The roles of the chemokines CXCL12 and CXCL16 in breast cancer

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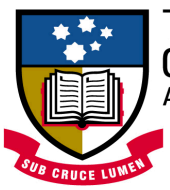

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I could write a volume equivalent to this thesis expressing my gratitude to everyone who has helped me to navigate my way to the end of my PhD studies, but for the sake of a forest somewhere, I'll keep it to a couple of pages.

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Amendments

p. xv "phospholipases C" should read "phospholipase C"

p. 35 In Table 1.4, the heading of the second column, "Role of CXCL16CXCR6" should read, "Role of CXCL16/CXCR6"

p. 53 In Section 2.4.4, the concentration of CXCL12 and CXCL16 used for stimulation of the cells should read "300ng/ml" and not "300μg/ml"

p. 107 In the first line, "angiogenesis with the tumour" should read "angiogenesis within the tumour"

p. 129 In the second line of the second paragraph, "4TX12 1 tumour-bearing were" should read "4TX12 1 tumour-bearing mice were"

p. 131 In the third-to-last line, "Type l IFN-γ-producing also" should read "Type I IFN-γ-producing T cells also"

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Abbreviations

300.19	a pre-B lymphocyte cell line
4T12Ala	4T1.2 cells transfected with the $CXCL12_{(Ala)}$::pEF DNA construct
4T12P2G	4T1.2 cells transfected with the $CXCL12_{(P2G)}$::pEF DNA construct
4TX12	4T1.2 cells transfected with the CXCL12::pEF DNA construct
4T16	4T1.2 cells transfected with the CXCL16::pEF DNA construct
4TΔ16	4T1.2 cells transfected with the $CXCL16_{(9-220)}$::pEF DNA construct
ADAM10	a disintegrin and metalloproteinase 10
ADCC	antibody-dependent cell-mediated cytotoxicity
APC	antigen presenting cell
APC-Cy7	allophycocyanin-cytochrome 7
ASMC	aortic smooth muscle cell
bFGF	basic fibroblast growth factor
BCIP/NBT	5-bromo-4-chloro-3′ indolylphosphate p-toluidine salt/Nitro Blue tetrazolium
BCX6	300.19 cells transfected with a CXCR6::pEF DNA construct
BSA	bovine serum albumin
CAM	cell adhesion molecule
CCL	CC chemokine
CCR	CC chemokine receptor
CTL	cytotoxic T lymphocyte
CXCL	CXC chemokine
CXCR	CXC chemokine receptor
DAB	3,3′-diaminobenzidine
DC	dendritic cell
DLN	draining lymph node
EC	endothelial cell
ECM	extracellular matrix
EF-1α	elongation factor-1α
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunosorbent spot assay
EMMPRIN	extracellular matrix protease inducer
EPC	endothelial progenitor cell
ER	oestrogen receptor
ERK	extracellular signal-regulated kinase

FCS	foetal calf serum
FITC	fluorescein isothiocyanate
GAPDH	glutaraldehyde 3-phosphate dehydrogenase
GM-CSF	granulocyte-macrophage colony stimulating factor
GRK	G protein-coupled receptor kinase
HBSS	Hank's balanced salt solution
HD	Hodgkin's disease
HER2	human epidermal growth factor receptor 2
HIF-1α	hypoxia-inducible factor-1α
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
HSC	haematopoietic stem cell
HUVEC	human umbilical vascular endothelial cell
IEL	intraepithelial lymphocyte
IFN-γ	interferon-γ
IKDC	interferon-producing killer dendritic cell
IL	interleukin
iNOS	inducible nitric oxide synthase
IRES	internal ribosome entry site
JAK	janus kinase
LDL	low density lipoprotein
LN	lymph node
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MQ H_2O	MilliQ H_2O
MRCRB	mouse red cell removal buffer
MSC	myeloid suppressor cell
NK cell	natural killer cell
NO	nitric oxide
NPC	nasopharyngeal carcinoma
oxLDL	oxidised low density lipoprotein
PA	PBS/Azide
PBA	PBS/BSA/azide
PBL	peripheral blood leukocyte
PBS	phosphate buffered saline

PCR	polymerase chain reaction
pDC	plasmacytoid dendritic cell
PDGF	platelet-derived growth factor
PE	phycoerythrin
PE-Cy7	phycoerythrin-cytochrome 7
pEF	pEF-IRES-puro6
pfp	perforin
PGC	primordial germ cell
PI3K	phosphoinositol 3-kinase
PIP3	phosphatidylinositol 3,4,5-phospate
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMS	N-methyl dibenzopyrazine methyl sulfate
PTEN	phosphatase and tensin homologue deleted on chromosome 10
RNAi	ribonucleic acid interference
RT-PCR	reverse transcription polymerase chain reaction
SCID	severe combined immunodeficient
SDF-1	stromal cell-derived factor-1
siRNA	small interfering ribonucleic acid
STAT	signal transducer and activator of transcription
TAA	tumour-associated antigen
TAM	tumour-associated macrophage
TCR	T cell receptor
TGF-β	transforming growth factor-β
Th	T helper
TLR	Toll-like receptor
TNF-α	tumour necrosis factor-α
Tr, T_{reg}	T regulatory
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
uPA	urokinase-type plasminogen activator
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VLA-4	very late antigen-4
XTT	sodium 3′-[1-(phenylaminocarbonyl)-3.4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate

Publications arising from this work

Manuscripts

Sharon A. Hampton-Smith & Shaun R. McColl. 'Overexpression of CXCL12 in an orthotopic model of breast cancer improves the efficacy of the anti-tumour immune response.' *Manuscript in preparation.*

Conference Proceedings

Adelaide Breast Development and Cancer Meeting, 2006 (Adelaide, Australia).

Title: Chemokines in breast cancer: whose side are they on anyway?

Oral Presentation

Adelaide Immunology Retreat II, 2006 (Adelaide, Australia).

Title: The role of the chemokine CXCL12 in breast cancer.

Oral presentation

Abstract

A growing body of work implicates chemokines and their receptors in the progression of various types of cancer, including breast cancer. However, as potent chemotactic factors for leukocytes, chemokines also have the potential to enhance anti-cancer immunity. Evidence suggests that the chemokine CXCL12 and its receptors may be important in a number of aspects of breast cancer progression and site-specific metastasis. Another chemokine, CXCL16, has been identified as a specific chemotactic factor for Type I-polarised T lymphocytes, which are major effectors of cell-mediated immunity and hence efficacious anti-tumour immune responses. The aim of this study, therefore, was to further elucidate the roles of CXCL12 and CXCL16 in breast cancer development and metastasis. To achieve this, wild-type CXCL12 and CXCL16 and antagonists of CXCL12 and CXCL16 activity, $CXCL12_{(P2G)}$ and $CXCL16_{(9\text{-}220)}$ respectively, were overexpressed in the 4T1.2 mouse model of breast carcinoma. Overexpression of wild-type CXCL12 potently inhibited both primary tumour growth and metastasis in this model. This was attributed to the induction of an anti-tumour response dependent, in part, on T cells, interferon-γ and the cytotoxic mediators perforin and TRAIL. This response was characterised by increased numbers of $CD11c^+$ cells in the tumour-draining lymph nodes and enhanced cytolytic activity of lymph node-derived effector cells against tumour cells. Unexpectedly, $CXCL12_{(P2G)}$ inhibited metastasis of tumour cells to the lungs of tumour-bearing mice, without affecting primary tumour growth. Intravenous injection of tumour cells revealed that $CXCL12_{(P2G)}$ expression could block metastatic steps occurring post tumour cell escape from the primary tumour, though a role for $CXCL12_{(P2G)}$ at earlier metastatic steps could not be ruled out. Further work is needed to clarify the precise stages of metastasis at which $CXCL12_{(P2G)}$ exerts its effects. No obvious effects on primary breast tumour growth were observed when CXCL16 or $CXCL16_{(9\text{-}220)}$ were overexpressed in tumour cells. Interestingly, $CXCL16_{(9\text{-}220)}$ expression inhibited experimental metastasis but not spontaneous metastasis. The findings of this study begin to shed light on the roles of CXCL12 and CXCL16 in breast cancer progression and also highlight the potential therapeutic applications of CXCL12, CXCL16 and/or their antagonists in the treatment of breast cancer and breast cancer metastasis.

CHAPTER 1

Introduction

CHAPTER 1: INTRODUCTION

1.1 Overview

Breast cancer is the most common malignancy occurring in women worldwide and represents approximately 1 million of the estimated 10 million cancers diagnosed in both sexes each year. It is the most common cause of cancer-related mortality in women throughout the world, claiming 375 000 lives in the year 2000[3]. Though it is primarily a disease found in women, breast cancer can also occur in men. The incidence in men is considerably lower than that in women and accounts for only 0.7% of breast cancer diagnoses[4]. However, the mortality rate of male breast cancer has not declined in recent years as has happened for female breast cancer, due to the lack of awareness and understanding of the disease[5].

Compared with patients with other malignancies, those with breast cancer have a relatively good overall survival rate, particularly when the cancer is diagnosed early. However, prognosis is significantly poorer if the disease is more advanced upon presentation: in the USA in 1994, the 5 year survival rate for localised disease was reported to be 97%, while that for metastatic disease was just 25%[3]. Thus, despite improvements in screening programs and early detection, more research is required to improve the outcome of patients presenting with advanced disease.

To this end, intensive efforts have been directed towards attaining a better understanding of the pathobiology of breast cancer: how it arises, evolves and progresses to metastatic disease. An improved knowledge of the mechanisms underlying metastasis in particular is essential, as this is the most dangerous aspect of breast cancer and also the most difficult to treat. Much research has focused on improving current therapies and developing new treatment modalities, and better understanding of the disease will provide insights into new therapeutic strategies that can be employed.

Immunotherapy holds great promise as a means to combat metastatic breast disease. The immune system is a powerful weapon ideally suited to targeting malignant cells: it has the ability to patrol the entire body for cancerous cells as well as the arsenal with which to destroy them. Harnessing the inherent power of the immune system of a patient is thus an

attractive prospect for treatment of breast cancer, and considerable research has focused on identifying ways to mobilise the anti-tumour response and overcome tumour immune evasion mechanisms.

In this vein, the chemokine family of chemotactic cytokines has been investigated as a potential source of immunotherapeutic mediators. The chemokines were originally identified based on their ability to guide the migration of leukocytes around the body, a property that makes them attractive as a means of targeting immune effector cells to the site of a tumour. Over the last six years, however, the chemokines have come under intense scrutiny for their role in promoting breast cancer progression. This followed the publication of a study by Muller and co-workers demonstrating that inhibiting the chemokine receptor CXCR4 could block breast cancer cell metastasis[6].

In this thesis, the pro- and anti-tumour effects of two chemokines, CXCL12 and CXCL16, are investigated. This research is conducted with a view to better understanding the influence of these chemokines on breast cancer progression and to provide insight into their potential as therapeutic agents to treat this very prevalent disease.

1.2 Breast cancer

1.2.1 Breast cancer progression

The mechanisms of breast tumour development and metastasis are extremely complex. For many years, the study of breast cancer was limited largely to the properties of the cancerous cells within a tumour. More recently, the focus has shifted to encompass the other components within a tumour, namely stromal cells, endothelial cells (EC) and infiltrating leukocytes, as the significance of their contribution to the growth of cancerous cells has been realised. Not surprisingly then, a huge array of genes, signalling molecules, growth factors and cells have been implicated in the progression of malignant breast disease.

The entity that we recognise as breast cancer is likely to include a range of distinct diseases, each with their own individual aetiology. Despite the differences in molecular mechanisms that give rise to individual malignant breast lesions, there are a few

characteristics common to most, if not all breast cancers, which are required for tumour development and metastasis. These 'hallmarks' of breast cancer, as outlined by Hanahan and Weinberg[1], include the following: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential (immortality), sustained angiogenesis and tissue invasion and metastasis (Figure 1.1). An additional hallmark, not specifically highlighted by Hanahan and Weinberg, is the requirement for 'normal' accessory cells in tumour development; cancerous cells must acquire the ability to co-opt and activate local stromal cells to promote malignant progression (Figure 1.1). Finally, the acquisition of genomic instability is crucial for the accumulation of the large number of genetic mutations required for the aforementioned characteristics to become manifest[1].

Of the eight hallmarks of cancer required for malignant progression, four of these (self-sufficient production of growth signals, insensitivity to anti-growth signals, evasion of apoptosis and immortality) directly pertain to the proliferative behaviour of tumour cells themselves. A well-studied example of a tumour-promoting growth signal in breast cancer is oestrogen. This has been demonstrated through success of the anti-oestrogen tamoxifen as a treatment modality for oestrogen receptor (ER)-positive breast cancers. Resistance to anti-growth signals can manifest as a result of mutations in any of a number of cell cycle regulators that allow malignant cells to progress through mitosis unchecked. For instance, deregulated expression of the cell cycle regulator Cyclin E is correlated with poor prognosis in human breast cancer[7] and mammary carcinogenesis in transgenic mice[8]. Mutations in the tumour suppressor p53 are associated with enhanced resistance to apoptosis in a number of cancers, including breast cancer[9]. The p53 protein is responsible for inducing cell death in response to irreparable DNA damage and/or mutations[10]. Thus eliminating p53 function is a considerable advantage for breast tumour cells, which frequently harbour multiple genetic mutations. In terms of immortality, activation of telomerase is a significant event in the majority of breast cancers[11]. Telomerase maintains the length of telomeres at the ends of chromosomes over many cell divisions, thus preventing end-to-end chromosome fusions, loss of karyotypic integrity and ultimately cell death. Telomerase is inactive in most normal adult cells, thus ensuring deletion of cells that have undergone multiple cell divisions and that potentially, have accumulated genetic mutations over time. Activation of telomerase allows breast cancer cells to continue dividing and accumulating genetic mutations, thus perpetuating the cancerous phenotype.

