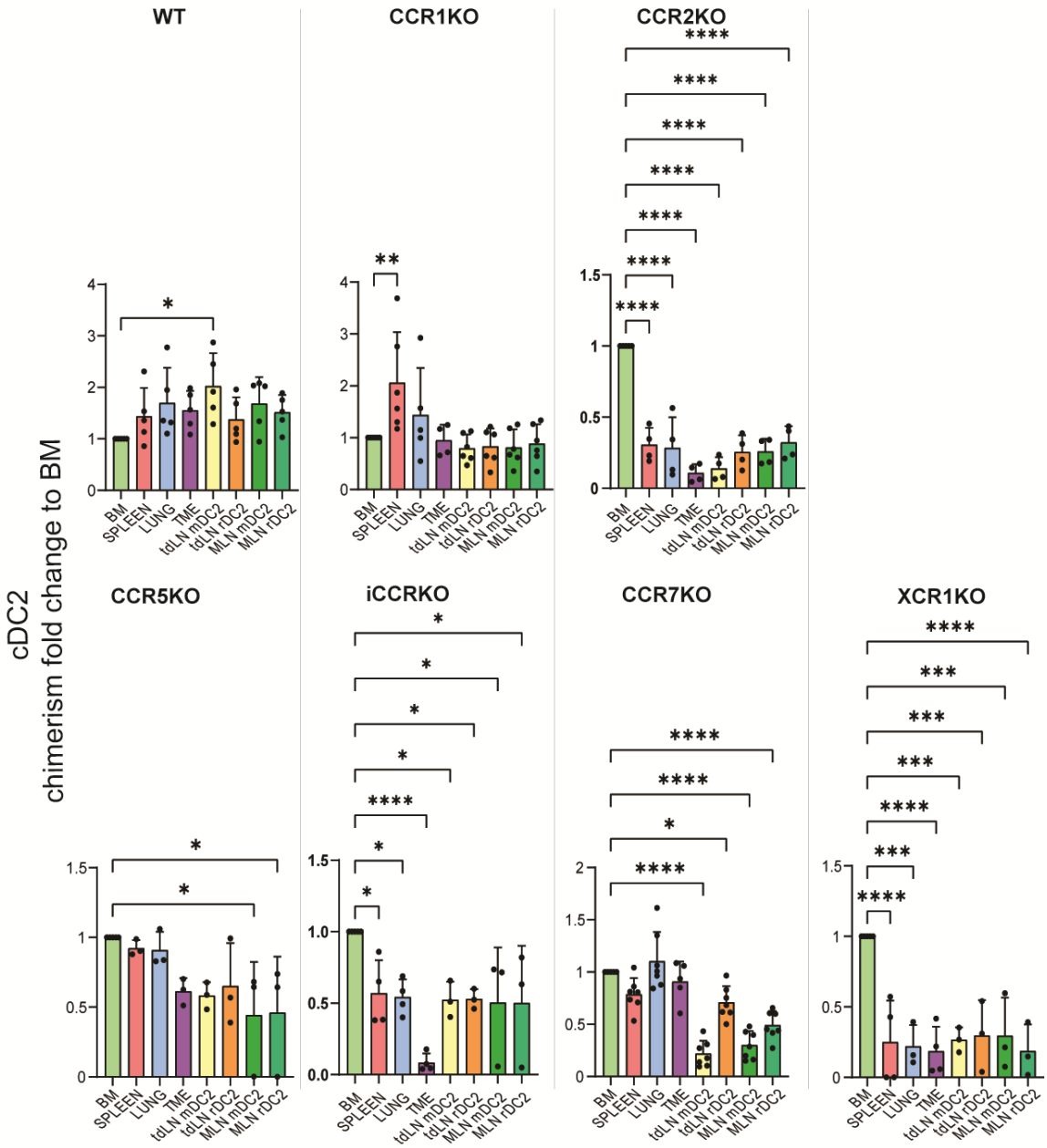


Figure 6-4- pre-cDC2 subsets have differential reliance on chemokine receptors to egress the BM and migrate systemically. *Donor bone marrow cells from wildtype (WT), CCR1KO, CCR2KO, CCR5KO, iCCRKO, CCR7KO and XCR1KO CD45.2 mice were lysed of red blood cells and transferred at a concentration of 1x10^6/100µLin PBS intravenously into Busulfan treated CD45.1 recipient mice. Percentage chimerism of cDCs in the bone marrow and systemic tissues was calculated by CD45.2 expression. Fold change of chimerism in each tissue compared to the bone marrow was calculated and One-way ANOVA with multiple comparisons was used to statistically test the chimerism fold change in each tissue compared to the bone marrow (BM). cDCs were identified by lineage (CD3, B220, NK1.1, SiglecF, F4/80)-, CD24+ (in the TME) and CD11c+. Then, BM, lung and TME cDCs were gated as CD103+ or CD11b+. Spleen cDCs were gated as CD8a+ or CD11b+. Lymph node mDC were MHCIIhi, CD103+ or CD11b+. Lymph node rDC were MHCIIhi, CD8a+ or CD11b+ *P<0.01**P<0.005***P<0.0005***P<0.0001*

CCR2 and iCCRs were required for preDCs to egress the bone marrow, as evidenced by the reduced chimerism cDC subsets from the bone marrow to the spleen Thus, to unpick which receptors were essential for cell migration once they had egressed the bone marrow, we compared the chimerism between the spleen and the rest of the peripheral tissues and lymph nodes (Figure 12.4). . Additionally, when lacking XCR1, chimerism of both monocytes and cDCs was drastically reduced from the BM to the spleen. Since these data are preliminary and both cell types were impacted, repeats are needed to confirm this. It is unclear if the lack of XCR1 impacted cell development, bone marrow egress or tissue infiltration. However, when comparing chimerism (fold change to the bone marrow) between the spleen and periphery, there were no differences seen in the chimerism of monocytes and cDC2s. On the other hand, XCR1 was essential for seeding of cDC1s in the lung, TME and mDC1 in the tdLN when XCR1KO pre-cDC1s were able to egress the bone marrow. Furthermore, an increased reliance on CCR2 was seen for tumour infiltrating monocytes. CCR2 was also needed for seeding of mDC1 in both the tdLN and MLN. Finally, there was a particular reliance on iCCRs on cDC1 and cDC2 infiltrating the TME but not the other peripheral tissues. Therefore, we present novel evidence that XCR1 is needed for cDC and monocyte precursors to exit the bone marrow and there exists tissue specificity with which receptors direct migration to each tissue. This is particularly evident with XCR1 reliance on tumour infiltrating cDC1 and iCCR reliance on both cDC1 and cDC2 tumour infiltration.

