The role of the atypical chemokine receptor CCX-CKR in progression and metastasis of cancer

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A thesis submitted to the University of Adelaide
in fulfilment of the requirements for the degree of
Doctor of Philosophy

May 2012
DECLARATION

I, Yuka Harata-Lee certify that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Yuka Harata-Lee, B.Sc. (Biomedical Sc.) (Hons)

2012
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<td>αMEM</td>
<td>Minimum Essential Medium Alpha</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CCX-CKR</td>
<td>Chemocentryx chemokine receptor</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
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<tr>
<td>CTLA</td>
<td>cytotoxic T-lymphocyte antigen</td>
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<tr>
<td>DARC</td>
<td>Duffy antigen receptor for chemokine</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DLN</td>
<td>draining lymph node</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DR</td>
<td>death receptor</td>
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<tr>
<td>DTT</td>
<td>DL-Dithiothreitol</td>
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<tr>
<td>E/F PBS</td>
<td>endotoxin-free phosphate buffered saline</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EGFR</td>
<td>epidermal growth factor receptor</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<td>EMT</td>
<td>epithelial-mesenchymal transition</td>
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<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
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<td>FCS</td>
<td>forward scatter</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>GOI</td>
<td>gene of interest</td>
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<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>GRK</td>
<td>G-protein coupled receptor kinase</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>ICCS</td>
<td>intracellular cytokine staining</td>
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<tr>
<td>IDO</td>
<td>indoleamine 2,3-dioxygenase</td>
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<tr>
<td>IF</td>
<td>immunofluorescence</td>
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<tr>
<td>IFN</td>
<td>interferon</td>
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<tr>
<td>IGF-1R</td>
<td>Insulin-like growth factor-1 receptor</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IMDM</td>
<td>Iscove’s Modified Dulbecco’s Medium</td>
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<tr>
<td>KO mice</td>
<td>knockout mice</td>
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<tr>
<td>LMP</td>
<td>low-molecular-weight protein</td>
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<td>LN</td>
<td>lymph node</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>mAB</td>
<td>monoclonal antibody</td>
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<td>MAPK</td>
<td>mitogen activated protein kinase</td>
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<td>MDSC</td>
<td>myeloid derived suppressor cell</td>
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<tr>
<td>MET</td>
<td>mesenchymal epithelial transition</td>
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<tr>
<td>MFI</td>
<td>mean fluorescent intensity</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MIC</td>
<td>MHC Class I chain-related molecules</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
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<td>MRCRB</td>
<td>mouse red cell removal buffer</td>
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<tr>
<td>MΦ</td>
<td>macrophage</td>
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<tr>
<td>NK cell</td>
<td>natural killer cell</td>
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<tr>
<td>NKT</td>
<td>natural killer T cell</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>PFA</td>
<td>paraformaldehyde</td>
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<td>PMA</td>
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</tr>
<tr>
<td>PMS</td>
<td>N-methyl dibenzopyrazine methyl sulphate</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethanesulphonyl fluoride</td>
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<tr>
<td>PNAd</td>
<td>peripheral node addressin</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>RAG</td>
<td>recombination activating gene</td>
</tr>
<tr>
<td>RG</td>
<td>reference gene</td>
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<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>ROI</td>
<td>reactive oxygen intermediate</td>
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<tr>
<td>RPLP0</td>
<td>ribosomal protein large P0</td>
</tr>
<tr>
<td>SCID</td>
<td>severe combined immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>shRNA</td>
<td>short-hairpin RNA</td>
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<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<td>SSC</td>
<td>side scatter</td>
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<td>TAM</td>
<td>tumour associated macrophage</td>
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<td>TAP</td>
<td>transporter associated with antigen processing</td>
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<td>TBS</td>
<td>Tris buffered saline</td>
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<td>Tc</td>
<td>cytotoxic T cell</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>Th</td>
<td>helper T cell</td>
</tr>
<tr>
<td>TIL</td>
<td>tumour infiltrating leukocyte</td>
</tr>
<tr>
<td>TMBS</td>
<td>tumour-bearing mouse serum</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>tumour necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>TSP</td>
<td>thrombospondin</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>wt</td>
<td>wildtype</td>
</tr>
<tr>
<td>XTT</td>
<td>2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilideinner salt</td>
</tr>
<tr>
<td>ZO</td>
<td>zona occudens</td>
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</tbody>
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PUBLICATIONS ARISING FROM THIS WORK

Manuscripts in preparation:


Harata-Lee Y., Comerford I., Bunting M.D., Li M., Bastow C., Smyth M.J., and McColl S.R., shRNA-mediated knockdown of atypical chemokine receptor, CCX-CKR leads to melanoma rejection through enhanced recruitment of anti-melanoma leukocytes.

Conference Proceedings:


Australian Society for Medical Research South Australian Meeting (2009): Poster Presentation entitled “The Atypical Chemokine receptor CCX-CKR suppresses the progression of murine melanoma.”
ABSTRACT

The significance of chemokine receptors CCR7, CCR9 and their ligands CCL19, CCL21, and CCL25 in various types of cancer including mammary carcinoma and melanoma has been highlighted over the last decade. The atypical chemokine receptor CCK-CKR is a high affinity receptor for these chemokine ligands but rather than inducing classical downstream signalling events promoting migration, it instead sequesters and targets its ligands for degradation. Therefore, CCX-CKR has been proposed to regulate chemokine bioavailability in vivo. This putative function of CCX-CKR to regulate the levels of pro-tumourigenic chemokines initially led to the hypothesis that local and systemic regulation of chemokine levels by CCX-CKR influences tumour growth and metastasis in vivo, and ultimately, targeting of CCX-CKR could be an effective cancer therapy. Three broad approaches were taken to investigate the role of CCX-CKR in tumour progression and metastasis including overexpression of the receptor on tumour cells, deletion from the mouse host and receptor expression knockdown in tumour cells. The results revealed that overexpression of CCX-CKR on 4T1.2 mouse mammary carcinoma cells inhibits orthotopic tumour growth. However, this effect could not be correlated with chemokine scavenging in vivo and was not attributed to host adaptive immunity from experiments performed during the course of the current study. On the other hand, overexpression of CCX-CKR on 4T1.2 cells also resulted in enhanced spontaneous metastasis and haematogenous metastasis in vivo. In vitro characterisation of tumourigenicity of 4T1.2 cells revealed that overexpression of CCX-CKR rendered them more invasive, less adherent to the ECM and to each other and more resistant to anoikis. These are established characteristics of cells which have undergone EMT and indeed, CCX-CKR overexpressing cells showed a typical expression pattern of EMT markers. In contrast, when endogenous expression of CCX-CKR is deleted in the mouse host, growth and metastasis of E0771 mammary carcinoma and B16 melanoma are inhibited, which is accompanied by elevated
levels of CCX-CKR ligands in tumours and relevant naïve tissues from CCX-CKR-deleted mice. Similarly, shRNA-mediated knockdown of endogenous CCX-CKR from B16 melanoma cells leads to the rejection of primary and secondary tumours. This effect is attributed to elevated levels of CCX-CKR ligands and CCR7+ and CCR9+ leukocytes in tumour tissues, which resulted in an overall enhancement of the host anti-tumour immune response. Consistent with these observations, growth of CCX-CKR knockdown tumours was comparable to that of control tumours in CCR7-deleted mice indicating host CCR7 dependency of CCX-CKR-mediated rejection of B16 melanoma. Together, findings from this study revealed important insights into the complex role of CCX-CKR in cancer progression and highlights CCX-CKR as a novel target for the development of more effective anti-melanoma therapies and potentially for the treatment of other types of cancer which affect millions of people worldwide.
CHAPTER 1: INTRODUCTION
CHAPTER 1: INTRODUCTION

1.1 Overview

Cancer is one of the leading causes of death worldwide with more than 7.5 million estimated mortalities and over 12 million newly diagnosed cases in 2008 (the latest available statistics). According to World Health Organisation statistics, the Australia/New Zealand region has the highest rate of new cancer cases in the world (when population age is normalised) in both males and females\(^1\). In the 5 most common cancers: prostate, colorectum, breast, melanoma and lung cancer, breast cancer is by far the most common cancer amongst females with over 12,000 new cases arising each year in Australia\(^2\). However, breast cancer can also develop in men, and males account for approximately 1% of deaths caused by breast cancer\(^2\). Prognosis of patients with breast cancer is relatively good compared with other types of cancer, particularly if it is diagnosed at an early stage. The 5-year survival rate for breast cancer patients with localised tumours is 98%, however this decreases by more than 15% if nodal metastasis is detected at time of diagnosis.

Melanoma is another common type of cancer in Australia in both males and females with more than 10,000 newly diagnosed cases each year. The most important measure of melanoma prognosis is the thickness of the lesion, and nodal biopsy is performed when a thick lesion (greater than 2mm) is excised. Although the 5-year survival for patients with localised melanoma is 99%, this decreases to 65% if spread is within the region of the primary melanoma, and further reduces to 15% if disease is widespread.

The cellular and molecular mechanisms involved in progression of cancer are extremely complex. Extensive research from various fields has started to unveil the mechanisms underlying the development and metastasis of cancer, and elucidated a number of important molecules involved in these processes. Although the knowledge gleaned from
these studies has enabled the development of therapies which can increase the survival rate of various cancers, further improvements are required to treat more advanced stages of cancer. The chemokine family is a group of molecules that was originally characterised as mediators of leukocyte migration that orchestrate immune responses. However, during the last decade they have attracted much attention as key players in various cancers, including breast cancer and melanoma. A better understanding of the mechanisms by which chemokines contribute to the progression and metastasis of tumours has potential to aid development of more effective means of early diagnosis and treatment.

1.2 Cancer biology

1.2.1 Hallmarks of cancer

Several critical properties of cancer cells distinguish them from normal healthy cells and enable them to grow, survive and disseminate within the host. For any cell to become a potentially lethal malignancy, it needs to acquire these cancer-specific properties. Tumour cells typically have undergone several genetic alterations including a point mutations and chromosomal disruptions, which result in modifications of various cellular characteristics. These “hallmarks of cancer”, as outlined by Hanahan and Weinberg\textsuperscript{3}, include: the ability to maintain growth signals, to evade growth suppressing signals, to enable limitless replication, to resist apoptotic signals, to reprogram energy metabolism, to induce angiogenesis, to evade host anti-tumour immune responses, and to activate invasive and metastatic processes (Figure 1.1). Each of these “hallmarks” will be depicted in detail below.
Figure 1.1: Hallmarks of cancer. There are 8 “hallmarks of cancer” which enable any transformed cells to establish as primary tumours and disseminate around the host to establish secondary tumours. They are distinct, yet complementary characteristics of cancer cells which give rise to complexity in our understanding of cancer biology. Underlying these hallmarks is genome instability, an inherent attribute of transformed cells, which further accelerates their acquisition of these hallmarks. Adapted and modified from ref. 3.
1.2.1.1 Maintenance of growth signals

The most fundamental and essential characteristic acquired by tumour cells is the ability to maintain proliferative signals, and tumour cells can achieve this in various ways. First, tumour cells are often capable of producing growth factors such as platelet-derived growth factor (PDGF) and transforming growth factor α (TGFα), and express growth factor receptors such as epidermal growth factor receptor family (EGFR)^4, which enable autocrine signalling of these pathways. Second, tumour cells can also stimulate surrounding cells, such as stromal fibroblasts^5, to produce various growth factors. Furthermore, genetic alterations can also result in interruption of signalling cascades downstream of growth signal receptors in such ways that a mitogenic signal is overactivated^6-8 or a negative-feedback loop to inhibit growth signalling pathways is disrupted^6,7,9.

1.2.1.2 Evasion of growth suppressing signals, contact inhibition and apoptosis

In normal cells overactivation of mitogenic pathways usually results in cell senescence and/or apoptosis^10. However, tumour cells often circumvent these cellular programs. For example, defects in cell cycle inhibitors, such as retinoblastoma and p53 proteins^11; regulators of contact inhibition, such as Merlin^12 and LKB1^13; anti-proliferative pathways, such as TGF-β signalling^14 are commonly observed in many types of cancer. Similarly, tumour cells often exhibit deregulation of apoptotic pathways normally activated upon overactivation of mitogenic responses or cell cycle arrest by up-regulating anti-apoptotic Bcl-2 and -XL proteins, and down-regulating pro-apoptotic factors, Bax and Bim^15.

Normal non-cancerous cells also experience growth suppression and/or undergo apoptosis when a certain number of successive cell divisions has occurred, leading to shortening of telomeres at the chromosomal extremities. However, many tumour cells overexpress the enzyme telomerase, which adds telomere repeats, preventing cell senescence/apoptosis^16.
This allows unlimited cell proliferation, effectively rendering the cells immortal. Furthermore, it was recently shown that telomerase, apart from its canonical function, can enhance proliferation and resistance to apoptosis\textsuperscript{17} and support repair of DNA damage\textsuperscript{18}.

1.2.1.3 Deregulation of cell metabolism

More recently, investigations have revealed that tumour cells utilise different energy metabolic pathways from non-cancerous cells. In cancer cells glucose metabolism is limited to glycolysis even in the presence of oxygen, i.e. aerobic glycolysis, and this is considered to be a result of tumour cell transformation which supports proliferation, survival and evasion of apoptosis. As aerobic glycolysis is an inefficient process of energy production, cancer cells achieve high efficiency in energy metabolism by up-regulation of glucose transporter, GLUT1 to increase uptake of glucose into cytoplasm and the reprogram of metabolic process is associated with oncogene activation and defect in tumour suppressor genes\textsuperscript{19-21}. It has also been shown that the up-regulation of GLUT1 can be driven not only by oncogenes but also under hypoxic conditions which is a feature of most tumours during progression\textsuperscript{22}.

1.2.1.4 Activation and maintenance of angiogenesis

Once cells achieve these oncogenic transformations and form a larger tumour, simple diffusion becomes insufficient to provide oxygen and nutrients to the tumour and to remove tumour-derived metabolites\textsuperscript{23}. In response, angiogenesis is stimulated. This process remains active at all times in tumour tissues whilst in normal tissues angiogenesis enters quiescence to avoid further branching of vessels\textsuperscript{24}. This chronic activation of angiogenesis in tumours occurs due to an imbalance between pro- and anti-angiogenic signalling such as vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1), respectively\textsuperscript{25}. 
1.2.1.5 Manipulation of immune system

In addition to supplying sufficient oxygen and nutrients to tumours, the newly formed blood vessels also provide a gateway for immune cells to enter tumour tissues where they may facilitate further angiogenesis. These leukocytes have also been shown to support tumour development, growth and metastasis by producing mutagenic factors, growth and survival factors, as well as factors promoting invasion and metastasis. Furthermore, although there is a long-standing concept of tumour immunosurveillance and an extensive body of evidence to suggest that under certain circumstances transformed cells can activate immune responses leading to resistance or rejection of developing tumours, it is also well established that tumour cells acquire the ability to manipulate and evade anti-tumour responses leading to tumour outgrowth. Recent advances in understanding the interplay between tumours and the immune system suggest that the outcome of this battle depends on the immunogenicity of the tumour itself. In some cases cellular transformation renders the tumour cells highly immunogenic leading to tumour rejection, while in other scenarios transformation leads to the generation of poorly immunogenic or highly immunosuppressive tumour cells leading to successful tumour progression. This concept is referred to as “cancer immunoediting” and will be discussed in further detail below.

1.2.1.6 Invasion and metastasis

The gateway created by neovasculature which allows leukocyte infiltration into tumours also provides the means whereby tumour cells exit their primary site and disseminate through the body, leading to metastases. This process is where the real danger of most cancers lies and also where the greatest difficulty lies in current cancer treatment. The detailed mechanisms underlying acquisition of metastatic characteristics by many types of tumour are still largely enigmatic. However, a significant research effort in the last two
decades has shed some light on our understanding of the process involved in the progression of primary tumour cells to highly invasive and motile disseminating cells which eventually colonise distant organs. Metastasis is a multistep process involving cascade of events: tumour cells first invade the surrounding tissues, then intravasate into lymph or blood vessels where a single cell or a small clump of cells detached from primary tumour travel within and to distant organs or tissues. Then, those cells are arrested in the capillaries of distant organs allowing them to extravasate into the organs and tissues, where they can proliferate and form secondary tumours (Figure 1.2). It is clear that transformed epithelial cells undergo several phenotypic and behavioural changes similar to a process called the “epithelial-mesenchymal transition (EMT)” which occurs during specific steps of embryogenesis and organ development.

The EMT is a biological process which allows polarised epithelial cells to assume mesenchymal phenotypes including enhanced motility and invasiveness, heightened resistance to apoptosis, and increased production of extracellular-matrix (ECM) components. As illustrated in Figure 1.3, EMT involves multiple alterations in cellular architecture and biochemical processes. EMT is initiated with dissociation of intercellular adhesion resulting from functional loss of homotypic adhesion molecules such as E-cadherin, claudins, occludins, zona occludens-1 (ZO-1) and desmoplakin followed by loss of microvilli and apical-basal polarity. The cells then undergo cytoskeletal rearrangement creating front-back polarity and up-regulation of alpha smooth muscle actin ($\alpha$-SMA) eventuating in enhanced cell motility. In the final stage of EMT, cells acquire heightened ability of invasion through basement membrane and stroma by producing matrix metalloproteinases (MMP). This last stage of EMT is also characterised by increased expression of ECM components such as fibronectin, vimentin, and collagen. N-cadherin is another important mesenchymal marker upregulated during EMT after down regulation of E-cadherin and this is referred to as “cadherin switch”.


Figure 1.2: Stages of tumour metastasis. A, transformed tumour cells acquire several hallmark features and establish as a primary tumour. An in situ tumour is surrounded by intact basement membrane. B, tumour cells induce chronic activation of angiogenesis to sustain oxygen and nutrient flow. Neovascularure provide a gateway for infiltration of leukocytes, which may further produce angiogenic and other tumour-promoting factors. C, genetic alterations in tumour cells can result in changes to cell motility, cell-cell and cell-ECM adherence and remodelling of ECM allowing tumour cells to invade surrounding tissue. D, highly invasive and motile cells enter lymphatic vessels where some tumours establish secondary tumours, or enter the circulation via the lymphatics. E, tumour cells can also enter directly into circulation, where they survive and disseminate. F, tumour cells eventually become arrested at capillary beds and extravasate into distant organ or tissue, G, where they establish secondary tumours. Adapted from ref.32.
Figure 1.3: Characteristics and process of EMT. The process of polarised epithelial cells changing to become mesenchymal cells is a complex multistep event which requires various alterations in cellular architecture and behaviour. It begins with loss of intercellular adhesion and apical-basal polarity, followed by cytoskeletal rearrangement and gain of front-back polarity, and then secretion of ECM-degrading enzymes. These highly motile and invasive cells can then enter circulation or lymphatics and reach secondary sites. It is believed that once in the parenchyma of distant tissue, these cells undergo MET in order to re-establish as solid tumours in the new environment. Adapted from ref. 35.
Another consequence of EMT which provides metastatic advantage to tumour cells is enhanced resistance to anoikis: detachment-induced apoptosis, which is triggered when healthy cells lose association with ECM\textsuperscript{36}. Although there is not yet solid evidence demonstrating a direct role for the EMT in conferring heightened resistance to anoikis, several studies have shown strong association between induction of EMT, particularly down regulation of E-cadherin, and resistance to anoikis\textsuperscript{36-39}. This mechanism allows tumour cells to survive after detaching from primary tumour and enter into lymphatic flow or circulation.

Although transformed epithelial cells undergo EMT and acquire mesenchymal traits which renders them highly motile, invasive, and resistant to anoikis, in order for them to re-establish in the distant tissues they need to re-acquire epithelial characteristics. This notion, termed the mesenchymal-epithelial transition (MET), is not yet fully understood, however some recent evidence indicates the importance of cell plasticity in successful metastatic colonisation\textsuperscript{40-42}. Reversion to an epithelial phenotype is believed to occur as a result of encountering a new microenvironment and the loss of signals originally responsible for inducing EMT at the primary tumour site\textsuperscript{34}. However, further investigation is necessary to elucidate when and how the switch between EMT and MET takes place.

1.2.2 Cancer immunoediting

During the last decade the importance and significance of the tumour microenvironment and cells present within the tumour have become evident. Contributors to successful tumour progression include endothelial cells, fibroblasts, cancer stem cells, and cells of haematopoietic origin\textsuperscript{3}. In particular, the involvement of the immune system in cancer biology has attracted much attention. As mentioned earlier, although there is a contribution of the immune response that is now considered to be one of the “hallmarks of cancer” that promotes tumour progression, it was originally hypothesised that the immune system
protected the host from developing tumours via “cancer immunesurveillance”\textsuperscript{30}. However, with advances in experimental technologies and animal models, there has been an accumulation of conflicting evidence indicating that activation of immune responses by transformed cells can result in different outcomes in different circumstances. Furthermore, in the beginning of the last decade, it was demonstrated that the cytokine, interferon-gamma (IFN-\(\gamma\)) together with lymphocytes not only functioned to prevent tumour development but also “edits” the immunogenic phenotype of tumour cells\textsuperscript{31} leading to tumour immune evasion and tumour progression. These observations led to the hypothesis of “cancer immunoediting” postulated by Dunn and colleagues which explains the paradoxical tumour outgrowth in an immunocompetent host\textsuperscript{29}. It was proposed that the process of cancer immunoediting occurs in a sequence of three phases: elimination, equilibrium and escape (Figure 1.4). Nascent transformed cells can be eliminated initially by immune effector cells, which results in immune selection and immune sculpting. This causes generation of tumour variants with decreased immunogenicity leading to the equilibrium phase. Eventually, further immune selection causes progression of tumours producing factors which induce immunosuppressive mechanism leading to the escape phase\textsuperscript{30}. However, in some circumstances tumours can directly enter equilibrium or escape phase, and external factors, such as environmental stress, aging, and an individual’s immune status can also change the directionality of the flow.

1.2.2.1 Elimination

The process of elimination is simply an eradication of developing tumours as a result of activated innate and adaptive immune responses. If this process reaches completion, the host remains free of cancer and represents the endpoint of the cancer immunoediting process. Cancer elimination requires orchestration of various leukocytes together with numerous effector molecules including cytokines. It is well-established that mice lacking
Figure 1.4: Cancer immunoediting. A, cancer immunoediting occurs after cellular transformation takes place and intrinsic tumour suppressing mechanisms fail. B, nascent transformed cells can be eliminated initially by various immune effector cells and effector molecules, which results in immune selection and immune sculpting. If elimination reaches its completion, this is the endpoint of immunoediting. C, however, immune sculpting can cause generation of surviving tumour variants with decreased immunogenicity leading to the equilibrium phase, where the immune system holds tumour growth “in check”. D, eventually, further immune selection causes generation of even less immunogenic tumour variants capable of producing factors which create an immunosuppressive environment leading to the escape phase. However, in some circumstances tumours can directly enter the equilibrium or escape phases, and external factors, such as environmental stress, aging, and individual’s immune status, can also change the directionality of the flow. Adapted from ref. 30.
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essential components of the innate or adaptive immune systems\textsuperscript{43-46}: αβT cells, γδ\textsuperscript{T} cells, natural killer (NK) cells\textsuperscript{46,49}, natural killer T (NKT) cells\textsuperscript{46,49}, IFN-γ\textsuperscript{31,50-53}, perforin\textsuperscript{51,54}, tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)\textsuperscript{43,55}, are more susceptible to the development of spontaneous and chemically-induced tumours.

Although the mechanisms by which the immune system interacts with tumour tissue and exerts its action has only recently begun to be revealed, it is clear from numerous investigations that successful anti-tumour immune responses involve a sequence of events: recognition of transformed cells by cells of innate immune system and limited killing of tumour cells, maturation and migration of antigen-presenting cells (APCs) and antigen presentation in the tumour draining lymph nodes (DLNs), generation of tumour-antigen specific T cells, and finally, migration/infiltration of effector T cells into tumour tissue where direct killing of tumour cells takes place\textsuperscript{56,57}.

Tumours can be recognised by the immune system in a few different ways. Tumour cells may express two types of antigens that the immune system can recognise: neo-antigens expressed as a result of genomic instability\textsuperscript{58} or self-antigens in abnormal levels and/or locations\textsuperscript{59}. However, expression of tumour antigens alone is often insufficient to evoke efficient anti-tumour immune responses, and the antigens need to be encountered in the context of danger signals. In tumour tissues, apoptotic and necrotic cells can release danger signals such as nucleic acid fragments\textsuperscript{60}, ECM components, heat shock proteins\textsuperscript{61,62} and uric acid\textsuperscript{63}, which activate pattern recognition receptors resulting in activation of macrophages and production of pro-inflammatory cytokines including IFN-γ. Those pro-inflammatory cytokines can recruit innate effector cells such as NK cells, NKT cells, and γδ\textsuperscript{T} cells which can further produce IFN-γ and these cells have shown to play a significant role in protecting from tumour initiation in various experimental
systems. Perforin-, FasL-, and TRAIL-mediated killing of tumour cells by NK cells causes further release of danger signals resulting in amplification of the signal. In addition, NK cells have also been shown to promote maturation and migration of dendritic cells (DCs) to tumour DLNs resulting in enhanced antigen presentation by DCs.

Danger signals from dying cells as well as IFNα/β and IFN-γ act as first maturation signals for DCs. In addition, dying tumour cells can also be phagocytosed by immature DCs which then migrate to tumour DLNs. Tumour antigens are processed by DCs and presented to CD4+ T cells on Class II MHC molecules and co-stimulation through CD40/CD40L potently up-regulates interleukin (IL)-12 expression which enhances further activation of CD4+ T cells. Activation of tumour-specific CD8+ T cells requires cross-presentation of tumour antigens on Class I MHC, co-stimulatory signals through ligation of CD28 on T cells and CD80 or CD86 which are upregulated on mature DCs, and inflammatory cytokines such as IL-12. CD4+ T cells are also required for survival, maintenance and cytotoxic activity of CD8+ T cells. In addition, it has also been shown that activated CD4+ T cells can induce polarisation of tumour associated macrophages (TAMs) towards IFN-γ-producing M1 macrophages (tumour inhibiting), instead of IL-10-producing M2 macrophages (tumour-promoting). M1 macrophages possess a greater ability to present antigen, enhanced expression of IL-12, and higher production of toxic intermediates such as nitric oxide (NO) and reactive oxygen intermediates (ROI) compared with M2 macrophages, which makes them potent effector cells in anti-tumour immunity.

The involvement of other leukocyte populations such as IL-4-producing type-2 T cells (T_H2 or T_C2) and IL-17-producing type 17 T cells (T_H17 or T_C17) in anti-tumour immunity is controversial and/or has not yet been fully investigated. For example, in type 2 responses, while IL-4 and T_C2 cells have been shown to have anti-tumourigenic effects, if IL-10 expression is upregulated in T_C2 cells, they can inhibit anti-tumour responses.
Furthermore, although T\textsubscript{H}17 cells have been shown to have positive impact on anti-tumour immunity in both human ovarian cancer\textsuperscript{80} and lung cancer\textsuperscript{81} and in a mouse model of melanoma\textsuperscript{82}, in other human cancers high levels of IL-17 and/or T\textsubscript{H}17 cells have been found to be associated with pro-tumourigenic outcomes\textsuperscript{83-85}. Thus, involvement of these cell populations in tumour immunity has started to be unveiled in recent years, however, further investigations are clearly required to elucidate their precise roles in different types of cancer environments.

1.2.2.2 Equilibrium

When tumour elimination does not reach completion, the surviving tumour cells enter the equilibrium phase, where the immune system holds tumour growth “in check” and sculpts (edits) the immunogenicity of the tumour cells, leading to selection for cells more resistant to immune attacks. During equilibrium tumour cells from the original transformation are eliminated by the immune system while genomic instability in newer variants of tumour cells may give rise to less immunogenic variants\textsuperscript{56}. Tumours in the equilibrium phase are essentially dormant and are clinically invisible for the life of the host, and hence, equilibrium can also represent a second endpoint of the cancer immunoediting process\textsuperscript{30}.