The importance of the enzyme was shown in a study where inhibition of telomerase increased the susceptibility of breast cancer cells to anti-cancer drugs in a manner that was dependent on the shortening of telomeres[12].

As well as altering their own behaviour, breast cancer cells must co-opt other normal cells to promote tumour progression. Tumour cell secretion of the cytokine transforming growth factor (TGF)-β is thought to promote activation and conversion of fibroblasts to a pro-malignant phenotype[13]. Although TGF-β has been shown to inhibit proliferation and transformation of normal breast epithelial cells, the activation of other oncogenic pathways can block the response of transformed cells to TGF-β inhibition and enhance the pro-tumorigenic properties of this cytokine[14]. At later stages of tumour growth, TGF-β can promote epithelial-to-mesenchymal transition of malignant cells, enhancing their invasive capabilites[14], and as mentioned above, TGF-β can enhance the pro-malignant activity of tumour stromal cells[13, 14]. These stromal cells may provide necessary growth factors as well as depositing ECM components that promote tumour growth and invasion[13]. Another stromal cell type important in breast tumour development is the tumour-associated macrophage (TAM), which can supply extracellular matrix (ECM)-degrading enzymes needed for tumour invasion[15]. In addition to promoting invasion, TAM-derived ECM-degrading enzymes can release growth factors sequestered in the surrounding ECM. TAM also promote breast tumour growth directly, through production of mitogenic cytokines such as epidermal growth factor (EGF)[15].

Perhaps the most striking contribution of normal cells to the growth of cancerous cells is that of EC. As a solid tumour, breast cancer requires a vascular network to supply oxygen and nutrients to the growing tumour mass. Tumour angiogenesis can be triggered by a number of factors including low oxygen tension, low pH, hypoglycaemia, increased pressure due to cell proliferation, inflammatory cell infiltration and genetic mutations[16]. Tumour blood vessels do not resemble the normal vasculature: they are highly disorganised and excessively permeable. They lack functional smooth muscle cells, which are important for vessel contractility, and perivascular cells, which protect vessels from changes in the environment and provide cues to control their metabolic needs[16]. Vascular endothelial growth factor (VEGF) is a major angiogenic cytokine. It promotes proliferation of EC and permeability of capillaries that are required for expansion and remodelling of

the vasculature. VEGF expression is induced by hypoxia-inducible factor (HIF)-1α under hypoxic conditions, as are often found in the centre of the growing tumour[17]. However, the dependence of breast tumour cells on VEGF is not absolute. Tumour cells can co-opt other angiogenic factors, such as basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF) for neovascularisation. Alternatively, some tumours can develop the ability to survive even in the absence of an ample blood supply[17].

The real danger of breast cancer lies in its ability to metastasise: distant metastases are the ultimate cause of 90% of cancer deaths[1]. Breast cancer commonly metastasises to the regional lymph nodes (LN), bone marrow, lung and liver[6]. Metastasis is a complex process of multiple steps, all of which are required for malignant cells to form secondary tumours. Malignant cells first need to escape from the primary tumour and invade surrounding tissue. This requires the expression of degradative enzymes, such as matrix metalloproteinases (MMP)[18] and urokinase type plasminogen activator (uPA)[19], that can break down ECM components and basement membranes. In addition, detached cancer cells must survive anoikis, a form of apoptosis triggered by the loss of anchorage-dependent survival signals. One way in which this can be achieved by breast cancer cells is through inactivation of E-cadherin and p53[20]. Interestingly, high level expression of extracellular matrix protease inducer (EMMPRIN), which can stimulate the production of MMP by both tumour and stromal cells[21], enhances the resistance of tumour cells to anoikis[22]. Thus expression of EMMPRIN might promote the invasive capacity of breast tumour cells by providing a means of moving through tissues, as well as the ability to survive whilst doing so.

Invasive capacity is considered to be the precursor of distant metastasis. Invasion of local tissue is required for malignant cells to reach a conduit for their passage to secondary sites. Two types of conduit are available for tumour cell metastasis (Figure 1.2): the blood vasculature (haematogenous metastasis) and the lymphatic vasculature (lymphatic metastasis). Angiogenesis, then, can serve a dual role in the progression of breast cancer by supplying oxygen and nutrients to the primary tumour, and by providing easy access to the circulation for metastatic cells. Upon arrival at a secondary tissue, a tumour cell needs to express appropriate adhesion molecules to permit it to adhere to the tissue endothelium and extravasate. Degradative enzymes are also required for the cell to cross the endothelial

basement membrane and migrate within the new tissue. Finally, to become established in its new niche, the tumour cell must be able to adhere to the local matrix components and to proliferate in its new environment to form a secondary tumour[1]. Lymphatic metastasis is the most direct route for breast tumour cells to reach the regional LN and induction of lymphangiogenesis by breast tumours has been shown to enhance metastasis to the LN[23]. Once in the LN, cancer cells can proliferate to form a secondary tumour from which metastatic cells can be shed. These cells eventually reach the bloodstream, thus embarking on the haematogenous route of metastasis to more distant anatomical locations. Indeed, enhanced lung metastasis in the absence of increased tumour angiogenesis has been attributed to the increase in lymphatic metastasis[23].

1.2.2 The immune response to breast cancer

One important aspect of breast tumour progression not included in the list of hallmarks outlined by Hanahan and Weinberg[1] is the ability to avoid destruction by the immune system. Tumour growth is an inherently inflammatory process because it inevitably involves tissue disruption. This leads to the release of pro-inflammatory signals in the form of cytokines and ECM fragments[24]. To continue growing to a clinically detectable size, a tumour must somehow prevent the mobilisation of a detrimental anti-tumour immune response, and a variety of mechanisms that achieve this end have been reported. Some tumours can even subvert the immune response to their advantage, using growth factors and degradative enzymes released by inflammatory cells to further their growth and invasion. However, successful evasion of immune-mediated eradication is likely to be the exception rather than the norm. It is thought that many tumours arise and are eliminated before they become evident due to constant surveillance of the tissues for signs of abnormality by the immune system[24].

1.2.2.1 Immunosurveillance

The theory of tumour immunosurveillance has enjoyed a renaissance in recent years. According to this hypothesis, tumours arise in tissues at a similar frequency to pathogenic infections, but are recognised and eliminated by the immune system before they become clinically evident. It is thought that intraepithelial lymphocytes (IEL), which are dispersed throughout epithelial tissues, may be responsible for surveying these tissues for

transformed cells[24]. NKT cells[25] and innate immune cells, including eosinophils[26] natural killer (NK) cells and possibly macrophages[25], also play a part in tumour immunosurveillance. IFN-γ and intact cytolytic functions are required for this process, as has been determined from studies in IFN-$\gamma^{-/-}$, perforin$^{-/-}$ and tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)$^{-/-}$ mice[25]. Recognition of nascent tumours is though to occur in two ways: through recognition of tumour-associated antigens (TAA) presented on major histocompatibility complex (MHC) molecules, and through recognition of tumour-expressed ligands of the NKG2D activating receptor expressed on NK cells and T cells[25]. Clearly, however, immunosurveillance of tumours is not a completely efficient process, otherwise cancer would be a disease unknown to medicine. It is thought that the immune response against a tumour selects for cells that are inherently less immunogenic; such cells overcome the immune response and grow into a clinically detectable tumour using both passive and active immune evasion mechanisms.

1.2.2.2 Immune evasion

Passive resistance mechanisms utilised by tumours can include down-regulation of MHC molecules or particular TAA[24, 25]. In human breast cancer, total loss or reduced expression of MHC class I antigens was frequently observed in primary tumours and LN metastases[27, 28], whereas these antigens were present in all normal breast tissue and benign lesions examined[27]. Moreover, mice receiving immunotherapy targeting the human Mucin 1 (MUC1) TAA escaped immune control through down-regulation of MHC class I molecules[29]. Mammary tumours from neu-transgenic mice transplanted into non-transgenic mice induced a strong anti-tumour response; however, escape variants emerged that had lost expression of the neu TAA[30]. This was attributed to the action of T cell-derived IFN-γ, because IFN-γ treatment of mammary tumour cells induced methylation of the neu promoter[31]. These antigen-loss variants also exhibited reduced expression of pro-inflammatory cytokines, chemokines and immunostimulatory chaperones and increased expression of tumour survival factors[32], suggesting that immune escape requires more than simple down-regulation of TAA. Indeed, down-regulation of MHC molecules and TAA would be expected to protect the tumour from T cell responses, but not from NK cell-mediated lysis, which is triggered by the loss of MHC molecules, and other innate immune

responses that are antigen-independent. Additional mechanisms are required, therefore, for a tumour to successfully evade the immune response.

1.2.2.3 Active Immunosuppression

As an alternative to 'hiding' from the immune system, tumours may actively blunt or disarm the immune response (Figure 1.3). Cytokines are important factors responsible for dampening immune responses. These molecules play a significant role in determining the polarisation and effector function of tumour-infiltrating leukocytes. Cytokines such as IL-12 and IFN-γ are responsible for the differentiation of T helper 1 (Th1) cells, the development of immunogenic dendritic cells (DC) and the activation of cytotoxic macrophages[33]. IFN-γ is also a major effector cytokine of $CD8^+$ effector T cells and NK cells[34]. It is therefore in the interests of the tumour to steer the cytokine balance away from this pro-immunogenic profile towards a more immunosuppressive profile. Such a cytokine profile induces the accumulation of regulatory T cells and other suppressor cells, and generates tolerogenic antigen presenting cells (APC) and pro-tumorigenic macrophages, the net effect of which is to enhance tumour growth while dampening deleterious immune responses.

Interleukin (IL)-10 and TGF-β are the two major cytokines associated with immunosuppression. IL-10 drives the differentiation of tolerogenic DC, which in turn produce more IL-10. This enables tolerogenic DC to induce T regulatory 1 (Tr1) cells, which also produce IL-10[35]. IL-10 promotes production of TGF-β by T_{reg} cells and enhances the responsiveness of T cells to the inhibitory effects of this cytokine[35]. However, it should be noted that the immunosuppressive role of IL-10 in anti-tumour responses is controversial[35]. In breast cancer, depletion of IL-10-producing γδ T cells was found to enhance anti-tumour cytotoxic T lymphocyte (CTL) and NK cell cytotoxic activity[36], while in another model, IL-10-transfected tumours were rejected by the immune system[37]. It is thought that the location of its expression determines how IL-10 influences the immune response: systemic IL-10 inhibits the development of adaptive immune responses in the secondary lymphoid organs, but localised IL-10 expression within the tumour enhances anti-tumour immunity[35].

TGF-β is an important differentiation factor for T regulatory (T_{reg}) cells, and is also a major product of these cells. As such, it is an important mediator of immune suppression. TGF-β inhibits T cell proliferation and differentiation of naïve T cells into Th1, Th2 or CTL effector cells, as well as B cell proliferation and activation[38]. TGF-β also inhibits macrophage effector functions and maturation of DC, which results in reduced antigen presentation by these cells[38]. The significance of TGF-β in breast cancer immunosuppression has been shown by studies where neutralisation of TGF-β restored T cell activity and cytotoxic function[39-41] and enhanced the efficacy of DC-based vaccines[42].

A critical factor determining the outcome of an anti-tumour immune response (i.e. immunogenic versus tolerogenic) is the type of immunostimulatory signals provided by APC, particularly DC. The nature of these signals depends on both the development and activation state of DC[43]. In breast cancer patients, the number of mature DC infiltrating tumours was correlated with improved relapse-free and overall survival[44]. However, maturation of DC does not always generate effective immunostimulatory APC, particularly when induced by tumour-cell derived factors[45]. Whether a DC induces activated or suppressive T cells seems to depend more upon the microenvironment in which the DC finds itself (e.g. surrounded by 'danger' signals in the form of TLR ligands versus an IL-10-rich milieu) and the kind of cytokines the DC itself secretes (e.g. IL-12 versus IL-10)[43].

Much attention has focused on the role of regulatory T cells (T_{reg}) in tumour-induced immunosuppression. T_{reg} cells are increased in breast cancer patients[46], and depletion of these cells improves anti-tumour responses in mouse models of breast cancer[47, 48]. T_{reg} can be classified broadly into 'natural' and 'induced' T_{reg} on account of their developmental pathway. Natural T_{reg} are generally $CD4^+CD25^+FoxP3^+$ and develop normally in the thymus as a distinct T cell population. Induced T_{reg} differentiate from naïve T cells in response to antigen stimulation in the absence of co-stimulatory molecules and/or the presence of cytokines such as IL-10 and TGF-β[49]. These cells constitute a number of regulatory T cell types, including Tr1 cells, T helper 3 (Th3), some γδ T cells and $CD8^+CD28^-$ T cells. Also, natural T_{reg}-like cells, which express FoxP3, can be induced to differentiate from naïve T cells[50]. T_{reg} are able to inhibit the functions of a variety of

leukocytes from both the innate and adaptive arms of immunity. They can suppress the activation and/or proliferation of naïve $CD4^+$ and $CD8^+$ T cells, and to a lesser extent, the function of effector T cells. They also inhibit the proliferation and effector functions of B cells, the cytolytic functions of NK and NKT cells, and the function and maturation of DC. This suppression can be mediated in various ways: through direct cell-cell contact and accessory molecules such as CTLA-4 and lymphocyte activation gene 3 (LAG3), through secretion of immunosuppressive cytokines such as IL-10 and TGF-β, and also through cytotoxic activity against effector T cells[50].