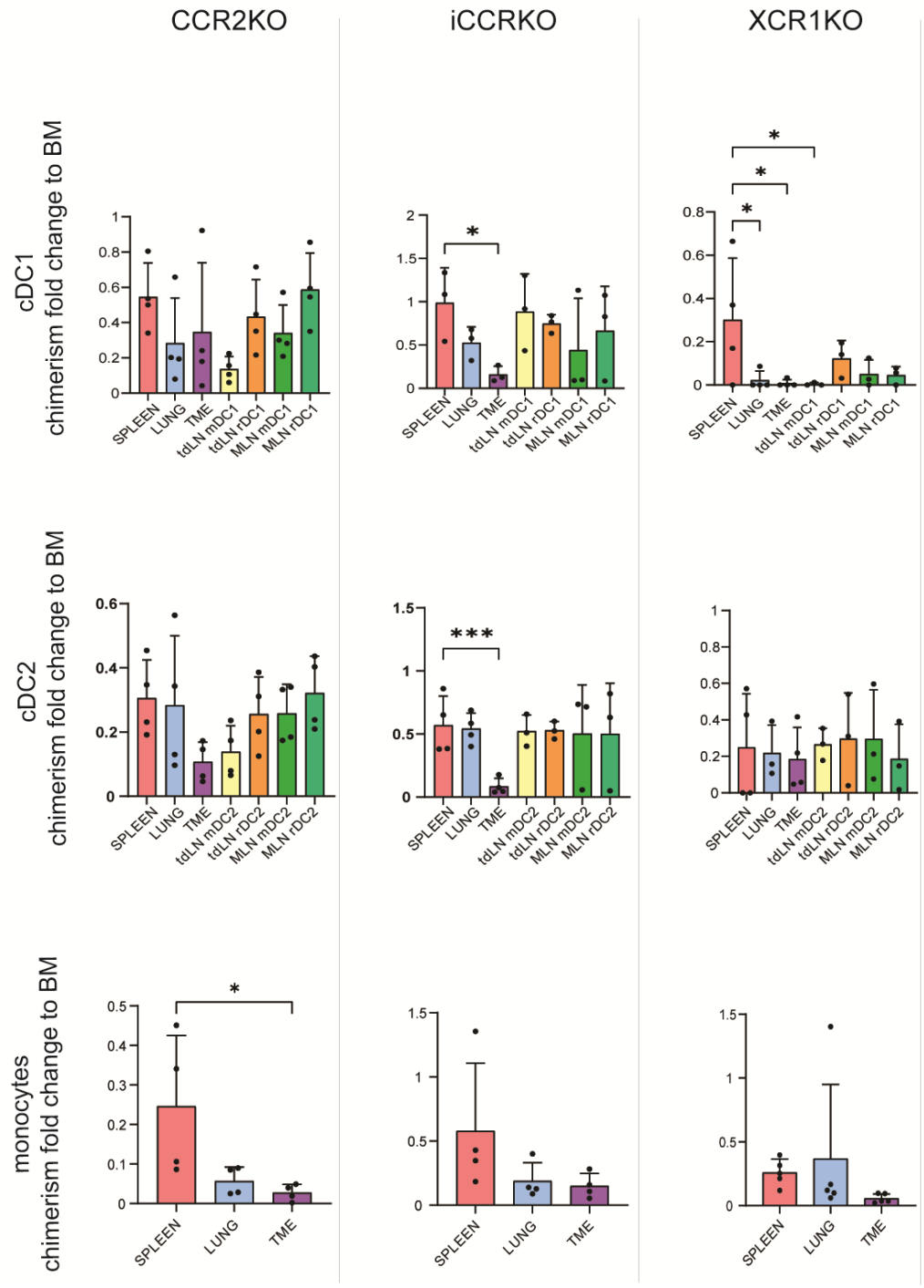


Figure 6-5 – preDC subsets have differential reliance on chemokine receptors to infiltrate tissues once they have egressed the bone marrow. *Donor bone marrow cells from wildtype (WT), CCR1KO, CCR2KO, CCR5KO, iCCRKO, CCR7KO and XCR1KO CD45.2 mice were lysed of red blood cells and transferred at a concentration of 1x10^6/100µLin PBS intravenously into Busulfan treated CD45.1 recipient mice. Percentage chimerism of cDCs in the bone marrow and systemic tissues was calculated by CD45.2 expression. Fold change of chimerism in each tissue compared to the bone marrow was calculated and One-way ANOVA with multiple comparisons was used to statistically test the chimerism fold change in each tissue compared to the spleen. cDCs were identified by lineage (CD3, B220, NK1.1, SiglecF, F4/80)-, CD24+ (in the TME) and CD11c+. Then, BM, lung and TME cDCs were gated as CD103+ or CD11b+. Spleen cDCs were gated as CD8a+ or CD11b+. *P<0.01**P<0.005***P<0.0005***P<0.0001.*