One of the clinical indications that tumours can enter a state of dormancy for an extended period of time was revealed when melanoma growth was observed in the transplanted organ of immunosuppressed organ recipients. The organ donor had been treated for melanoma 16 years prior to the transplant and was considered tumour-free\textsuperscript{86}. There have been several similar clinical cases reported since\textsuperscript{87-89} and the notion of tumour equilibrium was subsequently supported by experimental data \textit{in vivo}. Koebel and colleagues\textsuperscript{90} used a mouse model of chemically-induced sarcoma to demonstrate that adaptive immunity, but not innate immunity, is responsible for maintaining tumour equilibrium, and that these tumour cells remain highly immunogenic. Hence, unedited, but rare tumours which
spontaneously outgrew were poorly immunogenic suggesting that they had undergone editing. Similar observations have been made in another mouse model of squamous cell carcinoma induced by UVB irradiation\textsuperscript{91} and in a metastatic melanoma model using transgenic mice\textsuperscript{92}. In all experimental systems, depletion of CD8\textsuperscript{+} T cells (and other components of adaptive immunity in the case of Koebel \textit{et.al.}) from mice bearing dormant tumours led to their outgrowth, which indicates the significance of CD8\textsuperscript{+} T cells in tumour immunoediting.

1.2.2.3 Escape

Tumour cell variants which survive immune selection subsequently enter the escape phase. Tumour escape can occur as a result of both genomic instability and immune selection. As when tumour cells enter the equilibrium phase, genomic instability together with immune selection leave behind even less immunogenic cells or in some cases immunosuppressive variants. Those cell variants which acquire the ability to circumvent immune recognition and/or immune attack can now progress, leading to outgrowth.

There are several mechanisms by which tumour escape and outgrowth occur: by evading immune recognition, by avoiding immune cell-mediated cell lysis and by immune suppression. Random and spontaneous mutations in the genome of tumour cells can often cause loss of expression of tumour antigens\textsuperscript{93,94} or ligands for NKG2D\textsuperscript{95} resulting in loss of recognition by tumour-specific CD8\textsuperscript{+} T cells or NK cells. Loss of antigen processing or presentation machinery, such as TAP, LMP, MHC Class I, and β2 microglobulin, by tumour cells also leads to a failure to activate tumour-antigen-specific CD8\textsuperscript{+} T cells\textsuperscript{96,97}. Furthermore, the loss of responsiveness to IFNs observed in some tumour cells can result in dysfunction of antigen processing and presentation processes\textsuperscript{98}. Alternatively, in order to avoid immune cell-mediated killing, tumour cells can also acquire increased resistance to apoptosis by up-regulating anti-apoptotic proteins, such as Bcl-xL\textsuperscript{99} or increased resistance
to killing by expression of altered forms of death receptors, such as Fas, TRAIL receptors or death receptor (DR)-5\textsuperscript{100,101}.

A more active way by which tumour cells escape and outgrow is by suppression of the anti-tumour immune response. For example, some tumour cells are able to inhibit cytotoxic activity or to induce apoptosis of CD8\textsuperscript{+} T cells. Molecules of the B7 family are often expressed by various tumour cells\textsuperscript{102} and can be upregulated by IFN-γ\textsuperscript{103}. B7 molecules\textsuperscript{103,104} as well as human leukocytes antigen (HLA) molecules\textsuperscript{105,106} on tumour cells have been shown to dampen cytotoxicity or induce apoptosis of CD8\textsuperscript{+} T cells. Many tumour cells are also known to produce various soluble factors such as FasL, MHC Class I chain-related molecules (MICs), VEGF, type 2 cytokines IL-4 and IL-13, and immunosuppressive cytokines IL-10 and TGF-β. For example, FasL expression by tumour cells strongly correlates with T cell apoptosis, and FasL containing microvesicles isolated from cancer patients were able to reduce expression of the TCR and induce apoptosis of T cells \textit{in vitro}\textsuperscript{107}. Soluble MICs produced by tumour cells have also been shown to down-regulate NKG2D on NK cells resulting in inhibition of cytolyltic attack by NK cells\textsuperscript{108}. Type 2 cytokines IL-4 and IL-13 can be produced by many tumour cells leading to recruitment and development of M2 macrophages that further produce VEGF and suppressive cytokines\textsuperscript{93}.

VEGF produced by tumour cells plays multiple roles in suppression of immune response against tumours. Immature DCs, which produce indoleamine 2,3-dioxygenase (IDO) that catalyses tryptophan generating a series of metabolites to induce apoptosis of T cells\textsuperscript{109}, can be recruited to tumour tissues by VEGF\textsuperscript{110}. Another study has also shown that VEGF prevents DC differentiation and maturation by inhibiting nuclear factor-κB (NF-κB) in haematopoietic progenitor cells\textsuperscript{111}. VEGF has also been implicated to be one of the key molecules which enhance recruitment and activation of myeloid-derived suppressor cells
MDSCs are a heterogeneous population of immature myeloid cells capable of suppressing T cell activities either by nitrosylation of the TCR resulting in inhibition of T cell activation, or via production of TGF-β, leading to suppression of NK cell, or tumour-reactive T cells, and activation of regulatory T (T<sub>reg</sub>) cells.

TGF-β and IL-10 are potent immunosuppressive cytokines produced by tumour cells which generate an immunosuppressive microenvironment. IL-10 has been shown to inhibit DC function and cause skewing of T cell responses towards a type 2 response. As mentioned above, tumour-derived TGF-β has been shown not only to inhibit T and NK cell effector functions but also DC activation. Another crucial role of TGF-β is to activate and expand the T<sub>reg</sub> population. T<sub>reg</sub> cells are identified by expression of CD25 and intracellular FoxP3 and suppress T cell function during the induction and effector phases. Inhibition of T cell activity by T<sub>reg</sub> cells occurs though several mechanisms including: production of TGF-β and IL-10, induction of inhibitory DC development or expression of cytotoxic T-lymphocyte antigen-4 (CTLA-4) which binds to CD80 or CD86 on effector T cells, inhibiting their function. Heightened infiltration of T<sub>reg</sub> cells observed in human cancers is associated with poor prognostic outcomes and tumour regression after depletion of T<sub>reg</sub> cells in in vivo models further illustrate the significance of this population in tumour induced immune escape.

Thus, genomic instability, which is an inherent characteristic of tumour cells, allows gradual development of tumour cell variants that are poorly immunogenic or immunosuppressive. This model is now well accepted as the mechanism of tumour outgrowth in immunocompetent hosts or after immunotherapy in cancer patients. A major goal for future immunotherapies will be to enhance the elimination process to reach completion. It is clear that effective anti-tumour immune responses involve orchestration of various leukocytes, endothelial cells, fibroblasts and other stromal cells as well as
numerous factors which control these cells. The chemokine superfamily are key players in
the biology of all of these cell types and control immune responses as well as other aspects
of cancer biology such as proliferation, apoptosis, adhesion and migration. Therefore,
targeting of these molecules can be considered as potential future cancer strategies. The
next section will describe in more detail the biology of chemokines, particularly how they
relate to cancer biology.

1.3 The chemokine family

The chemokine superfamily is a structurally related group of cytokines whose fundamental
role is to direct leukocyte trafficking. Currently, there are nearly 50 chemokines identified
and they are classified into subfamilies according to their signature cysteine motif at the
N-terminus (Table 1.1). This classification gave rise to the initial nomenclature for
chemokines, CXC, CC, CX3C and C. In addition to this cysteine residue based
classification, chemokines can also be classified according to their expression pattern and
function. Homeostatic chemokines are constitutively expressed and are involved in
immune surveillance and homeostatic homing of leukocytes. Whereas, expression of
inflammatory chemokines is upregulated in response to immunological challenges to
coordinate innate and adaptive immune responses121.

Although best characterised for their critical role in directing cell migration/recruitment,
chemokines and their receptors have been implicated in many other biological processes
such as leukocyte activation, lymphoid organ development, control of T cell differentiation,
wound healing, angiogenesis/angiostasis, regulation of apoptosis and cell adhesion122-124.
Chemokines exert their biological effects by interacting with their cognate receptors which
are G-protein coupled receptors (GPCRs) consisting of seven transmembrane
Table 1.1: Chemokine nomenclatures.

<table>
<thead>
<tr>
<th>Systematic name</th>
<th>Human ligand</th>
<th>Mouse ligand</th>
<th>Cognate Receptor</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CXC chemokine group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL1</td>
<td>GROα/MGSA-α</td>
<td>GRO-1/ KC</td>
<td>CXCR2</td>
<td>I</td>
</tr>
<tr>
<td>CXCL2</td>
<td>GROβ/MGSA-β</td>
<td>GRO-2/MIP-2</td>
<td>CXCR2</td>
<td>I</td>
</tr>
<tr>
<td>CXCL3</td>
<td>GROγ/MGSA-γ</td>
<td>DCIP-1</td>
<td>CXCR2</td>
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<tr>
<td>CXCL4</td>
<td>PF4</td>
<td></td>
<td>CFXR3B</td>
<td>H</td>
</tr>
<tr>
<td>CXCL5</td>
<td>ENA-78</td>
<td>GCP-2/LIX</td>
<td>CXCR2</td>
<td>I</td>
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<td>GCP-2</td>
<td>Unknown</td>
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<td>PPBP</td>
<td>CXCR2</td>
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<tr>
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<td>IL-8</td>
<td>Unknown</td>
<td>CXCR1/CXCR2</td>
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<td>CXCL9</td>
<td>Mig</td>
<td>Mig</td>
<td>CXCR3A</td>
<td>I</td>
</tr>
<tr>
<td>CXCL10</td>
<td>IP-10</td>
<td>IP-10/CRG-2</td>
<td>CXCR3</td>
<td>I</td>
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<tr>
<td>CXCL11</td>
<td>I-TAC</td>
<td>I-TAC</td>
<td>CXCR3</td>
<td>I</td>
</tr>
<tr>
<td>CXCL12</td>
<td>SDF-1α/β</td>
<td>SDF-1/PBSF</td>
<td>CXCR4</td>
<td>H</td>
</tr>
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<td>CXCL13</td>
<td>BCA-1</td>
<td>BLC</td>
<td>CXCR5</td>
<td>H</td>
</tr>
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<td>CXCL14</td>
<td>BRAK/bolekine</td>
<td>BRAK</td>
<td>Unknown</td>
<td>I</td>
</tr>
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<td>CXCL15</td>
<td>Unknown</td>
<td>Lungkine/WECHE</td>
<td>Unknown</td>
<td>U</td>
</tr>
<tr>
<td>CXCL16</td>
<td>-</td>
<td>-</td>
<td>CXCR6</td>
<td>I</td>
</tr>
<tr>
<td><strong>C chemokine group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XCL1</td>
<td>Lymphotactin/SCM-1α/ATAC</td>
<td>Lymphotactin</td>
<td>XCR1</td>
<td>I/H</td>
</tr>
<tr>
<td>XCL2</td>
<td>SCM-1β</td>
<td>Unknown</td>
<td>XCR1</td>
<td>I/H</td>
</tr>
<tr>
<td><strong>CX3C chemokine group</strong></td>
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<td></td>
</tr>
<tr>
<td>CX3CL1</td>
<td>Fractalkine</td>
<td>Neurotactin/ABC</td>
<td>CX3CR1</td>
<td>I</td>
</tr>
<tr>
<td><strong>CC chemokine group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL1</td>
<td>I-309</td>
<td>TCA-3/P500</td>
<td>CCR8</td>
<td>I</td>
</tr>
<tr>
<td>CCL2</td>
<td>MCP-1/MCAF/TDCF</td>
<td>JE</td>
<td>CCR2</td>
<td>I/H</td>
</tr>
<tr>
<td>CCL3</td>
<td>MIP-1α/LD78α</td>
<td>MIP-1α</td>
<td>CCR1/CCR5</td>
<td>I</td>
</tr>
<tr>
<td>CCL3L1</td>
<td>LD78β</td>
<td>Unknown</td>
<td>CCR1/CCR5</td>
<td>I</td>
</tr>
<tr>
<td>CCL4</td>
<td>MIP-1β</td>
<td>MIP-1β</td>
<td>CCR5/CCR8</td>
<td>I</td>
</tr>
<tr>
<td>CCL5</td>
<td>RANTES</td>
<td>RANTES</td>
<td>CCR1/CCR3/CCR5</td>
<td>I</td>
</tr>
<tr>
<td>CCL6</td>
<td>Unknown</td>
<td>C10/MRP-1</td>
<td>Unknown</td>
<td>U</td>
</tr>
</tbody>
</table>
### Table 1.1 continued: Chemokine nomenclatures.

| CCL7  | MCP-3 | MCP-3 | CCR2 | I/H
|-------|-------|-------|------|------
| CCL8  | MCP-2 | MCP-2 | CCR1 | I/H
| CCL9/10 | Unknown | MRP-2/CCF18/MIP-1γ | Unknown | U
| CCL11 | Eotaxin | Eotaxin | CCR3 | I
| CCL12 | Unknown | MCP-5 | Unknown | U
| CCL13 | MCP-4 | Unknown | CCR1 | I/H
| CCL14 | HCC-1 | Unknown | CCR1 | H
| CCL15 | HCC-2/Lkn-1/MIP-1δ | Unknown | CCR1 | H
| CCL16 | HCC-4/LEC/LCC-1 | Unknown | CCR1 | H
| CCL17 | TARC | TARC/ABCD-2 | CCR4 | I/H
| CCL18 | DC-CK1/PARC/AMAC-1 | Unknown | Unknown | H
| CCL19 | MIP-3β/ELC/exodus-3 | MIP-3β/ELC/exodus-3 | CCR7 | H
| CCL20 | MIP-3α/LARC/exodus-1 | MIP-3α/LARC/exodus-1 | CCR6 | I/H
| CCL21 | 6Ckine/SLC/exodus-2 | 6Ckine/SLC/exodus-2/TCA-4 | CCR7 | I/H
| CCL22 | MDC/STCP-1 | ABCD-1 | CCR4 | I/H
| CCL23 | MPIF-1/CKβ8/CKβ8-1 | Unknown | CCR1 | I
| CCL24 | Eotaxin-2/MPIF-2 | MPIF-2 | CCR3 | I
| CCL25 | TECK | TECK | CCR9 | H
| CCL26 | Eotaxin-3 | Eotaxin-3 | CCR3 | I
| CCL27 | CTACK/ILC | ALP/CTACK/ILC/ESkine | CCR10 | H
| CCL28 | MEC | MEC | CCR10 | I

Adapted from ref. 121 and 223.

**Abbreviations:** I; Inflammatory, H; Homeostatic, U; Unknown
domains. They are classified based on the subfamily of chemokines with which they interact (Table 1.1). Interactions between chemokines and receptors can either be promiscuous (a receptor may bind a range of chemokines and some chemokines bind multiple receptors) or specific (some receptors bind only one particular chemokine and some chemokines bind only one receptor)\textsuperscript{121,125,126}. Upon binding of a chemokine to the extracellular domain of the receptor, the intracellular domain of the receptor undergoes a conformational change resulting in dissociation of the heterotrimeric G-protein $\beta\gamma$ subunits from the $\alpha$ subunit (Figure 1.5). These subunits activate downstream effector enzymes including phospholipases, kinases, and transcription factors, which eventually result in a range of cellular responses\textsuperscript{124}. Activation of phospholipase C causes mobilisation of calcium and activation of protein kinase C while activation of phosphoinositide 3-kinases (PI3K) results in induction of various downstream effectors\textsuperscript{127,128}. Both of these events lead to activation of actin-dependent cellular processes resulting in cell migration. Apart from inducing migration, activation of PI3K as well as mitogen activated protein kinase (MAPK) by chemokines is also known to play an important role in other cellular processes such as adhesion, degranulation, anti-apoptotic process, cell metabolism and gene expression\textsuperscript{128-131}. Another pathway activated by chemokine binding to the receptor is the JAK/STAT pathway. Although this pathway seems to be independent of $G\alpha$ activation\textsuperscript{132}, it appears to be important for activation of cell migration and adhesion, angiogenesis, and gene expression\textsuperscript{133-135}.

In addition to inducing chemotaxis and other cellular processes, chemokine receptor activation can also lead to receptor internalisation through either clathrin-mediated endocytosis or lipid raft and caveolae-dependent internalisation. Although little is known about lipid raft and caveolae-dependent mechanisms, it has been shown in certain types of cells that lipid raft and caveolae-dependent internalisation is used by CCR4\textsuperscript{136}, CCR5\textsuperscript{137-139} and CXCR4\textsuperscript{137}. Ligation of chemokine to the receptor triggers phosphorylation of the
Figure 1.5: Chemokine signalling and downstream effects. Chemokines exert their biological activities through cognate receptors, 7-transmembrane GPCRs. Ligation of chemokine to the receptor induces dissociation of heterotrimeric G protein subunits. These G proteins can activate PI3K, MAPK, PKC or PLC, which leads to amplification of second messengers. This results in modulation of actin-dependent cellular processes that ultimately induce chemotaxis. Chemokine binding also activates JAK/STAT pathway which results in expression of various genes. The activation of this pathway appears to be also important in adhesion and chemotaxis. Another consequence of chemokine receptor signalling is internalisation of the receptor through GRK activation and recruitment of β-arrestin, which leads to clathrin-mediated endocytosis. Internalised receptor is either degraded or recycled to the plasma membrane. Adapted from ref. 122.
intracellular loops and C-terminus by G protein-coupled receptor kinase (GRK), which recruits adaptor proteins such as β-arrestin which links the receptor and a clathrin lattice enabling the receptor endocytosis. The internalised receptor within endosome becomes dephosphorylated and either degraded or recycled back to the cell surface.

1.4 Involvement of chemokines in progression of cancer

As described above, apart from directing leukocyte migration, chemokines have been shown to display plethora of biological activities in homeostasis and immune defence as well as pathological states. Although a connection between inflammation and cancer has long been indicated, the work presented by Muller and colleagues in 2001 was perhaps the first to demonstrate the direct involvement of chemokines in tumour progression. Since then, extensive studies on the involvement of chemokines and chemokine receptors in cancer progression and metastasis have revealed multiple roles played by chemokine family in various cancers (Table 1.2). It is now clear that chemokines can be involved in any one or more stages of cancer progression depicted above as the “hallmarks of cancer”, including proliferation, survival, resistance to apoptosis, angiogenesis, invasion and metastasis, and cancer immunoediting. It has been shown in various studies that some oncogenic mutations can modulate the chemokine network by up/down-regulating chemokines and/or receptors. For those tumour cells which express chemokine receptors as a result of oncogenic transformation, chemokines can act either in an autocrine or paracrine manner to promote growth and survival, or metastasis. On the other hand, some tumour cells acquire the ability to produce chemokines which induce angiogenesis supporting growth and dissemination, or recruitment of leukocytes. Some tumour infiltrating leukocytes (TILs) can further promote tumour growth and invasion by producing various factors, while others can activate anti-tumour immune
Table 1.2: Involvement of chemokines in cancer progression and metastasis.

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>Receptor</th>
<th>Tumourigenic effects</th>
<th>Types of cancer</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL1-3, 5-8</td>
<td>CXCR1/2</td>
<td>angiogenesis, invasion and metastasis, growth, proliferation, survival</td>
<td>colorectal, lung, melanoma, pancreatic, prostate, renal cell</td>
<td>152-157</td>
</tr>
<tr>
<td>CXCL9-11</td>
<td>CXCR3</td>
<td>invasion, metastasis, survival proliferation</td>
<td>colorectal, melanoma, leukemia</td>
<td>158-160</td>
</tr>
<tr>
<td>CXCL12</td>
<td>CXCR4</td>
<td>angiogenesis, invasion, metastasis, growth proliferation, survival, DC recruitment</td>
<td>colorectal, lung, melanoma, leukemias, breast, ovarian and various others</td>
<td>141,160-165</td>
</tr>
<tr>
<td>CXCL13</td>
<td>CXCR5</td>
<td>invasion, metastasis, growth, proliferation</td>
<td>pancreas, colon, head and neck, leukemias and lymphomas</td>
<td>166-168</td>
</tr>
<tr>
<td>CCL3-5, 7, 16</td>
<td>CCR1</td>
<td>invasion, metastasis, angiogenesis, TAM and DC recruitment</td>
<td>breast, cervical, liver, lung, prostate, myeloma, leukemia</td>
<td>169-173</td>
</tr>
<tr>
<td>CCL2, 7, 12</td>
<td>CCR2</td>
<td>invasion, metastasis, angiogenesis, TAM and fibroblast recruitment</td>
<td>breast, glioma, lung, melanoma, prostate, myeloma</td>
<td>173-178</td>
</tr>
<tr>
<td>CCL5, 7, 11, 24, 26</td>
<td>CCR3</td>
<td>invasion, metastasis, angiogenesis, TAM and eosinophil recruitment</td>
<td>breast, cervical, melanoma, renal cell, lymphoma</td>
<td>169,179,180</td>
</tr>
<tr>
<td>CCL2, 3, 5, 17, 22</td>
<td>CCR4</td>
<td>invasion, metastasis, TAM and T cell recruitment</td>
<td>ovarian, lymphomas</td>
<td>181-183</td>
</tr>
<tr>
<td>CCL3-5, 8</td>
<td>CCR5</td>
<td>invasion, metastasis, TAM recruitment</td>
<td>breast cervical, lung pancreatic, prostate, myeloma</td>
<td>170,172,173,180, 184,185</td>
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<tr>
<td>CCL20</td>
<td>CCR6</td>
<td>invasion, metastasis, proliferation, DC recruitment</td>
<td>breast, colorectal, liver</td>
<td>186-190</td>
</tr>
<tr>
<td>CCL19, 21</td>
<td>CCR7</td>
<td>invasion, metastasis, survival</td>
<td>breast, colorectal, gastric, head and neck, lung melanoma, leukemia</td>
<td>141,191-201</td>
</tr>
<tr>
<td>CCL25</td>
<td>CCR9</td>
<td>invasion, metastasis, survival</td>
<td>melanoma, prostate</td>
<td>202-208</td>
</tr>
<tr>
<td>CCL27</td>
<td>CCR10</td>
<td>invasion, metastasis, growth, survival</td>
<td>melanoma, lymphomas</td>
<td>141,183.209</td>
</tr>
<tr>
<td>CX3CL1</td>
<td>CX3CR1</td>
<td>invasion, metastasis, survival</td>
<td>prostate</td>
<td>210</td>
</tr>
</tbody>
</table>

Adapted from ref.142.
Highlighted in red are chemokines and their receptors relevant to this study.
responses, as described above. Of particular relevance to this study, roles of the chemokine receptor CCR7 and its ligands, CCL19 and CCL21 in various aspects of cancer progression have been described. Overexpression of CCR7 was shown to enhance tumour formation in B16F1 melanoma model\(^{192}\). Although the mechanism of CCR7 action in these observed effects was not fully revealed in that report, other studies demonstrated that autocrine or paracrine activation of CCR7 on tumour cells by CCL19 and CCL21 protects tumour cells from apoptosis\(^{199}\), or induces proliferation of cancer cells\(^{201}\) in other cancer models. More extensive studies have also revealed involvement of CCR7 in tumour metastasis. Muller and colleagues showed that CCR7 is highly expressed in mammary carcinomas and melanoma but rarely detected in normal mammary gland tissue or in melanocytes\(^{141}\). They also found that CCL19 and CCL21 are highly expressed in organs such as lymph nodes, bone marrow, liver and lung where metastasis of those tumours is commonly observed. Furthermore, these ligands induced F-actin production and actin polymerisation required for pseudopodia formation and hence promote invasion of surrounding tissues and cell migration. It has since been shown in other types of cancer that expression of CCR7 and ligands has a significant association with the extent and location of metastasis\(^{191, 193, 195, 197, 198, 200}\). In both B16F1 and B16F10 melanoma models it was shown that overexpression of CCR7 facilitates LN metastasis\(^{192, 200}\). In our laboratory, CCL21 has been shown to promote breast cancer metastasis by rendering tumour cells more resistant to anoikis which is one of important features of cells undergoing EMT\(^{194}\). Therefore, CCR7 and its ligands not only promote metastasis, but it may also play roles from the early stages of tumour formation through to growth and survival of secondary tumours.

The role of CCR7 and its ligands in tumour progression has also been associated with regulation of the anti-tumour immune responses. Recently, it was demonstrated CCL21 secretion by B16F10 melanoma cells can mediate lymphoid neogenesis. In this study, CCL21-producing melanoma cells created an immunosuppressive tumour environment
characterised by lymphoid-like reticular stromal networks, high levels of TGF-β, T_{reg} cells, and MDSCs, leading to outgrowth of tumours\textsuperscript{196}. However, other studies have also shown that local expression of CCL19 or CCL21 leads to accumulation of mature DCs resulting in expansion of anti-tumour T cells\textsuperscript{211} and that better prognosis of patients with advanced colorectal carcinoma was associated with increased infiltration of CCR7\textsuperscript{+} T cells\textsuperscript{212}.

Although the role of CCR9 and CCL25 in cancer progression has not been investigated as extensively, there is evidence indicating that CCR9 signalling also mediates anti-apoptotic signals in prostate\textsuperscript{206} and breast cancer\textsuperscript{204}. In addition, consistent with its role in homeostasis, CCR9/CCL25 axis is strongly implicated in small intestinal metastasis of melanoma\textsuperscript{202,205,208} and increased motility and invasion in prostate\textsuperscript{207} and breast cancer\textsuperscript{203}.

1.5 Regulation of chemokines by atypical receptors

Since chemokines play a significant role in a wide range of biological processes both in homeostasis and pathological conditions, it is essential that precise regulation is exerted over chemokine biology. The activity of chemokines is regulated at many different levels including transcriptional control of chemokines and chemokine receptor expression\textsuperscript{213,214}, regulation of translation and secretion of chemokines, or translation and surface expression of the receptors\textsuperscript{215-220}. Other ways in which chemokine networks are regulated include activation or inactivation of chemokines by proteases, controlled secretion of chemokines from granular stores, and the proteolytic generation of receptor antagonists\textsuperscript{221}.

In other cytokine families, such as the TNF and IL-1 families, regulation is exerted by non-signalling receptors which control ligand bioactivity\textsuperscript{222}. Recently, it has become evident that similar receptors also regulate chemokine activities. In the past few years,
several chemokine receptors have been identified that differ from other ‘classical’ chemokine receptors in several important aspects, leading to their classification as ‘atypical’ chemokine receptors. While these receptors have structural homology to other chemokine receptors, and bind chemokines with high affinity, they do not couple to the downstream signal transduction pathways activated by other typical chemokine receptors. Therefore, these receptors do not support cell migration upon ligation and this has led to the proposition that they may act as ‘decoy’ receptors\textsuperscript{221, 222} (Figure 1.6). Although structure-function analysis of these receptors has not been extensively performed, clear structural differences between atypical and typical receptors have been described. Within the second intracellular loop, a conserved motif in typical chemokine receptors (Aspartic acid-Arginine-Tyrosine (DRY)) is either not present or altered in atypical receptors, and this motif is known to be important in coupling to G proteins\textsuperscript{222,223} (Figure 1.6). Currently, the definition of “atypical” chemokine receptors is the inability to activate conventional signalling responses upon ligand binding. Three atypical chemokine receptors have been identified and characterised according to this definition: D6, Duffy antigen receptor for chemokines (DARC), and Chemocentryx chemokine receptor (CCX-CKR) (Table 1.3), and characteristics and functions of each of these receptors will be discussed in the following sections.