Recently, a new cell type has come to the fore as a mediator of tumour immunosuppression. Myeloid suppressor cells (MSC), defined as $CD11b^+Gr1^+$ or $Gr1^+CD115^+F4/80^+$ in mice and $CD34^+CD33^+CD15^-CD13^+$ in humans, are a heterogeneous population of immature myeloid cells and myeloid precursors that accumulate in tumour-bearing patients and animals[51-53]. MSC can suppress anti-tumour immune responses either at the tumour site, or by influencing T cell priming in secondary lymphoid organs[51]. They impair T cell function through expression of arginase and inducible nitric oxide synthase (iNOS), the products of which can induce down-regulation of the T cell receptor (TCR) ζ-chain[54, 55], nitration of the TCR and inhibition of its peptide-MHC recognition capacity[56], and blockade of signalling intermediates required for the response of T cells to IL-2[57]. In a separate mechanism, MSC can suppress immune responses through production of TGF-β, which directly suppresses $CD8^+$ CTL[58]. In addition, MSC-derived IL-10 induces the development of antigen-specific T_{reg} cells, indicating that MSC can suppress immune responses indirectly[51]. MSC can inhibit NK cells as well, impairing their cytotoxicity by blocking their production of perforin and by inhibiting their ability to respond to IL-2[59].

1.2.2.4 Overcoming tumour-induced immunosuppression

Overcoming the immunosuppressive mechanisms of tumour cells is a key goal of immunotherapy. A variety of tactics have been employed to accomplish this and elicit effective tumour-specific immune responses. One strategy is to passively immunise breast cancer patients with monoclonal antibodies specific for breast TAA (e.g. trastuzumab (Herceptin), which targets the human epidermal growth factor receptor 2 (HER2) TAA[60]). In addition, a number of trials investigating the potential of cell-based vaccines, in

particular DC-based vaccines, have been conducted[61]. As yet, these therapies have achieved only modest success[60, 61], and further work is underway to optimise the induction of anti-tumour immunity.

One potential way of enhancing the efficacy of vaccination is to promote the co-localisation, and hence activation, of immune effectors such as DC and T cells, either in tumours or in secondary lymphoid organs. As chemotactic factors for leukocytes, chemokines are attractive candidates to achieve this end, and a few studies examining their potential in breast cancer therapy have been published[62-67]. The following Section provides an overview of the chemokines and describes the general properties and functions that have brought them to the attention of tumour immunologists.

1.3 The chemokine family

1.3.1 General properties of chemokines

The chemokines are a family of low molecular weight polypeptide chemotactic cytokines. This family is divided into four subgroups, CXC, CC, C and CX_3C chemokines, based on the number and position of the conserved cysteine residues at the amino-terminal end of the peptide[68] (Table 1.1). Chemokines may also be classified according to their function and expression pattern. Homeostatic chemokines are constitutively expressed, while expression of inflammatory chemokines is up-regulated in response to inflammatory stimuli such as lipopolysaccharide, interleukin (IL)-1 and tumour necrosis factor (TNF)-α[69].

With over 50 members identified to date, the chemokine family has been found to play a multitude of roles in biology, including cell recruitment, leukocyte activation, lymphocyte development and trafficking, lymphoid organ development, development of cell-mediated versus humoral immune bias, inflammation, wound healing, tumour metastasis, angiogenesis and angiostasis[68]. They exert their effects through their interactions with seven transmembrane domain G protein-coupled receptors (Table 1.2). Chemokine-chemokine receptor interactions can be quite promiscuous, with individual chemokines often binding multiple receptors, and receptors binding multiple chemokines. However, each receptor binds a unique panel of ligands[69, 70]. Although in recent years the sphere of

influence of the chemokine family has been found to encompass a wide variety of cell types, much of what is known about chemokine biology comes from studies of their effects on leukocytes. The activity of chemokines on leukocytic and non-leukocytic cell types is generally similar, but it should be kept in mind that extrapolation of the findings in leukocytes to non-leukocytic cell types may not be always valid.

1.3.2 Chemokine receptor signalling

Chemokines generally act as monomers with their N-termini governing the characteristics of receptor binding. The amino-terminus is typically the critical mediator of receptor triggering. Its length determines the ability of a chemokine to bind its receptor, and whether it will act as an agonist or an antagonist[69]. Binding of a chemokine to its receptor induces a conformational change that leads to dissociation of the receptor-associated heterotrimeric G proteins into α and $\beta\gamma$ subunits (Figure 1.4). These G protein subunits can then activate various effector enzymes, including phospholipases, which leads to inositol phosphate production and an increase in intracellular calcium, and protein kinases[71]. Phosphoinositol 3-kinases (PI3K) are also important downstream effectors of chemokine signalling[72], and both the class IA[73] and class IB[74] PI3K have been implicated in chemokine signalling. Activation of PI3K leads to induction of the downstream effectors Rho, Rac and Cdc42[75]. This results in modulation of actin-dependent cellular processes and increased expression and clustering of adhesion molecules[76], ultimately leading to cell chemotaxis.

Mitogen activated protein kinases (MAPK), also known as extracellular-signal regulated kinases (ERK), are activated in response to chemokine binding in a manner dependent on $G\beta\gamma$ signalling through Ras[2] (Figure 1.4). MAPK/ERK signalling is required for a variety of chemokine-induced responses, including degranulation[77], cell adhesion[78], chemotaxis[79], anti-apoptotic responses[80] and gene expression[81]. The requirement for chemokine-induced MAPK/ERK signalling does not seem to be absolute[2], however, and may depend on the particular cell type, the receptor activated and the functional response being examined.

Chemokine binding also induces activation of the JAK/STAT pathway (Figure 1.4). JAK1, 2 and 3 and STAT1, 3 and 5 have been implicated in chemokine receptor signalling, though the particular JAK and STAT activated may depend on the receptor transducing the

signal and/or the cell type examined[82-90]. JAK/STAT activation seems to be independent of $G\alpha_i$ activation[84, 88, 90]; however, whether or not JAK activation is required for $G\alpha_i$ activation seems to depend on the particular chemokine receptor studied[84, 91]. The biological significance of chemokine-induced JAK/STAT signalling has not been well characterised, but it appears to be important for cell adhesion and chemotaxis[83, 87], angiogenesis[83] and for induction of gene transcription[89].

Another outcome of chemokine signalling is receptor internalisation (Figure 1.4). This can occur via one of two pathways: clathrin-mediated endocytosis or lipid raft/caveolae-dependent internalisation[92]. Ligand binding to the receptor induces phosphorylation of serine and threonine residues in the C-terminal tail and intracellular loops by G protein-coupled receptor kinases (GRK). Adaptor proteins such as β-arrestin and adaptin-2 bind to these phosphorylated residues and link the receptors to a clathrin lattice. This enables receptor endocytosis, a process that requires the GTPase activity of dynamin. Once within the endosome, the receptor is dephosphorylated and either degraded, or recycled to the cell surface[92]. Little is known about the mechanism of lipid raft/caveolae-dependent internalisation, but CCR4, CCR5 and CXCR4 have been shown to use this internalisation route, at least in some cell types[92]. The functional role of receptor internalisation remains controversial, but a recent study suggests that it may be important for the fine-tuning of cell migration[93].

1.3.3 Biological functions of chemokines

Chemokines were initially identified as chemotactic factors for leukocytes, and accordingly, the way in which they influence this process has been the subject of intensive study. A critical requirement for chemotaxis is cell polarisation. Chemokine signalling induces actin polymerisation, which is manifested in the extension and retraction of lamellipodia, the driving force of cell movement, at the leading edge of the cell[94]. Polymerisation of actin is the result of the accumulation of phosphatidylinositol 3,4,5-phospate (PIP3), a product of PI3K, at the leading edge. There, PIP3 activates Rac and Cdc42, which in turn polymerise actin and contribute to cell polarisation and chemotaxis[94]. The negative regulator of PIP3 accumulation, phosphatase and tensin homologue deleted on chromosome 10 (PTEN), is restricted to the rear of the cell, thus ensuring a strong differential in PIP3 concentration within the cell and amplifying cell

polarity[72]. Chemokine signalling also induces expression of integrins on the cell surface and their clustering at the leading edge, again contributing to cell polarity[94]. This facilitates adhesion of the cell to surfaces and provides the traction necessary for migration along a chemokine gradient.

The mechanism by which chemokines facilitate leukocyte adhesion to endothelium and transendothelial migration has been well studied[95]. The egress of a leukocyte from the circulation is a process consisting of precise stages, namely tethering, rolling, activation, adhesion, margination, diapedesis (or extravasation) and migration within the tissue. Tethering and rolling are mediated by the selectin family of adhesion molecules expressed either by leukocytes (L-selectin) or the endothelium (E- and P-selectins). Chemokines are immobilised on the endothelial surface, and may be expressed constitutively by the tissue (e.g. CCL19 and CCL21 in the LN) or in response to inflammation (e.g. during tissue damage or infection). Chemokines signal a rolling leukocyte to up-regulate and activate integrins expressed on its surface, increasing their affinity for cell adhesion molecules (CAM) expressed by EC. This causes the cell to stop rolling and enables it to adhere firmly to the endothelium. The cell flattens itself against the endothelial surface ('margination') as it engages an increasing number of integrin-CAM interactions, before beginning diapedesis through the endothelial barrier into the tissue. Chemokines immobilised on ECM components guide the migration of the leukocyte within the tissue[95].

Chemokine signalling not only promotes cell migration, but also activates the effector function of various leukocytes. Chemokines can induce the production of microbicidal oxygen free radicals and enhance phagocytosis by macrophages[71, 96]. They can also promote synthesis of bioactive lipid mediators[71, 96] and degranulation of neutrophils[97, 98], eosinophils[99] and mast cells[75]. Chemokine-induced degranulation results in the release of MMP[100], which facilitate degradation of ECM components and enable leukocytes to migrate within the tissues to sites of inflammation.

Another major biological role of chemokines is the modulation of angiogenesis. Originally, chemokines of the CXC family were classified as angiogenic or angiostatic based on the presence or absence, respectively, of a Glu-Leu-Arg (ELR) motif N-terminal to the first conserved cysteine residue[68]. Two exceptions to this are CXCL12 and CXCL16, which are ELR-negative chemokines with pro-angiogenic properties. Moreover, it has been shown

that CCL21 is able to bind CXCR3 and exert angiostatic effects[101]. Thus it may be more meaningful to classify chemokines as angiogenic or angiostatic depending on which receptors they bind: chemokines that bind CXCR1, CXCR2, CXCR4 and CXCR6 promote angiogenesis, while those binding CXCR3 exert angiostatic effects. Apart from CCL21, other CC chemokines that influence angiogenesis include CCL1[102], CCL2[103], CCL11[104, 105], CCL15[106], CCL16[107] and CCL23[108]. All of these chemokines influence angiogenesis directly through interaction with their receptors expressed on EC. The CC chemokines are largely pro-angiogenic, with the exception of CCL11[104, 105] and CCL21 (discussed above), which are angiostatic. The pro-angiogenic effects of chemokines can be attributed to their ability to promote migration, invasion and differentiation of EC expressing their cognate receptors[102, 103, 106-111]. In some cases, chemokines can enhance EC production of other pro-angiogenic chemokines[107] and cytokines (e.g. VEGF[111]), as well as enhance sensitivity of EC to VEGF signalling[107]. Angiostatic chemokines generally regulate these same processes, but in a negative instead of a positive way[105, 110].

As mentioned previously, the chemokines CXCL12 and CXCL16 were selected for study in this thesis. Like the other members of the chemokine family, they mediate the basic cellular functions described above. Both chemokines are involved in the orchestration of immune responses, and both have been reported to facilitate cancer progression. The characteristics and biological functions of CXCL12 and CXCL16 are described more fully in the following sections, and the data published so far pertaining to their roles in cancer are summarised.

1.4 CXCL12/Stromal cell-derived factor (SDF)-1, CXCR4 and CXCR7

1.4.1 General properties of CXCL12 and its receptors

CXCL12, or stromal cell-derived factor (SDF)-1, is the most widely expressed chemokine. To date, six CXCL12 isoforms, which arise from a single gene through alternative splicing, have been identified in humans: CXCL12α, CXCL12β, CXCL12γ, CXCL12δ, CXCL12ε and CXCL12ϕ[112]. All exhibit chemotactic activity *in vitro*, though their tissue distribution patterns differ. CXCL12α and CXCL12β are the most widely expressed isoforms, found in primary and secondary lymphoid organs, heart, lung, lung, liver, pancreas, placenta, skeletal muscle and kidney, but not peripheral leukocytes[112]. Despite the variation in

expression patterns, the functional differences, if any, between the CXCL12 isoforms are not clear. A comparison of the α and β isoforms revealed differences in proteolytic processing by dipeptidyl-peptidase/CD26[113], which may differentially influence the half-life of the two isoforms and facilitate fine-tuning of the biological activity of CXCL12 *in vivo*. To date, only the CXCL12α and β isoforms have been identified in the mouse.