6.2.3 Developing an assay to knock out chemokine receptors in haematopoietic stem cells

We had access to a limited supply of chemokine receptor knockout mice that we could utilise for chimera development. As such, we aimed to develop a means to create chemokine receptor knockout HSCs that we could use to make bone marrow chimeras. Our experimental plan was to harvest bone marrow from congenic WT mice; expand their HSCs in culture; edit the HSCs to knockout the chemokine receptor of interest; and use these knockout HSCs to create bone marrow chimeras (Figure 12.5 A). Following the method from Ochi and colleagues (Ochi et al. 2021), we enriched bone marrow cell cultures for HSCs *in vitro* and confirmed that they included the LT-HSC subset, essential for chimera generation (Schoedel et al. 2016), when examined after two weeks in culture (Figure 12.5 B). When transducing cells, constructs can be designed to encode a resistance gene to the antibiotic puromycin. Therefore, when cells are treated with puromycin, only those successfully transduced will be able to resist the puromycin-induced death. The researchers had reported that a concentration of 1ug/ml of puromycin was used to enrich for transduced cells (Ochi et al. 2021) which we confirmed as a dose able to kill the majority of HSCs *in vitro* (Figure 12.5 C). We then attempted to transduce the HSCs using two GFP constructs, a puromycin-resistance non-GFP construct and compared the HSC GFP expression compared to a non-transduced control. HSCs expressed GFP when transduced with the two GFP constructs, although to a much higher level with PLKO-GFP compared to pLentiGFP (Figure 12.5 D). As expected, no GFP was expressed in HSCs in the non-transduced control and the cells transduced with the puromycin-resistance non-GFP construct. We are now in the process of optimising these transductions and testing our ability to knockout chemokine receptors in these cultures using CRISPR. Finally, we confirmed that after two weeks in culture that we could use these HSCs to create bone marrow chimeras. We injected congenic recipient mice with varying numbers of cultured HSCs or PBS as a control and, promisingly, we observed an increase in $CD8^+$ T-cell chimerism in the blood as the number of transferred cells increased (Figure 12.5 E). This chimerism was even higher at 12 weeks post injection. Then, subcutaneous B16ZsGreen-minOVA tumours were given to these chimeric mice at 12 weeks post injection and preDC, cDC and monocyte chimerism was measured in the bone marrow, spleen and TME. In all locations, cell chimerism could be identified and was influenced by the dose of HSCs injected (Figure 12.5 F). Based on this,

we have confirmed that we are able to culture HSCs *in vitro*, edit these HSCs and then use them to create chimeras. Once we have optimised our method of knocking out chemokine receptors by CRISPR we will be able to investigate the importance of these receptors in preDC migration systemically.

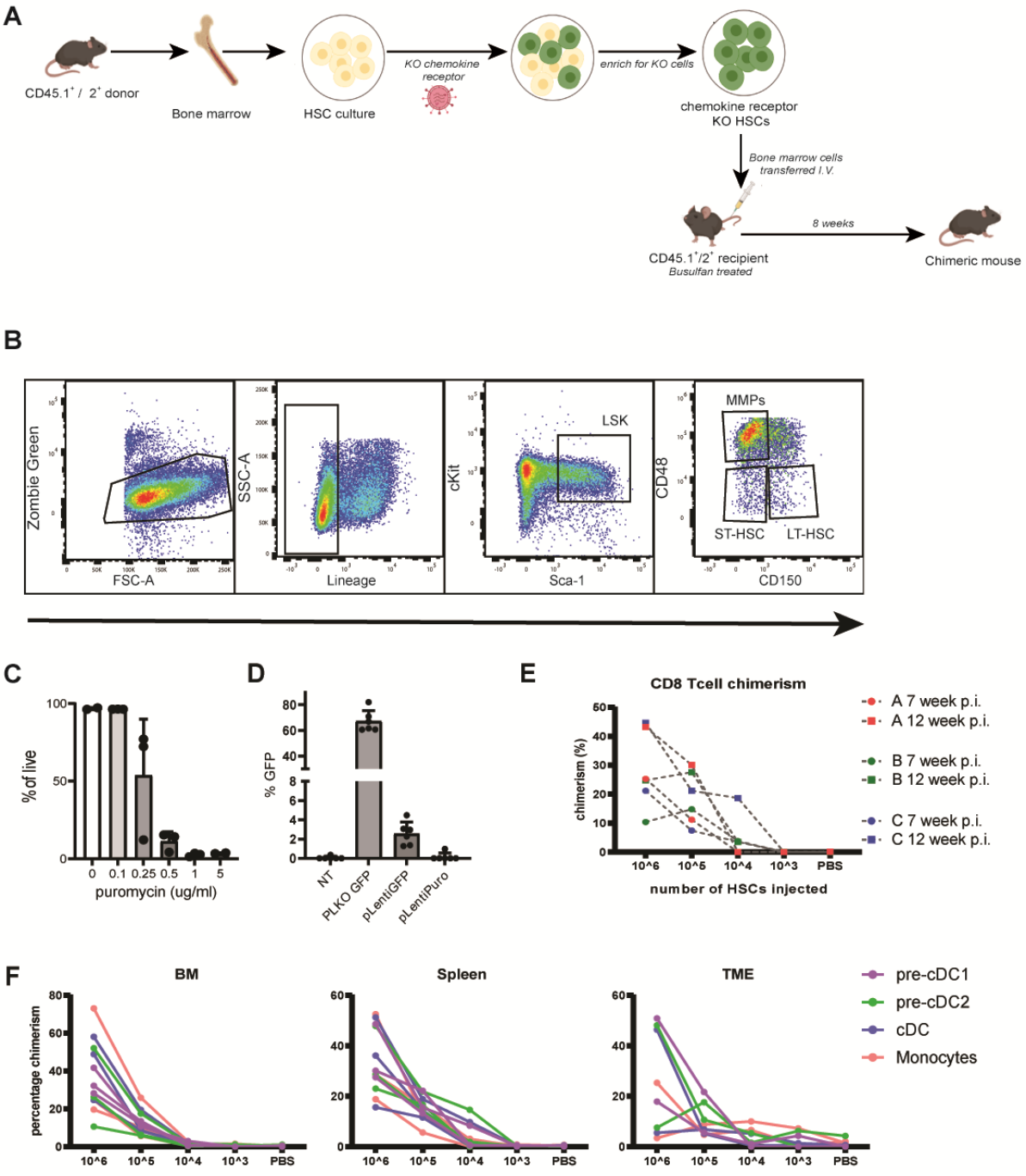


Figure 6-6 - HSCs can be expanded in culture, transduced, and create chimeras*. A) schematic displays experimental plan to use cultured HSCs, which have been edited, to make bone marrow chimeras B) Bone marrow cells were magnetically enriched for cKit+ cells and following the published method* (Ochi et al. 2021)*, HSCs were grown in culture. After two weeks of culture, cells were analysed by flow cytometry. HSCs were*

identified as single live lineage (CD3, Ly6G, CD11b, B220, Ter119)- cells and separated into cKIT+ Sca-1+ LSKs, cKIT+ Sca-1+ CD48+ CD150- MMPs, cKIT+ Sca-1+ CD48- CD150lo/- ST HSCs and cKIT+ Sca-1+ CD48- CD150-+ LT HSCs. C) HSC viability to increasing concentration of puromycin. D) % GFP of all cells following transduction with PLKO GFP, pLentiGFP, pLentPURO non-GFP control and a non-transducted (NT) control. E) after two weeks in culture, CD45.1+ HSCs were transferred intravenously into busulfan treated CD45.2+ recipient mice. CD8+ T-cell chimerism was measured by flow cytometry at 7- and 12-weeks post injection (p.i.), data points represent 3 biological replicates (A, B and C) per group. D) at 12 weeks p.i. chimeras were subcutaneously injected with B16zsGreen-minOVA cells. Bone marrow (BM), spleen and tumour (TME) were harvested at endpoint. percentage chimerism of preDCs, monocytes and cDCs were calculated.