However, it should be noted that there are other chemokine-binding receptors with high structural homology but experimental evidence has not been clear as to whether they can be categorised into this “atypical” group. For example, CCRL2, also known as, CRAM or HCR, has sequence homology and genomic localisation similar to CC-chemokine receptors\textsuperscript{224} and there are no similar residues to the DRY-motif found on the second intracellular loop\textsuperscript{225}. It is expressed on monocytes, neutrophils, mast cells, DCs, and B cells\textsuperscript{226-228}, and has been shown to bind CCL5 and CCL19, as well as the adipokine chemerin\textsuperscript{228-230}. CCRL2 is constitutively internalised and recycled\textsuperscript{230}, and \textit{in vitro} studies
Figure 1.6: *Typical vs. atypical chemokine receptor.* Both types of receptors have the same structure of extracellular N-terminal domain and loops, 7 transmembrane spanning domains, and C-terminal intracellular domain and loops. A, typical chemokine receptors have a canonical DRYLAIV motif in the second intracellular loop involved in G-protein coupling. Binding of chemokine activates signalling cascades, which leads to the various cell activities represented in Figure 1.5. B, in contrast, atypical chemokine receptors have either no or an altered DRY motif in the second intracellular loop, and appear incapable of triggering G-protein mediated signalling. Instead, these receptors can be internalised and recycled back to plasma membrane, suggesting their role as regulator of chemokine bioavailability in the microenvironment. *Adapted from ref. 222.*
### Table 1.3: Properties of atypical chemokine receptors.

<table>
<thead>
<tr>
<th>Chemokine ligands</th>
<th>Signalling receptors potentially regulated</th>
<th>Expression pattern</th>
<th>DRYLAIV motif</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D6</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL2</td>
<td>CCR1</td>
<td>· Lymphatic endothelial cells in afferent lymphatics from skin, gut, and lung</td>
<td></td>
</tr>
<tr>
<td>CCL3</td>
<td>CCR2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL4</td>
<td>CCR3</td>
<td>· Placenta</td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td>CCR4</td>
<td>· CNS</td>
<td></td>
</tr>
<tr>
<td>CCL7</td>
<td>CCR5</td>
<td>· B cell, DCs</td>
<td></td>
</tr>
<tr>
<td>CCL8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL11</td>
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<td>CCL12</td>
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<td></td>
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<td>CCL13</td>
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<td></td>
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</tr>
<tr>
<td>CCL22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>DARC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL1</td>
<td>CXCL1</td>
<td>· Erythrocytes</td>
<td></td>
</tr>
<tr>
<td>CCL2</td>
<td>CXCL2</td>
<td>· Vascular endothelial cells</td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td>CXCL3</td>
<td>· Lymphatic endothelial cells of skin lymphatic pre-collectors</td>
<td></td>
</tr>
<tr>
<td>CCL7</td>
<td>CXCL4</td>
<td>· High endothelial venules</td>
<td></td>
</tr>
<tr>
<td>CCL8</td>
<td>CXCL5</td>
<td>· Littoral cells lining splenic sinuses</td>
<td>No similar motif</td>
</tr>
<tr>
<td>CCL11</td>
<td>CXCL6</td>
<td>· Glomerular and peri-bronchiolar capillaries</td>
<td></td>
</tr>
<tr>
<td>CCL13</td>
<td>CXCL8</td>
<td>· Epithelium in lung and kidney collecting ducts</td>
<td></td>
</tr>
<tr>
<td>CCL14</td>
<td>CXCL9</td>
<td>· Purkinje neurons in the cerebellum</td>
<td></td>
</tr>
<tr>
<td>CCL16</td>
<td>CXCL10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL17</td>
<td>CXCL11</td>
<td></td>
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</tr>
<tr>
<td>CCL18</td>
<td>CXCL13</td>
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<td></td>
</tr>
<tr>
<td><strong>CCX-CKR</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL19</td>
<td>CCR7</td>
<td>· Widely expressed in tissues</td>
<td>DRYVAVT (human)</td>
</tr>
<tr>
<td>CCL21</td>
<td>CCR9</td>
<td>· Non-haematopoietic cells of thymus and LN</td>
<td>DRYWAIT (mouse)</td>
</tr>
<tr>
<td>CCL25</td>
<td>hCXCR5</td>
<td>· Epidermis</td>
<td></td>
</tr>
<tr>
<td>hCXCL13</td>
<td></td>
<td>· Immature DCs and T cells</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from ref. 222 and 231.
have demonstrated the association between expression of CCRL2 and reduced migration of the cells towards CCL19\textsuperscript{227}. Another study using CCRL2-deficient mice suggested a potential role of CCRL2 in trafficking of antigen-loaded DCs to draining LNs, but there is no direct indication that CCRL2 can function as a chemokine regulator\textsuperscript{232}.

Another candidate atypical receptor is CXCR7, formally known as RDC1, which also has sequence homology and genomic localisation similar to chemokine receptors but the DRYLAIV is replaced with DRYLSIT\textsuperscript{233}. CXCR7 binds to CXCL11\textsuperscript{234} and CXCL12 and it was initially shown to induce signalling events upon CXCL12 binding\textsuperscript{235}. However, this was not supported by subsequent independent studies\textsuperscript{234,236-239}. Later it was demonstrated that chemokine ligation to CXCR7 can induce alternative signalling pathways involving β-arrestin recruitment and MAPK activation\textsuperscript{240-242} resulting in regulation of cell survival and adhesion\textsuperscript{234,243-245}. Furthermore, some studies have also demonstrated that CXCR7 can heterodimerise with CXCR4, the typical receptor for CXCL12, resulting in alterations to CXCL12 signalling through $\mathrm{G}_\alpha$\textsuperscript{236,238,242}. More recently, CXCR7 was also reported to heterodimerise with EGFR in human prostate cancer cells, which result in activation of EGFR signalling independent of CXCL11 and CXCL12, leading to enhanced proliferation of cancer cells\textsuperscript{246}. Together these data suggest context dependency of downstream responses upon chemokine ligation to CXCR7.

The following sections will describe various studies on three atypical chemokine receptors which support the hypothesis that these receptors function as “chemokine scavenger”. However, it is important to note that some of the “typical” chemokine receptors (capable of chemokine-driven activation of signalling pathways leading to cellular responses), may also function to regulate chemokine bioavailability in certain context. For example, CXCR4 expressed by bone-marrow endothelial cells has been demonstrated to mediate internalisation and translocation of its ligand, CXCL12\textsuperscript{247}, CCR2 expressed on T cells and
monocytes which migrate across the blood-brain barrier is proposed to remove CCL2 from the cerebrospinal fluid\textsuperscript{248}, CXCR3B expressed by salivary-gland epithelial cells has been reported to sequester CXCL10 resulting in alteration of CXCR3-mediated response of neighbouring T cells\textsuperscript{249}. More recently, study conducted by Cardona and colleague\textsuperscript{250} also showed reduced binding of CCL3 to CCR1 in CCR2 knockout (KO) mice, suggesting that the elevated levels of CCR2 ligands in these mice down-regulated CCR1. The data from these studies further indicate the complexity associated with regulatory mechanisms within the chemokine network, and also suggest that categorisation of the “atypical” chemokine receptors, D6, DRAC and CCX-CKR as “professional” chemokine regulator may be more appropriate.

1.5.1 D6

D6 was originally cloned from placenta\textsuperscript{251} and haematopoietic stem cells\textsuperscript{252} and it binds broad range of inflammatory chemokines in the CC subfamily but not in other subfamily or homeostatic chemokines\textsuperscript{221}. High expression of D6 has been observed in lymphatic endothelial cells in the skin, gut and lung\textsuperscript{253} and placenta, as well as on leukocytes such as B cell and DCs\textsuperscript{254}. It has been shown that D6 is localised in intracellular stores associated with early and recycling endosomes and is constitutively internalised in a ligand-independent manner which is rapidly recycled back to cell membrane\textsuperscript{255,256}. The molecular mechanisms associated with internalisation and recycling of D6 has been controversial as to whether these processes involve β-arrestin and receptor phosphorylation\textsuperscript{255-257}. Nevertheless, these properties of D6 together with \textit{in vitro} evidence that D6-bound ligand is degraded once internalised\textsuperscript{255} led to its proposed function as chemokine scavenger. However, it should be noted that recent study conducted by Hansell and colleagues\textsuperscript{258} demonstrated cell autonomous function of D6 in innate-like B cells where expression of D6 is associated with suppression of CXCR5 function on these cells.
Nevertheless, the hypothesis of scavenger function by D6 has been supported by a number of in vivo studies using D6 KO mice. In models of cutaneous inflammation, D6 KO mice had increased levels of inflammatory chemokines, which are ligands for D6, at the site of inflammation as well as in draining lymph nodes, and the inflammatory pathology observed was more severe compared with wildtype (wt) counterparts. In those mice, the levels of inflammatory cytokines and the number of lymphocytes and APCs in inflamed tissue as well as lymph nodes were dramatically elevated. Furthermore, it has also been shown that deletion of D6 leads to increased susceptibility to cutaneous tumour development in response to chemical carcinogenesis. The same study also showed that while deletion of D6 was sufficient to render invasive squamous cell carcinoma-resistant mouse strains susceptible, while transgenic expression of D6 in keratinocytes protects a susceptible strain from tumour formation. Consistent with these observations, it was recently demonstrated that deletion of D6 also leads to enhanced susceptibility to colitis and carcinogen-induced colon cancer compared with wt mice. These data supported the hypothesis that D6 acts as a decoy receptor which removes inflammatory chemokines from the site of inflammation to regulate the inflammatory response.

1.5.2 DARC

DARC was originally found as an entry receptor for some malaria parasites, but later found to be a seven transmembrane receptor with 40% homology to typical chemokine receptors but missing DRY motif on the second intracellular loop. DARC binds various inflammatory chemokines in both CC and CXC subfamilies, but ligand binding does not support downstream signalling or chemotaxis. DARC expressed on erythrocytes has been suggested to act as chemokine “sink” or chemokine “reservoir” based on two major observations: that plasma levels of CCL2 in humans lacking DARC expression on erythrocytes is reduced compared with individuals with DARC expressing erythrocytes.
and that DARC ligands injected into DARC KO are rapidly cleared from the plasma of these mice. Apart from erythrocytes, expression of DARC has also been observed in vascular endothelial cells\textsuperscript{272} as well as high endothelial venules of lymph nodes\textsuperscript{273} which implied a potential role of DARC in vascular biology. \textit{In vitro} studies have shown that DARC can internalise its ligands\textsuperscript{272} and is also capable of transporting the ligands from the sub-luminal to the luminal face across endothelial cells (transcytosis)\textsuperscript{270,271}. Interestingly, CXC chemokines that are ligands for DARC are known to be angiogenic but it does not bind angiostatic CXC chemokines\textsuperscript{223}. Consistent with this, \textit{in vivo} studies using transgenic mice overexpressing DARC on vascular endothelial cells showed inhibition of angiogenesis in response to CXCL3\textsuperscript{274} and overexpression of DARC on tumour cells also resulted in reduced tumour-associated angiogenesis which inhibited metastasis\textsuperscript{275,276}. In prostate cancer model it was shown that tumours grown in DARC-deficient mice had higher levels of angiogenic DARC ligands in the tumour environment which was associated with enhanced tumour growth\textsuperscript{277}. Another interesting observation was made by Bandyopadhyay and colleagues where they showed that DARC can bind the tetraspanin CD82 on tumour cells inducing cell senescence and inhibiting metastasis\textsuperscript{278}. This indicates that chemokines may not be the only group of molecules which can interact with DARC.

\textit{1.5.3 CCX-CKR}

The most recently described atypical chemokine receptor is CCX-CKR. It is expressed widely in a number of tissues. In humans, the original study by Gosling and colleagues\textsuperscript{271} indicated that CCX-CKR mRNA is expressed by DCs, lymphocytes, the spleen, and lymph nodes, although their RT-PCR data may not be a true reflection of the expression of CCX-CKR mRNA due to the absence of appropriate negative controls. Subsequently, Towson and colleagues\textsuperscript{272} demonstrated that CCX-CKR mRNA is expressed in heart, lung,
small intestine, colon and skeletal muscle but not in leukocytes in humans by Northern blot analysis which is not affected by genomic DNA contamination. This study also showed that CCX-CKR mRNA is expressed in many organs of mice including the heart, lung, and testis by Northern blot and RT-PCR with appropriate controls. Unlike D6 and DARC which bind various inflammatory chemokines, CCX-CKR interacts with the homeostatic chemokines CCL19, CCL21, and CCL25 in mouse and in humans, CXCL13 is also a ligand for human CCX-CKR albeit with low affinity. These chemokines, when bound to typical receptors, CCR7 CCR9 or CXCR5, are involved in leukocyte homing to secondary lymphoid organs, and migration to the small intestine, migration of thymocytes and architecture of secondary lymphoid organs. However, these ligands do not trigger a typical downstream signalling cascade when bound to CCX-CKR, and modifications to the DRY motif in the second intracellular loop is also present. Recent in vitro studies have demonstrated that CCX-CKR has the capacity to internalise CCL19 which was subsequently degraded. Notably, unlike the other functional chemokine receptors which become desensitised by ligand stimulation, CCX-CKR does not become desensitised but instead, ligand sequestration by CCX-CKR become enhanced following exposure to its ligands. Although lack of reliable antibodies against CCX-CKR protein has made it difficult to study the function of this receptor, the development of genetically-modified mice has assisted our understanding of CCX-CKR function in vivo in recent years. Heinzel and colleagues used a CCX-CKR reporter mouse to demonstrate that CCX-CKR is expressed by non-haematopoietic cells of thymus, LNs and epidermis, and a subset of DCs in CCX-CKR-deficient mice have reduced ability to home from the skin to the draining lymph nodes. More recently, in vivo studies using CCX-CKR-deficient mice has revealed that the absence of CCX-CKR leads to elevated levels of ligands in serum and LNs, as well as in the central nervous system (CNS) during experimental autoimmune encephalomyelitis (EAE), a mouse model of multiple sclerosis. This was associated with
increased severity and earlier onset of EAE and skewing of the $T_H$ cell response towards $T_H17^{283}$. Together, these data indicate that CCX-CKR functions to control bioavailability of homeostatic chemokines and regulate adaptive immune responses in vivo.

1.6 The research project

Growing evidence indicates an important role for chemokines and their receptors in progression and metastasis of various types of cancers suggesting that strategies to manipulate chemokines may be viable therapeutic options in various cancers. In particular, the homeostatic chemokine receptor CCR7 and its ligand CCL19 and CCL21 have been strongly implicated in progression and metastasis of many tumours. In addition, the CCR9/CCL25 axis has also been implicated in progression and metastasis of prostate cancer and melanoma.

Evidence from in vitro and in vivo studies suggests that the atypical receptor CCX-CKR functions as a regulator of these ligands, CCL19, CCL21 and CCL25 by scavenging and degrading these chemokines during homeostasis and in inflammatory conditions. However, the significance of chemokine regulation by CCX-CKR in tumour biology is poorly understood. Therefore, in this study the role of CCX-CKR in tumour progression and metastasis was investigated by testing the following hypotheses.

**Hypothesis 1**: That overexpression of the atypical chemokine receptor CCX-CKR in tumour cells will inhibit tumour growth and metastasis in vivo.

**Hypothesis 2**: That deletion or knockdown of expression of the atypical chemokine receptor CCX-CKR will promote tumour growth and metastasis in vivo.

To test these hypotheses, the following experimental aims were established.
Aim 1.1: To generate and characterise murine tumour cell lines overexpressing CCX-CKR.

Aim 1.2: To investigate the effect of CCX-CKR overexpression on tumour progression and metastasis in vivo.

Aim 2.1: To investigate the effect of CCX-CKR deletion in vivo on tumour progression and metastasis.

Aim 2.2: To generate and characterise murine cell lines expressing shRNA constructs targeting CCX-CKR.

Aim 2.3: To investigate the effect of CCX-CKR knockdown on tumour progression and metastasis in vivo.

Together, findings from these investigations were predicted to provide a better understanding of the roles of CCX-CKR and chemokine networks in tumour biology, which may lead to development of novel therapeutics for various cancers.
CHAPTER 2: MATERIALS AND METHODS
CHAPTER 2: MATERIALS AND METHODS

2.1 Reagents and materials

2.1.1 Plasmids

The expression plasmid pEF-IRES-puro6 (pEF) was a kind gift from Dr. Dan Peet at the University of Adelaide and the coding sequence of murine CCX-CKR insert was previously cloned into pEF (CCX>pEF) by Drs. Jeremy Swann and Sharon Williams in our laboratory. The lentiviral packaging plasmids: pREV, psPAX2, and pMD2.G were purchased from Addgene (MA, USA). All following shRNA cassettes were inserted into lentiviral plasmid pLKO.1. Short-hairpin RNA (shRNA) constructs targeting murine CCX-CKR were purchased from Thermo Fisher Scientific (MA, USA) and green fluorescent protein (GFP) shRNA construct was purchased from Addgene. The sequences of each shRNA are shown in Table 2.1. All plasmids were purified using Plasmid Maxi Kit (QIAGEN, Hilden, Germany).

2.1.2 Oligonucleotides

Primers used for quantitative PCR (qPCR) are listed in Table 2.2. All primers were purchased from Geneworks (South Australia (SA), Australia) and reconstituted with sterile MilliQ water to 100 μM as stock concentration and diluted to 20 μM as working concentration.

2.1.3 Antibodies

Antibodies and streptavidin conjugates used in flow cytometry, Western blot analysis, immunofluorescent staining and in vivo neutralisation are listed in Table 2.3.
Table 2.1: Sequence of shRNA used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<td>GAGGTCATTTGCTTTTT</td>
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Highlighted in red is the sense and antisense target sequence of the indicated gene, and the hairpin loop is highlighted in blue.

Table 2.2: Sequence of primers used in this study.

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<th>Target gene</th>
<th>Forward Primer</th>
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<td>RPLP0</td>
<td>AGATGCAAGAGATCCGCA</td>
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<td>GAPDH</td>
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<td>CCR9</td>
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<td>CCX-CKR</td>
<td>AATGCTAGGTGCACTCCCATCT</td>
<td>GCCGATTTCCAGCATCTGA</td>
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<td>E-cadherin</td>
<td>CGGTTCTCCTGATTGCTTTCG</td>
<td>CTTCCGAAAAGAGGCTGCCTC</td>
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<tr>
<td>Fibronectin</td>
<td>ACAGAGCTCAACTCCCTGA</td>
<td>GGTGTGCTCTCTGGTTCCTC</td>
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<td>Vimentin</td>
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<td>GTCAGGCTTGGAAACGTC</td>
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<td>N-cadherin</td>
<td>AGCGCAGTCTTACCAGAGG</td>
<td>TCGCTGCTTCTCATACTGAACCTT</td>
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</table>

Highlighted in red is the sense and antisense target sequence of the indicated gene, and the hairpin loop is highlighted in blue.
Table 2.3: Antibodies and streptavidin conjugates used in this study.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Conjugate</th>
<th>Species</th>
<th>Application</th>
<th>Concentration</th>
<th>Source</th>
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<td>FC</td>
<td>50 μg/ml</td>
<td>Dr. Joe Chiba</td>
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<tr>
<td>Chemokines</td>
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<td>ELISA capt.</td>
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<tr>
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<tr>
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<tr>
<td></td>
<td>—</td>
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<tr>
<td></td>
<td></td>
<td>Goat</td>
<td>ELISA det.</td>
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<tr>
<td>Cytokines</td>
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<tr>
<td></td>
<td></td>
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<td>CD45</td>
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### Table 2.3 continued: Antibodies and streptavidin conjugates used in this study.

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<tr>
<td>Rabbit IgG HRP</td>
<td>Goat</td>
<td>WB</td>
<td>1:20000</td>
<td>Thermo Fisher</td>
</tr>
<tr>
<td>Rabbit IgM FITC</td>
<td>Rabbit</td>
<td>IF</td>
<td>10 µg/ml</td>
<td>Rockland</td>
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<tr>
<td>Streptavidin conjugates</td>
<td></td>
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<tr>
<td>— HRP — ELISA</td>
<td>—</td>
<td>—</td>
<td>0.1 µg/ml</td>
<td>Rockland</td>
</tr>
<tr>
<td>— — PerCP-Cy5.5</td>
<td>—</td>
<td>FC</td>
<td>1.3 µg/ml</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>— — Alexa Fluor 546</td>
<td>—</td>
<td>IF</td>
<td>2 µg/ml</td>
<td>Life Technologies</td>
</tr>
</tbody>
</table>

**Concentrations indicated are working concentrations.**

**Abbreviations:** AH; Armenian hamster; ELISA capt; ELISA capture, ELISA det; ELISA detection, FC; flow cytometry, GSH; golden Syrian hamster, ICCS; intracellular cytokine staining, IF; immunofluorescence, NS; not specified, WB; western blot.
2.1.4 Mice

Wt Balb/c mice, C57Bl/6 mice and severe combined immunodeficiency (SCID) mice were purchased from Animal Resource Centre (WA, Australia) or University of Adelaide Waite campus Laboratory Animal Services (LAS; SA, Australia). CCX-CKR-deficient (CCX-CKR−/−) mice were provided by Dr. Robert Nibbs at the University of Glasgow (Glasgow, United Kingdom) and CCR7-deficient (CCR7−/−) mice were provided by Prof. Heinrich Korner at the Menzies Research Institute (Tasmania, Australia). All mice were housed at the University of Adelaide Laboratory Animal Services (LAS) and SCID, CCX-CKR−/−, CCR7−/− mice were kept in pathogen free conditions in the barrier rodent area of the LAS.

The recombinase activating gene-1 deficient (RAG-1−/−) mice and RAG-2 deficient mice crossed with common γ-chain deficient (RAG-2 x γc−/−) mice were housed at the Peter MacCallum Cancer Centre animal facility (Victoria, Australia) in appropriate conditions.

All animal experiments were conducted in accordance with institutional and national regulations, under approval by the University of Adelaide or Peter MacCallum Cancer Centre.

2.1.5 Solutions and buffers

2.1.5.1 Basic solutions

Phosphate buffered saline (PBS), 10x Tris buffered saline (TBS), 80% glycerol, MilliQ water were obtained from the Technical Services Unit (TSU) at the School of Molecular Biomedical Science (SA, Australia) unless otherwise stated. Endotoxin-free (E/F) PBS was obtained from the Media Production Unit at the Institute of Medical and Veterinary Sciences (IMVS; SA, Australia) and stored at 4°C.
2.1.5.2 Nuclease-free water

Diethylpyrocarbonate (DEPC; Sigma-Aldrich, MO, USA) was diluted to 0.1% (v/v) in MilliQ water, incubated overnight at room temperature and then autoclaved.

2.1.5.3 Formaldehyde solutions

4% formaldehyde solution (w/v) was prepared by dissolving paraformaldehyde (PFA) in PBS at 55°C with stirring overnight. 1% formaldehyde solution (w/v) was prepared by diluting 4% formaldehyde solution with PBS. These solutions were stored at 4°C for short term or -20°C for long term storage.

2.1.5.4 FACS staining buffer

Bovine serum albumin (BSA; Sigma-Aldrich) and NaN₃ were added to PBS to a final concentration of 1% BSA (w/v) and 0.04% NaN₃ (w/v) and was stored at 4°C.

2.1.5.5 Binding buffer for scavenging assay

RPMI-1640 (RPMI; Life Technologies) was supplemented with 4 mM HEPES (IMVS) and 1% BSA.

2.1.5.6 PBS/Tween

Polyoxyethylene-sorbitan monolaurate (Tween 20; Sigma-Aldrich) was added to PBS to a final concentration of 0.05% (v/v) and the solution mixed thoroughly.

2.1.5.7 XTT:PMS

2,3-Bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilideinner salt (XTT 1 mg/ml; Sigma-Aldrich) was combined with N-methyl dibenzopyrazine methyl sulphate (PMS; 1.25 mM; Sigma-Aldrich) in a 50:1 ratio.
2.1.5.8 Complete 2x Iscove’s Modified Dulbecco’s Medium (IMDM)

Powdered IMDM (Life Technologies, NY, USA) was made up to a 2x concentrate solution according to manufacturer’s instructions. Complete 2x IMDM was prepared by adding 5% foetal bovine serum (FBS; Sigma-Aldrich), 0.2 U/ml penicillin/gentamycin (IMVS) and 0.2 U/ml Fungizone (IMVS) to incomplete 2xIMDM.

2.1.5.9 DNA extraction buffer

192 ml of 0.2 M Na₂PO₄ was combined with 8 ml of 0.1% Triton X-100 (v/v; Sigma-Aldrich) and pH was adjusted to 7.8.

2.1.5.10 DNA staining solution

20 µg/ml propidium iodide (PI; Sigma-Aldrich) and 200 µg/ml RNase (TSU) were added to PBS.

2.1.5.11 Complete Lysis Buffer

Complete lysis buffer was prepared by combining 50 mM Tris-HCl (pH 7.4), 1% Igepal/NP-40 (Sigma-Aldrich), 150 mM NaCl, 1 mM EDTA, 1.5 mM MgCl₂, 2M Na₃VO₄, 50 mM NaF, 1 mM phenylmethanesulphonyl fluoride (PMSF; Sigma-Aldrich) and 1% protease inhibitor cocktail (Sigma-Aldrich).

2.1.5.12 2x Loading buffer

Loading buffer was prepared by combining 100 mM Tris-HCl (pH 6.8), 2% sodium dodecyl sulphate (SDS; TSU), 20% glycerol, 100 mM DL-Dithiothreitol (DTT; Sigma-Aldrich) and 0.01% bromophenol blue (Sigma-Aldrich).
CHAPTER 2: Materials and Methods

2.1.5.13 Polyacrylamide gels

4% stacking gel was prepared by combining 6.1 ml of MilliQ water, 1.3 ml 30% acrylamide/bis (Bio-Rad Laboratories, CA, USA), 0.1 ml 10% SDS, 2.5 ml 0.5M Tris-HCl (pH 6.8), 70 µl of 10% ammonium persulphate and 14 µl TEMED (Sigma-Aldrich).

12% running gel was prepared by combining 3.4 ml of MilliQ water, 4.0 ml 30% acrylamide/bis, 0.1 ml 10% SDS, 2.5 ml 1.5M Tris-HCl, pH 8.8, 70 µl 10% ammonium persulphate and 70 µl TEMED.

2.1.5.14 TBS/Tween

Tween 20 was added to TBS to a final concentration of 0.05% (v/v) and the solution was mixed thoroughly.

2.1.5.15 Digestion buffer

Dulbecco’s Modified Eagle Medium (DMEM; Life Technologies) was supplemented with 5% FBS, 2.5 mM CaCl₂, 10mM HEPES and 0.2 U/ml penicillin/gentamycin. DNase (30 U/ml; Sigma-Aldrich) and collagenase IA (1 mg/ml; Sigma-Aldrich) were added on the day of use.

2.1.5.16 Mouse red cell removal buffer (MRCRB)

155 mM NH₄Cl and 170 mM Tris-HCl (pH 7.65) were combined in a 9:1 ratio. pH was adjusted to 7.2 and the solution was filter sterilised before use.

2.1.5.17 Recombinant protein diluent for sequential ELISA

0.5% skim milk, 0.1% BSA and 0.005% Tween 20 were added to PBS.
2.1.5.18 Diluent for sequential ELISA

0.1% BSA and 0.005% Tween 20 were added to PBS.

2.1.5.19 Lymphocyte restimulation medium

20 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich), 1 nM ionomycin (Life Technologies) and GolgiStop (1:1500 dilution; BD Biosciences) were added to RPMI containing 10% FBS, 200 mM L-glutamine (IMVS), 27 mM 2-β mecaptoethanol (Sigma-Aldrich) and 0.2 U/ml penicillin/gentamycin.

2.2 Tissue culture and generation of genetically modified cell lines

2.2.1 4T1.2 cells

The murine mammary carcinoma cell line 4T1.2 was derived from a Balb/cfC3H mouse and was a kind donation from Assoc. Prof. Robin Anderson (Peter MacCallum Cancer Centre). Cells were maintained in Minimum Essential Medium Alpha (αMEM; Life Technologies) supplemented with 10% FBS and 0.2 U/ml penicillin/gentamycin and cultured at 37°C in 5% CO₂. Cells were passaged every 2-3 days by rinsing the flasks with sterile PBS (TSU) and then incubating with 10 mM EDTA in PBS for 10 minutes at 37°C in 5% CO₂ to detach the adherent cells from flasks.