CXCL12 is the ligand for the chemokine receptor CXCR4, which has attracted much attention for its role as a co-receptor for entry of X4 HIV strains into host cells[114]. Like its ligand, CXCR4 has a broad expression pattern, being found in brain, primary and secondary lymphoid organs, stomach, intestine and kidney. It is expressed on the majority of leukocytes, as well as on EC[111, 115-118], various epithelial cells[119-121], cells of the central nervous system[122] and primordial germ cells[123, 124]. The expression of both CXCR4 and CXCL12 is regulated by hypoxia[125, 126]. This is important for recruitment of haematopoietic progenitor cells to hypoxic niches in the bone marrow and for recruitment of circulating endothelial progenitor cells for neovascularisation and repair of ischaemic tissues[126]. Hypoxic regulation of CXCR4 and CXCL12 also has significance for the growth and metastasis of tumours, which frequently become hypoxic. As discussed in Section 1.4.3, CXCR4 and CXCL12 both have pro-tumorigenic properties, and the up-regulation of this receptor/ligand pair by hypoxia may confer a growth advantage on malignant cells[125, 127].

For many years it was thought that the interaction between CXCL12 and CXCR4 was monogamous. However, recent work by two groups has identified the orphan G protein-coupled receptor RDC1 as a receptor for CXCL12[128, 129]. To acknowledge this, it has been proposed that RDC1 be renamed CXCR7 in accordance with the chemokine receptor nomenclature. Though there is agreement that CXCL12 does indeed bind CXCR7, controversy remains regarding the expression pattern of CXCR7 and the functional consequences of the CXCL12-CXCR7 interaction. One group reported that CXCR7 was expressed on various human leukocyte subpopulations and that CXCL12 could induce CXCR7-dependent chemotaxis of peripheral blood leukocytes[128, 130]. The second group, in contrast, identified surface CXCR7 expression only on tumour cell lines, human EC, and mouse foetal liver cells[129]. They also reported that CXCL12 ligation of CXCR7 did not lead to the typical chemokine-induced responses of calcium mobilization and

chemotaxis[129]. The reason for the inconsistencies in the findings of the two groups is not apparent, and the precise function and expression pattern of CXCR7 await further clarification. What is clear is that work on CXCL12 and CXCR4, both previous and current, must be interpreted carefully in light of the identification of CXCR7 as a receptor for CXCL12.

A single amino acid substitution in the N-terminus of the CXCL12 peptide sequence converts CXCL12 into a potent antagonist of its wild-type activity. The $CXCL12_{(P2G)}$ analogue of CXCL12 has an amino acid sequence identical to that of CXCL12 with the exception of a glycine substituted for the proline residue at position 2. This analogue has only 3-fold lower binding affinity for the CXCL12 receptor CXCR4 than wild-type CXCL12. However, the amino acid substitution results in a complete loss of activity, as shown by the inability of the analogue to induce calcium mobilization and receptor internalisation in the human CEM T cell line. Moreover, $CXCL12_{(P2G)}$ is able to inhibit chemotaxis of CEM cells towards CXCL12[131].

1.4.2 Physiological functions of CXCL12

1.4.2.1 Functions of CXCL12 in the haematopoietic system

The significance of the role of CXCL12 and CXCR4 in physiology is suggested by the high degree of evolutionary conservation across species: they share 98 and 94% homology between human and mouse, respectively[69]. A CXCL12 homologue has also been identified in the lower vertebrate *Xenopus laevis*, which is capable of inducing the migration of human lymphoblastic cells expressing CXCR4[132]. Further support for the crucial biological role of this chemokine/receptor pair has come from studies of CXCL12 and CXCR4 knockout mice. For both, the genetic deletion is lethal, and the mice display similar severe disruptions to haematopoiesis, cardiac development[133] and vascularisation of the gastrointestinal tract[134, 135]. In addition, CXCR4 knockout mice display abnormal cerebellar development[135].

Not surprisingly, then, multiple functions for CXCL12 and CXCR4 have been identified. Though it was originally characterised as a growth stimulatory factor for pre-B cells[136], CXCL12 has since been found to influence a variety of cells of both haematopoietic and

non-haematopoietic origin (Table 1.3). CXCL12 is highly expressed in the bone marrow where it is critical for the engraftment[137] and retention[138] of haematopoietic stem cells (HSC). Blockade of CXCR4 by the small molecule inhibitor AMD3100 has been shown to be an effective strategy for mobilising HSC for transplantation[139-141]. As well as stimulating growth of pre-B cells[136], CXCL12 and CXCR4 serve to retain B cell and myeloid progenitors within the bone marrow microenvironment[142]. Despite expressing CXCR4 throughout their maturation, B cells are only responsive to CXCL12 at certain stages of their development[143]. This property is thought to enable B cells to migrate from the primary to the secondary lymphoid organs and to position themselves optimally within secondary lymphoid organs during activation[144].

CXCL12 plays a role in the survival, expansion and differentiation of early thymocytes within the thymus. Thymic precursors in foetal blood are responsive to CXCL12, which is thought to induce their migration to and population of the thymic anlage[145]. Immature, but not mature, thymocytes respond to CXCL12[146], which may be important for their recruitment to the cortex for positive selection[147]. There is also evidence to suggest that CXCL12 and CXCR4 are required for thymic egress. Blockade of CXCR4 or elimination of the CXCL12 gradient leads to retention of mature T cells within the thymus, indicating that CXCL12 may provide a chemorepellent signal to induce emigration of such cells to the periphery[148].

In addition to its effects on haematopoietic progenitors, B cells and thymocytes, CXCL12 is a potent chemotactic factor for mature lymphocytes, monocytes[149], mature DC[150], NK cells[151, 152], NKT cells[153] and megakaryocyte progenitors[154]. In mature T cells, the action of CXCL12 is not limited to induction of chemotaxis. This chemokine has been implicated in co-stimulation of T cells upon encounter with antigen, though the signalling mechanisms underlying this process are only just beginning to be elucidated. Recent work from Karen Hedin's lab has shown that CXCL12 stimulates physical association of CXCR4 and the TCR[81]. CXCL12 up-regulates activation markers on T cells in a CXCR4-dependent manner and promotes their proliferation and production of IFN-γ, IL-2 IL-4 and IL-10[81, 155]. In contrast to the findings outlined above, Peacock et al. found that anti-CD3 stimulation of $CD4^+$ T cells reduced surface CXCR4 expression and inhibited CXCL12-induced chemotaxis[156]. The reason for this discrepancy is not clear, however differences in

T cell activation protocols could be one explanation. Others have reported that CXCR4 is down-regulated upon TCR activation[157, 158], but in those experiments, CXCR4 expression was examined days after TCR stimulation, rather than within hours as in the studies described above. One study demonstrated that CXCL12 induced apoptosis of Jurkat T cells by induction of surface Fas/CD95 and intracellular FasL/CD95L expression[159]. Since activation-induced cell death is important for the resolution of immune responses[160], it may be that one role of CXCL12 in T cell activation is to sensitise activated cells to apoptosis at late stages of the immune response. IL-2 is an important mediator of this phenomenon, in addition to its role in inducing clonal expansion of T cells at the initiation of an immune response[161]. Thus down-regulation of CXCR4 may be an important step to allow antigen-experienced T cells to avoid activation-induced cell death and instead enter the memory pool.

1.4.2.2 Functions of CXCL12 in non-haematopoietic tissues

The action of CXCL12 and CXCR4 is not restricted to the haematopoietic system (Table 1.3), as evidenced by the severe defects in multiple organs arising in $CXCL12^{-/-}$ and $CXCR4^{-/-}$ mice. Indeed, disruptions to the vascularisation of the gastrointestinal tract in these mice provided the first indication of a role for CXCL12 and CXCR4 in angiogenesis[134]. Expression of both CXCL12[117, 162] and CXCR4[111, 115, 118] is detectable in EC; CXCL12 can also reciprocally up-regulate expression of VEGF[116, 163]. CXCL12 is a potent chemotactic factor for EC[162] and promotes endothelial tube formation *in vitro*[116, 117, 162], as well as neovascularisation in various *in vivo* models of angiogenesis[111, 116, 117]. CXCL12 not only acts on differentiated EC but also recruits endothelial progenitor cells (EPC) to ischaemic tissues: CXCL12 gene transfer into ischaemic muscle tissues was found to mobilize EPC into the peripheral blood, increase capillary density in, and augment recovery of blood perfusion to, these tissues[164].

CXCL12 and CXCR4 are important for migration of a variety of non-haematopoietic progenitor cells. They are required for primordial germ cell (PGC) migration and colonisation of the gonads in both zebrafish[165, 166] and mice[123, 124]. Muscle progenitors express CXCR4 and CXCL12 is expressed along the migratory routes and at the target sites of these cells[167]. Development of the retina is dependent on the interaction of CXCL12 and CXCR4, where they provide important guidance signals for axons. Unlike its

effect on leukocytes and other cells, CXCL12 does not seem to have any direct chemoattractive or chemorepulsive effects on axons, rather it acts by antagonising the repellent effects of Slit-2 in a CXCR4-dependent manner[168, 169].

Within the central nervous system, CXCL12 and CXCR4 are also key players, as is indicated by the developmental defects in the cerebellum of CXCR4 knockout mice. CXCR4 is expressed on a variety of neural cells, and its ligation by CXCL12 leads to enhanced migration and proliferation, stimulation of cytokine production and even the production of pain through excitation of nociceptive neurons (reviewed in ref. 122). Epithelial cells of the alveoli, bladder and mammary tissue are responsive to CXCL12 and express the CXCR4 receptor[120]. Human colon epithelial cells also express CXCR4 and produce CXCL1 and CXCL8 in response to CXCL12 stimulation[170, 171]. Moreover, down-regulation of CXCR4 induces differentiation of these cells, and may play a role in maintenance and renewal of the colonic epithelium[172]. CXCL12 may play a role in trophoblast survival during pregnancy as cultured human cytotrophoblasts treated with anti-CXCR4 antibody displayed reduced cellularity[173]. CXCL12 is also important for recruitment and differentiation of osteoclast precursors[174, 175], and it has been associated with the osteolytic bone disease characteristic of multiple myeloma[176].

1.4.3 CXCL12 and CXCR4 in cancer

In a recent landmark study, CXCR4 was shown to be expressed by various human breast cancer cells and cell lines[6]. When injected either intravenously or into the mammary fat pad of severe combined immunodeficient (SCID) mice, these CXCR4-expressing cells metastasized to organs where CXCL12 was highly expressed, namely the lungs, liver, LN and bone marrow. This migration pattern mimics the metastatic pattern typical of human breast cancer. A subsequent study of human breast carcinomas found that increased expression of CXCR4 correlated with higher incidence of LN metastases[177]. CXCR4 has since been found to be expressed on a variety of cancer cells and cell lines including cervical cancer[178], colorectal cancer[179, 180], head & neck cancer[181], nasopharyngeal carcinoma[182], non-small cell lung carcinoma[183], melanoma[184, 185], neuroblastoma[186, 187], oral squamous cell carcinoma[188, 189], pancreatic cancer[190, 191], renal carcinoma[127, 192], rhabdomyosarcoma[193] and prostate cancer[194, 195]. Such widespread expression of CXCR4 in cancerous tissues is compelling evidence for the involvement of the CXCL12-CXCR4 axis

in malignant progression. The mechanisms by which this receptor-ligand pair influence tumour growth and metastasis are only beginning to be understood, but it is apparent that CXCL12 and CXCR4 have a variety of tumour-promoting functions. For the purposes of this thesis, these functions will be examined in the context of breast cancer, though they have been reported in other forms of cancer as well.

CXCL12 and its receptors are not only involved in processes specific to metastasis. Indeed, many of their functions promote primary tumour growth and progression in addition to facilitating the metastasis of malignant cells. Various studies have demonstrated a role for CXCL12 in promoting breast tumour cell proliferation[196-199]. Oestrogen can enhance tumour cell expression of CXCL12[198, 200], or CXCL12 can be supplied by stromal components to act in a paracrine fashion to enhance tumour cell proliferation[196, 199].

Another pro-tumour property of CXCL12 is its ability to promote cell survival and prevent apoptosis. For example, treatment of mice bearing glioblastoma or medulloblastoma with the CXCR4 inhibitor AMD3100 inhibited tumour growth *in vivo* by increasing apoptosis of tumour cells[201]. Very little has been reported regarding the ability of CXCL12 to directly promote survival of breast cancer cells, although CXCL12 was found to prevent apoptosis of cultured MDA-MB-231 cells upon withdrawal of serum from the culture medium[202]. Interestingly, introducing the novel atypical CXCL12 receptor, CXCR7, into the recently redefined human melanoma cell line MDA-MB-435[203] enhanced cell survival and reduced apoptosis under low serum conditions *in vitro*[129]. This cell line produces endogenous CXCL12[204], indicating that the CXCL12-CXCR7 interaction could be a relevant means by which cancer cells gain a survival advantage. Specifically antagonizing CXCR7 inhibited tumour growth in mouse models of lung carcinoma and lymphoma[129], though this effect is yet to be reported in breast cancer.