6.3 Discussion

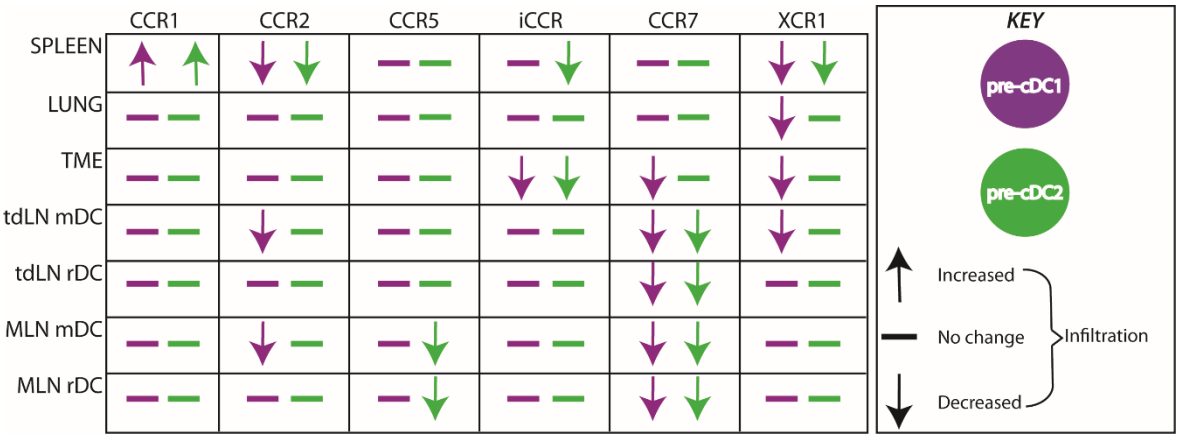
Bone marrow chimeras fit our criteria to measure preDC migration. The assay is competitive and sensitive. Furthermore, it allows the development of preDCs from both WT and KO HSCs and hence preDCs can egress when they are at the correct developmental stage and there will be a continual replenishment of haematopoiesis of both donor and recipient cells. Using Busulfan, a less toxic approach than total-body irradiation, recipient mice were prepared for engraftment. Subsequently, bone marrow from donor cells was injected intravenously and after 8-weeks, chimeric mice were used for further experimentation. Chimerism was measured by the percentage of cells originating from donor cells, identified by congenic markers. Since chimerism varied from recipient to recipient, we needed a means to normalise data between recipients. As such we decided to calculate the percentage chimerism of cells in each tissue and subsequently calculate the fold change compared to the chimerism within the bone marrow. We chose the bone marrow to normalise to since it is the source of haematopoietic precursors and potentially a subset of preDCs remain localised here as they develop in to bone marrow resident cDCs. However, it is important to note that different methods of normalisation should be analysed to confirm our findings. Each analysis method comes with its own limitations. For example, with our current method of normalisation to bone marrow cDCs, this does not consider the importance of the chemokine receptor for the maturation of preDC to cDCs within the bone marrow in general. We considered including an HSC panel for flow cytometry analysis and calculate HSC chimerism as a baseline. However, if the chemokine receptor is required for haematopoiesis, this will impact our analysis. Alternatively, we could calculate the average chimerism of multiple bone marrow immune cells as a baseline, but this also comes with the limitation of not considering the importance of the chemokine receptor in cell development. Thus, no method is ideal but analysing the data by multiple approaches may

clarify if we have identified a chemokine receptor which has importance in haematopoiesis, preDC maturation or BM egress and then tissue infiltration.

To confirm the accuracy of chimeras in measuring immune cell migration we initially measured monocytes. As with our previous experiments, we used monocytic CCR2 dependent migration as a control for assay sensitivity. As expected, CCR2 and iCCR deficiency diminished monocytic migration to peripheral tissues. Interestingly, a similar result was observed when donor cells lacked XCR1. As far as we are aware, this requirement for monocytes has not been identified. To understand if XCR1 was needed for monocytes to egress the bone marrow or to seed peripheral tissues, we compared chimerism to the spleen where migrating cells in the blood pass through. Here, we identified no significant difference between monocyte chimerism in the spleen, lung, and tumour. This suggests that tissue infiltration is not impacted by XCR1 depletion and therefore conclude that a XCR1 likely contributes to monocyte development within the bone marrow or egress from the bone marrow.

With this we were assured that chimeras provided an accurate and sensitive approach to investigate haematopoietic cell migration and, as such, we began to unpick the receptors required for preDC migration peripherally. Summarised data can be visualised in Table 12.1 and is discussed below.

Table 6-1– Summary table displaying the impact of chemokine receptor knockout on preDC tissue infiltration



CCR2 was required for both precursors to egress the bone marrow and seed peripheral tissues. This supports previous evidence that during homeostasis CCR2 is required to seed the lung with cDC1 and cDC2 (Nakano et al. 2017). In comparison, previous reports (Cabeza-Cabrerizo, Minutti, et al. 2021a; Pereira da Costa et al. 2023a) identified that CCR2 was only

required to seed inflamed lungs during influenza infection. So, we compared preDC chimerism in the lung to the spleen to gain an idea of which part of preDC migration was impacted by CCR2. Indeed, CCR2 was not needed for lung tissue infiltration since no significant difference was observed between spleen and lung chimerism. This suggests that the sensitivity of this migration assay has allowed for the detection that CCR2 is involved in preDC bone marrow egression even during homeostasis but not infiltration to the lung. Interestingly, compared to the spleen, CCR2 KO pre-cDC1s had reduced ability to seed the tdLN and MLN with mDC1 yet this was not the case with mDC2. Again, this supports our hypothesis that cDC subsets utilise different mechanisms to migrate.