2.2.2 B16 cells

The murine melanoma cell line B16 was derived from a C57B1/6 mouse and was a kind donation of Prof. Mark Smyth. Cells were maintained in RPMI supplemented with 10% FBS, 200 mM L-glutamine and 0.2 U/ml penicillin/gentamycin and cultured at 37°C in 5% CO₂. Cells were passaged every 2-3 days by rinsing the flasks with sterile PBS and then
incubating with trypsin/EDTA in PBS (TSU) for 3 minutes at 37°C in 5% CO₂ to detach the adherent cells from flasks.

2.2.3 E0771 cells

The murine mammary carcinoma cell line E0771 was derived from a C57BL/6J mouse and was a kind donation of Assoc. Prof. Robin Anderson. Cells were maintained in DMEM supplemented with 10% FBS, 200 mM L-glutamine and 0.2 U/ml penicillin/gentamycin and cultured at 37°C in 5% CO₂. Cells were passaged every 2-3 days by rinsing the flasks with sterile PBS and then incubating with trypsin/EDTA in PBS for 3 minutes at 37°C in 5% CO₂ to detach the adherent cells from flasks.

2.2.4 HEK293T cells

The human embryonic kidney cell line HEK293T was maintained in DMEM supplemented with 10% FBS, 200 mM L-glutamine and 0.2 U/ml penicillin/gentamycin and cultured at 37°C in 5% CO₂. Cells were passaged every 2-3 days by rinsing the flasks with sterile PBS and then incubating with trypsin/EDTA in PBS for 3 minutes at 37°C in 5% CO₂ to detach the adherent cells from flasks.

2.2.5 Transfection of 4T1.2 cells

4T1.2 cells were harvested and 2 x 10⁵ cells were cultured overnight in the wells of a 24-well tray (BD Biosciences, NJ, USA). The medium was replaced with serum-free OptiMEM (Life Technologies), and the cells were then transfected with either CCX>pEF or empty pEF using Lipofectamine 2000 (Life Technologies) according to the manufacturer’s instructions. After a 6-hour transfection incubation, Opti-MEM was replaced with complete αMEM (Section 2.2.1) and cells were cultured overnight before selection with 3 μg/ml puromycin (Merck, Darmstadt, Germany) was commenced. The transfectants were kept in medium containing puromycin for 2 weeks before being
subjected to further analysis.

2.2.6 Lentiviral transduction of B16 cells

Lentiviral supernatants were produced using HEK293T packaging cells which were transfected with one of the CCX-CKR shRNA or GFP shRNA-encoding lentiviral vectors together with psPAX2, pREV and pMD2-G packaging plasmids using Lipofectamine 2000 reagent according to manufacturer’s instructions. Briefly, 3 x 10⁶ HEK293T cells were cultured overnight in a 10 cm dish and the medium was replaced with Opti-MEM containing 5% FBS. DNA plasmids/Lipofectamine mixture in 1ml Opti-MEM was added to the cells and incubated overnight. The medium was replaced with complete DMEM (Section 2.2.4) and lentivirus-containing supernatants were harvested 48 hours post-transfection. Supernatants were filtered through a 0.45 μm-pore syringe filter (Sartorius AG, Goettingen, Germany) and polybrene (Sigma-Aldrich) was added to a final concentration of 8μg/ml.

For transduction, 1.5 x 10⁵ B16 cells were cultured overnight in 6-well trays (BD Biosciences). The culture medium was replaced with 2ml of filtered lentiviral supernatant harvested above containing polybrene and incubated for 6 hours. The viral supernatant was removed and replaced with complete RPMI (Section 2.2.2) and cells were cultured overnight before selection with 2 μg/ml of puromycin was commenced. The transductants were kept in medium containing puromycin for at least 2 weeks before being subjected to further analysis.
2.3 in vitro assays

2.3.1 qPCR

Total RNA was extracted from 5-10 x 10^6 cells using 1 ml of TRI Reagent (Life Technologies) according to the manufacturer’s instructions. Briefly, chloroform was added to the whole cell lysate in TRI Reagent and the aqueous phase was separated from the organic phase by centrifugation. RNA present in the aqueous phase was precipitated using isopropanol and washed with 75% ethanol (w/v). In order to further clarify the product, RNA resuspended in nuclease-free water (Section 2.1.5.2) was incubated with 2 M NaCl and 100% ethanol for 1 hour and washed with 75% ethanol (w/v). The concentration of RNA was determined using a Nanodrop 2000 (Thermo Fisher Scientific) and 4 μg of RNA was treated with TURBO DNA-free (Life Technologies). The concentration of RNA was again determined and the purity was confirmed (A260/280=1.9-2.0) using the Nanodrop 2000. One microgram of DNase-treated RNA was added to reverse transcription reaction using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Basel, Switzerland) with or without reverse transcriptase according to the manufacturer’s instructions. All reagents were prepared using nuclease free water.

The cDNA obtained was subjected to qPCR reactions using SYBR Green I master mix (Roche Applied Science). Each reaction contained 5 μl master mix, 600 nM each of forward and reverse primers, 1 μl cDNA and 3.4 μl nuclease-free water. The qPCR reactions were performed using the Light Cycler 480 system (Roche Applied Science) according to the manufacturer’s instructions. C_T values were generated by the software and the mRNA level of the gene of interest (GOI) was calculated as a ratio to a reference gene (RG) using the formula; 1/2^{(C_T(RG)-C_T(GOI))}. The samples with a C_T value greater than 35 were considered “not detectable”. The melting curve of each product was also analysed to confirm the specificity of the product. The absence of genomic DNA contamination was
confirmed by using samples of reverse transcription reaction performed without reverse transcriptase.

2.3.2 Flow cytometric analysis for chemokine receptors

Cells were harvested and immediately fixed with 4% formaldehyde solution (Section 2.1.5.3) for 10 minutes at room temperature and then resuspended at 4 x 10^6 cells/ml in FACS staining buffer (Section 2.1.5.4). Either 50μl (for CCR7 and CCR9 staining) or 100 μl of cells (for CCX-CKR staining) were incubated with antibodies at appropriate concentrations (Table 2.3) for 30 minutes at room temperature. Cells were then incubated with 50 μl of Alexa Fluor 488-conjugated anti-rat IgG or PE-conjugated anti-mouse IgG (Table 2.3) for 30 minutes in the dark on ice. Stained cells were acquired on an LSRII (BD Biosciences) and data were analysed using FlowJo software (TreeStar Inc., OR, USA).

2.3.3 Scavenging assay and enzyme linked immunosorbent assay (ELISA)

Binding buffer (Section 2.1.5.5) was prepared containing either 10 or 5 ng/ml of recombinant mouse CCL19 (R&D Systems, MN, USA). 4T1.2 cells were prepared at 1 x 10^6 cells/ml and 200 μl were aliquoted into tubes. The cells were resuspended with 200 μl of binding buffer containing recombinant CCL19 and incubated at 37°C for 3 hours during which time the cells were resuspended by inversion of the tubes every 30 minutes. After the incubation tubes were centrifuged and 100 μL of the supernatants were collected for detection of remaining CCL19 by ELISA.

On the previous day, a 96-well EIA/RIA high binding plate (Corning Life Science, NY, USA) was coated with 100 μl of anti-CCL19 capture antibody (Table 2.3) prepared in 100mM NaHCO3 coating buffer and incubated at 4°C overnight. The wells were blocked with 200 μl/well of 3% BSA in PBS for 1 hour. Recombinant CCL19 was prepared at 300 ng/ml, serially diluted 1:2 with 1% BSA/PBS to generate a standard curve. 100 μl of each
concentration as well as sample supernatants were added to the wells and incubated for 90 minutes. The plate was next incubated with 100μl of biotin-conjugated anti-CCL19 (Table 2.3) for 1 hour and then with 100 μl streptavidin-conjugated horseradish peroxidase (strep-HRP; Table 2.3) for 30 minutes at room temperature. 200 μl of TMB substrate solution (eBioscience) were then added to the wells and incubated in the dark at room temperature until an appropriate level of colour developed. Finally, 50 μl of 3M HCl solution was added to stop the reaction. Between each step wells were washed with PBS/Tween (Section 2.1.5.6) 3 times and the plate was incubated at 37°C unless otherwise stated. The absorbance was measured at 450 nm on a Biotrak II Plate reader (GE Healthcare, England, United Kingdom). The concentration of each sample was calculated from the standard curves generated using GraphPad Prism (GraphPad Software Inc., CA, USA).

2.3.4 Analysis of anchorage-dependent cell growth

Cells were prepared at 1 x 10^5 cells/ml (4T1.2) or 0.75 x 10^5 cells/ml (B16) and 25 μl of the cell suspension was added to the wells of 24-well trays containing 500 μl complete medium (Section 2.2.1 and 2.2.2). The cells were allowed to proliferate at 37°C in 5% CO₂ for 7 days. On days 0, 2, 5 and 7, 100μl of media were transferred to 96-well tray and 50 μl of XTT:PMS (Section 2.1.5.7) were added to each well. Colour was allowed to develop for 2 hours at 37°C and OD was measured at 490 nm (ref 650 nm) on a Biotrak II Plate reader.

2.3.5 Analysis of anchorage-independent cell growth

1.4% and 0.6% Bacto Agar (BD Biosciences) solutions were prepared in MilliQ water and combined with complete 2 x IMDM (Section2.1.5.8) in a 1:1 ratio to make 0.7% and 0.3% Bacto Agar solutions. Wells of 6-well trays were coated with 1ml of 0.7% agar/IMDM. Cells were prepared at 1 x 10^4 cells/ml in complete medium (Section 2.2.1 and 2.2.2) and 50 μl were added to 1ml of 0.3% agar/IMDM which was overlaid onto the layer of 0.7% agar/IMDM.
agard/IMDM. The trays were incubated at 37°C in 5% CO₂ for 2 weeks or until colonies formed. The colonies were visualised by adding 1 ml of 0.005% crystal violet (Ajax Finechem, NSW, Australia) solution in PBS to each well and incubating the tray for 1 hour at 37°C. The number of colonies in each well was determined by scanning the tray using Umax MagicScan (Techvill Inc., TX, USA) and quantified on the Quantity One Version 4.3.1 (Bio-Rad Laboratories).

2.3.6 Adhesion assay

4T1.2 cells were prepared at 1 × 10⁷ cells/ml in PBS and Calcein-AM (Life Technologies) was added to the cells at 1:500 dilution and incubated for 30 minutes at 37°C. The stained cells were washed with PBS and resuspended at 2 × 10⁵ cells/ml in serum-free αMEM. The wells of a 96-well tray were coated with either 0.5 mg/ml or 0.1 mg/ml Matrigel and allowed to set. The wells were then blocked with 150 μl of αMEM containing 1% BSA for 1 hour before 100 μl of cell suspension were added to each well. After a 30-minute incubation at 37°C, the tray was scanned on a Typhoon 9400 (GE Healthcare) before and after unbound cells were washed 3 times with PBS. Some wells were blocked with 150 μl of αMEM containing 1% BSA without Matrigel to measure background adhesion of cells and the fluorescent intensity of these wells was subtracted from appropriate sample wells. Percentage adhesion was calculated as (fluorescent intensity after washes/fluorescent intensity before washes) x 100.

2.3.7 Homotypic adhesion assay

4T1.2 cells were prepared at 3 × 10⁵ cells/ml in complete αMEM and 100 μl were added to a 96-well tray and cultured overnight to form a monolayer. On the following day, culture medium was replaced with 150 μl of αMEM containing 1% BSA to block wells. Freshly harvested cells were prepared at 1 × 10⁷ cells/ml in PBS and Calcein-AM was added to the cells at 1:500 dilution and incubated for 30 minutes at 37°C. The stained cells were washed
with PBS, resuspended at either $1 \times 10^5$ or $2 \times 10^5$ cells/ml and 100 µl was added to appropriate wells. After a 90-minute incubation at 37°C, the tray was scanned on a Typhoon 9400 before and after bound cells were fixed with 4% formaldehyde solution and unbound cells were washed away with PBS. Some wells were blocked with 150 µl of αMEM containing 1% BSA without cell monolayer to measure background adhesion of cells and the fluorescent intensity of these wells was subtracted from appropriate sample wells. Percentage adhesion was calculated as (fluorescent intensity after washes/fluorescent intensity before washes) x 100.

2.3.8 Invasion assay

4T1.2 cells were prepared at $2 \times 10^6$ cells/ml in αMEM containing 0.1% BSA and combined with neat Matrigel (BD Biosciences) at 1:1 ratio. The upper side of transwell membranes (8 µm pores; Corning Life Science) were coated with 50 µl of cell/Matrigel mixture and allowed to set at 37°C for 30 minutes. The lower chambers were filled with 500 µl of αMEM with or without 1% mouse serum harvested from 4T1.2 tumour-bearing mice (TBMS) and the upper chambers were inserted and filled with αMEM to avoid drying. The trays were incubated for 24 hours at 37°C in 5% CO$_2$. Cells left on the upper side of the membranes were scraped off with cotton buds before fixing the cells on bottom side of the membranes with 100% ethanol. The fixed cells were stained with 2% Toluidine blue (Sigma-Aldrich) and the membranes were cut off from transwell inserts and mounted onto glass slides. The number of cells on the bottom side of membranes as well as at the bottom of lower chambers were estimated by counting the number of cells in 5 random fields under microscope. The percentage of invaded cells was calculated as (no. of invaded cells/no. of cells added in transwell) x 100.

2.3.9 Migration assay

The lower chambers of blind well chambers (Neuro Probe, MD, USA) were filled with 200 µl...
μl of αMEM with or without 1% TBMS. Polycarbonate membranes (8 μm pores; Neuro Probe) were inserted into lower chambers assembled with upper chambers. 4T1.2 cells were prepared at 1 x 10^6 cells/ml in αMEM containing 0.1% BSA and 50 μl were added to the upper chamber. Blind well chambers were incubated for 6 hours at 37°C and chambers were disassembled. The cells migrated to the lower chambers were transferred to a 12-well tray and cultured until colonies formed. The colonies were fixed with 4% formaldehyde solution and stained with 0.06% Giemsa stain (Sigma-Aldrich) in PBS. The number of colonies in each well was determined by scanning the tray using Umax MagicScan and quantified on the Quantity One Version 4.3.1. The percentage of migrated cells was estimated as (no. of migrated cells/no. of cells added in upper chamber) x 100.

2.3.10 Anoikis assay and PI staining of DNA extracted cells

4T1.2 cells were prepared at 1 x 10^5 cells/ml and 2ml of cells were cultured in either Ultra-low attachment (Corning Life Science) or standard tissue culture 6-well trays with or without 5% FBS for 24 hours at 37°C in 5% CO₂. In order to measure apoptosis, the cells were subjected to Western blot analysis on lysates (Section 2.3.11) as well as PI staining. For PI staining, harvested cells were incubated in 10mM EDTA in PBS for 10 minutes at 37°C to separate any cell aggregates. The suspended cells were fixed in cold 75% ethanol (w/v) and then incubated in 100μl DNA extraction buffer (Section 2.1.5.9) for 5 minutes at room temperature. DNA remaining in the cells was stained with DNA staining solution (Section 2.1.5.10) for 30 minutes. The cells were washed between each step with PBS except after DNA extraction. The stained cells were acquired on FACSCanto (BD Biosciences) and the data were analysed using FlowJo software.

2.3.11 Western blot analysis

Cells harvested from the anoikis assay (Section 2.3.10) were washed once in cold PBS, resuspended in 100 μl complete lysis buffer (Section 2.1.5.11) and incubated on ice with
agitation for 30 min before being centrifuged at 13,000xg for 10 minutes at 4°C. A bicinchoninic acid protein assay was then performed using a BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer’s instructions to determine the protein concentration. Samples were prepared by combining lysates (60 μg total protein) with an equal volume of 2x loading buffer (Section 2.1.5.12) and then loaded on 12% polyacrylamide gels (Section 2.1.5.13). Gels were cast in a Bio-Rad 1.0 mm vertical slab gel unit according to manufacturer’s instructions and were run at 200 V. Proteins were then transferred from the polyacrylamide gel to a PVDF membrane using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories) as instructed by the manufacturer. The membrane was blocked with 5% skim milk in TBS/Tween (Section 2.1.5.14) at room temperature for 1 hour and incubated overnight with primary antibody (anti-PARP and anti-Caspase-3) at appropriate concentrations (Table 2.3) in 5% BSA in TBS/Tween at 4°C. The secondary antibody (Table 2.3) in 5% skim milk in TBS/Tween was added onto the membrane and incubated for 1 hour and then covered with ECL detection solution (Sigma-Aldrich) as specified by the manufacturer. The membrane was washed 3 times with TBS/Tween between each step. The blot was exposed to X-ray film (AGFA, Mortsel, Belgium) and developed using an Ilfospeed 2240 X-ray processor (Ilford, Marly, Switzerland). The membrane was then stripped with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific) and was reprobed for E-cadherin and β-actin (Table 2.3) using the same method described above. Western blot detection was performed by Dr. Julie Brazzatti.

2.4 in vivo assays

2.4.1 Primary tumour growth and spontaneous metastasis

Cells were harvested and resuspended at 1 x 10^7 cells/ml (4T1.2 and E0771) or 2 x 10^7
cells/ml (B16) in sterile E/F PBS (Section 2.1.5.1) and kept on ice. Syngeneic mice aged between 6 and 8 weeks old were anaesthetised by isoflurane (Abbott Laboratories, Illinois, U.S.A) inhalation and 10 μl of cell suspension were injected into a mammary fat pad (4T1.2 and E0771) or subcutaneously into the left flank (B16) using a 50 μl Gastight glass syringe (Hamilton Company, NV, USA). Tumour growth was monitored every 2 days and the perpendicular diameters of each tumour were measured using digital callipers (Mitsuyo, Kanagawa, Japan). The tumour sizes were calculated as multiples of shortest and longest diameters. Mice were sacrificed once the tumour diameter reached 15 mm or became ulcerated. Tumours were removed and tumour weight was measured using analytical balance. The experiments using RAG-1−/− and RAG-2 x γc−/− were performed by Prof. Mark Smyth.

For the 4T1.2 model, when the mice were sacrificed, lungs were also harvested and fixed in 4% formaldehyde solution overnight. The lungs were passed though a 30% sucrose solution twice, each for at least 2 hours, before being immersed in 75% ethanol (w/v) and stored at 4°C. To quantify the extent of metastasis, lobes of the lungs were separated and the number of metastatic nodules was counted under a stereomicroscope.

2.4.2 Haematogenous metastasis

Cells were harvested and resuspended at 2 x 10^6 cells/ml (4T1.2 and B16) or 1 x 10^6 cells/ml (E0771) in sterile E/F PBS (Section 2.1.5.1) and kept on ice. Syngeneic mice aged between 6 and 8 weeks old were placed in a holder and 100 μl cell suspension was injected into the tail vein using 29G1/2 insulin syringe (BD Biosciences). On day 14 post-injection the lungs were removed and fixed in 4% formaldehyde solution overnight, then passed though 30% sucrose solution twice, each for at least 2 hours, before being immersed in 75% ethanol (w/v) and stored at 4°C. To quantify the extent of metastasis, lobes of the lungs were separated and the number of metastatic nodules was counted under a
For B16 CCX-CKR knockdown experiments, the images of the lung lobes were captured on a Leica MZ16FA Stereomicroscope (Leica Microsystems, Wetzlar, Germany). To quantify the metastatic burden on these mice, the lungs were minced and digested in digestion buffer (Section 2.1.5.15) for 1 hour at 37°C during which time the tissue was homogenised by repeated pipetting every 15 minutes. The resulting cell suspension was passed through a cell strainer (BD Biosciences) to remove any tissue debris. Erythrocytes were removed using MRCRB (Section 2.1.5.16) and 1/50 of the cell suspension was cultured in 6-well trays in the presence of puromycin until colonies formed. The colonies were then fixed with 4% formaldehyde solution and stained with Calcein-AM diluted 1:500 in PBS. The trays were scanned on Typhoon 9400 to measure the fluorescent intensity of each well.

2.4.3 Haematogenous metastasis survival assay

C57Bl/6 mice were injected with either $2 \times 10^5$ cells or $5 \times 10^5$ cells into the tail vein. The health of mice was monitored for signs of distress such as laboured breathing, hunched, ruffled fur, reduced movement, and mice were culled when weight loss reached 20%. Survival curves were plotted using GraphPad Prism. *This assay was performed Prof. Mark Smyth.*

2.4.4 In vivo intravasation assay

Female Balb/c mice were injected with 4T1.2 cells into the mammary fat pad as described above (Section 2.4.1). On days 14, 21 and 28 post-injection mice were sacrificed and blood was collected from the right atrium into heparin treated tubes. Erythrocytes were removed using MRCRB (Section 2.1.5.16) and the remaining cells were cultured in a 6-well tray in the presence of puromycin until colonies formed. The colonies were then fixed with 4%
formaldehyde solution and stained with 0.06% Giemsa stain (Sigma-Aldrich) in PBS. The number of colonies was counted manually.

2.4.5 In vivo neutralisation of CCL21

Mice were injected with B16 cells, subcutaneously as described above (Section 2.4.1). On days -1, 6 and 13 post-tumour cell injection, mice received 500 µg of either normal rabbit IgG or anti-CCL21 antibody (Table 2.3) intraperitoneally. Mice were monitored, sacrificed and tissues were analysed as described above (Section 2.4.1).

2.5 Ex vivo assays

2.5.1 Preparation of single cell suspensions from tissue

For ex vivo analysis of tumours and lungs, tissues harvested from mice were weighed, minced and digested in digestion buffer (Section 2.1.5.15) for 1 hour at 37°C during which time the tissue was homogenised by repeated pipetting every 15 minutes. The resulting cell suspension was passed though a cell strainer to remove any tissue debris. Cells were centrifuged and supernatants were transferred into fresh tubes containing protease inhibitor cocktail (1/100; Sigma-Aldrich) for chemokine and cytokine analysis by sequential ELISA (Section 2.5.2). Erythrocytes were removed from the cell suspension and the remaining cells were subjected to flow cytometric analysis (Section 2.5.3).

For analysis of mammary fat pads, both of the fourth mammary fat pads were removed from 6-8 weeks old female mice, weighed and placed in bead beating tubes (MoBio, CA, USA) containing 10% glycerol and protease inhibitor cocktail (1/100) in PBS. Tissues were homogenised using a Precellys 24 (Bertin Technologies, Yvelines, France) and supernatants were collected into fresh tubes after centrifugation for chemokine analysis by
sequential ELISA (Section 2.5.2).

2.5.2 Sequential ELISA on tissue homogenate supernatants

Sequential ELISAs for chemokines were performed in the order of CCL19, CCL21, and CCL25, and for cytokines; IL-17, IL-4, IL-12 and IFN-γ. 96-well EIA/RIA high binding plates were coated with 50 µl of appropriate capture antibodies (Table 2.3) prepared in 100 mM NaHCO₃ coating buffer and incubated at 4°C overnight. All the subsequent steps were performed one plate at a time in sequence. First, the wells were blocked with 150 µl of 5% skim milk in TBS for 1 hour before recombinant proteins and sample supernatants were added. For standard curves, recombinant CCL19 (300 ng/ml), CCL21 (750 ng/ml; R&D system) and CCL25 (500 ng/ml; R&D Systems) or recombinant IL-17 (10 ng/ml; eBioscience), IL-4 (10 ng/ml; eBioscience), IL-12 (50 ng/ml; eBioscience) and IFN-γ (50 ng/ml; eBioscience) were prepared together in recombinant protein diluent (Section 2.1.5.17) and serially diluted 1:2 in U-bottom 96-well tray (BD Biosciences). 50µl of each concentration as well as sample supernatants were added to appropriate wells and the plate was incubated for 1 hour. The recombinant protein mix and sample supernatants in the first plate were sequentially transferred from one plate to the next. The plates were then incubated with 50 µl of biotin-conjugated antibodies (Table 2.3) followed by strep-HRP (Table 2.3) prepared in diluent (Section 2.1.5.18) for 1 hour and 30 minutes, respectively. 100 µl of TMB substrate solution were then added to the wells and incubated in the dark until the appropriate level of colour developed. Finally, 50 µl of 3M HCl solution was added to stop the reaction. Between each step wells were washed with PBS containing 0.05% Tween20 (Sigma-Aldrich) 3 times and the plate was incubated at room temperature unless otherwise stated. The absorbance was measured at 450 nm on Biotrak II Plate reader. The concentration of each sample was calculated from the standard curves generated using GraphPad Prism.
2.5.3 Antibody labelling of cell preparations for flow cytometric analysis

2.5.3.1 Cell surface staining with unconjugated and directly conjugated antibodies

Cells were resuspended at 4 x 10⁶ cells/ml in FACS staining buffer (Section 2.1.5.4) and Fcγ receptors were blocked with mouse γ-globulin (200 μg/ml; Rockland) at room temperature. Anti-CCR7 and -CCR9 antibodies were prepared at appropriate concentrations (Table 2.3) and 50 μl of cells were incubated with 10μl of diluted antibody at room temperature. Alexa Fluor 647-conjugated anti-rat IgG (Table 2.3) was pre-absorbed with normal mouse serum (1/50 dilution) and mouse γ-globulin (100 μg/ml) in the dark at room temperature before 50 μl were added to the cells and incubated on ice. In order to block free binding sites on anti-rat IgG, cells were incubated with 50 μl of rat γ-globulin (400 μg/ml; Rockland) at room temperature. 10 μl of directly conjugated antibodies (Table 2.3) were then added and incubated in the dark on ice. In the case where biotin-conjugated antibody was used, cells were further incubated with 50 μl of streptavidin-conjugated PerCPCy5.5 (Table 2.3) in the dark on ice. All incubations were for the duration of 30 minutes and cells were washed with 200μl of FACS staining buffer (Section 2.1.5.4) between each step. Before acquisition, cells were resuspended with 200 μl FluoroGold (Fluorochrome LLC., CO, USA) diluted at 1:20 in PBS to identify live and dead cells. The cells were acquired on an LSRII and the data was analysed using FlowJo software.

2.5.3.2 Intracellular cytokine staining (ICCS)

Cells were resuspended at 4 x 10⁶ cells/ml in 50 μl of restimulation medium (Section 2.1.5.19) and incubated for 4 hours at 37°C in 5% CO₂. Cells were washed twice in 200 μl of PBS and stained with LIVE/DEAD UV Fixable Dead Cell Stain (Life Technologies) according to the manufacturer’s instructions. Cells were again washed twice with 200μl of FACS staining buffer (Section 2.1.5.4). Fcγ receptors were then blocked with mouse
γ-globulin (200 μg/ml) at room temperature. Cells were stained with 10 μl of biotinylated anti-CD8 antibody (Table 2.3) prepared in PBS and washed twice with 200 μl of FACS staining buffer. 10 μl of diluted streptavidin-PerCP Cy5.5 was then added and incubated in the dark. Cells were then washed twice in 200 μl PBS containing 0.04% NaN₃ (w/v) and then fixed and permeabilised using 100 μl Cytofix/Cytoperm kit (BD Biosciences) according to manufacturer’s instructions for 20 minutes at 4°C. Cells were then washed twice in 200 μl of 1x Perm/Wash buffer (BD Biosciences), resuspended in 50 μl 1X Perm/Wash buffer before incubation with 10 μl of anti-IFN-γ (Table 2.3) prepared in 1x Perm/Wash buffer in the dark. Cells were then washed twice in cold PBS and resuspended in 200 μl of 1% formaldehyde solution (Section 2.1.5.3). All incubations were for the duration of 30 minutes at 4°C unless otherwise stated. The cells were acquired on an LSRII and the data were analysed using FlowJo software. ICCS was performed by Dr. Iain Comerford.