A defining feature of malignant tumours is their ability to invade surrounding tissues. The ability of CXCL12 and CXCR4 to enhance breast tumour growth and metastasis is frequently associated with their ability to induce invasion of breast cancer cells *in vitro*[197, 205]. CXCL12 can promote adhesion of breast cancer cells to the ECM components fibronectin and collagen[206], and knockdown of either CXCL12[204] or CXCR4[197, 205] is sufficient to block invasion of breast cancer cells through Transwells coated with ECM. Invasion also may be facilitated through MMP induction: both MMP2 and MMP9 are

produced by breast cancer cells in response to CXCL12 stimulation[207]. In addition, CXCL12 can induce breast cancer cell invasion though endothelial monolayers in a CXCR4-dependent manner[208, 209]. This highlights the potential role of CXCL12, acting through CXCR4, in the entry of metastatic cells into the circulation and their extravasation at secondary sites.

Although there is considerable evidence for the angiogenic function of CXCL12 and CXCR4, and indeed, that this function is a significant factor in the progression of various cancers, there is little direct evidence of a role for CXCL12/CXCR4 in vascularisation-dependent progression of breast cancer. It has been shown that VEGF up-regulates CXCR4 expression in a breast cancer cell line, and that these cells migrate towards CXCL12 in a VEGF-dependent manner[210]. While these data point to an interplay between CXCL12/CXCR4 and VEGF as important for migration of breast tumour cells, no link to the angiogenic functions of these molecules was demonstrated in this system. In another study, fibroblasts isolated from invasive human breast carcinomas were implanted along with breast carcinoma cells into mice, and these fibroblasts enhanced tumour growth more effectively than fibroblasts isolated from normal mammary tissue[196]. This effect was in part dependent on CXCL12 secretion by the fibroblasts, which recruited endothelial progenitors and promoted vascularisation of the tumours[196]. This work demonstrates the potential for CXCL12 produced by host stromal cells to contribute to tumour vascularisation.

Somewhat paradoxically, one other way in which CXCL12 may serve to promote tumour progression is by enhancing immune evasion, although very little has been published in this area, particularly with respect to breast cancer. A recent report showed that $CXCR4^{hi}$ MDA-MB-231 cells expressed lower basal and IFN-γ-inducible levels of MHC class II molecules than did $CXCR4^{lo}$ cells[211]. CXCL12 was able to down-regulate MHC class II mRNA expression in both cell types, an effect that was reduced in CXCR4 siRNA-expressing cells. While by no means conclusive, these data implicate CXCL12 in assisting tumour cells to 'hide' from T cells by down-regulating their expression of antigen-presenting molecules. A handful of studies have implicated CXCL12 in immune evasion in other forms of cancer. Antagonism of CXCR4 with the small molecule inhibitor T22 enhanced the susceptibility of melanoma cells to CTL killing[212]. Moreover, CXCL12

induced expression of CCL5 by melanoma cells, which in turn induced apoptosis of tumour-infiltrating lymphocytes[213]. These data suggest that CXCL12 may not only promote immune evasion by tumour cells, but also actively induce immune suppression. Further support for this idea came from a study of ovarian cancer. Ovarian tumour cells were found to express high levels of CXCL12, which resulted in the recruitment of plasmacytoid dendritic cell (pDC) precursors to the tumour mass where they were protected from apoptosis in a CXCR4-dependent manner. These pDC precursors induced IL-10 production by T cells, which inhibited T cell activation and tumour destruction[214].

A small number of studies have investigated the potential anti-tumour properties of CXCL12 by inducing its overexpression in various models of cancer, including fibrosarcoma[215], ovarian carcinoma[215], leukaemia[216], myeloma[217], lung carcinoma[218], colon carcinoma[218] and melanoma[216, 218, 219]. In the majority of cases, CXCL12 expression induced anti-tumour immune responses that were dependent on T cell activity[215-218]. In one study of melanoma, however, engineered expression of high levels of CXCL12 inhibited the anti-tumour immune response[219]. This effect was attributed to the ability of high levels of CXCL12 to induce chemorepulsion of tumour-specific CTL away from the tumour mass, and it was proposed that such a phenomenon might represent a tumour immune evasion strategy[219]. Accumulation of such high levels of CXCL12 in a physiological setting has not been demonstrated and seems unlikely. However, the data suggest that in an immunotherapeutic setting, care should be exercised in selecting the dose of CXCL12 to be used to generate an optimal anti-tumour response. To date, no studies have investigated the effect of CXCL12 overexpression in breast cancer cells.

1.5 CXCL16 and CXCR6

1.5.1 General properties of CXCL16 and CXCR6

CXCL16 is one of the most recently identified chemokines. Whilst it is a member of the CXC subfamily, CXCL16 shows highest amino acid sequence homology to the CC chemokine CCL4 [220], although it has significant structural homology to the transmembrane chemokine CX_3CL1. Like CX_3CL1, CXCL16 is comprised of a globular extracellular chemokine domain attached via a mucin-like stalk to a transmembrane domain and a short intracellular cytoplasmic tail[221]. CXCL16 is expressed *in vivo* in transmembrane form, but

it can also be cleaved by proteases such as ADAM10, releasing the chemokine domain as a soluble factor[222, 223].

CXCL16 is expressed by DC[221], macrophages, B cells[220], and bone marrow stromal cells[224], and at lower levels on aortic smooth muscle cells[222, 225], EC[222, 226] and T cells[227]. It binds to the receptor CXCR6, which can act as a co-receptor for certain strains of HIV[228, 229]. Expression of CXCR6 so far seems to be limited to activated $CD8^+$ and $CD4^+$ T cells[230], particularly Type I polarised T cells[231], natural killer[229] and NKT cells[221], B cells at low levels[229], plasma cells[224], human skin-derived DC and both immature and mature blood monocyte-derived DC[232].

1.5.2 Physiological functions of CXCL16

Various biological roles for CXCL16 have been reported (Table 1.4). The expression pattern of CXCR6 on Type I polarised T cells and the induction of CXCL16 by the Type I cytokine IFN-γ[222, 233, 234], suggests that this chemokine/receptor pair has a significant role in cell-mediated immunity. Further support for this hypothesis comes from evidence that CXCL16 induces IFN-γ production by $CD8^+$ T cells, at least *in vitro*[235], suggesting that CXCL16 and IFN-γ form a positive feedback loop to perpetuate Type I immune responses. In addition, the expression pattern of CXCL16 by various APC and its cognate receptor on T cells raises the possibility that the CXCL16/CXCR6 interaction may contribute to the activation of T cells, though this has yet to be verified. Certainly, $CXCR6^+$ T cells adhere firmly to cells expressing surface CXCL16, in a manner that does not require CXCR6 signalling, suggesting direct involvement of the chemokine and its receptor in T cell activation[236]. In addition, CXCL16 is capable of binding to bacteria, facilitating their uptake and destruction by phagocytes[237].

Undoubtedly, CXCL16 and CXCR6 are required for the recruitment of various leukocytes as part of the immune response. Several studies have demonstrated that blockade of the CXCL16-CXCR6 interaction inhibits recruitment of lymphocytes to sites of inflammation[238-240]. CXCR6 is frequently associated with leukocytic infiltrates in inflammatory hepatitis[241-243], and indeed, $CXCR6^{-/-}$ mice are less susceptible to the disease[244]. Elevated CXCL16 expression has been documented in the synovia of

rheumatoid arthritis patients, and $CXCR6^+$ cells are frequently observed in inflamed joints[245-247]. Moreover, an anti-CXCL16 antibody reduces the clinical arthritis score of mice with collagen-induced arthritis, with associated reduction of inflammatory cell infiltration and bone destruction in the synovium[246]. Of course, not all CXCL16/CXCR6-mediated immune responses are deleterious. CXCL16 and CXCR6 are important in the development of protective immune responses to acute bacterial infections. This was demonstrated in a model of acute *Salmonella* infection where depletion of CXCL16 resulted in increased bacterial colonisation of the spleen and liver and reduced IFN-γ production by splenic lymphocytes[238].

Interestingly, under homeostatic conditions, the majority of $CXCR6^+$ cells in the liver are NKT cells, which appear to 'patrol' hepatic sinusoids. CXCR6 deficiency leads to reduced survival of these cells, though not their patrolling speed or pattern [244]. The exact function of these cells is not clear, however CXCL16 expression has been associated with increased NKT cell infiltration of cardiac allografts and graft tolerance[248]. Thus, it seems that under certain conditions, CXCL16 recruits inflammatory cells, while under other conditions, CXCL16 recruits cells that have an immunoregulatory role.

Within the vasculature, CXCL16 has been found in both aortic smooth muscle cells (ASMC)[222, 225] and EC[222, 226]. Recent work revealed that CXCL16 could enhance the proliferation, migration and tube formation *in vitro* of HUVEC[249], suggesting that this chemokine may have angiogenic properties. The ability of CXCL16 to induce vascularisation *in vivo* is yet to be demonstrated, however.

The expression of CXCL16 on macrophages has significance for the pathogenesis of atherosclerosis. Minami et al. cloned CXCL16 as a surface receptor for oxidised low density lipoproteins (oxLDL) on macrophages, and demonstrated that it is localised to lipid-laden macrophages present in atherosclerotic plaques[250]. Smooth muscle cells from human atherosclerotic lesions also have been found to express CXCL16, and cultured ASMC up-regulate CXCL16 expression and oxLDL uptake in response to IFN-γ[233]. The authors of these studies concluded that CXCL16 has a pro-atherosclerotic effect, but subsequent findings have led others to dispute these conclusions. In one study, reduced plasma CXCL16 was found to correlate with coronary artery disease[251], suggesting a

possible atheroprotective effect for CXCL16. Indeed, CXCL16$^{-/-}$ LDL receptor$^{-/-}$ double knockout mice display accelerated atherosclerosis[252]. Macrophages from CXCL16$^{-/-}$ mice have a reduced capacity to bind and internalise oxLDL *in vivo*, indicating that CXCL16-scavenging of oxLDL is a relevant process in atherosclerosis, and may actually have a protective effect[252].

1.5.3 CXCL16 and CXCR6 in cancer

To date, very little is known about the role of this chemokine receptor/ligand pair in tumorigenesis. CXCL16 and CXCR6 expression have been reported in cell lines derived from Hodgkin and Reed-Sternberg cells from Hodgkin's disease (HD). It was suggested that CXCL16 production by HD tissues may lead to the recruitment of CXCR6-expressing plasma cells that are commonly found in the inflammatory cell infiltrates characteristic of HD[253].

Two groups have looked at the CXCL16/CXCR6 axis in human colorectal cancer. One study demonstrated that CXCL16 protein expression in TAM is suppressed compared with that in macrophages in normal adjacent tissue[254]. Further work revealed a similar trend in CXCR6 expression, with suppression in tumour tissue compared to normal tissue observed in 41% of cases[255]. The second group found that CXCL16 expression by colorectal tumour cells correlated with increased infiltration of the tumours by $CD4^+$ and $CD8^+$ lymphocytes and an improved prognosis[256]. This highlights the potential role of CXCL16 and CXCR6 in the anti-tumour immune response. It is possible that reduced expression of CXCL16 by TAM and tumour cells could inhibit recruitment and activation of CXCR6-expressing T cells, thereby providing a mechanism by which the tumour can evade their destructive cytolytic activity.

Interestingly, CXCR6 may have an anti-tumour function independent of the immune response. Sasai and colleagues reported that 3 to 4 per cent of mice lacking one or both functional copies of CXCR6 develop medulloblastoma[257]. Tumours from these mice displayed elevated activity of the sonic hedgehog signalling pathway, and were inhibited by a small molecule inhibitor of Smoothened, a signalling mediator within this pathway. These data point to a potential anti-tumour function of CXCR6 through blockade of the

sonic hedgehog mitogenic pathway, although a direct effect of CXCR6 signalling on this pathway is yet to be demonstrated.

It should be noted that the anti-tumour effect of CXCL16 and CXCR6 may not be universal for all tumours. In contrast to the observations of TAM in human colorectal cancer, immunohistochemical analysis of mouse fibrosarcomas revealed increased CXCL16 expression by resting TAM compared to normal macrophages[258], suggesting that in this context, TAM CXCL16 expression provides a survival advantage to the tumours. Ludwig et al. found that CXCL16 was highly expressed and continuously shed by human glioma cells, in contrast to normal brain tissue. Since CXCL16 induced proliferation of mouse glial cells and CXCR6 mRNA could be detected in glioma cells, it was hypothesised that CXCL16 could act in an autocrine fashion to enhance the growth and/or survival of these cells[259]. CXCL16 could also promote tumour growth indirectly, as activated T cells recruited to the tumour site may produce factors that promote the survival and growth of tumour cells. This was postulated to be the case in Kaposi's sarcoma[260].

There is some indication that the CXCL16-CXCR6 axis could play a role in metastasis, at least in some forms of cancer. Functional CXCR6 was detected in several human nasopharyngeal carcinoma (NPC) cell lines. CXCR6 was also detected in biopsies of regional and metastatic NPC from human patients, though expression in the primary tumour was only observed in patients with liver metastasis[261]. A subsequent study reported that CXCL16 was required for migration of mammary epithelial tumour cells induced by Akt1, and that Akt1 deficiency led to reduced lung metastasis of these cells[262]. Furthermore, Seidl et al. detected *de novo* expression of CXCR6 protein in primary melanomas and melanoma metastases, though it was not expressed in melanoma cell lines[263]. This highlights the need for care in extrapolating results from cancer cell lines to primary tissues. It also raises the possibility that CXCL16 and CXCR6 may play a more significant role in tumorigenesis than studies of tumour cell lines have revealed thus far.