Next, we analysed iCCRKO chimeras. Intriguingly, pre-cDC1 were not inhibited from egress when they lacked iCCR despite also lacking CCR2. In Chapter 1 we demonstrated an increase in CCR2 and decrease in CCR5 as preDCs differentiated and we theorised that CCR5 may be involved in preDC bone marrow retention, as it does with macrophages (Seyfried et al. 2021a). Therefore, based on our chimerism data we considered that the absent CCR5 reduces preDC retention and this removes the impact of the absent CCR2. This supports our hypothesis that a balance between CCR5 and CCR2 allows the preDCs to remain localised to the bone marrow during development and able to egress when they are ready to. Interestingly, CCR5 KO alone did not induce an increase in preDC egress, therefore, the balance in expression between low CCR2 and high CCR5 and not CCR5 expression alone may be involved in pre-cDC1 retention.

Pre-cDC1s from iCCRKO donors were able to seed all tissues except from the TME, suggesting that one, or multiple of the iCCR receptors in combination, specifically allows pre-cDC1s to infiltrate the TME. In contrast, iCCRKO pre-cDC2s had reduced ability to egress from the bone marrow, potentially pointing to a greater reliance on CCR2 to egress. Once in the blood, all tissues were seeded equally with pre-cDC2 except the TME which also required both infiltrating preDC subsets to express iCCRs. Of the iCCR ligands, CCL3 significantly increased both preDC subsets in our chemokine overexpression assays (Chapter 2). Therefore, we suggest that CCL3 may increase preDC recruitment via interaction with its receptors CCR1 and CCR5. Alone, these receptors were dispensable for both preDCs to enter the TME, therefore a combination of CCR1 and CCR5 expression may be required.

Alone, CCR5 was not essential for pre-cDC1 migration but improved pre-cDC2s seeding the MLN with rDC2 and mDC2. Additionally, pre-cDC1 and pre-cDC2 did not rely on CCR1 alone to egress or infiltrate. Interestingly, CCL5 is a ligand for both CCR1 and CCR5 and had previously been suggested to induce cDC1 tumour infiltration (Böttcher et al. 2018a), however, our data here supports our chemokine overexpression analysis that CCL5 does not induce infiltration of either preDC subset.

Next, CCR7 was investigated. As expected, CCR7KO preDCs could migrate to most peripheral tissues but not seed lymph nodes with mDC1 and mDC2. However, supporting our evidence and others (Novak et al. 2007; Shields et al. 2010) that CCL21 in the TME differentially recruits cDC1, CCR7KO pre-cDC2 and not pre-cDC1 were able to seed the TME. We also found that lymph node rDC seeding relied on CCR7; rDC1 in the tdLN and MLN and rDC2 in the MLN and to a lesser extent in the tdLN, were reduced when precursors lacked CCR7. Importantly, this adds to the evidence presented in a recent publication demonstrating that, in CCR7 KO chimeras, the spatiotemporal control of lymph node rDC1 within the paracortex required CCR7 (Ugur et al. 2023a). Here, rDC1 were suggested to be trapped in the medulla, close to the blood vessels, and unable to migrate within the tissue. Thus, CCR7 is likely to have an important role in the infiltration of resident precursors in the lymph node tissue. Although this finding does not allow us to create specific means to inhibit rDC in the lymph node without inhibiting mDC, is has provided novel evidence of the signals controlling rDC infiltration and may allow us to develop techniques to specifically inhibit them in the future.

Lastly, as with monocytes, XCR1 expression was needed for both preDC subsets to egress the bone marrow. This supports the evidence that XCR1 has a particular role during development in the bone marrow that supports their egress. Of the few that egressed, pre-cDC2 infiltration was equal peripherally. In contrast, infiltration of cDC1 in the lung, TME and mDC in the tdLN were reduced. Supporting this, overexpression of XCL1 in the TME (Chapter 2) increased cDC1 infiltration, however, this did not reach significance. Together these data suggest that on top of being vital for monocyte and preDC egress from the bone marrow, certain tissues, including the TME in our model, require XCR1 on pre-cDC1s for their infiltration.

Overall, the data presented in this chapter has provided novel evidence demonstrating the differential chemokine receptors that preDCs use to egress the bone marrow and infiltrate peripheral tissues and lymph nodes. Using this assay, we provide a means to investigate

preDC migration to all tissues during different contexts. Based on evidence that influenza infection in the lung adapts the requirement of preDCs to use CCR2 to infiltrate the tissue (Cabeza-Cabrerizo, Minutti, et al. 2021a; Pereira da Costa et al. 2023a), we aim to investigate this using influenza-infected chimeras. Additionally, there exists potential for different chemokines being involved in preDC seeding between tumour types, as such we aim to create chimeric mice to study multiple models of cancer. Furthermore, a limitation of this study is that we did not include healthy skin tissue as a model of a location proximal to the tumour. Including this in future studies will allow us to identify if there exist differences in preDC migration patterns to the TME compared to healthy tissue despite being located proximally. Finally, a benefit of these chimeric mice compared to adoptive transfers is their longevity, allowing longer-term models of infection and cancer to be investigated. These chimeras will allow the migration of all haematopoietic immune cells to be studied and as such we have optimised an assay that will contribute to the field by allowing tissue-specific migration pathways to be unpicked in homeostasis, inflammation, and cancer.

Nonetheless, there exist only a few available chemokine receptor knockout mice to be used for chimera creation. Therefore, we decided to optimise a means to engineer HSCs *in vitro* that subsequently can be used to create chimeras. Although in the early stages of optimisation, we have confirmed the ability to grow and transduce cKit^{+} bone marrow HSCs in culture. These HSCs could successfully engraft recipient mice and create long-term chimeras. We are now aware of the conditions needed to maintain HSC growth and the dose of puromycin needed to select transduced HSCs, therefore, we are now in the stages of optimising HSC transductions with CRISPR vectors in order to knockout additional chemokine receptors. Using this, we will be able to understand the reliance on the chemokine system for endogenous preDC migration.