2.5.3.3 Analysis of flow cytometric data

In order to analyse TILs in tumour homogenates, gate was first set to include all the populations potentially found in tumour tissue but exclude cell debris as recognised by the FSC-A and SSC-A profile. Splenocyte preparation was also included at the time of acquisition as a guide to set the leukocyte gate. Cells in this gate were further gated to exclude multi-cell events as recognised by the FSC-A and FSC-H profile. Next gate was set for FluoroGold negative cells to ensure only live-cell event were analysed for the expression of various leukocyte markers. The gating strategy used is shown in Figure 2.1 with representative plots.

2.5.4 Immunofluorescence (IF) staining of tissue sections

The tissues harvested from mice were embedded in Tissue-Tek OCT (Sakura Finetek, South Holland, Netherlands) and frozen in liquid nitrogen. Sections used for IF were fixed
in cold 100% acetone for 10 minutes and air dried before being stored overnight at -20°C. All incubations were performed in a humid chamber at room temperature and slides were washed between each step by three changes through TBS for 2 minutes per change. Slides were re-hydrated in TBS, blocked by incubating with 2% normal mouse serum and 2% normal goat serum in TBS for 30 minutes. Primary antibodies (Table 2.3) were added to each section and incubated for 1 hour. The primary antibodies were detected with either anti-rat Alexa Fluor 488, anti-rat Alexa Fluor 647 or streptavidin-conjugated Alexa Fluor 546 (Table 2.3) secondary antibodies incubated on sections for 45 minutes. If sections required the use of two purified rat anti-mouse primary antibodies specific for different antigens then available sites on the first primary and secondary combination were blocked with 2% normal rat serum followed by addition of the second primary-secondary combination. Upon completion, stained slides were air dried and coverslips were mounted using fluorescence preserving Vectashield mounting medium (Vector Laboratories, CA, USA). Images were acquired using a Leica SP5 spectral scanning confocal microscope (Leica Microsystems) and LAS AF software (Leica Microsystems). Preparation of tissue sections and IF staining were performed by Mark Bunting.

2.6 Statistical analysis

All statistical tests were performed using GraphPad Prism version 5.00 for Windows, GraphPad Software (CA, USA, www.graphpad.com).
Figure 2.1: Gating strategy used for the analysis of TILs in tumour homogenate preparation.
CHAPTER 3: THE EFFECT OF CCX-CKR OVEREXPRESSION ON PROGRESSION OF MAMMARY CARCINOMA
3.1 Introduction

Previous in vitro and in vivo studies suggest that CCX-CKR functions as a scavenger or decoy receptor for chemokines, CCL19, CCL21 (ligands for CCR7) and CCL25 (ligand for CCR9), thereby regulating the level and activity of these chemokines, and therefore, the activity of CCR7 and CCR9, during homeostasis and under inflammatory conditions. Furthermore, previous studies also indicate that these receptors and their ligands can have pro-tumour effects. This suggests that the expression of CCX-CKR in the tumour environment may regulate tumour growth and metastasis. However, the significance of chemokine regulation by CCX-CKR on tumour growth and metastasis had not been investigated prior to the onset of this study. Therefore, the initial aim of this project was to investigate the effect of CCX-CKR overexpression in tumour cells on tumour growth and metastasis.

3.2 Characterisation of 4T1.2 mammary carcinoma model

In order to investigate the effect of overexpression of CCX-CKR on growth and metastasis of mammary carcinoma, the 4T1.2 murine model of mammary carcinoma was selected. This model was chosen as it spontaneously metastasises to various tissues including lung and bone, which closely resembles the metastatic pattern of human breast cancer. Thus, 4T1.2 cells were first characterized in terms of their expression of chemokines and chemokine receptors relevant to CCX-CKR. qPCR analysis indicated that both CCR7 and CCR9 were expressed by 4T1.2 cells at the mRNA level (Figure 3.1A). The surface
expression of these receptors was confirmed by flow cytometric analysis of the cells using receptor-specific monoclonal antibodies (mAbs) (Figure 3.1B and C). However, the endogenous expression of CCX-CKR was detected only at the limit of detection of the highly sensitive qPCR assay (Figure 3.1A). Note that at the time of experimentation no antibody specific for mouse CCX-CKR was available. In addition, ELISA analysis revealed that CCL19, CCL21 and CCL25 in the culture supernatant of 4T1.2 cells were below the limit of detection (data not shown).

3.3 Generation and characterization of CCX-CKR overexpressing 4T1.2 cell lines

Low level expression of CCX-CKR in 4T1.2 cells suggested that this was a suitable system to test the effect of CCX-CKR overexpression. Therefore, 4T1.2 cells were stably transfected with a plasmid construct encoding mouse CCX-CKR. This construct, CCX>pEF or empty plasmid was transfected into 4T1.2 cells. Cells were selected for stable transfectants with puromycin for 2 weeks before being subjected to experimentation and continued to be cultured in the presence of puromycin at all times during this study. The transfected cells were first tested for expression of CCX-CKR. The qPCR indicated that cells transfected with CCX-CKR (4TCCX) expressed approximately a 14-fold higher level of CCX-CKR mRNA compared with empty vector transfected cells (4TpEF) (Figure 3.2).

Since a major function reported for CCX-CKR is chemokine scavenging281, the transfected 4T1.2 cells were also tested for their ability to scavenge CCL19. Cells were incubated with 2 different concentrations of recombinant mouse CCL19 for 3 hours, the supernatants collected and assessed for levels of remaining CCL19 by ELISA. 4TCCX cells exhibited a pronounced ability to scavenge CCL19 compared with control cells (Figure 3.3), indicating that CCX-CKR is functionally expressed at the protein level by 4TCCX cells at
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Figure 3.1: Expression of chemokine receptors CCR7, CCR9 and CCX-CKR by 4T1.2 cells. A, expression of chemokine receptor mRNA detected by qPCR. Total RNA extracted from 4T1.2 cells was reverse-transcribed to generate cDNA, which was then subjected to qPCR analysis using primers specific for CCR7, CCR9, CCX-CKR and GAPDH. The mRNA level of each target gene was calculated as a ratio to GAPDH. Bars represent the mean ±SEM of two experiments performed in duplicate. B, surface expression of chemokine receptors detected by flow cytometry. 4T1.2 cells were fixed with 4% formaldehyde in PBS, then labelled with either rat anti-muCCR7 or rat anti-muCCR9 antibody. The purified antibodies were detected using Alexa488-conjugated anti-ratIgG secondary antibody. Data shown are the mean fluorescent intensity (MFI) of cells labelled with specific antibody after the MFI of isotype control was subtracted. Bars represent the mean ±SEM of 3 independent experiments, each performed in duplicate. C, representative histograms from B. Shaded area; isotype-matched control antibody, solid line; chemokine receptor-specific antibody.
Figure 3.2: Expression of CCX-CKR by transfected 4T1.2 cell lines. Total RNA extracted from transfected cells was reverse-transcribed to generate cDNA, which was then subjected to qPCR analysis using primers specific for CCX-CKR and GAPDH. The mRNA level of the target gene was calculated as a ratio to GAPDH. Bars represent the mean ±SEM of 2 independent experiments, each performed in duplicate. Statistical analysis was performed using unpaired t-test.
Figure 3.3: *In vitro scavenging of CCL19 by CCX-CKR transfected 4T1.2 cells.* 4T1.2 cells were resuspended with buffer containing either 10 or 5 ng/ml of recombinant CCL19. The cells were incubated for 3 hours, and 100 μl of the supernatants were collected for detection of remaining CCL19 by ELISA. As an additional control, CCL19 was incubated under the same conditions in the absence of cells to gauge potential non-specific degradation of CCL19 and the mean concentration of these control samples were set as 0% scavenging to normalise experimental samples. Bars represent the mean ±SEM of 3 independent experiments, each performed in triplicate. Statistical analysis was performed using unpaired t-test.
a level capable of significantly altering the chemokine microenvironment.

Before assessing the effect of CCX-CKR knockdown on 4T1.2 tumour growth and metastasis *in vivo*, the effect of increased expression of CCX-CKR on the growth properties of these cells was examined *in vitro*. This was assessed in two different ways. First, anchorage-dependent growth of cells was tested by measuring the reduction of the tetrazolium salt XTT by metabolically active cells. Second, anchorage-independent growth was tested by examining colony formation in semi-solid medium created with Bacto-agar. Data from these experiments revealed no significant difference in growth properties of 4TpEF and 4TCCX cells (Figure 3.4), indicating that the increase of CCX-CKR expression had no effect on *in vitro* growth of 4T1.2 cells.

3.4 The effect of CCX-CKR overexpression on progression of 4T1.2 tumours *in vivo*

In order to investigate the effect of CCX-CKR on *in vivo* growth and metastasis of mammary carcinoma, CCX-CKR overexpressing 4T1.2 cells or control cells were injected into the mammary fat pad of syngeneic Balb/c mice. The perpendicular diameters of palpable tumours were measured and recorded every second day using digital callipers and approximately 4 weeks post-tumour injection, mice were sacrificed and tumours were weighed. As shown in Figure 3.5A, from day15 onwards 4T1.2 tumour overexpressing CCX-CKR were significantly smaller than control tumours. This was also shown by measurement of final tumour weights where 4TCCX tumours were significantly smaller than control tumour at the end point of these experiments (Figure 3.5B).

As mentioned above, 4T1.2 tumours spontaneously metastasise to various sites including the lungs. Therefore, when mice were sacrificed at the end of each experiment, lungs were
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Figure 3.4: *In vitro growth of transfected 4T1.2 cells*. **A**, anchorage-dependent growth. Cells were prepared at 1 x 10^3 cells/500 μl/well in 24-well trays and incubated for 7 days. On days 0, 2, 5, and 7, level of metabolically active cells was measured by adding XXT:PMS into the media and the absorbance at 490 nm (reference 650 nm) was determined. Bars represent the mean ± SEM of 3 independent experiments, each performed in triplicate. **B**, anchorage-independent growth. The wells of 6-well trays were covered with 0.7% Bacto-agar made up in complete IMDM. The top layer containing 500 cells with 0.3% agar in IMDM was overlaid on the 0.7% agar layer. The tray was incubated for 10 days and the resulting colonies were visualised with 0.005% crystal violet solution and the number of colonies was determined using Quantity One software. Bars represent the mean ± SEM of 3 independent experiments, each performed in triplicate. Statistical analysis was performed using two-way ANOVA for A and unpaired t-test for B.
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Figure 3.5: Growth and metastasis of CCX-CKR overexpressing 4T1.2 mammary tumours in syngeneic Balb/c mice. A, growth curve of orthotopic tumours. Female Balb/c mice aged between 6 and 8 weeks were injected with 1 x 10⁶ cells into the mammary fat pad. The perpendicular diameters of each tumour was measured every 2 days from day 7 post-injection. Tumour sizes are presented as multiple of shortest and longest diameters. B, final tumour weight. When one of the tumour diameters was reached 15mm, mice were sacrificed and tumours were harvested and weighed. C, spontaneous metastasis of CCX-CKR overexpressing 4T1.2 mammary tumours. When mice were sacrificed, lungs were also harvested. The lobes of lungs were separated and metastatic nodules on the surface were counted under a stereomicroscope. Data points (A) and bars (B and C) represent the mean ± SEM of 4 independent experiments, each performed with 10 mice per group. Each point (B and C) represents the data from individual mice. Statistical analysis was performed using two-way ANOVA for A and unpaired t-test for B and C.
also removed for assessment of spontaneous metastasis. The lungs from tumour-bearing mice were fixed and the 5 lobes were separated before being examined for surface metastatic nodules using a stereomicroscope. Unexpectedly, the number of surface nodules on the lungs of 4TCCX tumour-bearing mice was significantly greater than those of 4TpEF tumour-bearing mice (Figure 3.5C). To determine whether the increased metastasis was dependent on or independent of the primary tumours, experiments were performed to assess the ability of the cells to colonise lungs when they were injected directly into the tail veins of Balb/c mice. As shown in Figure 3.6, 4TCCX cells were able to colonise lungs more efficiently compared with control 4TpEF cells upon intravenous administration.

From these data it is clear that overexpression of CCX-CKR significantly alters progression and metastasis of mammary carcinoma in the 4T1.2 model. The original hypothesis of this investigation was founded on the assumed function of CCX-CKR to scavenge its ligands which have been shown in our laboratory\textsuperscript{194} and by others to have pro-tumour effects\textsuperscript{192,198,199,201,203,204,206,207}. Given its function as chemokine scavenger, and as 4TCCX cells showed higher scavenging ability than control cells \textit{in vitro} (Figure 3.3), the effects of overexpression of CCX-CKR on chemokine scavenging \textit{in vivo} when expressed on 4T1.2 tumours was tested. Tumours harvested from mice at the end of experiments were homogenised in PBS and supernatants were assayed for levels of CCX-CKR ligands, CCL19, CCL21 and CCL25. As shown in Figure 3.7, levels of these chemokines in tumours from 4TCCX injected mice were not different from the control tumours, although the level of CCL21 in 4TCCX tumour had a statistically non-significant trend to be lower than in control tumours (Figure 3.7B). Although there are several possible interpretations of these data which will be discussed in chapter 5, one of the implications is that the effect of CCX-CKR overexpression on tumour progression in this model may be independent of its proposed function as chemokine scavenger. Therefore, possible novel functions of CCX-CKR in this system were investigated.
Figure 3.6: Lung colonisation of CCX-CKR overexpressing 4T1.2 cells via haematogenous route. Female Balb/c mice aged between 6 and 8 weeks were injected with $1 \times 10^5$ cells intravenously into the tail vein. On day 14 post tumour injection mice were sacrificed and lungs were harvested. The lobes of lungs were separated and metastatic nodules on the surface were counted under a stereomicroscope. Each point represents the data from individual mice and bars represent the mean ± SEM of 2 independent experiments, each performed with 8 mice. Statistical analysis was performed using unpaired t-test.
Figure 3.7: Levels of CCX-CKR ligands in the CCX-CKR overexpressing 4T1.2 tumour microenvironment. The tumours harvested from mice were minced and homogenised in PBS. The homogenate supernatant was tested to measure the levels of A, CCL19, B, CCL21 and C, CCL25 by sequential ELISA. Concentrations of chemokines were normalised to each tumour weight. Each point represents the data from individual mice and bars represent the mean ±SEM of 10 mice per group. Statistical analysis was performed using unpaired t-test.
3.5 The effect of CCX-CKR overexpression on host anti-tumour immune response

The data above indicate that overexpression of CCX-CKR inhibits orthotopic tumour growth but enhances metastasis, however this effect was not associated with demonstrable chemokine scavenging in vivo. Therefore, the next aim was to explore alternative mechanisms whereby overexpression of CCX-CKR inhibited primary tumour growth and enhanced metastasis. Since one of the well-characterised functions of chemokine/chemokine receptors is regulation and activation of the immune response, the effect of CCX-CKR overexpression on anti-tumour immune responses was examined.

To determine whether the immune system played a role in CCX-CKR-mediated inhibition of tumour growth, tumour microenvironment of 4TpEF and 4TCCX was examined. First, the levels of cytokines important in three major arms of inflammatory response, type 1, 2 and 17 were analysed by ELISA. As shown in Figure 3.8, no differences in levels of cytokines, IFN-γ, IL-4 or IL-17, in tumour microenvironment of 4TpEF and 4TCCX were observed. Next, tumour cell suspensions were labelled for various leukocyte markers to measure the level of tumour infiltration by leukocytes. Similar frequencies of CD45$^+$ cells were observed in both 4TpEF and 4TCCX tumours (Figure 3.9 A and B). Furthermore, when this leukocyte population was dissected into different types of lymphocytes; B cells, T cells, NK and NKT cells and myeloid cells; myeloid-derived suppressor cells (MDSCs), dendritic cells and macrophages, there was no significant difference between the two groups in these populations (Figure 3.9 C and D).

In addition, to confirm the observation above from different perspective, the growth of CCX-CKR overexpressing tumours and control tumours in severe combined immunodeficiency (SCID) mice was monitored. The inhibitory effect of CCX-CKR overexpression on orthotopic tumour growth was still evident in these mice which lack functional T cells and B cells (Figure 3.10). These data indicate that T and B cells do not
Figure 3.8: Levels of inflammatory cytokines in the CCX-CKR overexpressing 4T1.2 tumour microenvironment. The tumours harvested from mice were minced and homogenised in PBS. The homogenate supernatant was tested to measure the levels of IFN-γ, IL-4, and IL-17 by sequential ELISA. Concentrations of chemokines were normalised to each tumour weight. Each point represents the data from individual mice and bars represent the mean ±SEM of 4 mice per group. Statistical analysis was performed using unpaired t-test.
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Figure 3.9: Levels of tumour infiltrating leukocytes in CCX-CKR overexpressing 4T1.2 tumours. The tumours harvested from mice were minced and digested using collagenase IA cocktail. Erythrocytes were removed from the single cell suspension before Fcγ receptors were blocked and the cells were labelled for combinations of CD3 and B220, CD4 and CD8, Gr-1 and CD11b, CD3 and NK1.1, IA/IE and F4/80, IA/IE and CD11c or CD45 alone. A, percentage of tumour homogenate cells that were CD45+. B, number of CD45+ cells per milligram of tumour tissue. C, percentage of tumour homogenate cells that were positive for indicated markers. D, number of cells that were positive for indicated markers per milligram of tumour tissue. Each point represents the data from individual mice and bars represent the mean ±SEM of 9 mice per group. Statistical analysis was performed using unpaired t-test.
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A

% of CD45<sup>+</sup> cells in tumours

4TpEF  4TCCX

B

Number of CD45<sup>+</sup> cells/mg of tumour

4TpEF  4TCCX

C

% of leukocytes in tumours

B220<sup>+</sup>  CD3<sup>+</sup>  CD4<sup>+</sup>  CD8<sup>+</sup>  NK1.1<sup>+</sup>  CD4<sup>+</sup>CD3<sup>+</sup>  Gr<sup>+</sup>CD11b<sup>+</sup>  CD11c<sup>+</sup>MHCII<sup>+</sup>  F4/80<sup>+</sup>MHCII<sup>+</sup>

4TpEF  4TCCX

D

Number of leukocytes/mg of tumour

B220<sup>+</sup>  CD3<sup>+</sup>  CD4<sup>+</sup>  CD8<sup>+</sup>  NK1.1<sup>+</sup>CD3<sup>+</sup>  Gr<sup>+</sup>CD11b<sup>+</sup>  CD11c<sup>+</sup>MHCII<sup>+</sup>  F4/80<sup>+</sup>MHCII<sup>+</sup>

4TpEF  4TCCX
essentially contribute to CCX-CKR-mediated inhibition of tumour growth. Together, these data suggest that it is unlikely that inhibition of primary 4T1CCX tumour growth is mediated through changes in chemokine ligand levels in the tumour microenvironment or altered anti-tumour immunity. This suggest that overexpression of CCX-CKR may have effects on other intrinsic characteristics of 4T1.2 cells, although it still not clear how CCX-CKR overexpression mediates inhibition of tumour growth. Given the observation that overexpression of CCX-CKR aggravated spontaneous and haematogenous metastasis, it is possible that the overexpression of CCX-CKR renders the 4T1.2 cells intrinsically more malignant, i.e. more motile and invasive, which may cause rapid shedding of cells from the primary tumours resulting in smaller primary tumours with extensive metastatic nodules. Therefore, the effect of CCX-CKR overexpression on these aspects of tumour cell biology of 4T1.2 cells was next explored.

3.6 The effect of CCX-CKR overexpression on malignancy of 4T1.2 cells

As previously discussed, tumour progression and metastasis is a complex, multi-step process involving various factors and cellular responses. The possibility of any novel direct involvement of CCX-CKR in tumour cell biology is yet to be demonstrated in the literature, thus further investigation was conducted to determine whether overexpression of CCX-CKR affects cellular characteristics and responses involved in different stages of tumour progression.

3.6.1 Intravasation of CCX-CKR overexpressing 4T1.2 cells

The process of spontaneous metastasis first involves detachment of cells from the primary tumour which then invade through the ECM and intravasate through endothelial cells into circulation. Once in the circulation the cells disseminate until they become arrested in
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Figure 3.10: Growth of CCX-CKR overexpressing 4T1.2 tumours in SCID mice. A, growth curve of orthotopic tumours. Female SCID mice aged between 6 and 8 weeks were injected with $1 \times 10^5$ cells into the mammary fat pad. The perpendicular diameters of each tumour were measured every 2 days from day 7 post-injection. Tumour sizes are presented as multiple of shortest and longest diameters. B, final tumour weight. When one of the tumour diameters was reached 15 mm, mice were sacrificed and tumours were harvested and weighed. Data points (A) and bars (B) represent the mean ±SEM of 10 mice per group. Each point (B) represents the data from individual mice. Statistical analysis was performed using two-way ANOVA for A and unpaired t-test for B.
capillary beds of secondary site where they extravasate into the secondary tissue to form new tumours. The in vivo data above (Figure 3.5C) imply that overexpression of CCX-CKR may influence one or more of these steps in the initial process of metastasis. The haematogenous metastasis experiments (Figure 3.6) indicated that CCX-CKR also plays a role in later steps of metastasis. Therefore, in order to examine whether overexpression of CCX-CKR influenced the initial steps of metastasis, the ability of 4TCCX tumour cells to escape from the primary tumour and to intravasate into the circulation was assessed in vivo. The growth of orthotopic tumours was monitored as previously and on days 14, 21 and 28, blood of tumour-bearing mice was collected from their right atrium and the circulating tumour cell frequency was assessed by enumerating puromycin-resistant colonies grown in culture. On days 14 and 21 no significant differences between the two groups were apparent. However, on day 28 a significantly higher number of tumour cells were recovered from the blood of 4TCCX tumour-bearing mice compared with 4TpEF tumour-bearing mice (Figure 3.11). However, it should be noted that although highly unlikely, it is possible that the cells recovered from the blood on day 28 post tumour-injection originated from the metastatic deposits from the lung. Nevertheless, this data imply that overexpression of CCX-CKR plays a role in one or more of the initial steps of tumour metastasis including 1) detachment from the primary tumour, 2) invasion, 3) migration and/or 4) survival in the blood stream. Therefore, the effect of CCX-CKR overexpression on these aspects of cellular behaviour was examined in vitro.

3.6.2 Adhesion, invasion and migration of CCX-CKR overexpressing 4T1.2 cells

First, to examine whether CCX-CKR overexpression has any effect on the ability of the cells to detach from primary tumour, ECM adhesion and cell-cell adhesion of 4T1.2 cells were examined. Cells were added to wells coated with different concentrations of Matrigel which mimics the ECM environment. As shown in Figure 3.12,
Figure 3.11: Intravasation and survival of CCX-CKR overexpressing 4T1.2 tumour cells in blood circulation. Female Balb/c mice aged between 6 and 8 weeks were injected with $1 \times 10^5$ cells into the mammary fat pad. On days 14, 21 and 28 mice were sacrificed and blood was collected from right atrium into heparin treated tubes. Erythrocytes were removed from each sample and the remaining cells were plated into 6-well trays and cultured for 7 days in the presence of puromycin. Colonies formed in the wells were visualised by 0.06% Giemsa staining and counted. Each point represents the data from individual mice and bars represent the mean ±SEM of 2 independent experiments, each performed with 3 mice per group per time point. Statistical analysis was performed using unpaired t-test.
**Figure 3.12:** ECM adhesion of CCX-CKR overexpressing 4T1.2 cells in vitro. Cells were stained with Calcein-AM and prepared at $2 \times 10^5$ cells/ml in serum-free αMEM. The wells of 96-well tray were coated with either 0.5 mg/ml or 0.1 mg/ml Matrigel and then blocked with 1% BSA before 100 μl of cells were added to each well. After a 30-minute incubation at 37°C, the tray was scanned before and after unbound cells were washed away. The fluorescent intensity of wells coated only with BSA was subtracted from appropriate wells as background. Percentage of adhesion was calculated as (fluorescent intensity after washes/fluorescent intensity before washes) x 100. Bars represent the mean ±SEM of 2 independent experiments, each performed in triplicate. Statistical analysis was performed using unpaired t-test.
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CCX-CKR-overexpressing cells were less able to adhere to Matrigel. Furthermore, when cells of a given cell line were added to wells coated with a monolayer of the same cell line, CCX-CKR-overexpressing 4T1.2 cells were less capable of homotypic adhesion compared with control cells (Figure 3.13). Next, the ability of 4T1.2 cells to invade through tissue was tested using Matrigel. Cells were seeded within the Matrigel layer and assayed for a response to mouse serum harvested from 4T1.2 tumour-bearing mice (TBMS). After 24 hours, significantly higher numbers of CCX-CKR-overexpressing cells had invaded through Matrigel compared with control cells (Figure 3.14). Furthermore, when motility of the cells was compared in blind well chambers, a significantly higher number of CCX-CKR-overexpressing cells than control cells migrated through the pores of polycarbonate membranes to the lower chambers containing TMBS (Figure 3.15A and B). Together, these data indicated that overexpression of CCX-CKR inhibits 4T1.2 cell adhesion to the ECM and to each other, while increasing their invasion and motility.

3.6.3 Resistance of CCX-CKR overexpressing 4T1.2 cells to anoikis

The above data show that overexpression of CCX-CKR results in a reduced ability of 4T1.2 cells to attach to the ECM and to neighbouring cells as well as in increasing invasion through the ECM and motility. The next key step in the process of metastasis is dissemination through the circulation. As discussed earlier, tumour cells often acquire resistance to anoikis, detachment-induced apoptosis, which enables them to survive in circulation after losing attachment to ECM. Therefore, whether the overexpression of CCX-CKR had an effect on anoikis of 4T1.2 cells was determined by measuring survival of these cells in suspension. Two approaches were taken to examine the effect of CCX-CKR overexpression on the ability of the cells to resist anoikis: Western blot analysis was performed to detect pro-apoptotic proteins PARP and Caspase-3 and flow cytometric analysis of propidium iodide (PI) stained cells where small fragmented DNA had been
Figure 3.1: Homotypic adhesion of CCX-CKR overexpressing 4T1.2 cells in vitro. Cells were prepared at $3 \times 10^5$ cells/ml in complete αMEM and 100 μl was added to 96-well tray and cultured overnight to form a monolayer. On the following day, culture medium was replaced with 1% BSA to block wells and then 100 μl of freshly harvested cells, either at $1 \times 10^5$ or $2 \times 10^5$ cells/ml, stained with Calcein-AM were added. After a 90-minute incubation at 37°C, the tray was scanned before and after bound cells were fixed with 4% formaldehyde solution and unbound cells were washed away. The fluorescent intensity of wells coated only with BSA was subtracted from appropriate wells as background. Percentage of adhesion was calculated as ($\text{fluorescent intensity after washes/fluorescent intensity before washes}$) x 100. Bars represent the mean ±SEM of 2 independent experiments, each performed in triplicate. Statistical analysis was performed using unpaired t-test.
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Figure 3.14: Invasion through ECM by CCX-CKR overexpressing 4T1.2 cells in vitro. Cells were prepared at 2 x 10^6 cells/ml in αMEM containing 0.1% BSA and combined with Matrigel at 1:1 ratio. The upper side of transwell membrane was coated with cell:Matrigel mixture. The lower chambers were filled with αMEM with or without 1% TBMS and the trays were incubated for 24 hours at 37°C. Number of cells invaded through the transwell membrane was counted under microscope and the number of cells in no TBMS controls was subtracted from each count as a background. The percentage of invaded cells was estimated as (no. of cells invaded/no. of cells added) x 100. Bars represent the mean ±SEM of 3 independent experiments, each performed at least in duplicate. Statistical analysis was performed using unpaired t-test.
Figure 3.15: Migration of CCX-CKR overexpressing 4T1.2 cells in vitro. The lower chamber of blind well was filled with αMEM with or without 1% TBMS. Cells were prepared at 1 x 10^6 cells/ml in αMEM containing 0.1% BSA and added to the upper chamber. Blind wells were incubated for 6 hours at 37°C, and then cells migrated through the polycarbonate membrane were transferred to 12-well trays and cultured until colonies formed. Number of colonies was counted under microscope and the number of cells in no TBMS controls was subtracted from each count as a background. The percentage of migrated cells was estimated as (no. of cells migrated/no. of cells added) x 100. A, images of colonies grown in wells of 12-well tray. B, percentage of cells migrated. Bars represent the mean ±SEM of 3 independent experiments, each performed in triplicate. Statistical analysis was performed using unpaired t-test.
extracted. In both cases cells were cultured in wells coated with a hydrogel layer to prevent the cells from adhering, thereby creating anoikis-inducing conditions. The pro-apoptotic protein poly (ADP-ribose) polymerase (PARP) is a DNA repair protein which is cleaved in apoptotic cells. Another pro-apoptotic protein Caspase-3 is a key molecule involved in Caspase cascade activated in apoptotic cells. The presence of cleaved form of these proteins indicates that the observed changes in cell death are due to apoptotic processes and not necrotic processes. Overexpression of CCX-CKR led to inhibition of apoptosis regardless of whether the cells were cultured under adherent conditions or prevented from adhering in the absence of serum (Figure 3.16). Thus, it was not clear from these data whether CCX-CKR overexpression specifically inhibits anoikis. When apoptosis of these cells was assessed by PI staining, the levels of specific anoikis were plotted by subtracting the percentage apoptosis in adherent cells from suspension cells. These data indicate that overexpression of CCX-CKR inhibits anoikis both in the presence and absence of serum (Figure 3.17). Together, this demonstrates that overexpression of CCX-CKR inhibits both apoptosis in general and increases resistance to anoikis.