1.6 The Research Project

As described in the preceding sections, CXCL12 and CXCL16 and their receptors are implicated in tumour progression and metastasis, or in processes that may be co-opted by tumours to enhance their progression, such as cell survival, proliferation, migration and

angiogenesis. In addition, these chemokines can participate in the orchestration of immune responses. An intriguing dichotomy of function for CXCL12 and CXCL16 in malignancy thus emerges: do they act to promote tumour development, or to enhance anti-tumour immune responses? Either way, the literature indicates that these chemokines have the potential to affect breast cancer progression, and this led to the following hypotheses:

(1) that expression of wild-type CXCL12/CXCL16 by breast tumour cells will influence the progression of breast cancer *in vivo.*

(2) that expression of the respective antagonists of these chemokines by breast tumour cells will exert an opposing influence to wild-type chemokine expression on the progression of breast cancer *in vivo*.

To test these hypotheses, the experimental aims were as follows:

(1) To develop DNA constructs expressing wild-type chemokines and their respective antagonists.

(2) To characterize the 4T1.2 mouse model of breast cancer in terms of chemokine receptor/ligand expression and function.

(3) To generate and characterize chemokine- and antagonist-expressing cell lines.

(4) To investigate the effects of chemokine and antagonist expression on tumour progression *in vivo*.

The results of the experimental investigation of the aforementioned hypotheses and the implications of these findings will be addressed in the subsequent chapters of this thesis.

Table 1.1 Chemokine nomenclature[a, b].

Systematic Name	Human Ligand	Mouse Ligand	Receptor(s)
CXC family			
CXCL1	GROα/MGSAα	Gro/KC?	CXCR2>CXCR1
CXCL2	GROβ/MGSAβ	Gro/KC?	CXCR2
CXCL3	GROγ/MGSAγ	Gro/KC?	CXCR2
CXCL4	PF4	PF4	CXCR3b
CXCL5	ENA-78	LIX?	CXCR2
CXCL6	GCP-2	CKα-3	CXCR1, CXCR2
CXCL7	NAP-2	Unknown	CXCR2
CXCL8	IL-8	Unknown	CXCR1, CXCR2
CXCL9	MIG	MIG	CXCR3a
CXCL10	IP-10	IP-10/CRG-2	CXCR3a
CXCL11	I-TAC	I-TAC	CXCR3a
CXCL12	SDF-1α/β	SDF-1 α/β	CXCR4, CXCR7
CXCL13	BLC/BCA-1	BLC/BCA-1	CXCR5
CXCL14	BRAK	BRAK	Unknown
(CXCL15)	Unknown	Lungkine	Unknown
CXCL16	CXCL16	CXCL16	CXCR6
C family			
XCL1	Lymphotactin/SCM1α/ATAC	Lymphotactin	XCR1
XCL2	SCM1β	Unknown	XCR1
CX_3C family			
CX_3CL1	Fracktalkine	Neurotactin	CX_3CR1
CC family			
CCL1	I-309	TCA-3/P500	CCR8
CCL2	MCP-1/MCAF/TDCF	JE?	CCR2
CCL3	MIP-1α/LD78α	MIP-1α	CCR1, CCR5
CCL3L1	LD78β	Unknown	CCR1, CCR5
CCL4	MIP-1β	MIP-1β	CCR5
CCL5	RANTES	RANTES	CCR1, CCR3, CCR5
(CCL6)	Unknown	C10/MRP-1	Unknown
CCL7	MCP-3	MARC?	CCR1, CCR2, CCR3
CCL8	MCP-2	MCP-2?	CCR3
(CCL9/10)	Unknown	Unknown	Unknown
CCL11	Eotaxin	Eotaxin	CCR3
(CCL12)	Unknown	MCP-5	CCR2
CCL13	MCP-4	Unknown	CCR2, CCR3
CCL14	HCC-1	Unknown	CCR1
CCL15	HCC-2/Lkn-1/MIP-1δ	Unknown	CCR1, CCR3
CCL16	HCC-4/LEC/LCC-1	LCC-1	CCR1
CCL17	TARC	TARC	CCR4
CCL18	DC-CK1/PARC/AMAC-1	Unknown	Unknown
CCL19	MIP-3β/ELC/exodus-3	MIP-3β/ELC/exodus-3	CCR7
CCL20	MIP-3α/LARC/exodus-1	MIP-3α/LARC/exodus-1	CCR6
CCL21	6Ckine/SLC/exodus-2	6Ckine/SLC/exodus-2	CCR7
CCL22	MDC/STCP-1	ABCD-1	CCR4

Systematic Name	Human Ligand	Mouse Ligand	Receptor(s)
CC family (continued)			
CCL23	MPIF-1/CKβ8/CKβ8-1	Unknown	CCR1, CCR12
CCL24	Eotaxin-2/MPIF-2	Unknown	CCR3
CCL25	TECK	TECK	CCR9
CCL26	Eotaxin-3	Unknown	CCR3
CCL27	CTACK/ILC	ALP/CTACK/ILC/ESkine	CCR10
CCL28	MEC	Unknown	CCR3/CCR10

a, Adapted from refs 264 & 75.
b, Abbreviations: *AMAC*, alternative macrophage activation-associated CC chemokine; *ATAC*, activation-induced chemokine-related molecule; *BCA*, B cell attracting chemokine; *BLC*, B lymphocyte chemoattractant; *BRAK*, breast and kidney expressed chemokine,; *CK*, chemokine; *CTACK*, cutaneous T cell-activating chemokine; *DC-CK*, dendritic cell-derived CC chemokine; *ELC*, EBL-1-ligand chemokine; *ENA*, epithelial neutrophil activating; *GCP*, granulocyte chemotactic protein; *GRO*, growth-related oncogene; *HCC*, human CC chemokine; *ILC*, IL-11 receptor alpha-locus chemokine; *IP*, IFN-γ inducible protein; *I-TAC*, IFN-inducible T cell alpha chemoattractant; *KC*, keratinocyte chemoattractant; *LARC*, liver and activation-regulated chemokine; *LCC*, liver-specific CC chemokine; *LEC*, liver-expressed chemokine; *LIX*, LPS-induced chemokine; *Lkn*, leukotactin; *MCAF*, monocyte chemotactic and activating factor; *MCP*, monocyte chemoattractant protein; *MDC*, macrophage-derived chemokine; *MEC*, mucosae-associated epithelial chemokine; *MGSA*, melanoma growth stimulatory activity; *MIG*, monokine induced by IFN-γ; *MIP*, macrophage inflammatory protein; *MPIF*, myeloid progenitor inhibitory factor; *MRP*, MIP-related protein; *NAP*, neutrophil-activating peptide; *PARC*, pulmonary and activation-regulated chemokine; *PF*, platelet factor; *RANTES*, regulated on activation normal T cell expressed and secreted; *SCM*, single C motif; *SDF*, stromal-derived factor; *SLC*, secondary lymphoid tissue chemokine; *STCP*, stimulated T cell chemoattractant protein; *TARC*, thymus and activation-regulated chemokine; *TCA*, T cell activation gene; *TDCF*, tumour-derived chemotactic factor; *TECK*, thymus-expressed chemokine.

Table 1.2 The chemokine receptors and their biological functions[a].

NOTE: This table is included on page 33 of the print copy of the thesis held in the University of Adelaide Library.

a, Adapted from ref 70.

Table 1.3 The biological functions of CXCL12 and CXCR4[a].

Cell Type/Tissue	Role of CXCL12	References
HSC	Promotes engraftment and retention in the bone marrow	137-141
B cells	Growth factor for early B cells	136
	Optimal localisation of B cells in secondary lymphoid organs	144
Thymocytes	Population of thymic anlage	145
	Thymocyte development	147
	Thymic egress	148
T cells	Co-stimulation of $CD4^+$ T cell activation	81, 155, 265
	HIV co-receptor	114
Mature leukocytes	Chemotaxis	149-154
Endothelial cells	Induction of VEGF expression	115, 116, 162, 163, 266
	Increased response to VEGF	
	Chemotaxis	
	Vascularisation	
Endothelial precursors	Mobilisation	267
	Vascularisation in response to ischaemia	164
PGC	Migration	123, 124, 165, 166
	Colonisation of gonads	
Tissue progenitors	Migration during development	167
Retinal axons	Survival & migration	168, 169, 268
Nervous system	Migration & proliferation of neural cells	122
	Induction of pain through triggering of nociceptive neurons	
Epithelial cells	Renewal of colonic epithelium?	172
	Ca^{2+} flux (alveolar epithelial cells)	120
Trophoblasts	Cytotrophoblast survival	173
Osteoclasts	Recruitment & differentiation	174, 175

a, Abbreviations: *HIV*, human immunodeficiency virus; *HSC*, haematopoietic stem cell; *PGC*, primordial germ cell; *VEGF*, vascular endothelial growth factor.

Table 1.4 The biological functions of CXCL16 and CXCR6[a].

Cell Type/Tissue	Role of CXCL16/CXCR6	References
Type 1 polarised T cells	Recruitment to sites of inflammation	238-240, 246
NKT cells	Immunosurveillance of liver? Graft tolerance	244 248
Macrophages	Atherosclerosis	234, 250, 252
Endothelial cells	Proliferation Chemotaxis Tube formation	249
ASMC	Atherosclerosis	225, 233, 269
PBL	HIV/SIV co-receptor	228, 229, 270

a, Abbreviations: *ASMC*, aortic smooth muscle cells; *HIV*, human immunodeficiency virus; *PBL*, peripheral blood leukocytes; *SIV*, simian immunodeficiency virus.