Based on our hypothesis and previous suggestions (Zeng et al. 2013b) that preDCs are predestined during development to migrate to specific tissues, our data has provided novel and intriguing evidence supporting this idea. By unpicking these specific signals, we may be able to interfere with preDC migration, inhibiting it during autoimmunity or promoting it in cancer, infection and vaccination responses.

7 Final discussion and future directions

The data presented in this thesis has demonstrated our contribution to understanding the specific signals involved in allowing preDCs to egress the bone marrow, migrate systemically and seed peripheral tissues and lymph nodes with cDCs. We have presented data which aligns with previous publications as well as novel evidence explaining preDC migration. Our main findings are summarised in Figure 13.1 and discussed below.

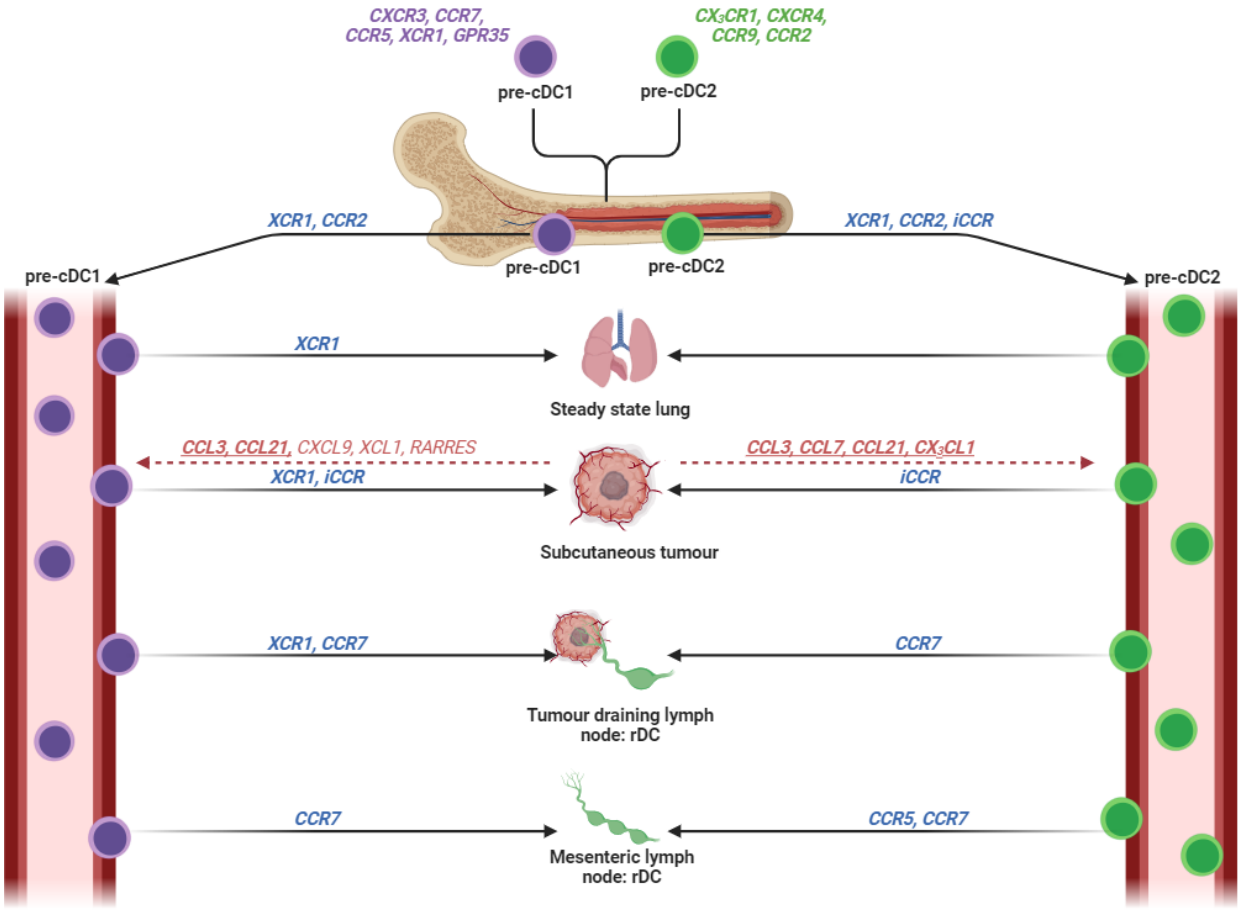


Figure 7-1- preDC migration is controlled chemokine receptors that induce tissue-specific migration. *This schematic summarises the main findings of this thesis. Chemokine receptors differentially expressed on bone marrow pre-cDC1 are shown in purple, while those in green represent receptors more highly expressed on pre-cDC2. Receptors shown in blue indicate those used by preDC subsets to migrate to specific tissues, as shown by the black arrows. The red dotted arrow highlights the chemokines that may be used therapeutically to increase preDC recruitment in a subcutaneous tumour model. Chemokines in bold and underlined induced significant increases in cDC1 or cDC2 while chemokines in regular font showed a trend to increasing the subset.*

7.1 preDC chemokine receptor expression

Defining the mechanisms involved in preDC migration will contribute to our basic understanding underpinning cDC biology as well as providing potential routes to exploit the chemotaxis of preDCs. Based on previous publications, and the migration of other immune cells, we hypothesised that preDC migration might be controlled by tissue-specific mechanisms. In this case, preDCs would be predestined within the bone marrow pre-

egression and migration. As such, understanding these mechanisms could allow us to enhance preDC migration to 'cold' TMEs, which lack cDCs, and vaccination areas while inhibiting migration to tissues experiencing autoimmune responses or allergies. Thus, we aimed to unpick the chemotaxis of preDCs by initially defining the expression of chemokine receptors on preDC subsets.

In Chapter 1 we confirmed, using publicly available data, the distinct expression of chemokine receptors of pre-cDC1 and pre-cDC2. While CX_3CR1 was previously associated with pre-cDC2s and CXCR3, CCR5, CCR2 and XCR1 on pre-cDC1s (Cook et al. 2018a; Lança et al. 2022; Schlitzer et al. 2015b), We identified that pre-cDC1s express higher levels of CXCR3, CCR7, CCR5, GpR35 and XCR1 compared to CX_3CR1, CXCR4, CCR9 and CCR2 on pre-cDC2s. In addition, we found CCR1 and CXCR2 are expressed on preDCs in general. Next, we used iCCR mice to analysis expression of CCR1, CCR2, CCR3 and CCR5 on preDC subsets. Here we confirmed the differential expression of CCR2 between pre-cDC1 and pre-cDC2s and CCR5 trended higher on pre-cDC1s. Accordingly, preDC subsets utilise distinct chemokine receptors suggesting their migration patterns vary.