3.6.4 The effect of CCX-CKR overexpression on EMT of 4T1.2 cells

The in vitro characteristics displayed by CCX-CKR overexpressing 4T1.2 cells revealed above are typical features of tumour cells which have undergone EMT, including loss of adhesion, enhanced motility and invasiveness through ECM and increased resistance to anoikis. Previous studies have shown that parental 4T1.2 cells have undergone EMT to a greater extent than other less malignant mammary carcinoma cell lines. However, it was also shown that EMT can be manifested to different degrees in different cell lines. Therefore, in order to assess whether overexpression of CCX-CKR has accelerated EMT in 4T1.2 cells, changes in expression of EMT markers on these cells were examined.

Expression of E-cadherin, an important molecule involved in homotypic adhesion which is
Figure 3.16: Resistance to anoikis by CCX-CKR overexpressing 4T1.2 cells in vitro measured by western blot for pro-apoptotic proteins. Cells were prepared at 1 x 10^5 cells/ml and 2ml of cells were cultured in either Ultra-low attachment or standard tissue culture 6-well trays with or without 5% FCS. After a 24-hour incubation at 37°C, cells were harvested and lysed. Lysates were analysed by Western Blot for the presence of pro-apoptotic full length PARP, apoptotic cleaved PARP (top panel), as well as inactive and active forms of Caspase-3 (middle panel) to measure the levels of apoptosis. The blot was reprobed with anti-β-actin as a loading control (bottom panel). Image is a representative blot of three independent experiments. This experiment was conducted in collaboration with Dr. Julie Brazzatti.
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Figure 3.17: Resistance to anoikis by CCX-CKR overexpressing 4T1.2 cells in vitro measured by DNA extraction and PI staining. A, levels of anoikis in 4T1.2 cells cultured in different conditions. Cells were prepared at $1 \times 10^5$ cells/ml and 2 ml of cells were cultured in either Ultra-low attachment or standard tissue culture 6-well trays with or without 5% FCS. After a 24-hour incubation at 37°C, cells were harvested and fixed in 70% ethanol before the small fragmented DNA was extracted. The cells were stained for the remaining DNA with PI and the stained cells were subjected to flow cytometric analysis where the population with low PI level was gated as apoptotic. The percentage of apoptotic cells in adherent controls was subtracted from the percentage of apoptotic cells in suspension. Bars represent the mean ±SEM of 2 independent experiments, each performed in duplicate. B, representative histograms for PI staining. Statistical analysis was performed using unpaired t-test.
expressed highly in normal epithelial cells, was found to be significantly reduced in CCX-CKR-overexpressing cells at both mRNA and protein levels (Figure 3.18A). This was consistent with the earlier observation that homotypic adhesion of these cells was decreased compared with control cells. Furthermore, other markers expressed at low levels in normal epithelial cells including fibronectin and vimentin were increased in CCX-CKR-overexpressing cells compared with control cells (Figure 3.18B). However, while CCX-CKR-overexpressing cells demonstrated a clear trend towards an increased level of N-cadherin expression compared with control cells, this did not reach statistical significance (Figure 3.18B). Together these data suggest that overexpression of CCX-CKR enhances malignancy by accelerating the EMT of 4T1.2 cells.

3.7 Summary

To elucidate the role of CCX-CKR in cancer progression, the effect of CCX-CKR overexpression on tumour growth and metastasis in 4T1.2 mammary carcinoma model was investigated. Characterisation of the 4T1.2 model indicated that the signalling receptors relevant to CCX-CKR were expressed while there was no detectable CCX-CKR, making this a suitable model for studying the effect of overexpression of this receptor. Transfection of 4T1.2 cells with mouse CCX-CKR led to increased expression of CCX-CKR in stable transfectants which inhibited orthotopic tumour growth while having no effect on *in vitro* growth. However, experiments performed during the course of this study indicated that this effect is not associated with chemokine scavenging *in vivo* and was not attributed to host adaptive immunity. In order to fully elucidate the mechanism of action of CCX-CKR in 4T1.2 model, further experimentation is required to explore possible functions of CCX-CKR yet to be demonstrated previously, which will be discussed in detail in chapter 5.
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Figure 3.18: Expression of epithelial-mesenchymal transition (EMT) markers by CCX-CKR overexpressing 4T1.2 cells. A, expression of E-cadherin at protein (left) and mRNA (right) levels. Lysates were prepared from 4TpEF and 4TCCX cells and analysed by Western blot (performed by Dr. Julie Brazzatti) for the presence of E-cadherin (top panel). The blot was reprobed with anti-β-actin as a loading control (bottom panel). Image is a representative blot of three independent experiments. Total RNA was also extracted from 4TpEF and 4TCCX cells was reverse-transcribed to generate cDNA, which was then subjected to qPCR analysis using primers specific for E-cadherin and GAPDH. The mRNA level of the target gene was calculated as a ratio to GAPDH. B, expression of other EMT markers at mRNA level. The cDNA prepared above was also used to detect vimentin, fibronectin, N-cadherin in qPCR analysis. Bars represent the mean ±SEM of one experiment performed in triplicate. Statistical analysis was performed using unpaired t-test.
A key observation was that overexpression of CCX-CKR enhanced spontaneous metastasis as well as haematogenous metastasis in vivo. In vitro characterisation of tumourigenicity of the cells revealed that overexpression of CCX-CKR rendered them more invasive, less adherent to ECM and to each other and more resistant to anoikis. These are established characteristics of cells undergone EMT\textsuperscript{34} and, indeed, CCX-CKR overexpressing cells showed typical expression pattern of EMT markers. Although the precise molecular mechanisms underlying the role of CCX-CKR in rendering 4T1.2 cells more malignant is still to be elucidated, the data presented in this chapter imply possible novel functions of CCX-CKR in tumour progression.
CHAPTER 4: THE EFFECT OF DELETION AND KNOCKDOWN OF CCX-CKR ON PROGRESSION OF MELANOMA
CHAPTER 4: THE EFFECT OF DELETION AND KNOCKDOWN OF CCX-CKR ON PROGRESSION OF MELANOMA

4.1 Introduction

The data presented in the previous chapter strongly indicate possible novel functions for CCX-CKR in tumour biology which may be independent of its known function as chemokine scavenger. However, those experiments determined the effect of exogenous expression of CCX-CKR on tumour progression and metastasis, and the role of endogenously expressed CCX-CKR in tumour biology is yet to be determined. To address this, two broad approaches were taken. First, role of CCX-CKR expressed by the host during tumour progression was examined using CCX-CKR\(^{-/-}\) mice. Second, the effect of CCX-CKR expression knockdown in tumour cells themselves on tumour growth and metastasis was also examined using short-hairpin RNA (shRNA) targeting CCX-CKR mRNA. These experiments could not be conducted in the 4T1.2 model syngeneic to Balb/c, as these cells express low level of CCX-CKR (near the limit of detection; Figure 3.1A) and CCX-CKR\(^{-/-}\) mice were only available on the C57Bl/6 background.

4.2 The effect of CCX-CKR deletion in the host on progression of mammary carcinoma and melanoma

In order to investigate the role of CCX-CKR \textit{in vivo} during tumour progression, CCX-CKR\(^{-/-}\) mice were utilised. These CCX-CKR\(^{-/-}\) mice had been backcrossed to the C57Bl/6 background for over 10 generations and had been confirmed to be syngeneic to C57Bl/6 strain using a genome-wide single nucleotide polymorphism (SNP) analysis (Prof. Alan Baxter, James Cook University, personal communication) eliminating the possibility
of minor antigens differing between wt and knockout mice. Two murine tumour models that are syngeneic to the C57Bl/6 strain were chosen to study the function of CCX-CKR in tumour biology: the E0771 mammary carcinoma model and the B16 melanoma model. Prior to initiating these studies, CCX-CKR<sup>+/−</sup> mice were analysed for the levels of CCX-CKR ligands in relevant tissues: the mammary fat pads, skin and lungs in order to determine whether CCX-CKR controlled chemokine bioavailability at these sites. ELISA analysis revealed that levels of bioavailable CCL21 were significantly elevated in all of tissues examined from CCX-CKR<sup>+/−</sup> mice compared with wt mice (Figure 4.1A, B and C). There was no significant difference between wt and CCX-CKR<sup>+/−</sup> mice in the levels of CCL19 in mammary fat pads or levels of CCL25 in lungs (Figure 4.1A and C). CCL25 in mammary fat pads and skin, and CCL19 in skin and lung were below the limit of detection (data not shown).

These alterations in CCX-CKR ligands at sites of primary tumour growth and metastasis in CCX-CKR<sup>+/−</sup> mice strongly suggest that deletion of CCX-CKR in the host could have a significant effect on tumour growth and metastasis. Therefore, the effect of CCX-CKR deletion in the host on the growth of mammary tumours and melanoma was examined. E0771 mammary carcinoma cells were injected into the mammary fat pads of wt or CCX-CKR<sup>+/−</sup> female mice and tumour growth was monitored. As shown in Figure 4.2A, the growth of E0771 tumours in CCX-CKR<sup>+/−</sup> mice was significantly inhibited compared with these same tumours grown in wt mice. A similar effect was observed with B16 melanoma cells where growth of subcutaneously injected B16 melanomas was inhibited in CCX-CKR<sup>+/−</sup> mice compared with wt mice (Figure 4.2B).

Unlike the 4T1.2 model, E0771 and B16 tumours do not consistently undergo spontaneous metastasis to specific tissues. Therefore, the effect of CCX-CKR deletion on lung metastasis by E0771 and B16 cells was examined using the haematogenous metastasis
Figure 4.1: Levels of CCX-CKR ligands in various tissues of CCX-CKR−/− mice. A, levels of ligands in mammary fat pads. 4th mammary fat pads were harvested from female mice and homogenised in PBS containing glycerol and protease inhibitor. B, levels of bioavailable ligands in skin. Strips of abdominal skin were immersed in PBS containing protease inhibitor for 3 hours at 4°C. C, levels of ligands in lungs. Lungs harvested from mice were minced and homogenised in PBS containing protease inhibitor. For all tissues, the supernatants were assayed to measure the levels of CCL19, CCL21 and CCL25 by sequential ELISA. Concentrations of chemokines were normalised to each tissue weight. Each point represents the data from individual mice and bars represent the mean ±SEM of 8 mice per group for A, and 4 mice per group for B and C. Statistical analysis was performed using unpaired t-test.
Figure 4.2: Growth of E0771 mammary tumours and B16 melanoma in syngeneic C57Bl/6 CCX-CKR<sup>−/−</sup> mice. A, growth curve (left) and final tumour weight (right) of E0771 orthotopic tumours. Female mice aged between 6 and 10 weeks were injected with 1 x 10<sup>5</sup> cells into the mammary fat pad. B, growth curve (left) and final tumour weight (right) of B16 subcutaneous tumours. Male mice aged between 6 and 10 weeks were injected subcutaneously with 2 x 10<sup>5</sup> cells into the left flank. For both models, the perpendicular diameters of each tumour was measured every 2 days from day 7 post-injection. Tumour sizes are presented as multiple of shortest and longest diameters. When one of tumour diameters was reached 15mm, mice were sacrificed and tumours were harvested and weighed. Data points (left) and bars (right) represent the mean ± SEM of 2 independent experiments, each performed with at least 6 mice per group (E0771) or of 3 independent experiments, each performed with at least 6 mice per group (B16). Each point (right) represents the data from individual mice. Statistical analysis was performed using two-way ANOVA for left panels and unpaired t-test for right panels.
model. For both tumour models, colonisation of lungs following intravenous administration was significantly inhibited in CCX-CKR\(^{-/-}\) mice compared with wt mice when lungs were examined on day 14 post-injection (Figure 4.3A and B). Together, these data clearly indicate that CCX-CKR expressed in the host plays a substantial role in promoting the progression and metastasis of murine tumours.

In order to examine whether deletion of host CCX-CKR alter the levels of CCX-CKR ligands in the tumour microenvironment, tumour homogenate supernatants were analysed by ELISA. Significantly higher levels of CCL21 were detected in both E0771 and B16 tumours grown in CCX-CKR\(^{-/-}\) than in wt mice (Figure 4.4). In E0771 tumours the levels of CCL19 was below the limit of detection in both wt and CCX-CKR\(^{-/-}\) mice, and there was a statistically non-significant trend towards elevated levels of CCL25 in tumours from CCX-CKR\(^{-/-}\) compared with wt mice (Figure 4.4A). In B16 tumours, significantly higher levels of CCL25 were detected in tumours from CCX-CKR\(^{-/-}\) mice, while there was no significant difference in the levels of CCL19 (Figure 4.4B).

These data clearly indicated that the absence of host CCX-CKR significantly inhibits tumour growth and metastasis and this effect is associated with the levels of CCX-CKR ligands in tumours as well as naïve tissues. This implies that CCX-CKR may function as chemokine scavenger in vivo which leads to inhibition of tumour growth and metastasis in E0771 and B16 models. Investigation to reveal the precise mechanism by which tumour growth and metastasis are inhibited in CCX-CKR\(^{-/-}\) mice was not pursued further during the course of this study. This was due to a remarkable observation made through another investigation conducted concurrently and the focus of the study was shifted to examine the role of endogenous CCX-CKR on tumour cells themselves as described below.
Figure 4.3: Lung colonisation of E0771 and B16 cells in C57Bl/6 CCX-CKR<sup>−</sup> mice via haematogenous route. Mice aged between 6 and 8 weeks were injected with either A, 1 x 10<sup>5</sup> of E0771 or B, 2 x 10<sup>5</sup> of B16 cells intravenously into the tail vein. After 2 weeks mice were sacrificed and lungs were harvested. The lobes of lungs were separated and metastatic nodules on the surface were counted under a stereomicroscope. Each point represents the data from individual mice and bars represent the mean ± SEM of 8 mice (E0771) or of 2 independent experiments, each performed with at least 9 mice per group (B16). Statistical analysis was performed using unpaired t-test.
Figure 4.4: Levels of CCX-CKR ligands in E0771 and B16 tumour microenvironment in CCX-CKR−/− mice. The tumours harvested from mice were minced and homogenised in PBS containing protease inhibitor. The homogenate supernatants were tested to measure the levels of CCL19, CCL21 and CCL25 by sequential ELISA. Concentrations of chemokines were normalised to each tumour weight. A, CCL21 and, CCL25 in E0771 tumours. B, CCL19, CCL21 and CCL25 in B16 tumours. Each point represents the data from individual mice and bars represent the mean ±SEM of 8 mice per group (E0771) or of 6 mice per group (B16). Statistical analysis was performed using unpaired t-test.
4.3 Generation and characterisation of CCX-CKR knockdown B16 cell lines

While the investigation of the role of CCX-CKR in tumour progression using CCX-CKR−/− mice provided valuable observations, it was also important to examine whether CCX-CKR endogenously expressed by tumour cells themselves played a role in tumour progression. To begin addressing this question, E0771 mammary carcinoma cell line and B16 melanoma cell line were screened for endogenous expression of CCX-CKR by qPCR. The data showed that CCX-CKR mRNA was expressed at higher level in B16 cells than E0771 cells (Figure 4.5). Note that the breast cancer cell lines 4T1.2 and EMT6.5 (both syngeneic to Balb/c) were negative for CCX-CKR expression (data not shown). Therefore, the B16 melanoma model was selected for further investigation to determine the effect of CCX-CKR knockdown on tumour progression and metastasis.

In order to knockdown expression of CCX-CKR in B16 cells, RNA interference (RNAi) system was utilised. This involved production of lentivirus encoding short-hairpin RNA (shRNA) targeting different sequences within CCX-CKR mRNA. B16 cells were transduced with lentiviruses encoding shRNAs targeting CCX-CKR or an off-target shRNA targeting an irrelevant GFP sequence as a control. Cells were selected for stable transductants with puromycin for 2 weeks before being subjected to experimentation and maintained in the presence of puromycin at all times during this study. The transduced cells were first tested for the expression level of CCX-CKR. qPCR data indicated that cells transduced with CCX-CKR shRNA constructs #6 and #7 (B16CCXkd 6 and B16CCXkd 7) expressed a significantly lower level of CCX-CKR compared with control GFP shRNA transduced cells (B16CTLkd) and wt B16 cells (Figure 4.6A). However, CCX-CKR expression in cells transduced with the shRNA constructs #5 and #8 was not significantly different from B16CTLkd (Figure 4.6A). In addition, the transduced B16 cells were also labelled with a mouse mAb raised against human CCX-CKR. This antibody (2F11) was
Figure 4.5: Endogenous expression of CCX-CKR by mouse cancer cell lines. Total RNA extracted from each cell line was reverse-transcribed to generate cDNA, which was then subjected to qPCR analysis using primers specific for CCX-CKR and RPLP0. The mRNA level of the target gene was calculated as a ratio to RPLP0. Bars represent the mean ±SEM of 2 independent experiments, each performed in duplicate.
Figure 4.6: Expression of CCX-CKR by transduced B16 cell lines. A, expression of CCX-CKR mRNA detected by qPCR. Total RNA extracted from transduced cells was reverse-transcribed to generate cDNA, which was then subjected to qPCR analysis using primers specific for CCX-CKR and GAPDH. The mRNA level of the target gene was calculated as a ratio to GAPDH. Bars represent the mean ±SEM of 2 independent experiments, each performed in duplicate. B, surface expression of CCX-CKR detected by flow cytometry. B16 cells were fixed with 4% formaldehyde in PBS, then labelled with either mouse anti-hCCX-CKR antibody or isotype matched control antibody. The purified antibodies were detected using PE-conjugated goat anti-muIgG secondary antibody. Data shown are MFI of cells labelled with specific antibody after the MFI of isotype control was subtracted Bars represent the mean ±SEM of 2 independent experiments, each performed in duplicate. C, representative histograms from B. Statistical analysis was performed using unpaired t-test.
one of the panel of mAb recently developed by Jo Chiba and his colleagues (Tokyo
University of Science, Japan) by DNA immunisation and in vivo electroporation of plasmid
construct encoding human CCX-CKR. These antibodies were generously made
available towards the end of this project. Cross-reactivity of the 2F11 antibody with mouse
CCX-CKR was demonstrated in our laboratory using HEK293 cells transiently transfected
with a mouse CCX-CKR encoding plasmid and compared with a human CCX-CKR
encoding plasmid (Comerford, unpublished). Labelling of transduced B16 cells with 2F11
further suggested that expression of CCX-CKR in B16CCXkd 6 and 7 was significantly
lower than B16CTLkd confirming the data obtained by qPCR (Figure 4.6B and C).

Before assessing the effect of CCX-CKR knockdown on B16 tumour growth and
metastasis in vivo, the effect of reduced expression of CCX-CKR on the growth properties
of these cells in vitro was tested. This was assessed in two different ways: one where
anchorag-dependent growth of cells was assayed by measuring the reduction of the
tetrazolium salt XTT by metabolically active cells; and secondly, measurements of
anchorag-independent growth were assayed by quantifying colony formation in
semi-solid medium created with Bacto-agar. Data from these experiments revealed no
significant differences in the growth properties of B16CTLkd, B16CCXkd 6 and
B16CCXkd 7 cells (Figure 4.7), indicating that the knockdown of CCX-CKR expression
had no effect on in vitro growth of B16 cells.

4.4 The effect of CCX-CKR knockdown on B16 melanoma progression in vivo

4.4.1 Growth of CCX-CKR knockdown B16 tumours in syngeneic mice

In order to investigate the effect of CCX-CKR knockdown on in vivo growth of melanoma,
CCX-CKR knockdown cells or control cells were injected subcutaneously into syngeneic
Figure 4.7: In vitro growth of transduced B16 cell lines. A, anchorage-dependent growth. Cells were prepared at 1 x 10^3 cells/500 μl/well in 24-well trays and incubated for 7 days. On days 0, 2, 5, and 7, level of metabolically active cells was measured by adding XXT:PMS and the absorbance at 490nm (reference 650nm) was determined. Bars represent the mean ± SEM of 2 independent experiments, each performed in triplicate. B, anchorage-independent growth. The wells of 6-well trays were covered with 0.7% Bacto-agar made up in complete IMDM. The top layer containing 500 cells with 0.3% agar in IMDM was overlaid on the 0.7% agar layer. The tray was incubated for 10 days and the resulting colonies were visualised with 0.005% crystal violet solution and the number of colonies was determined using Quantity One software. Bars represent the mean ± SEM of 3 independent experiments, each performed in triplicate. Statistical analysis was performed using two-way ANOVA for A and unpaired t-test for B.
C57Bl/6 mice. Measurement of the perpendicular diameter of palpable tumours revealed that, initially, CCX-CKR knockdown B16 tumours (6 and 7) were moderately larger than control tumours (Figure 4.8A and B). However, at approximately day 11 the growth of CCX-CKR knockdown tumours plateaued and started to decrease resulting in substantial attenuation of tumour burden compared with control tumours (Figure 4.8A and B). Interestingly, these tumours were morphologically quite distinct from control tumours. The latter appeared black in colour and soft in texture as normally observed in the B16 melanoma model. In contrast, CCX-CKR knockdown tumours appeared opaque white in colour and had a firm texture (Figure 4.8C). Since B16CCXkd 6 and 7 showed similar growth rate and morphological features in vivo, subsequent more detailed investigations were performed using the B16CCXkd 6 cell line only. Analysis of growth of these tumours over extended time period revealed that by approximately 20 days post-tumour injection, mice injected with CCX-CKR knockdown B16 cell were completely free of palpable tumours (Figure 4.8D).

To determine whether CCX-CKR knockdown alters the levels of ligands in the tumour microenvironment, supernatants of tumours harvested on days 10 and 17 post-injection were assayed for CCL19, CCL21 and CCL25. Although there was no significant difference in the levels of CCX-CKR ligands between CCX-CKR knockdown and control tumours harvested on day10, by day 17 the levels of all three ligands were significantly elevated in CCX-CKR knockdown tumours compared with control tumours (Figure 4.9). This indicated that CCX-CKR expressed by B16 may have an in vivo role as chemokine scavenger as previously described.

4.4.2 Metastasis of CCX-CKR knockdown B16 tumours in syngeneic mice

Next, the effect of CCX-CKR knockdown on the ability of B16 cells to colonise lungs upon intravenous injection was assessed. Lungs harvested from mice injected with
Figure 4.8: Growth of CCX-CKR knockdown B16 melanomas in syngeneic C57Bl/6 mice. A, growth curve of subcutaneous tumours. C57Bl/6 mice aged between 6 and 10 weeks were injected subcutaneously with $2 \times 10^5$ cells into the left flank. The perpendicular diameters of each tumour was measured every 2 days from day 7 post-injection. Tumour sizes are presented as multiple of shortest and longest diameters. Data points represent the mean ± SEM of 6 mice per group. B, representative images of tumours harvested at the end point of A. C, growth curve of subcutaneous tumours with later time points (conducted in collaboration with Prof. Mark Smyth). Data points represent the mean ± SEM of 5 mice per group. Statistical analysis was performed using two-way ANOVA.
Figure 4.9: Levels of CCX-CKR ligands in the CCX-CKR knockdown B16 tumour microenvironment. The tumours harvested from mice were minced and homogenised in PBS containing protease inhibitor. The homogenate supernatants were tested to measure the levels of A, CCL19, B, CCL21 and C, CCL25 by sequential ELISA. Concentrations of chemokines were normalised to each tumour weight. Each point represents the data from individual mice and bars represent the mean ±SEM of 6 mice per group (day 10) or of 3 independent experiments each performed with 6 mice per group (day 17). Statistical analysis was performed using unpaired t-test.
CCX-CKR knockdown B16 cells appeared to have large white nodules on the surface while lungs from control B16 cells injected mice displayed the small black nodules usually observed in this model (Figure 4.10). In order to quantify the pulmonary tumour burden, fractions of lung homogenates were cultured in the presence of puromycin and the outgrowing colonies were enumerated. The data revealed an increased tumour burden on day 14 post-injection in the lungs of mice receiving CCX-CKR knockdown B16 cells (Figure 4.11). Interestingly, when survival of mice was monitored after two different doses of B16 cell were administered intravenously, all of the mice receiving control B16 cells were moribund by day 20 (high dose) or day 30 (low dose) post-injection (Figure 4.12A and B). In contrast, all mice injected with CCX-CKR knockdown cells were healthy on day 100 post-injection regardless of the dose of cells (Figure 4.12). Furthermore, post mortem analysis of mice injected with control B16 cells revealed that the lungs displayed extensive B16 growth while lungs from mice receiving CCX-CKR knockdown cells were free of tumour nodules (data not shown, personal communication with Prof. Mark Smyth). These data suggest that knockdown of CCX-CKR enhances initial colonisation of the lungs but that these tumours are unable to effectively continue to grow in the lung.

4.5 The effect of CCX-CKR knockdown on anti-tumour immune responses

4.5.1 The anti-tumour immune responses to B16 subcutaneous tumours

The data above indicate that knockdown of CCX-CKR inhibits melanoma growth and prevents progression of both primary and secondary tumours. Since CCX-CKR knockdown tumours appeared opaque white in colour and morphologically resembled lymph nodes, it was hypothesised that knockdown of CCX-CKR in B16 cells promotes infiltration of anti-tumour leukocytes, thus allowing immune-mediated destruction of melanoma. Therefore, the effect of CCX-CKR knockdown on anti-tumour immune
Figure 4.10 Lung colonisation of CCX-CKR knockdown B16 cells via haematogenous route. C57Bl/6 mice aged between 6 and 8 weeks were injected with $2 \times 10^5$ cells intravenously into the tail vein. On day 14 post-tumour injection mice were sacrificed and lungs were harvested. The lobes of lungs were separated and images were captured using Leica MZ16FA Stereomicroscope. Shown are representative images from 2 independent experiments, each performed with at least 6 mice per group.
Figure 4.1 Metastatic burden on the lungs bearing CCX-CKR knockdown B16 nodules. The lungs harvested from mice injected with B16 cells were minced and digested using collagenase IA cocktail. Erythrocytes were removed from the single cell suspension and 1/50 of cells were cultured in 6-well trays in the presence of puromycin for 4 days. The colonies formed in the wells were fixed and stained with Calcein-AM. The trays were scanned on Typhoon 9400 and the fluorescent intensity of each well was quantified using Quantity One software. Each point represents the data from individual mice and bars represent the mean ± SEM of 6 mice per group with 3 naïve mice. Statistical analysis was performed using unpaired t-test.
Figure 4.12 Survival of mice bearing lung nodules of CCX-CKR knockdown B16 cells. C57Bl/6 mice were injected with either A, $2 \times 10^5$ or B, $5 \times 10^5$ cells intravenously into the tail vein. The clinical state of mice was monitored and mice were culled when they became moribund. Data points represent the proportions of surviving mice during the course of experiment performed with 5 mice per group. Statistical analysis was performed using log-rank test. This experiment was conducted in collaboration with Prof. Mark Smyth.
responses was investigated.