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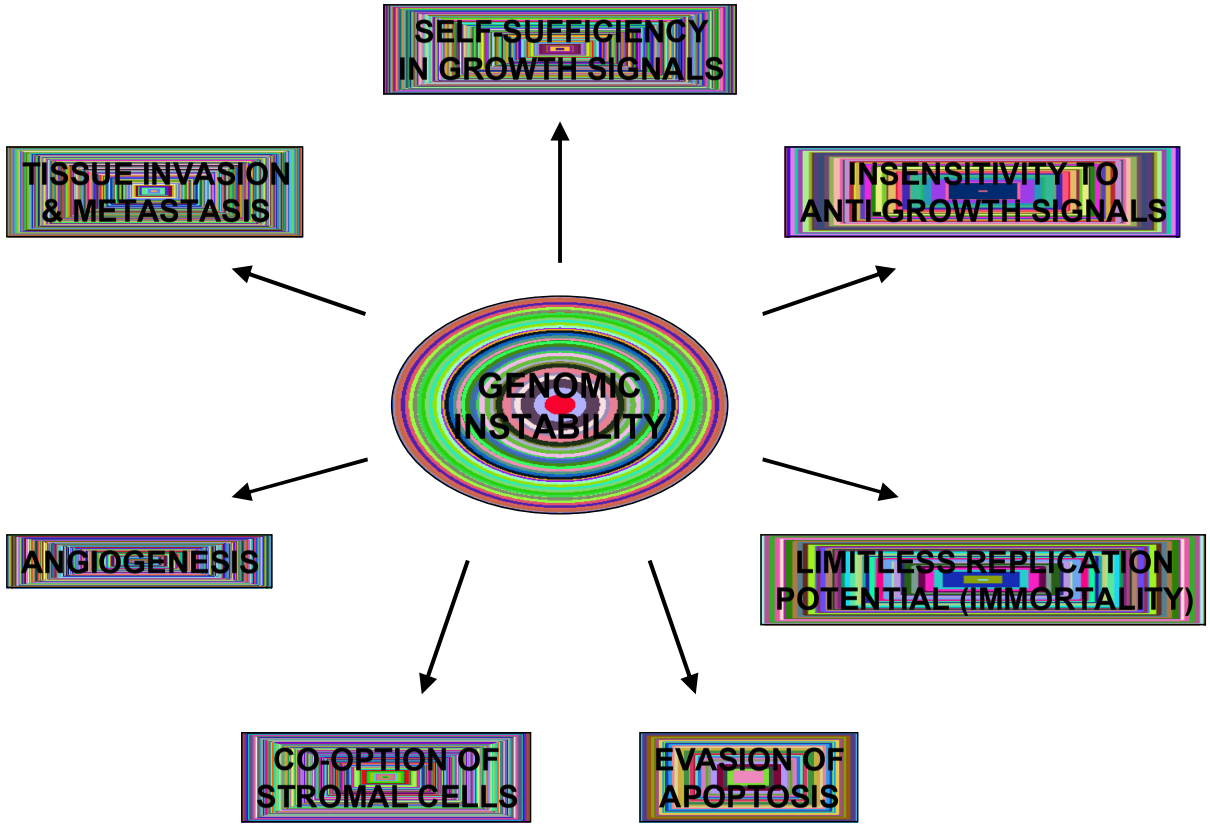
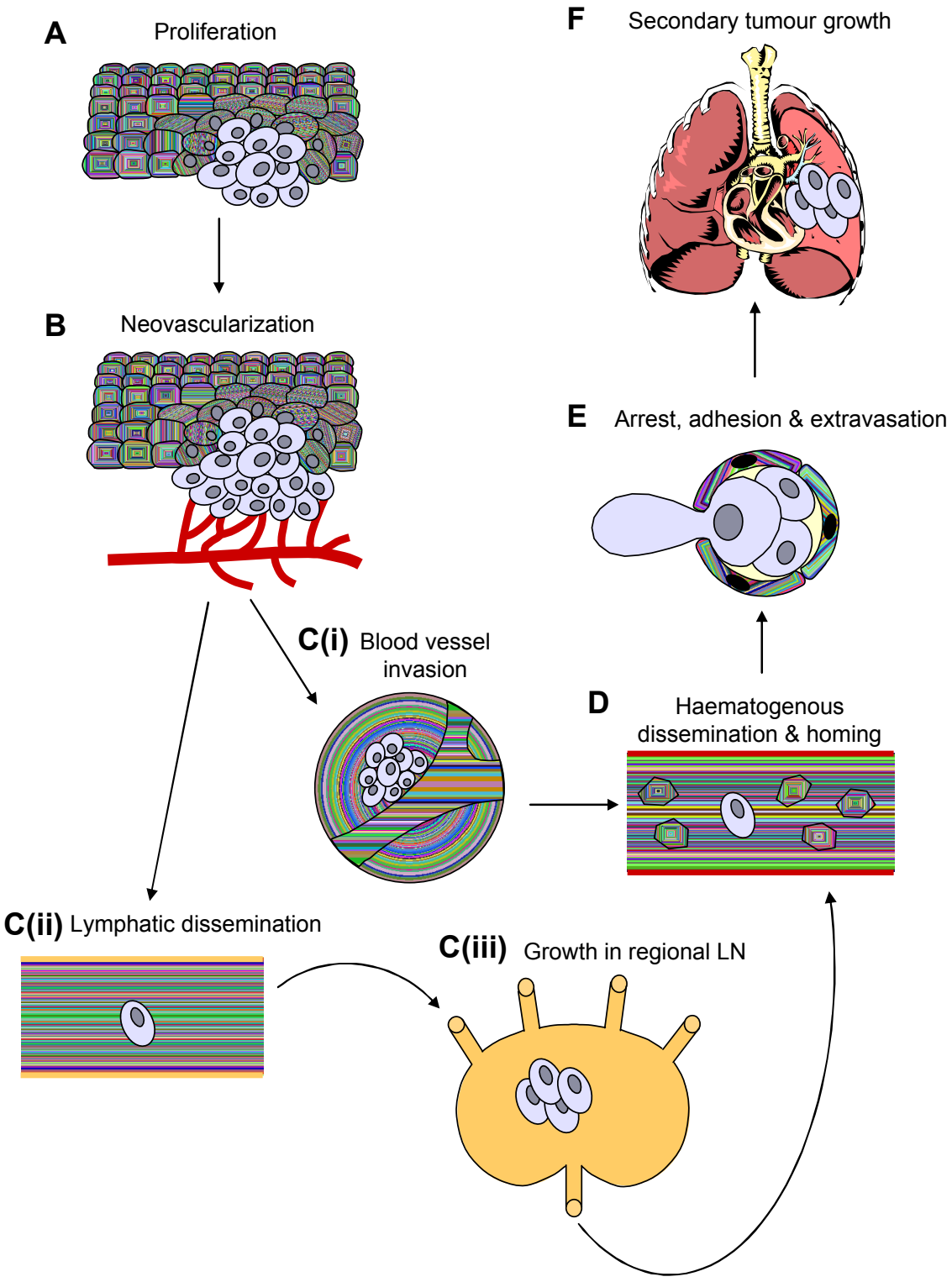


Figure 1.1 *The hallmarks of breast cancer.*

Breast cancer includes a range of distinctdiseases, each with their own individual aetiology. Despite the huge array of genes, signalling molecules, growth factors and cells implicated in breast cancer, a few characteristics are common to most, if not all breast cancers. These eight 'hallmarks'o f breast cancer, as outlined by Hanahan and Weinberg[1], are summarised in the Figure above and are described more fully in the text. The hallmarks are depicted as arising from genomic instability within malignant cells; this is crucial for the accumulation of the large number of genetic mutations required for malignant transformation.

Figure 1.2 *The steps of breast cancer metastasis.*

(A) Breast tumour cells proliferate to form a primary tumour by developing the ability to evade apoptosis and stimulate their own unlimited growth. **(B)** Tumour cells either directly or indirectly (via stromal cells) induce angiogenesis to supply oxygen and nutrients to support the growing tumour mass. **(C)** To invade through the tissue, malignant cells must acquire the ability to survive without anchorage-dependent signals. Also, they must induce the production of ECM degradative enzymes that allow them to move through the tissue. *i*, Tumour cells can invade through basement membranes directly into blood vessels. *ii & iii*, Alternatively, tumour cells can enter the draining lymphatics and migrate to the local DLN, where they can become established and form secondary tumours (lymphatic metastasis). **(D)** Tumour cells can arrive in the circulation (haematogenous metastasis) either through direct intravasation at the tumour site, or through shedding from secondary LN tumours into the efferent lymphatics. These cells are carried through the body to distant metastatic sites. **(E)** Malignant cells arrest in the vasculature of a distant organ, adhere to the vessel wall and extravasate into the tissue. This requires the expression of degradative enzymes that facilitate tumour cell passage through the endothelial basement membrane and into the underlying tissue. **(F)** The final stage of distant metastasis is tumour cell survival and proliferation within the new tissue. This may involve autocrine mechanisms and recruitment of growth-supporting accessory cells. *DLN*, draining lymph node; *ECM*, extracellular matrix; *LN*, lymph node. *Adapted from ref 271.*


A
Proliferation
B
Neovascularization
C(i) Blood vessel invasion
C(ii) Lymphatic dissemination
C(iii) Growth in regional LN
D
Haematogenous dissemination & homing
E
Arrest, adhesion & extravasation
F
Secondary tumour growth

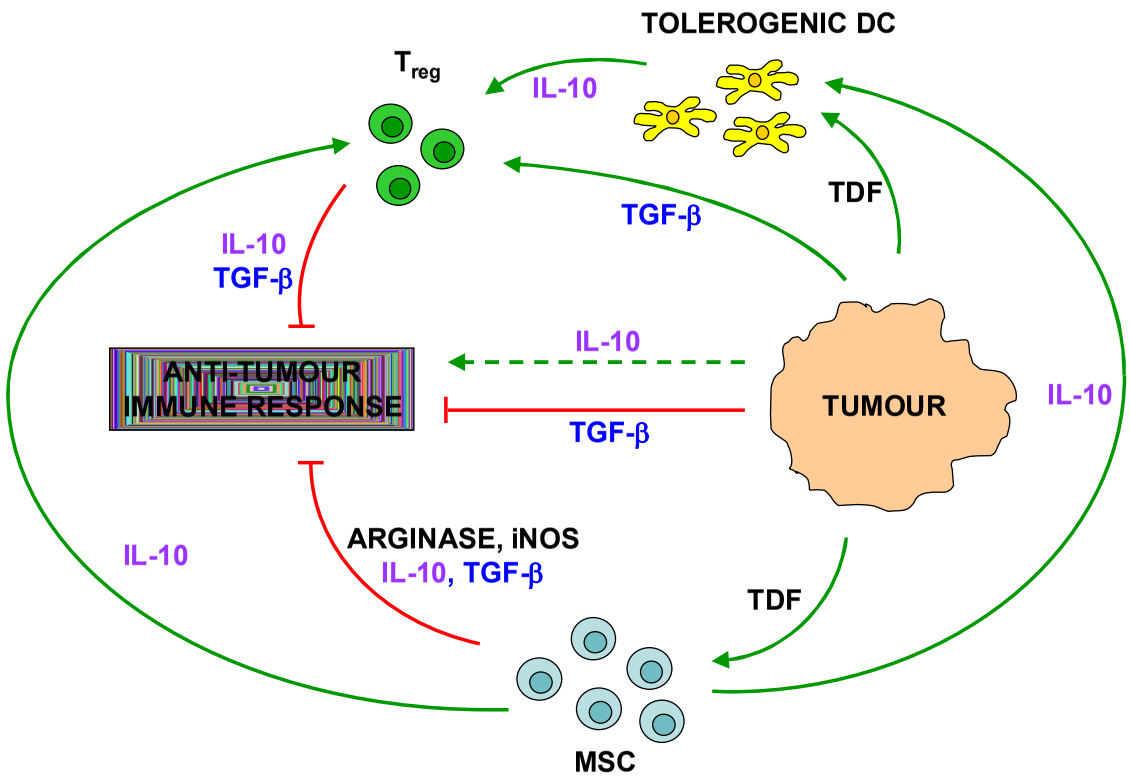


Figure 1.3 *Immunosuppressive networks induced by breast tumours.*

Breast cancers need to evade the immune response if they areto become clinically evident and life -threatening tumours. Immunosuppressive cytokines such as TGF-β and other tumour-derived factors can promote the generation of tolerogenic DC and T_{reg}. Tolerogenic DC in turn promote the differentiation of T_{reg}, which can suppress active anti-tumour immune responses. Tumour-derived factors also stimulate the accumulation of MSC. These cells produce immunosuppressive cytokines, as well as iNOS and arginase, the products of which inhibit productive anti-tumour immune responses. Although IL-10 is generally regarded as an immunosuppressive cytokine, there is some evidence to suggest that IL-10 localised to the tumour site can enhance anti-tumour immune responses (depicted by a dashed arrow). *Green arrows*denote positive effects, *red bars*denote inhibitory effects. *DC*, dendritic cell; *IL-10*, interleukin-10; *iNOS*, inducible nitric oxide synthase; *MSC*, myeloid suppressor cell; T_{reg}, regulatory T cell; *TDF*, tumour-derived factors; *TGF*-β, transforming growth factor-β.

NOTE: This figure is included on page 41 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.4 *Chemokine signalling.*

Binding of a chemokine to its receptor induces a conformational change that leads to dissociation of the receptor-associated heterotrimeric G proteins into α and β γsubunits. These G -protein subunits can then activate various downstream signalling molecules including phosphoinositol 3-kinase (PI3K), protein kinase C (PKC) and mitogen-activated protein kinases (MAPK), as well as phospholipase C (PLC), which leads to inositol phosphate production and an increase in intracellular calcium. The overall effect of this signalling is to modulate actindependent cellular processes and increase expression and clustering of adhesion molecules, ultimately leading to cell chemotaxis and/or degranulation. Chemokine binding also stimulates tyrosine phosphorylation of the receptor and activation of the JAK/STAT pathway. This, together with MAPK activation, leads to modulation of gene expression. Finally, ligand binding induces internalisation of chemokine receptors through either clathrin-mediated endocytosis or, in some cases, a lipid raft/caveolae-dependent mechanism (not shown). Internalised receptors are then degraded or recycled to the cell surface.

Adapted from ref 2.

CHAPTER 2

Materials & Methods

CHAPTER 2: MATERIALS & METHODS

2.1 Subcloning of chemokine constructs

2.1.1 Chemokine DNA constructs

The three CXCL12 constructs used in this study were variants of the murine CXCL12 protein. CXCL12 was identical to wild-type CXCL12 protein. $CXCL12_{(P2G)}$, an antagonist CXCL12 of activity, was a mutant form of the CXCL12 protein in which the second proline residue of the mature peptide was mutated to a glycine residue[131]. $CXCL12_{(Ala)}$ was a putative biologically inactive form of CXCL12 in which the first two conserved cysteines (Cys9 and Cys11) of the mature peptide were replaced with alanine residues, effectively destroying the tertiary structure of the molecule. The two CXCL16 constructs used in this study were variants of the murine CXCL16 protein. CXCL16 was identical to wild-type membrane-bound CXCL16. $CXCL16_{(9-220)}$, an antagonist of CXCL16 activity, lacked the coding sequence for the first eight amino acids of the mature CXCL16 protein (but retained the signal sequence) and was expressed in membrane-bound form. All five chemokine proteins were tagged at the carboxyl-terminus with six histidine residues.

2.1.2 Cloning vector

The five histidine-tagged chemokine constructs used in this study were expressed in the pEF-IRES-puro6 (pEF) vector (Figure 2.1). This vector, a variation of the pEFIRES-P vector[272], was a kind gift from Dr. Dan Peet in the School of Molecular and Biomedical Science at the University of Adelaide. In pEF-IRES-puro6, the constitutive human elongation factor (EF)-1α promoter controls expression of the chemokine construct and a puromycin selection marker, which are transcribed as a bicistronic mRNA separated by an internal ribosome entry site (IRES).

2.1.3 Polymerase Chain Reaction

Polymerase chain reaction was used to amplify the $CXCL12_{(Ala)}$ coding sequence without the KDEL peptide motif and to amplify the CXCL16 coding sequence with the nucleotides encoding a C-terminal 6x histidine residue tag appended. The mCXCL12(His) and the mCXCL16FLH primer sets (Table 2.1) were used to amplify the $CXCL12_{(Ala)}$ and

CXCL16 sequences, respectively. The 5′ primers contained an EcoRI restriction site while the 3′ mCXCL12(His) primer contained a SpeI restriction site and the 3′ mCXCL16FLH-R primer contained an EcoRV restriction site. These sites were incorporated to facilitate subcloning of the DNA cassettes into the multiple cloning site of pEF. The polymerase chain reaction was carried out in a final volume of 25µl and contained 5µl 5′ primer (5µM), 5µl 3′ primer (5µM), 2.5µl 10x Mg^{2+}-free EXT buffer and 1.25µl $MgCl_2$ (50mM) (Finnzymes, Espoo, Finland), 1µl dNTPs (10mM) (Amersham Biosciences, Buckinghamshire, England, UK), 0.25µl DyNAzyme EXT polymerase (Finnzymes) and 125ng of template DNA. The reaction was carried out on a Programmable Thermal Controller (MJ Research Inc., Waltham, MA, USA) under the following conditions: 94°C for 2 minutes, then 15 cycles of 94°C for 30 seconds, 60°C for 45 seconds and 72°C for 1 minute, followed by a final extension step of 72°C for 10 minutes.