Excitingly, we identified that as preDCs develop from non-committed subsets, they increase expression of CCR2 while downregulating CCR5. With this, we hypothesised that a balance of low CCR2 and high CCR5 expression is involved in the retention of preDCs during development and subsequently egress from the bone marrow when they have differentiated and the balance shifts. CCR2 has previously been suggested to be upregulated on preDCs in the bone marrow, and more readily during peripheral infection, which enhances the CXCR4 dependent egress of preDCs (Pereira da Costa et al. 2023b). Additionally, CCR5 has been shown to retain macrophages within the bone marrow (Seyfried et al. 2021b). With this, we theorised that the balance between CCR2 and CCR5 expression on preDCs adapts throughout preDC development and may be involved in controlling bone marrow egress.

Finally, we aimed to identify if chemokine receptor heterogeneity within preDC subsets was associated with tissue specific migration, supporting previous publications (Zeng et al. 2013b). Here we provided novel evidence that within preDC subsets, groups of cells express heterogeneous combinations of chemokine receptors. We recognised this in the bone marrow and hypothesised that these combinations could show preDCs predestined to migrate to certain tissues. So, to understand if the heterogeneity within preDC subsets

represents this predefined migration or just stages of preDC development, we analysed chemokine receptor expression combination in the periphery. Overall, we identified heterogeneity between and within preDC subsets in the periphery suggesting that certain chemokine receptors were associated with preDC infiltration to certain tissues and that this heterogeneity is inherent to preDCs rather than just a feature of development. To characterise these tissue specific subsets further, we have sorted preDCs to analyse them by scRNAseq. Here, we aim to further define these clusters of preDC subsets in terms of chemokine receptor, integrin and tissue homing receptor expression. Characterising which preDCs are destined to each tissue will contribute to our overall aim of exploiting preDC chemotaxis to specifically control preDC migration.

7.2 Therapeutically increasing cDCs within the TME

Promoting cDC numbers and function within the TME is an exciting approach to tackle resistance to current T-cell based immunotherapies for cancer. Thus, in Chapter 2, we screened chemokines for their ability to increase cDC subsets within the TME. Despite not measuring the endogenous signals involved in preDC recruitment and not accounting for different tissue/tumour types, it allowed us to measure the therapeutic potential of certain chemokines in improving cDCs within subcutaneous tumours. In our model, cDC1s were increased by CCL3, CCL21 and potentially XCL1 and RARRES while cDC2s were increased by CCL3, CCL7, CCL21, CX_3CL1 and slightly with CXCL9. Supporting this, previous publications have also identified chemokine signals which increase intratumoural cDC: CCL3 (Diao et al. 2010a); XCL1 (Böttcher et al. 2018a); CCL21 (Novak et al. 2007; J. D. Shields et al. 2010b). Additionally, the CXCL9 receptor CXCR3 was important in cDC1 infiltration to melanoma tumours (Cook et al. 2018a). On the other hand, CCL4 (Spranger, Bao, and Gajewski 2015b; Williford et al. 2019b) and CCL5 (Böttcher et al. 2018a) increased cDC recruitment in other studies which did not align with our data. These differences could be explained by differences in tumour type and potentially preDC tissue specific migration also applies to tumour type or tissue origin of the tumour.We have identified CX_3CL1 to be associated with cDC2 tumour infiltration, supporting our previous finding the pre-cDC2 differentially express CX_3CR1 compared to pre-cDC1. Additionally, selective recruitment of cDC2s was identified when CCL7 was overexpressed, which has not previously been identified. Thus, we have created a screen that has allowed us to unpick the chemokines associated with preDC recruitment to the subcutaneous TME. However, it is important to note that our screen does not demonstrate direct recruitment of preDCs in response to these

chemokines, rather an association with increased chemokines and frequency of the cDC subsets and thus the therapeutic potential of chemokines. Our screen may highlight the ability of chemokines to induce indirect recruitment of preDCs; promote the retention of cDCs; or indirectly support their maturation. To test if these chemokines are directly increasing preDC recruitment to the TME we could test the chemokine overexpressing tumours in our chimeric mice and ask: if the preDC lacks the chemokine receptor, is the effect of the chemokine overexpression ablated?

Additionally, these chemokine-induced changes to the cDC compartment may influence the balance of T-cell subsets within the TME and tdLN. Since both $CD8^+$ and $CD4^+$ T-cells, in combination with lack of Treg, are important to the anti-tumour immune response, the ideal chemokine therapeutic would recruit both subsets. Thus, analysis of the T-cell compartment will be vital to understand which cDC increasing agent corresponds to increased $CD8^+$ and non-Treg $CD4^+$ T-cells. This will be particularly important in the case of XCL1 and RARRES which, in our model, slightly increased cDC1s but significantly decreased cDC2s. Whether a decrease in cDC2s induces decreased Tregs and therefore promotes the anti-tumour response, or the decrease in effector $CD4^+$ T-cells abrogates the response, will be essential to understand.

It is likely that tumour-type will be an important factor associated with which chemokines are involved in preDC migration. Thus, we are currently optimising a universal means to measure preDC migration in multiple different, and more relevant, tumour models. Therefore, our future plans involve creating chemokines linked to CBDs (Ishihara et al. 2019b; Williford et al. 2019c), to therapeutically localise chemokines to the TME of multiple tumour models as well as test the ability of chemokines to augment anti-PD-1 therapy by increasing cDC infiltration. This will allow us to understand if different chemokines have different abilities to augment immunotherapies depending on the tumour type or if exploiting the system allows us to develop a universal therapy.