Two approaches were taken to examine leukocyte infiltration into tumours; flow cytometric analysis of tumour homogenates and immunofluorescent (IF) staining of tumour sections. For flow cytometry tumours harvested on days 10 and 17 post-injection were digested and the homogenates were labelled with antibodies to detect CD4$^+$ and CD8$^+$ T cells, NK cell DCs and macrophages. Significantly higher frequencies of all of these leukocyte subsets were apparent in CCX-CKR knockdown B16 tumours on both day 10 and 17 compared with control tumours (Figure 4.13 A and B). Frequencies of infiltrating leukocytes were higher in tumours harvested on day 10 than on day 17 in both control and CCX-CKR knockdown tumours, although the difference between the two groups was greater on day 17 (Figure 4.13). These data were supported by IF staining of tumour sections. The CCX-CKR knockdown tumour sections showed greater staining for CD4, CD8, F4/80, CD11b and CD11c compared with control tumours (Figure 4.14). Together, these data clearly demonstrate that knockdown of CCX-CKR in B16 melanoma cells enhances infiltration of leukocytes into B16 tumours.

Since significant differences were observed in the levels of CCX-CKR ligands in the microenvironment of CCX-CKR knockdown and control tumours, whether these differences affect the frequencies of infiltrating leukocytes expressing the signalling receptors, CCR7 and CCR9 was examined by flow cytometry. Significantly higher frequencies of CCR7-expressing CD4$^+$ and CD8$^+$ T cells, NK cells, DCs and macrophages were found in CCX-CKR knockdown B16 tumours compared with control tumours (Figure 4.15A). Interestingly, CCR9 expression was also detected on these cells as previously demonstrated by few other studies$^{289-292}$, and the frequencies of CCR9$^+$ leukocytes were elevated in CCX-CKR knockdown B16 tumours (Figure 4.15B).

Further analysis of tumour infiltrating leukocytes was carried out in order to determine
Figure 4.13 Levels of tumour infiltrating leukocytes in CCX-CKR knockdown B16 tumours. The subcutaneous tumours harvested on day 10 or 17 post-tumour injection were minced and digested using collagenase IA. Erythrocytes were removed from the single cell suspension before Fcγ receptors were blocked and the cells were labelled for combinations of CD4 and CD8, CD49b and NK1.1, IA/IE and CD11c, or IA/IE and F4/80. A, day10 and B, day17. Each point represents the data from individual mice and bars represent the mean ± SEM of 6 mice per group. Statistical analysis was performed using unpaired t-test.
Figure 4.14: Immunofluorescent staining of CCX-CKR knockdown tumour sections for tumour infiltrating leukocytes. The subcutaneous tumours harvested on day 17 post-tumour injection were embedded into OCT and frozen in liquid nitrogen. The tumour sections were fixed and blocked with 2% normal mouse serum and 2% normal goat serum. The sections were then stained for CD4, CD8, F4/80, CD11b or CD11c. Images were acquired using Leica SP5 spectral scanning confocal microscope and LAS AF software. Shown are representative images from 2 independent experiments, each performed with 3 mice per group. This experiment was conducted in collaboration with Mark Bunting.
Figure 4.15: Levels of tumour infiltrating leukocytes expressing CCR7 or CCR9 in the CCX-CKR knockdown B16 tumours. The subcutaneous tumours harvested on day 17 were minced and digested using collagenase IA cocktail. Erythrocytes were removed from the single cell suspension before Fcγ receptors were blocked. The cells were labelled for CCR7 or CCR9 together with the combinations of CD4 and CD8, CD49b and NK1.1, IA/IE and CD11c or IA/IE and F4/80. A, CCR7 expressing leukocytes and B, CCR9 expressing leukocytes. Each point represents the data from individual mice and bars represent the mean ±SEM of 6 mice per group. Statistical analysis was performed using unpaired t-test.
whether any particular leukocyte populations played significant role in CCX-CKR knockdown-mediated inhibition and rejection of B16 tumours. It is well established that the presence of neoplastic cells triggers activation of cell-mediated immune response involving an increase in the levels of type 1 cytokines, IFN-γ and IL-12, and IFN-γ producing CD8⁺ T cells play an important anti-tumour effector function²⁹,²⁹³. Therefore, the levels of cytokines in the tumour microenvironment were first examined. ELISA analysis of tumour homogenate supernatants revealed that significantly higher levels of IFN-γ were present in CCX-CKR knockdown tumours compared with control tumours at both days 10 and 17 post-injection (Figure 4.16A). Although there was no detectable IL-12 in tumours harvested on day 10 post-injection, CCX-CKR knockdown tumours from day 17 contained elevated IL-12 levels compared with control tumours (Figure 4.16B). Furthermore, another inflammatory cytokine, IL-17, was also found at a higher level in CCX-CKR knockdown tumours harvested on day 17 post-injection compared with control tumours (Figure 4.16C).

Next, the frequency of IFN-γ producing CD8⁺ T cells in tumour infiltrating lymphocytes was assessed. Cells in tumour homogenates were treated with ionomycin/PMA/golgi stop to activate the production of cytokines and to arrest secretion, before antibody labelling of intracellular cytokines and CD8. Flow cytometric analysis showed that in CCX-CKR knockdown tumours a higher proportion of lymphocytes were CD8⁺ T cells producing IFN-γ (Figure 4.17). Another characteristic of type 1 immune response involving inflammatory cytokines IFN-γ and IL-12 is polarisation of macrophages towards proinflammatory M1 as opposed to anti-inflammatory M2 macrophages, as characterised by their expression of CD206 and CD124²⁹⁴-²⁹⁶. Within the macrophage population in CCX-CKR knockdown B16 tumours, an approximately 3 times higher frequency of M1 macrophages (CD206⁻CD124⁻) was present on day 10 which increased to 10 times higher frequency by day 17 compared with M2 macrophages (CD206⁺CD124⁺) (Figure 4.18).
CHAPTER 4: The effect of deletion and knockdown of CCX-CKR on progression of melanoma

Figure 4.16: Levels of inflammatory cytokines in the CCX-CKR knockdown B16 tumour microenvironment. The subcutaneous tumours harvested from mice were minced and digested using collagenase IA. The homogenate supernatant was tested to measure the levels of A, IFN-γ, B, IL-12 and C, IL-17 by sequential ELISA. Concentrations of chemokines were normalised to each tumour weight. Each point represents the data from individual mice and bars represent the mean ±SEM of 6 mice per group. Statistical analysis was performed using unpaired t-test.
Figure 4.17: Levels of IFN-γ producing CD8+ T cells in CCX-CKR knockdown B16 tumours. The subcutaneous tumours harvested on day 10 or 17 post-tumour injection were minced and digested using collagenase IA. Erythrocytes were removed from the single cell suspension. Cells were activated with PMA/ionomycin/Golgi stop for 4 hours before Fcγ receptors were blocked and the cells were labelled for cell surface CD8. The cells were then fixed and permeabilised, and intracellular IFN-γ was detected. A, percentages of IFN-γ+CD8+ cells on day 10 and 17. Each point represents the data from individual mice and bars represent the mean ±SEM of 6 mice per group. B, representative plots for lymphocytes labelled for CD8 and IFN-γ. Statistical analysis was performed using unpaired t-test. This experiment was conducted in collaboration with Dr. Iain Comerford.
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However, in control tumours the ratios of M1 to M2 macrophages were approximately 1 on day 10 which increased to 3 by day 17 (Figure 4.18). The data above indicate an increase in activated leukocyte infiltration that associated with the observed lymph node-like morphology of CCX-CKR knockdown B16 tumours. Two of the ligands for CCX-CKR, CCL19 and CCL21 have been shown to play pivotal roles in initiating the formation of tertiary lymphoid-like structures\textsuperscript{196,297}. As these chemokines are more abundant in CCX-CKR knockdown B16 tumours, it was hypothesised that the elevated levels of these chemokines may lead to ectopic lymphoid neogenesis within tumours. To test this hypothesis, CCX-CKR knockdown tumours were examined by IF labelling ER-TR7, gp38 and PNAd. ER-TR7\textsuperscript{+} reticular fibroblasts are found in secondary lymphoid organs\textsuperscript{298}, gp38 is expressed by lymph node stromal cells\textsuperscript{299} and PNAd is a lymphatic endothelial cell marker\textsuperscript{300}. Analysis of CCX-CKR knockdown tumours showed increased staining for all three markers compared with control tumour sections (Figure 4.19). Overlaying staining for these markers also indicated that these areas contained organised structures in CCX-CKR knockdown tumours while there was no such organisation found in control tumours (Figure 4.19).

4.5.2 Anti-tumour immune responses to secondary B16 tumours in lungs

Since opaque white nodules were also observed on the lungs of mice that received intravenous injection of CCX-CKR knockdown B16 cells and those mice survived for at least 100 days, the effect of CCX-CKR knockdown on immunity to secondary tumours was also examined. Lungs were harvested from mice injected with either CCX-CKR knockdown B16 cells or control cells on day 14 post-injection. The data indicated that significantly higher numbers of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells, NK cells as well as DCs were found in the lungs colonised with CCX-CKR knockdown B16 tumours compared with lungs colonised with control tumours (Figure 4.20). However, there was no difference in
Figure 4.18: Ratio of M1/M2 macrophages in CCX-CKR knockdown B16 tumours. The subcutaneous tumours harvested on day 10 or 17 post-tumour injection were minced and digested using collagenase IA. Erythrocytes were removed from the single cell suspension before Fcγ receptors were blocked and the cells were labelled for CD11b, F4/80, CD206 and CD124. CD11b\(^+\)F4/80\(^+\) cells were pre-gated for analysis of macrophages A, ratios of M1:M2 macrophages on day 10 and 17. Each point represents the data from individual mice and bars represent the mean ±SEM of 6 mice per group. B, representative plots for macrophages labelled for CD206 and CD124. Statistical analysis was performed using unpaired t-test.
Figure 4.19: Immunofluorescent staining of CCX-CKR knockdown tumour sections for lymphoid tissue markers. The subcutaneous tumours harvested on day 17 post-tumour injection were embedded into OCT and frozen in liquid nitrogen. The tumour sections were fixed and blocked with 2% normal mouse serum and 2% normal goat serum. The sections were then stained for reticular fibroblasts (ER-TR7), gp38 and PNAd. Images were acquired using Leica SP5 spectral scanning confocal microscope and LAS AF software. Shown are representative images from 2 independent experiments, each performed with 3 mice per group. This experiment was conducted in collaboration with Mark Bunting.
**Figure 4.20:** Levels of infiltrating leukocytes in the lungs bearing CCX-CKR knockdown B16 tumour nodules. The lungs harvested from mice injected with B16 cells were minced and digested using collagenase IA. Erythrocytes were removed from the single cell suspension before Fcγ receptors were blocked and the cells were labelled for combinations of CD4 and CD8, CD49b and NK1.1, IA/IE and CD11c, or IA/IE and F4/80. Each point represents the data from individual mice and bars represent the mean ± SEM of 6 mice per group and 3 naïve mice. Statistical analysis was performed using unpaired t-test.
the number of macrophages between the lungs bearing CCX-CKR knockdown tumour nodules and control tumour nodules (Figure 4.20). Further analysis of lungs bearing B16 tumour nodules also showed significantly higher levels of CCR7+ and CCR9+ expressing CD4+ and CD8+ T cells, NK cells, DCs and macrophages in the lungs bearing CCX-CKR knockdown tumour nodules compared with control tumour-bearing lungs (Figure 4.21). These data suggest that knockdown of CCX-CKR in B16 cells enhances infiltration of CCR7+ and CCR9+ leukocytes not only to subcutaneous tumours but also to the tissues involved in secondary tumour growth.

4.6 The role of CCR7 and CCL21 in CCX-CKR knockdown–mediated enhancement of anti-tumour immune responses

The data above show unequivocal evidence that when endogenous expression of CCX-CKR in B16 cells is knocked down, primary and secondary tumour growth is inhibited and eventually rejected and this is associated with marked enhancement of inflammatory leukocyte infiltration at the site of tumour growth. However, the mechanism whereby reduced expression of CCX-CKR in B16 cells results in such a striking effect on anti-tumour immunity is not yet clear. Therefore, the next aim of the investigation was to explore the mechanisms underlying how reduced expression of CCX-CKR enhances anti-tumour immune responses.

Firstly, to confirm that the observed effect of CCX-CKR knockdown on B16 tumour growth inhibition and rejection is dependent upon the host immune system, growth of CCX-CKR knockdown and control tumours were monitored in RAG-1−/− mice, lacking mature B and T cells, and RAG-2 x γc−/− mice, lacking functional B, T and NK cells. In both RAG-1−/− and RAG-2 x γc−/− mice growth of CCX-CKR knockdown tumours was significantly enhanced compared with CCX-CKR knockdown tumours in wt mice.
Figure 4.21: Levels of infiltrating leukocytes expressing CCR7 or CCR9 in the lungs bearing CCX-CKR knockdown B16 tumour nodules. The lungs harvested from mice injected with B16 cells were minced and digested using collagenase IA cocktail. Erythrocytes were removed from the single cell suspension before Fcγ receptors were blocked. The cells were labelled for CCR7 or CCR9 together with the combinations of CD4 and CD8, CD49b and NK1.1, IA/IE and CD11c or IA/IE and F4/80. A, CCR7 expressing leukocytes and B, CCR9 expressing leukocytes. Each point represents the data from individual mice and bars represent the mean ±SEM of 6 mice per group and 3 naïve mice. Statistical analysis was performed using unpaired t-test.
indicating that the adaptive immune system is critically involved in the rejection of CCX-CKR knockdown tumours (Figure 4.22). Growth of CCX-CKR knockdown tumours were enhanced to approximately the same extent in RAG-1⁻/⁻ and RAG-2 x γc⁻/⁻ mice indicating that NK cells do not contribute to the rejection of these tumours. Together, these data indicate, first, that T and B lymphocytes, but not NK cells, play critical roles in CCX-CKR knockdown-mediated inhibition and rejection of B16 tumours, and second, because the inhibition of growth of CCX-CKR knockdown tumours was not completely reversed in RAG-1⁻/⁻ and RAG-2 x γc⁻/⁻ mice, that additional factors beyond T and B cells inhibit B16 melanoma growth when CCX-CKR is knocked down.

The data above indicate that knockdown of CCX-CKR in B16 cells results in elevated levels of its ligands, CCL19, CCL21 and CCL25 in microenvironment of in vivo tumours, which was associated with enhanced recruitment of CCR7⁻ and CCR9-expressing leukocytes to sites of tumour growth. In order to demonstrate the functional significance of these observations, the effect of ligand neutralisation and deletion of CCR7 on growth of CCX-CKR knockdown tumours was examined. Note that these experiments could not be conducted on the CCR9/CCL25 axis because of a lack of suitable reagents.

First, the effect of depletion of CCL21 in mice bearing B16 tumours was tested. Neutralising anti-mouse CCL21 previously raised and tested in our laboratory was administered into mice every 7 days from one day before B16 cells were injected subcutaneously. As shown in Figure 4.23, neutralisation of CCL21 significantly inhibited the growth of control tumours but showed no effect on the growth of CCX-CKR knockdown tumours.

The effect of deletion of CCR7 on the growth of CCX-CKR knockdown tumours was then assessed. Consistent with the previous observation by Shields and colleagues that the growth of control B16 tumours in CCR7⁻/⁻ mice was significantly inhibited (Figure 4.24A).
Figure 4.22: *Growth of CCX-CKR knockdown B16 tumours in immunodeficient mice.* RAG-1⁻/⁻, RAG-2xγc⁻/⁻ mice and wt mice were injected subcutaneously with 2 x 10⁵ cells into the left flank. The perpendicular diameters of each tumour were measured every 2 days from day 6 post-injection. Tumour sizes are presented as multiple of shortest and longest diameters. Data points represent the mean ±SEM of 5 mice per group. Statistical analysis was performed using two-way ANOVA. *This experiment was conducted in collaboration with Prof. Mark Smyth.*
Figure 4.23: Growth of CCX-CKR knockdown B16 tumours in CCL21-neutralised mice. C57Bl/6 mice were injected subcutaneously with $2 \times 10^5$ cells into the left flank. These mice also received intraperitoneal injections of either normal rabbit IgG or anti-mouse CCL21 on the day before, day 6 and 13 post-tumour injection. The perpendicular diameters of each tumour were measured every 2 days from day 7 post-injection. Tumour sizes are presented as multiple of shortest and longest diameters. Data points represent the mean ± SEM of at least 6 mice per group. Statistical analysis was performed using two-way ANOVA.
At the same time, the growth of CCX-CKR knockdown tumours in CCR7\(^{-/-}\) mice was significantly enhanced compared with wt mice (Figure 4.24B), and it was comparable to the growth of control tumours in CCR7\(^{-/-}\) mice (Figure 4.24C). Together these data suggest that the observed effect of CCX-CKR knockdown-mediated inhibition and rejection of B16 tumour growth is a host CCR7-dependent effect, although CCL21 does not appear to be essentially involved.

### 4.7 Summary

In order to uncover the functions of CCX-CKR in vivo during cancer progression and metastasis, the effect of CCX-CKR deletion in the mouse host on mammary tumour and melanoma progression and metastasis was initially investigated. The data indicated that deletion of CCX-CKR from the host results in significant inhibition of primary tumour growth as well as lung colonisation upon intravenous administration of tumour cells in both mammary tumour (E0771) and melanoma (B16) models. It was also shown that this effect may be attributed to elevated level of at least one of the ligands, CCL21 in tumours and naïve tissues harvested from CCX-CKR\(^{-/-}\) mice. Further investigations focused on the effect of reduced expression of CCX-CKR in tumour cells on cancer progression and metastasis. The B16 melanoma model was selected on the basis of a high level of endogenous expression of CCX-CKR and these cells were subjected to shRNA-mediated knockdown.

Transduction of B16 cells with lentivirus encoding shRNA targeting CCX-CKR led to significant reduction in CCX-CKR expression in stable transductants which resulted in marked inhibition of subcutaneous tumour growth and tumour rejection. Intravenous administration of CCX-CKR knockdown cells also resulted in rejection of tumours in the lung leading to increased survival of mice. The unusual morphology of CCX-CKR
Figure 4.24: Growth of CCX-CKR knockdown B16 tumours in CCR7\textsuperscript{-/-} mice. A, CCR7\textsuperscript{-/-} and wt mice were injected subcutaneously with 2 x 10\textsuperscript{5} cells into the left flank. The perpendicular diameters of each tumour were measured every 2 days from day 7 post-injection. Tumour sizes are presented as multiple of shortest and longest diameters. B, growth of CCX-CKR knockdown tumours in CCR7\textsuperscript{-/-} mice compared with wt mice. C, growth of CCX-CKR knockdown tumours compared with control tumours in CCR7\textsuperscript{-/-} mice. Data points represent the mean ± SEM of 2 independent experiments, each performed with at least 4 mice per group. Statistical analysis was performed using two-way ANOVA.
knockdown tumours and lung nodules prompted examination of the cellular composition of CCX-CKR knockdown B16 tumours and lungs bearing CCX-CKR knockdown nodules. Flow cytometric analysis of tumour and lung homogenates and IF staining of tumour sections revealed a substantial increase in various tumour infiltrating leukocyte populations. The levels of inflammatory cytokines were also significantly elevated in microenvironment of CCX-CKR knockdown tumours compared with control tumours. IF staining of tumour sections also indicated that ectopic lymphoid neogenesis may be occurring in CCX-CKR knockdown tumours. The importance of the anti-tumour immune responses in CCX-CKR knockdown-mediated inhibition and rejection of tumour growth was confirmed using immunocompromised mice, which indicated that T cells but not NK cells play a significant role in this system.

Further analysis of CCX-CKR knockdown tumours suggested that reduced expression of CCX-CKR in B16 cells results in elevated levels of chemokine ligands in the microenvironment, suggesting an in vivo role as a chemokine scavenger. In addition, this increase in the levels of ligands was strongly associated with the high frequencies of CCR7 and CCR9 expressing leukocyte infiltration into CCX-CKR knockdown tumours and the lungs bearing CCX-CKR knockdown nodules. Consistent with this finding, a reversal of CCX-CKR knockdown-mediated inhibition of tumour growth was observed in CCR7\textsuperscript{+/−} mice, although there was no effect of CCL21 neutralisation on the growth of CCX-CKR knockdown tumours. This indicated that CCX-CKR knockdown-mediated rejection of B16 tumours was at least partially host CCR7-dependent but may not essentially involve CCL21. Although the involvement of other ligands, CCL19 and CCL25, and the other signalling receptor, CCR9 in CCX-CKR knockdown-mediated effect on B16 tumour growth and metastasis is yet to be investigated, there is strong evidence suggesting a role for CCX-CKR as a chemokine scavenger in vivo which regulates the levels of its ligands in tissues. Therefore, knockdown of CCX-CKR expression in tumour cells may disrupt the
balance of chemokines in tumours, resulting in substantial infiltration of anti-tumour immune cells leading to tumour rejection. These results are discussed in more detail in chapter 5.
CHAPTER 5: DISCUSSION
5.1 Introduction

The significance of the chemokine receptors CCR7$^{141,191,193-200}$ and CCR9$^{202,205-208}$, and their ligands, CCL19, CCL21, and CCL25 in various types of cancer including mammary carcinoma and melanoma has been highlighted over the last decade and it has been shown that these chemokine/receptor axes clearly make an important contribution to both tumour growth and metastasis. The atypical chemokine receptor, CCK-CKR is a high-affinity receptor for these chemokines but rather than inducing classical downstream signalling events promoting migration, it instead sequesters and targets its ligands for degradation. Therefore, CCX-CKR has been shown to regulate chemokine bioavailability in vivo$^{279-281,283}$. These observations posed a potentially important question: can local and systemic regulation of chemokines by CCX-CKR influence tumour growth and metastasis in vivo, and ultimately, can targeting of CCX-CKR be an effective cancer therapy? Prior to the onset of this study, no investigations examining the role of CCX-CKR in cancer progression and metastasis had been reported. Therefore, three approaches were taken to investigate whether CCX-CKR plays a role in cancer progression and metastasis: 1) overexpression of CCX-CKR in cancer cells, 2) deletion of CCX-CKR from host tissue, and 3) knockdown of CCX-CKR expression in cancer cells. Each of these approaches provided insights into the complex role of CCX-CKR in cancer progression and together, demonstrated that CCX-CKR can influence various stages of cancer progression.

Interestingly, the data obtained from approaches 1) and 3) revealed contradictory results, where both increased and decreased expression of CCX-CKR on 4T1.2 cells and B16 cells, respectively, led to inhibition of tumour growth. Detailed analysis of tumours in both systems, however, demonstrated that CCX-CKR may display quite distinct functions...
CHAPTER 5: Discussion

depending on the type of expression (exogenous or endogenous) and the type of tumours (mammary carcinoma or melanoma). In 4T1.2 cells where expression of CCX-CKR was forced, CCX-CKR did not appear to function as chemokine scavenger as previously proposed. Instead, overexpression of CCX-CKR led to acceleration of EMT of the 4T1.2 cells, in an as yet to be characterised manner, resulting in enhanced metastasis of 4T1.2 cells. On the other hand, in B16 melanoma cells which endogenously express CCX-CKR, knockdown of its expression by shRNA resulted in increased levels of its ligands in tumour tissue, suggesting that CCX-CKR endogenously expressed in B16 cells may function as chemokine scavenger. The excess chemokines present as a result of the reduced expression of CCX-CKR in B16 tumours was associated with enhanced recruitment of CCR7+ and CCR9+ anti-tumour leukocytes which, in turn, led to enhanced anti-tumour immune response resulting in tumour rejection. The following sections will now discuss the results from these studies.

5.2 Role of exogenously-expressed CCX-CKR on progression and metastasis of cancer

Overexpression of CCX-CKR on the murine mammary carcinoma 4T1.2 cells led to inhibition of primary tumour growth and the hypothesis that this was due to chemokine scavenging via CCX-CKR (i.e. CCX-CKR overexpressed on 4T1.2 cells mops up tumour-promoting chemokines in the microenvironment, thereby inhibiting tumour growth) was tested. However, no differences were observed in the levels of CCX-CKR ligands in tumour samples when CCX-CKR-overexpressing tumours were compared with control tumours harvested on day 28. This may indicate that the effect of CCX-CKR overexpression on tumour progression in this model is independent of its proposed function as chemokine scavenger, and that CCX-CKR on 4T1.2 cells has an alternative
function. However, there are three possible scenarios whereby no difference would be detected in chemokine levels between control and CCX-CKR-overexpressing tumours, even if CCX-CKR is functioning as chemokine scavenger on 4T1.2 cells. First, it is possible that during growth *in vivo*, CCX-CKR expression is reduced or lost over time, which may result in similar levels of CCX-CKR ligands being apparent in both control and CCX-CKR overexpressing 4T1.2 tumours by day 28 post-tumour injection. Second, if a feedback loop exists for production of chemokines, this may have confounded the apparent levels of chemokines in CCX-CKR overexpressing tumours. Third, the endogenous CCX-CKR on 4T1.2 cells, though expressed at very low level, has already reached its maximum functional level of chemokine scavenging and that further expression has no effect on the levels of chemokines in the tumour microenvironment. Nevertheless, further experimentation is required to assess whether any one of the above hypotheses is correct, and from the data obtained during the current study, it is more likely that CCX-CKR overexpressed on 4T1.2 cells does not alter chemokine levels in tissues but it has an alternative function that results in inhibition of tumour growth.

This gives rise to a possibility that CCX-CKR has novel function yet to be characterised. Like other atypical receptors such as DARC, which can interact with tetraspanin CD82, and CCRL2, which can bind chemerin (an adipokine), CCX-CKR may also be able to interact with non-chemokine molecules yet to be identified. If novel ligands for CCX-CKR are of a tumour-promoting nature and CCX-CKR acts as a scavenger for those molecules, this could result in inhibition of growth of CCX-CKR overexpressing tumours (Figure 5.1A). On the other hand, if novel ligands are of a tumour-suppressing nature, the possibility of CCX-CKR signalling leading to suppression of tumour growth cannot be ruled out (Figure 5.1B). If CCX-CKR interacts with a membrane-bound molecule (as in DARC’s interaction with CD82), it is also possible that CCX-CKR influences cells of tumour stroma through their surface protein-protein interactions, such as
Figure 5.1: Proposed mechanisms of CCX-CKR-mediated inhibition of 4T1.2 tumour growth. A, CCX-CKR may be able to scavenge yet to be identified ligands and these novel ligands may be of tumour-promoting nature. Lack of tumour-promoting factors in the microenvironment leads to inhibition of tumour growth. B, CCX-CKR may be able to transduce signals from tumour-inhibiting factors through yet to be characterised pathways. Overexpression of CCX-CKR amplifies such signals resulting in inhibition of tumour growth. C, CCX-CKR may be able to interact with membrane-bound molecules expressed on cells of tumour stroma, such as endothelial cells or leukocytes. Such interactions may lead to inhibition of angiogenesis or activation of innate immune cells, resulting in inhibition of tumour growth.
A Novel soluble ligands – scavenging

- Tumour-promoting factors
- Scavenging of tumour-promoting factors

B Novel soluble ligands – signalling

- No effect of tumour-inhibiting factors
- Signalling of tumour-inhibiting factors

C Interaction with membrane-bound molecules

- Active endothelial cells
  - No activation of NK cells
- Inactivation of endothelial cells
  - Activation of NK cells
inhibiting angiogenesis or activating leukocytes (Figure 5.1C). Although CCX-CKR-mediated inhibition of tumour growth was not abrogated in SCID mice lacking functional T and B cells which indicated that the effect of CCX-CKR was independent of adaptive immunity, the possibility of the involvement of innate immunity still remains. For example, although no differences were observed in the level of NK cell infiltration between control and CCX-CKR-overexpressing tumours, it is possible that CCX-CKR interacts with a membrane-bound molecule on the surface of NK cells. Such an interaction may lead to activation or enhanced cytotoxic activity of NK cells resulting in inhibition of CCX-CKR-overexpressing primary tumour growth.

Interestingly, in addition to inhibiting primary tumour growth, CCX-CKR overexpression on 4T1.2 cells enhanced metastasis of the cells. This is inconsistent with the results of a previous study by Feng and colleagues\(^\text{302}\) which demonstrated that overexpression of CCX-CKR protein in human breast cancer cell lines resulted in inhibition of spontaneous metastasis in athymic mice and that expression of CCX-CKR in human breast cancer negatively-correlated with LN metastasis and positively-correlated with patient survival. However, in that study the effect of CCX-CKR overexpression was examined using a few clones of transfectants which displayed high CCX-CKR expression. Those clones showed impaired proliferation and invasion \textit{in vitro} which suggests that the observed effect on spontaneous metastasis in athymic mice may have been due to clonal artefacts which are often associated with cells derived from single cell clones. Moreover, the demonstrated correlation between CCX-CKR expression and LN metastasis or disease prognosis was examined using an anti-CCX-CKR antibody which is not well-characterised for specificity and has only been used by those authors. Thus, their data and conclusions remain uncertain.

In the present study using a pooled population of transfectants, \textit{in vitro} analysis indicated
the association between overexpression of CCX-CKR and high motility and invasiveness, low adherence, and higher resistance to anoikis in 4T1.2 cells. In line with these malignant characteristics, CCX-CKR-overexpressing 4T1.2 cells displayed a molecular expression pattern typical of EMT markers. These observations give rise to the possibility that CCX-CKR-overexpressing tumours may more readily shed from the primary tumours and thus disseminate earlier than control 4T1.2 cells; hence, these tumours grow slower but metastasise faster than control tumours. It should also be noted that the difference observed in the number of metastasis on the lung may be due to changes in the localisation of metastatic lesions, rather than a reduction in the number of metastasis. Nevertheless, the question of how overexpression of CCX-CKR induces EMT in 4T1.2 cells remains. Several studies have demonstrated that activation of PI3K/Akt\textsuperscript{303-307} and/or MAPK/Erk\textsuperscript{308-310} pathways play the central role in inducing EMT in various types of cancer cells and these signalling pathways are also known to play an important role in chemokine receptor signalling\textsuperscript{128-131}. Although CCX-CKR has been shown to be incapable of inducing calcium mobilisation upon ligand binding in CCX-CKR-expressing cells\textsuperscript{280,282}, its ability to induce signalling events may be context-dependent as seen in another candidate atypical chemokine receptor, CXCR7. Although CXCR7 was reported to be incapable of inducing downstream signalling upon ligand binding in various types of cells\textsuperscript{234,237,238}, in different systems activation of downstream signalling pathways has been detected, including phosphorylation of Akt in a prostate cancer cell line\textsuperscript{245}, MAPK activation in HEK293 cells and migration of vascular smooth muscle cells\textsuperscript{240}. Interestingly, in prostate cancer cells, overexpression of CXCR7 led to upregulation of fibronectin, cadherin-11 and CD44 (mediators of cell-cell\textsuperscript{311,312} and cell-ECM adhesion\textsuperscript{313}, respectively), and siRNA knock down of CXCR7 resulted in downregulation of these genes, indicating that analogous to the observation with respect to CCX-CKR, CXCR7 may regulate expression of some of the EMT markers and/or families of cell adhesion.
molecules. Given the limited reports on intracellular biochemistry of CCX-CKR, and hence, limited understanding of downstream effects of this receptor, it is possible that alternative pathways, such as PI3K/Akt or MAPK/Erk, are activated downstream of CCX-CKR in 4T1.2 cells, and that activation of those pathways leads to regulation of genes involved in progression of the EMT resulting in enhanced metastasis of CCX-CKR-overexpressing tumours (Figure 5.2A).

Another possibility when considering how CCX-CKR may enhance metastasis of 4T1.2 cells is that it may form complexes with other membrane-bound receptors. It is well established that chemokine receptors can form homodimers or heteromers with other chemokine receptors, or other membrane-bound molecules from different families. When two chemokine receptors form a heteromer, it can result in activation of downstream pathway distinct from monomer activation, as seen in CCR2/CCR5 dimerisation. Furthermore, the atypical chemokine receptor, DARC and the candidate atypical receptor, CXCR7 have been shown to form heteromers with the typical chemokine receptors, CCR5 and CXCR4, respectively. Consequences of multimerisation can be context dependent, particularly with the CXCR7/CXCR4 heteromer where both enhancement and impairment of downstream signalling has been observed in different cells. Therefore, CCX-CKR may also be capable of forming heteromers with other chemokine receptors on 4T1.2 cells. If overexpression of CCX-CKR results in increased formation of these heteromers which alters signalling pathways downstream of typical chemokine receptors, these alterations may lead to activation of EMT-inducing pathways without direct signalling by CCX-CKR (Figure 5.2B).

Chemokine receptors have also been shown to form heteromers with cell surface molecules from different families. In our laboratory, for example, it was shown that CXCR4 interacts with the insulin-like growth factor-receptor 1 (IGF-R1) which enables IGF-1 to
trigger signalling pathways downstream of CXCR4\textsuperscript{322}. Therefore, it is also possible that CCX-CKR associates with another cell surface receptor on 4T1.2 cells. If CCX-CKR interacts with a known EMT-inducing receptor, such as the TGF-β receptor (TGF-β is a potent inducer of EMT\textsuperscript{306,325,326}), it may lead to enhanced activation of EMT-inducing pathways, such as the PI3K or MAPK pathways resulting in highly metastatic cells when CCX-CKR is overexpressed (Figure 5.2C).

In order to fully understand how CCX-CKR overexpressed on 4T1.2 cells may act to inhibit primary tumour growth while enhancing metastasis which is associated with accelerated EMT, it is essential that a better understanding of the behaviour of CCX-CKR on the cell surface and the intracellular events downstream of CCX-CKR potentially triggered in 4T1.2 cells is achieved. In fact, there are several aspects of CCX-CKR function which need to be further explored: whether observed effects of CCX-CKR on primary tumour growth and metastasis are dependent on CCL19, CCL21 and/or CCL25, whether CCX-CKR interacts with non-chemokine molecules yet to be identified, whether CCX-CKR is capable of activating alternative signalling pathways yet to be identified, and whether CCX-CKR forms heteromers with other chemokine receptors and/or other membrane-bound molecules which result in altered signal transduction. While the data obtained from overexpression of CCX-CKR in 4T1.2 cells implied possible novel function(s) of this receptor in tumour biology, it is important to note that the experimental system used in this study involved genetic modification to exogenously express a receptor on cells which do not otherwise express this receptor. In this type of experimental system, it is possible that the observed effect of tumour growth inhibition and accelerated EMT in CCX-CKR overexpressed tumours occurred for reasons unrelated to CCX-CKR function. Nevertheless, it appears that CCX-CKR may play a significant role in tumour growth and metastasis, and therefore better understanding of CCX-CKR function in tumour biology in more physiologically relevant settings, i.e. examination of the function of
**Figure 5.2:** Proposed mechanisms of CCX-CKR-mediated activation of the EMT in 4T1.2 cells. A, CCX-CKR may be capable of transducing uncharacterised signalling in 4T1.2 cells. B, CCX-CKR may form a heteromer with another chemokine receptor and synergistically activate downstream signalling. C, CCX-CKR may form a heteromer with an EMT-activating membrane receptor, such as the TGF-β receptor.
endogenously-expressed CCX-CKR in tumour progression, was also examined.

5.3 Role of endogenously-expressed CCX-CKR in progression and metastasis of cancer

Recently developed CCX-CKR–/– mice have enabled investigation of the role of CCX-CKR in physiological settings. Previous studies and the present study revealed that deletion of CCX-CKR leads to elevated levels of CCX-CKR ligands in various tissues including secondary lymphoid organs, serum, mammary fat pad, skin and lung. These observations support previous studies demonstrating mRNA expression of CCX-CKR in secondary lymphoid organs, skin and lung, and imply that CCX-CKR may be also expressed in the mammary fat pad of wt mice during homeostasis and that CCX-CKR may function to regulate its ligands at those locations. In the current study, growth and metastasis of the C57Bl/6-derived E0771 mammary carcinoma and B16 melanoma were significantly inhibited in CCX-CKR–/– mice, and higher levels of CCL19, CCL21 and CCL25 were present in those tumours when grown in CCX-CKR–/– mice. These data imply two possibilities as to how the tumour growth and metastasis are inhibited in CCX-CKR–/– mice. First, excess CCX-CKR ligands may directly act through CCR7 and/or CCR9 expressed on tumour cells. Second, those ligands may act on host cells such as tumour endothelial cells, fibroblasts and/or leukocytes, which indirectly inhibit tumour growth and metastasis in CCX-CKR–/– mice. Further investigation is certainly required to understand the mechanism of inhibition of tumour growth and metastasis in CCX-CKR–/– mice, which will be discussed in more detail below.

On the other hand, when endogenous expression of CCX-CKR was knocked down in B16 melanoma, complete tumour inhibition/rejection was observed and elevated levels of CCX-CKR ligands were detected. Because of the magnitude of the inhibitory effect of
knockdown of CCX-CKR on B16 melanoma growth, the focus of the study shifted to characterise this effect in more detail. Although there were differences between the studies where CCX-CKR was deleted in the host and where it was knocked down in the tumour, such as the physical appearance of subcutaneous and lung tumours and the extent of tumour growth inhibition, similarities in the overall effect suggest that a similar mechanism may operate, at least partially, in both systems.

The majority of previous studies have demonstrated tumour-promoting roles of CCL19, CCL21 and/or CCL25 in various cancers\textsuperscript{140,191-199,201,204-207}. However, some evidence also implies a tumour-inhibiting effect of CCR7 and CCL19/CCL21 attributed to anti-tumour leukocyte infiltration. For instance, in metastatic colorectal cancer high levels of infiltration by CCR7\textsuperscript{+} CD8\textsuperscript{+} T cells was indicative of better survival and prognosis\textsuperscript{212}. In a mouse model of lung cancer, intratumoural delivery of CCL21 resulted in enhanced infiltration of IFN-\(\gamma\)\textsuperscript{+} T cells leading to inhibition of tumour growth\textsuperscript{327}. Furthermore, proinflammatory M1 macrophages with anti-tumour activities are characterised by expression of CCR7 along with expression of IFN-\(\gamma\) and NO\textsuperscript{294-296}. Indeed, in the current study the data generated in RAG-1\textsuperscript{-/-} and RAG-2\textsuperscript{γc-/-} mice demonstrated that adaptive immunity plays a critical role in rejection of CCX-CKR knockdown B16 tumours. At the same time, in those experiments, inhibition of adaptive immune system failed to completely reverse the growth of CCX-CKR knockdown tumours suggesting that factors in addition to the adaptive immune response also play a role in rejection of CCX-CKR knockdown B16 tumours. Examination of TILs supported these observations and indicated that CCX-CKR knockdown B16 tumours contained substantially more inflammatory cytokines including IFN-\(\gamma\) and had a greater infiltration of various leukocytes of both the innate and adaptive immune systems including IFN-\(\gamma\)-producing CD8\textsuperscript{+} T cells and M1 macrophages. Although expression of CCR7 or CCR9 on those populations of leukocytes was not directly determined, more CCR7- and CCR9-expressing leukocytes were found in
CCX-CKR knockdown B16 tumours. In addition, it has previously been shown that CCR7 and its ligands not only function to direct migration but also to protect effector T cells from apoptosis through the Akt/PI3K pathway in squamous cell carcinoma\textsuperscript{328}. Together, the data indicate a possible association between low CCX-CKR expression on tumour cells, high bioavailability of its ligands in the microenvironment, increased recruitment and survival of leukocytes known to be important in anti-tumour immunity, and tumour regression.

From the observations outlined above it was hypothesised that the excess chemokines in CCX-CKR knockdown tumours causes enhanced recruitment of CCR7/CCR9-expressing anti-tumour leukocytes leading to tumour rejection. However, treatment of mice with neutralising anti-CCL21 antibody did not alter the inhibitory effect of CCX-CKR knockdown on tumour growth. While this implies that rejection of CCX-CKR knockdown tumours may be independent of CCL21 function, examination of CCX-CKR knockdown tumour growth in CCR7\textsuperscript{−/−} mice indicated that the effect is, at least partially, host CCR7-dependent. However, it must also be stated that in these experiments, both neutralisation of CCL21 and deletion of CCR7 from the host also caused significant inhibition of control tumour growth. This latter observation is consistent with the results of a recent study conducted by Shields and colleagues\textsuperscript{196}, which demonstrated that growth of wt B16F10 melanoma in CCR7\textsuperscript{−/−} mice and growth of CCL21-knockdown B16F10 melanoma in wt mice were significantly inhibited. However, Shields and colleagues also showed that overexpression of CCL21 by B16F10 cells resulted in enhanced melanoma growth in wt mice and concluded that CCL21 produced by B16F10 cells recruited $T_{\text{reg}}$ cells and MDSCs in a host CCR7-dependent manner and created an immunosuppressive environment that supported tumour growth, observations that are inconsistent with those of the present study. While the reasons for this difference are difficult to determine without specific experimentation, it is probably due to differences in the levels of CCL21 detected in tumour tissues between the two studies. In their study CCL21-overexpressing B16F10
tumours contained approximately 7 pg of CCL21 per milligram of tissue. This is equivalent to the level of CCL21 detected in control B16 tumours in the present study (approximately 6 and 9 pg/mg of tissue on days 10 and 17, respectively). On the other hand, CCX-CKR knockdown B16 tumours contained more than 2-fold and 10-fold more CCL21 on day 10 and 17 post-injection, respectively, compared with control tumours. The data from the present study and those of Shields and colleagues\(^\text{196}\) allow a number of hypotheses to be proposed. The differences in the levels of CCL21 and the rates of tumour growth imply that the concentration of CCL21 for optimal recruitment of T\(_\text{reg}\) cells and MDSCs may be different from that required for CD8\(^+\) T cells and M1 macrophages. Although future experimentation is required to test this hypothesis, the optimal level of CCL21 required for recruitment of immunosuppressive leukocytes may be lower, as seen in CCL21-overexpressing B16F10 tumours in the study by Shields and colleagues\(^\text{196}\) and control B16 tumours in the present study, than that for optimal recruitment and/or maintenance of tumour cell-specific CD8\(^+\) T cells and M1 macrophages as seen in CCX-CKR knockdown tumours (Figure 5.3A). Furthermore, in CCR7\(^-/\) mice regardless of the level of CCL21 in the tumour microenvironment, the absence of CCR7 on host T\(_\text{reg}\) cells, MDSCs, CD8\(^+\) T cells and M1 macrophages impedes their migration to tumour tissues, resulting in presence and activity of only CCR7-independent leukocytes and this results in a similar rate of tumour growth in control and CCX-CKR knockdown tumours in CCR7\(^-/\) mice (Figure 5.3B). On the other hand, in CCL21-neutralised mice, while depletion of CCL21 from control tumours inhibits recruitment of T\(_\text{reg}\) cells and MDSCs, thereby inhibiting tumour growth through CCR7-independent CD8\(^+\) T cells, the extent of CCL21-neutralisation may not have been enough to reduce the level of CCL21 in CCX-CKR knockdown tumours, and hence, CCR7\(^+\) CD8\(^+\) T cells and M1 macrophages were still able to infiltrate and exert their effector functions (Figure 5.3C). Although the anti-CCL21 antibody used in this study was
fully characterised in vitro and that migration of CCR7-overexpressing B300.19 cells was inhibited in transwell assay when recombinant CCL21 in lower chamber was incubated with the antibody (data not shown), it is still probable that the level of CCL21 neutralisation in vivo for the B16 melanoma model was not adequate.

In addition, when considering the effect of anti-CCL21 neutralisation on CCX-CKR knockdown tumour growth, the possibility that the second CCR7 ligand, CCL19, may compensate for the loss of CCL21 cannot be ignored. Although there has not been direct evidence indicating these two chemokines can functionally compensate for each other, normal architecture and cellular composition was observed in thymus of CCL19−/− mice, which suggested that CCL21 may compensate for the lack of CCL19 in the thymus329. Therefore, it is possible that CCL21 was effectively neutralised in anti-CCL21 antibody-treated mice but the presence of CCL19 compensated and recruited CCR7+ anti-tumour leukocytes. This may be a reason why the effect of CCX-CKR knockdown is not reversed in anti-CCL21-treated mice, while a clear reversal of the effect was observed in CCR7−/− mice. Taken together, these hypotheses, if proven, have potentially important implications for any proposed therapy targeting this axis in melanoma.

Interestingly, knockdown of CCX-CKR on B16 tumours not only resulted in significantly enhanced infiltration of anti-tumour leukocytes, but also led to generation of lymphoid-like structure in tumour tissue. It is well-established that CCL19 and CCL21 induce lymphoid neogenesis in various inflammatory conditions such as autoimmunity and infection297,330-332. In particular, it has been shown that CCL21 induces this process by recruiting CCR7+ lymphoid tissue inducer cells which drive the maturation of lymphoid stroma297,333. In addition, CCL19 and CCL21 produced in LN paracortex also function to guide the interaction between CCR7+ T cells and APCs, which are essential for activation of effector responses334. Although the level of CCR7+ lymphoid tissue inducer cells in B16
Figure 5.3: **CCX-CKR knockdown tumour microenvironment in CCR7\textsuperscript{−/−} and CCL21-neutralised mice.** A, in wt mice low level of CCL21 in control B16 tumours is optimal for recruitment of T\textsubscript{reg} cells and MDSCs which suppress proliferation and activation of CD8\textsuperscript{+} T cells (infiltration by CCR7-independent migration), whereas in CCX-CKR knockdown B16 tumours, high level of CCL21 is optimal of CCR7 dependent recruitment of CD8\textsuperscript{+} T cells and M1 macrophages leading to enhanced anti-tumour immunity. B, in CCR7\textsuperscript{−/−} mice regardless of CCL21 levels in tumour microenvironment, no CCR7-dependent recruitment of immune cells, thereby CCR7-independent CD8\textsuperscript{+} T cells infiltration in both tumours leading intermediate anti-tumour immunity. C, when anti-CCL21 antibody is administered to mice, control tumours no longer contain enough level of CCL21 to recruit T\textsubscript{reg} cells and MDSCs which inhibit CD8\textsuperscript{+} T cells, whereas in CCX-CKR knockdown tumours still high enough level of CCL21 is present for CCR7 dependent recruitment of CD8\textsuperscript{+} T cells and M1 macrophages leading to enhanced anti-tumour immunity.
CHAPTER 5: Discussion

### A

**B16CTLkd**

- **wt mice**
  - Low CCL21
  - CCR7-dependent recruitment of immunosuppressive cells

**B16CCXkd**

- **High CCL21**
  - CCR7-dependent recruitment of anti-tumour immune cells

### B

**CCR7⁻ mice**

- No CCR7-dependent recruitment of immunosuppressive cells

### C

**CCL21 neutralised mice**

- CCL21 level is too low to induce CCR7-dependent recruitment of immunosuppressive cells

- CCL21 level is still high enough to recruit anti-tumour immune cells
tumours was not examined and interaction of CCR7$^+$ T cells and APCs in the tumours was not directly determined, it is likely that the excess CCL19 and CCL21 present in CCX-CKR knockdown tumours not only function as chemoattractants to recruit effector cells into tumours, but also to induce generation of organised lymphoid structure which ensures efficient antigen presentation and maturation of anti-tumour effector T cells to take place within tumour tissue.

Thus far, the data presented in this study suggests, although do not unequivocally prove, that CCX-CKR expressed endogenously by B16 cells may function as chemokine scavenger as originally proposed. Thus, the data indicate association between reduced expression of CCX-CKR on tumour cells, elevation of CCX-CKR ligands CCL19 and CCL21 in tumour tissues, high levels of CCR7$^+$ anti-tumour leukocytes, and tumour rejection. It is interesting to note, however, that in the current study CCX-CKR knockdown tumours were larger than control tumours at an early stage of tumour development. This was not due to a high influx of lymphocytes since CCX-CKR knockdown tumours at early stage were also larger than control tumours in RAG-1$^{-/-}$ and RAG-2$\gamma c^{-/-}$ mice. Although the levels of CCX-CKR ligands in early stage tumours were not examined during this study, it is possible that as observed at the later time points, higher levels of the ligands are present at this early stage in CCX-CKR knockdown tumours. Therefore, CCX-CKR ligands present in CCX-CKR knockdown tumours may initially support tumour growth, as previously shown$^{199,201,204,206}$, before the immune response become fully activated until activities of anti-tumour immune cells eventually override the tumour-supporting effect of these chemokines.

Similar effects were observed on the ability of CCX-CKR knockdown B16 cells to form tumour nodules in lungs upon intravenous administration. Initially, a higher metastatic burden was detected in lungs of CCX-CKR knockdown tumour-injected mice. However,
those mice became free of lung tumours when autopsied on day 100 post-injection, whereas mice bearing control tumours survived only for 2-4 weeks depending on the dose with extensive tumour nodules on the lungs. Although the kinetics of tumour burden on these mice were not monitored at regular intervals, it is likely that the metastatic burden by CCX-CKR knockdown tumours initially increased but then plateaued and started to decrease from a later time point eventuating in clearance of lung nodules, as seen in primary tumour growth. Due to time constrains, lungs bearing B16 tumours were not as extensively analysed as were the subcutaneous tumours. However, morphology of tumour nodules and elevated infiltration by CCR7+ and CCR9+ leukocytes in CCX-CKR knockdown tumour-bearing lungs clearly indicates that a similar mechanism to that in subcutaneous tumour inhibition may operate in lung tumour nodules. Further analysis of tumour-bearing lungs will be necessary to determine the levels of CCX-CKR ligands, cytokines and presence of specific anti-tumour leukocytes, and haematogenous metastasis in immunocompromised mice and in CCR7+/− mice, as shown in primary tumours. However, observations from these experiments only relates to growth of secondary tumours within the lungs once cells are seeded in the lung tissue. Therefore, in order to determine the function of CCX-CKR knockdown on metastatic capacity, other malignant characteristics, such as invasiveness, motility and survival in circulation also need to be examined.

Investigations conducted in this study have revealed a remarkable effect of CCX-CKR deletion in the host and of CCX-CKR knockdown in tumour cells on tumour progression and metastasis. In both systems significantly higher levels of CCX-CKR ligands were observed in tumour tissues, whether it resulted from an absence of CCX-CKR in host tissues or tumour cells. Although it is possible that elevated levels of CCX-CKR ligands in tissues of primary tumour and metastasis and tumour tissues themselves in CCX-CKR+/− mice results in similar anti-tumour effects to that seen in the knockdown system, there are two major differences in observations made between the two systems which implies that
different mechanisms of inhibition of tumour growth and metastasis operate in CCX-CKR−/− mice. First, the morphology of primary tumours and lung tumour nodules of CCX-CKR−/− mice was black in colour and soft in texture, not white and firm as seen in CCX-CKR knockdown tumours (data not shown) and second, although growth of primary tumours and lung nodules were inhibited, unlike the CCX-CKR knockdown tumours, they were not rejected in CCX-CKR−/− mice. Thus, in the knockdown system, it was shown that high levels of CCX-CKR ligands in CCX-CKR knockdown tumours were associated with high presence of CCR7+ effector cells and lymphoid tissue inducer cells which presumably led to effective anti-tumour response and lymphoid neogenesis at tumour tissues (hence white colour in appearance) and eventually resulted in rejection of tumours. From the data obtained during the current study using CCX-CKR−/− mice, whether the observed effects are dependent on the adaptive immune system, CCX-CKR ligands, or host CCR7 and CCR9, as seen in the knockdown system was not examined due to time constraints. Further investigation of the role of endogenously-expressed CCX-CKR in tumour progression and metastasis is essential to determine the involvement of immune system, CCX-CKR ligands and their signalling receptors by examining tumour growth and metastasis in immunocompromised mice lacking CCX-CKR, CCL19+/−/CCL21−/− mice lacking CCX-CKR, and CCR7−/− or CCR9−/− mice lacking CCX-CKR. The data obtained from these experiments will provide a better perspective of the mechanism underlying the effect of CCX-CKR deletion on tumour progression and metastasis.

5.4 Concluding remarks and future perspectives

This study has begun to reveal the role of CCX-CKR in tumour progression and metastasis. Increased expression of CCX-CKR on 4T1.2 mammary carcinoma cells resulted in inhibition of primary tumour growth and enhanced metastasis, while reduced expression of
CCX-CKR on B16 melanoma cells led to rejection of subcutaneous tumours and metastatic nodules and absence of CCX-CKR in the host also resulted in inhibition of subcutaneous tumour growth and formation of metastatic nodules. Further analysis of how the manipulation of CCX-CKR expression on tumour cells revealed that CCX-CKR and its ligands may play distinct roles in each model. Although the mechanism whereby exogenously expressed CCX-CKR on 4T1.2 cells inhibits primary tumour growth and promotes metastasis by enhancing progress of the EMT was not fully elucidated, the data obtained from this study implied potential novel functions yet to be demonstrated, including possible non-chemokine ligand(s), the possibility of activating alternative signalling pathways and possible crosstalk with other chemokine receptors or membrane-bound proteins to alter downstream signalling. There are still too many unknowns about the mode of action of CCX-CKR in these cells and extensive molecular and biochemical analyses are required to assess a number of possible mechanisms. Although the three approaches taken led to somewhat contradictory outcomes, it is clear from the data that different mechanism of CCX-CKR operates in different experimental system and different types of tumour model.

The results of the study using CCX-CKR knockdown in melanoma cells clearly indicated that CCX-CKR may be targeted for melanoma therapy, although it is essential to unequivocally determine the mechanism of CCX-CKR knockdown-mediated rejection of primary and secondary tumours. Conceivably, therapies could block expression/function of CCX-CKR in melanoma in the hope of enhancing anti-tumour immune responses to reject established tumours in patients. For such application, it is necessary to determine that CCX-CKR is expressed by individual human melanomas. Gene array data deposited in Gene Expression Omnibus Profiles (GEO Profiles: GDS3489, GDS1965, GDS1989) shows that CCX-CKR is expressed to varying degrees in melanoma tissues isolated from various patients. The approach taken to block CCX-CKR expression in
established melanoma may be a “gene-silencing therapy” whereby an RNAi construct targeting CCX-CKR (either siRNA shRNA or miRNA) is delivered to tumour cells. Although a number of studies and clinical trials have generated some promising results\textsuperscript{337}, obstacles still remain regarding delivery (non-viral or viral), off-target effects, limited duration of silencing, and general toxicity and safety\textsuperscript{338}. Another approach for blocking the function of CCX-CKR could be administration of a neutralising antibody. However, systemic administration of CCX-CKR neutralising antibody may also have off-target effects by interacting with host CCX-CKR in different tissues. Therefore, development of highly effective targeted delivery of antibody to tumour tissues may be required.

In conclusion, this study has certainly extended understanding of the function of the atypical chemokine receptor CCX-CKR in tumour biology. Although a great deal remains to be explored regarding the function and mechanisms of action of this receptor, the hypothesis that CCX-CKR regulates bioavailability of chemokine ligands in the tumour microenvironment which in turn regulates tumour progression and metastasis has been partially supported, at least in melanoma. If this hypothesis is fully proven, manipulation of CCX-CKR expression can be explored in other types of cancer as well as other pathological conditions where CCL19, CCL21 and/ or CCL25 have been implicated. In addition, investigations of other atypical chemokine receptors can also be extended for various diseases where chemokine ligands of these receptors are known to be important. Nevertheless, findings from this study has shed light on a novel potential target for more effective anti-melanoma therapies and potentially against other types of cancer which affect millions of people worldwide.
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