7.3 preDC migration is tissue specific

Previous *in vitro* assays, adoptive transfer and chimera experiments have provided insight into which chemokine receptors control preDC migration. Sensitivity and consistency were important factors in the design of our assay, but we also wanted to create a universal system to measure preDC migration systemically and in various contexts. Thus, in Chapter 3 we demonstrated that, despite some success, based on lack of sensitivity and consistency, we ruled out the use of total body knockout mice and adoptive transfers. This led us to the

development of bone marrow chimeras which we described in Chapter 4. Bone marrow chimeras allowed a competitive setting that was sensitive enough to identify requirements of chemokine receptors without complimentary pathways masking the effect of the receptor knockout. Additionally, with the constant replenishment of HSCs from donor knockout mice, this assay will allow us to investigate migration in long term models of infection and cancer. Furthermore, this assay will allow us to measure the migration of all haematopoietic immune cells peripherally during multiple contexts. Additionally, we are intrigued to investigate chemokine receptor dependent spatiotemporal regulation of preDC and cDCs. For example, which receptors induce the accumulation of cDC1 niches in the TME which prevent T-cell exhaustion (Dähling et al. 2022b)?; which receptors are important for the niche of developing preDCs within the bone marrow (Williams et al. 2017b)? and which niches within the bone marrow enrich for preDCs which upregulate different chemokine receptors and tissue homing genes?

Using this assay, we identified the chemokine receptors which may be important to bone marrow egress and tissue infiltration. Our novel data suggest a reliance on XCR1 for both monocytes and preDCs to egress the bone marrow. We hypothesised this based on the reduced chimerism from bone marrow to spleen where circulating preDCs accumulate. Further analysis will hopefully unpick the role of XCR1 in preDC retention and egress. Additionally, monocytes sand pre-cDC2s are not known to express XCR1, thus, repeating this analysis with XCR1 chimeras is required before making solid conclusions. However, it could be that during monocyte and cDC precursor development, XCR1 is required for endogenous development. By culturing XCR1 KO precursors at distinct stages of development, we may be able to understand at which stage XCR1 is important for cell differentiation.

Additionally, lack of CCR2 prevented both preDC subsets from egressing the bone marrow, supporting the previously published work that CCR2 was needed for preDCs to egress the bone marrow in homeostasis (Pereira da Costa et al. 2023b). Furthermore, CCR2 KO preDCs which exited the bone marrow were not required for infiltration into the lung, supporting the previous work that CCR2 is only required for lung infiltration during influenza infection (Cabeza-Cabrerizo, Minutti, et al. 2021b; Pereira da Costa et al. 2023b). On the contrary, CCR2 KO mice were previously used to show that in homeostasis preDCs require CCR2 to infiltrate the healthy lung (Nakano et al. 2016b). This may be explained by lack of preDC egress from the bone marrow rather than an impact on preDC infiltration to the healthy

lung. Interestingly, despite also lacking CCR2, iCCRKO had different impacts on preDC migration. The requirement of CCR2 on preDC bone marrow egress was abrogated in pre-cDC1s, but not pre-cDC2s, when they lacked iCCR. From this we hypothesised that since CCR5 was expressed during early preDC development (Chapter 1) and was involved in macrophage bone marrow retention (Seyfried et al. 2021b), potentially the lack of CCR5 here inhibits pre-cDC1 bone marrow retention allowing them to egress in a non-CCR2 dependent manner. The difference here between pre-cDC1 and pre-cDC2 may suggest that pre-cDC2s have an increased reliance of CCR2 to egress or our analysis has contamination of CCR2 dependent cells such as monocytes. Furthermore, Nakano and colleagues (Nakano et al. 2016b) also reported that preDCs do not require CCR7 to migrate, with the use of CCR7 KO mice. However, using this more sensitive approach, we identified a novel mechanism for CCR7 in allowing rDC infiltration in the lymph nodes. As expected mDC in lymph nodes were reduced when preDCs lacked CCR7 and, interestingly, rDC1 and rDC2 also required CCR7 on preDCs to seed the lymph nodes which supports the spatiotemporal control of rDC1 by CCR7 expression (Ugur et al. 2023a).

In terms of TME infiltration, cDC1s were reduced intratumorally when they lacked CCR7, supporting the ability of CCL21 to induce both subsets to infiltrate in our model and others (J. D. Shields et al. 2010b). XCR1 was important to seed the TME with pre-cDC1 and not pre-cDC2, supporting the differential ability of XCL1 to recruit pre-cDC1s in our model and others (Böttcher et al. 2018b). Additionally, TME infiltration of both preDC subsets required iCCR, but not CCR2. This suggests that additional receptors here were needed for preDC migration to the TME. Since CCR1 and CCR5 alone were not required for preDC TME infiltration but their ligand CCL3 induced cDC1 and cDC2 accumulation, we hypothesised that a combination of CCR1 and CCR5 on both preDC subsets, induces TME infiltration to CCL3 in our subcutaneous model.

Lastly, we demonstrated our optimisation of HSC engineering to allow us the ability to investigate the role of multiple more chemokine receptors, and other genes, in controlling preDC development and migration. Using this, we could investigate the importance of genes in the homeostatic control of haematopoiesis and seeding of peripheral tissues. Overall, the data presented in this chapter supports the hypothesis that expression of chemokine receptors induces a bias for preDCs have migration to specific tissue and this bias is subset specific. Additionally, we have demonstrated novel evidence explaining the

usage of chemokine receptors in seeding peripheral tissues and lymph nodes with a cDC compartment.

7.4 Conclusions

Despite the infromation presented in this theiss, it is imortant to note the overall limitations of this work. Firstly all experiments were carried out in male C57/BL6 SPF mice, and it is therefore likely that strain, age, sex and micrbiome bias have contributed to our conclusions. Following confirmation of these results, future work should include testing our hypothesis considering these factors. Next, this work has a lack of relevant human evidence, something that will have to investigated to understand the significance of this project to human biology. Ideally, analysis of preDCs from human bone marrow or preDCs expanded from peripheral blood HSCs will allow us to ask our questions in relevance to the human system.

To conclude, this project has contributed to, and expanded on, our understanding of the heterogeneity between preDC subsets, developed a universal assay for the migration of preDCs to be charactierised and provided some understanding of the therapeutic potential of chemokines to augment immunotherapy by increasing cDCs within the subcutaneous TME. From this, and the literature, we conclude that preDC migration is controlled by tissue, subset and context specific mechanisms that we have started to clarify.

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