Overlap extension PCR was used to generate the $CXCL16_{(9-220)}$ construct. The mCXCL16FLH-F and mCXCL16SP-R PCR primers (Table 2.1) were used to amplify the signal sequence of CXCL16, while the mCXCL16A-F and mCXCL16FLH-R primers (Table 2.1) were used to amplify the nucleotide sequence encoding amino acid residues 9 to 220. Sequence analysis software (SignalP v3.0; http://www.cbs.dtu.dk/services/SignalP/[273, 274]) was employed to verify that deletion of the eight N-terminal amino acids of the CXCL16 protein would not alter the cleavage site of the signal peptide. The mCXCL16SP-R primer was designed such that it included a region complementary to the 5′ end of the antagonist portion of CXCL16. The reaction mix for the first round of amplification of the signal peptide and antagonist portions of CXCL16 was the same as described above, with the following cycling conditions: 94°C for 2 minutes, then 15 cycles of 94°C for 30 seconds, 57°C for 45 seconds and 72°C for 1 minute, followed by a final extension step of 72°C for 10 minutes. For the second round of amplification, reaction mixes were as described above, with 1.25µl each of the two first round reactions used as template and mCXCL16FLH-F and mCXCL16FLH-R as primers. Cycling conditions were as described for the first round of amplification.

2.1.4 Subcloning

The $CXCL12_{(Ala)}$ PCR product, the CXCL12- and $CXCL12_{(P2G)}$-containing pCR2.1-TOPO constructs and the pEF cloning vector were subjected to restriction digest with EcoRI and SpeI restriction enzymes (New England Biolabs, Beverly, MA, USA) in a 1:10 dilution of Eco Buffer (New England Biolabs) for 2 hours at 37°C. The CXCL16 and $CXCL16_{(9-220)}$ PCR products and pEF were digested under similar conditions with EcoRI and EcoRV (New England Biolabs). Fragments were then separated by agarose gel electrophoresis through a 1.2% agarose gel and the desired bands excised. DNA was purified from the gel using the Ultra Clean DNA purification kit (MoBio Laboratories Inc, Solana Beach, CA, USA), according to the manufacturer's instructions. The purified chemokine constructs were ligated into the digested pEF vector in a final reaction volume of 10µl: 1µl 10x TL-1 ligation buffer and 1µl T4 DNA ligase (Geneworks, Adelaide, SA, Australia), 3µl purified chemokine DNA, 1µl pEF DNA and 4µl H_2O. Reactions were incubated overnight at 4°C. The ligation mixtures were then used to transform chemically competent TOP10 *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA). Cells were incubated with the ligation mixture on ice for up to 2 hours. Cells were then subjected to heat shock at 37°C for 3 minutes followed by 10 minutes on ice. Cells were then allowed to recover in Luria Broth at 37°C for 45 minutes before being plated on Luria agar plates supplemented with 25µg/ml ampicillin. Plates were incubated at 37°C overnight.

2.1.5 Identification of positive clones

Overnight cultures of potential positive clones were set up in 10ml Luria broth with 25µg/ml ampicillin and plasmid mini-preps were performed the following day using the alkaline lysis method. Briefly, 1.5ml of culture were spun down and supernatant removed. Cells were resuspended in 100µl buffer P1 (Section 2.6.2.1), lysed in 100µl buffer P2 (Section 2.6.2.2), and cell debris was precipitated from solution with 100µl buffer P3 (Section 2.6.2.3). Debris was spun down for 8 minutes at 4°C, and plasmid DNA-containing supernatant was transferred to a fresh eppendorf tube. DNA was washed with 100% ethanol and resuspended in 20µl milliQ H_2O. DNA concentration was determined on a BioRad SmartSpec 3000 spectrophotometer (BioRad Laboratories, Hercules, CA, USA). Positive clones were identified by subjecting plasmid DNA to restriction digest with EcoRI and SpeI (CXCL12 constructs) or EcoRI and EcoRV (CXCL16 constructs) and separating

the fragments generated by agarose gel electrophoresis. The presence of the CXCL12, $CXCL12_{(P2G)}$, $CXCL12_{(Ala)}$, CXCL16 or $CXCL16_{(9-220)}$ coding sequence was confirmed by sequencing plasmid DNA from positive clones. Sequencing reactions contained 6µl Big Dye Terminator Ready Reaction Mix (Version 3; Perkin-Elmer, Foster City, CA, USA), 2µl primer (pEF-F or pEF-R at 5µM; Table 2.1), and 250ng plasmid DNA made up to a final volume of 20µl with H_2O. Cycling conditions were as follows: 94°C for 2 min, followed by 25 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 65°C for 10 minutes, then a final extension step of 65°C for 10 minutes. DNA was precipitated with 60% isopropanol, pelleted by centrifuging at 14000rpm for 25 minutes at -9°C, and then washed in 70% ethanol, before being vacuum dried and sent to the Institute of Medical and Veterinary Science (IMVS; Adelaide, SA, Australia) for analysis on an ABI 373A DNA sequencer (Perkin-Elmer).

2.2 Generation of transfected cell lines

2.2.1 Cells

The murine breast epithelial cancer cell line 4T1.2 was derived from a spontaneously arising mammary carcinoma in a Balb/cfC3H mouse, and was a kind donation from Dr. Robin Anderson (Peter MacCallum Cancer Institute, Melbourne, Vic, Australia). Cells were cultured in complete Alpha MEM (Section 2.6.1.2) and incubated at 37°C in 5% CO_2. Cells were passaged every 2-3 days by rinsing flasks with sterile phosphate buffered saline (PBS; endotoxin-free) and then incubating flasks with 10mM EDTA in PBS (endotoxin-free) at 37°C in 5% CO_2 for 10 minutes to remove adherent cells. Cells were passaged for no more than 6 weeks.

The murine pre-B lymphocyte cell line 300.19 was cultured at 37°C in 5% CO_2 in complete RPMI (Section 2.6.1.6). Cells were passaged every 2-3 days, and cells were maintained in culture for no more than 6 weeks.

2.2.2 Transfection

4T1.2 cells were removed from tissue culture flasks with 10mM EDTA as described above and washed once in iced PBS. Aliquots of 370µl containing 5 x 10^6 cells were placed into

chilled electroporation cuvettes (BioRad) and incubated on ice with 20-40μg of plasmid DNA for 10 minutes. Cells were electroporated with a BioRad Gene Pulser apparatus at 300V and 960μF. Cells were then returned to ice for 10 minutes before being resuspended in 15ml of pre-warmed complete Alpha MEM and placed at 37°C in 5% CO_2. Two to three days later, selection with 6μg/ml puromycin (Calbiochem, La Jolla, CA, USA) was commenced. Individual clones of stably transfected cells were obtained by limiting dilution. Cells were assayed by ELISA for chemokine production as described in Section 2.4.3. For each of the 5 clones secreting the greatest amounts of chemokine protein, equal numbers of cells were taken, pooled and cultured for further experiments.

2.3 Mouse model

2.3.1 Animals

Female 6-8 week old Balb/c mice were bred and maintained at the University of Adelaide Animal Facility (Adelaide, SA, Australia). Severe combined immunodeficient (SCID; C.B-17-Igh-1^{b}-Prkdc^{scid}) mice and athymic (Nude) mice on the Balb/c background (BALB/c-Foxn1^{nu}) were bred at the Animal Resources Centre (ARC; Canning Vale, WA, Australia) and maintained at the University of Adelaide Animal Facility. Female 6-8 week old Jα18$^{-/-}$ mice and 9-16 week old pfp$^{-/-}$, TRAIL$^{-/-}$ and IFN-γ$^{-/-}$ mice (all on the Balb/c background) were obtained from the Peter MacCallum Cancer Centre (Melbourne, Vic, Australia) and maintained at the University of Adelaide Animal Facility.

2.3.2 Tumour model

Tumour cells were removed from flasks with PBS containing 10mM EDTA and resuspended in PBS at a final concentration of 1 x 10^7 cells/ml. Mice were anaesthetized by halothane inhalation and 1 x 10^5 cells in a 10μl volume were injected into the fourth mammary fat pad using a 50μl Hamilton syringe (Hamilton, Reno, NE, USA). Tumour growth was assessed every 2 days and tumour diameter was measured using digital callipers (Mitutoyo, Japan). Tumour volume was calculated using the equation width^2 x length/2 to approximate the volume of a spheroid. Mice were euthanised once tumour diameter reached 15mm or tumours became ulcerated. To quantify metastasis, lungs were fixed for 5 days in 4% formaldehyde in PBS. Lungs were passed through two 30% sucrose

baths over 2 days before being immersed in 70% ethanol. Lungs were dissected into five lobes and surface metastases were counted under a dissecting microscope. For correlation of tumour weight with numbers and proportions of total, $CD4^+$ and $CD8^+$ splenocytes, spleens were removed, minced and macerated through 70μm nylon sieves (BD, Franklin Lakes, NJ, USA) to generate single cell suspensions in complete RPMI. Splenocytes were centrifuged and treated with mouse red cell removal buffer (MRCRB; Section 2.6.2.8) for 5 minutes at 37°C before being washed and resuspended in PBS/BSA/Azide (PBA; Section 2.6.2.10). Splenocytes were then stained with fluorochrome-conjugated anti-mouse CD4 or anti-mouse CD8 antibodies for flow cytometric analysis (see Section 2.4.1 and Table 2.2).

For the experimental metastasis experiments, tumour cells were harvested as described and resuspended to 2.5×10^6 or 3.75×10^6 cells/ml in PBS. Cells in a 200μl volume were injected into the tail vein using a 0.5ml insulin syringe (BD). After 14 days, mice were sacrificed and the lungs were harvested for enumeration of surface metastases as described above.

For the cytotoxicity experiments, mice received 10^6 cells in a 25μl volume of PBS in each hind foot pad. Draining (popliteal) LN were removed 7 days later, and lymphocytes extracted for use as effector cells in *in vitro* cytotoxicity assays.

For analysis of early stage tumours and draining lymph node (DLN) by immunohistochemistry, flow cytometry, intracellular cytokine staining, ELISPOT or cytokine bead array, mice received 1×10^5 cells by mammary fat pad injection or 1×10^6 cells by foot pad injection as described above, as indicated. Tissues were harvested at the indicated time points for analysis.

2.4 Analytical and functional assays

2.4.1 Flow cytometric staining

2.4.1.1 Cell preparation:

Cell lines: Cells were washed once in PBS and resuspended at 4×10^6 cells/ml in PBS/BSA/azide.

LN and tumour cells: Female 6-8 week old Balb/c mice were injected in both hind foot pads with 1 x 10^6 cells per foot pad. Seven days later, tumours and tumour-draining popliteal LN were harvested. Tumours were minced in 5mg/ml collagenase solution (Section 2.6.2.5) and incubated 1 hour at 37°C. Tumours were then macerated through a 70µm nylon sieve to generate single cell suspensions and washed in incomplete RPMI. LN cells were macerated through a 70µm nylon sieve to generate single cell suspensions. All cells were resuspended at 4 x 10^6 cells/ml in PBA.

2.4.1.2 Staining procedure

Cells were incubated at room temperature with 20µg/ml mouse gamma globulin (Rockland, Gilbertsville, PA, USA) for at least 20 minutes. Cells were added in 50µl aliquots to a 96-well V-bottom tray (Costar, Corning, NY, USA). Primary antibodies and isotype-matched controls were prepared at the appropriate concentrations in PBA (Table 2.2) and added to the appropriate wells. Cells were incubated with primary antibody for 30 minutes at room temperature, before being washed in 200µl PBA. If required, cells were then incubated for 30 minutes at 4°C with 50µl streptavidin-conjugated phycoerythrin (Rockland) diluted 1:150 in PBA and washed in 200µl PBA. Cells were washed in 200µl of PBS containing 0.04% sodium azide (PA; Section 2.6.2.9), before being resuspended in 150µl PA containing 2.5µM hydroxystilbamidine (Invitrogen Molecular Probes, Eugene, OR, USA) to distinguish non-viable cells. Cells were analysed on the same day on an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA).

2.4.2 Reverse-transcriptase PCR

Total cell RNA was extracted using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions and treated with RNase-free DNase (1U/µl; Promega, Madison, WI, USA). A total of 5µg DNase-treated RNA was reverse transcribed using random hexamers (Promega) and the reverse-transcriptase Superscript III (200U/µl; Invitrogen), as directed by the manufacturer. The cDNA obtained was treated with DNase-free RNase A (10mg/ml; Sigma, St Louis, MO, USA) for 1 hour at 37°C, and 1.25µl was used as template in subsequent PCR reactions. The sequences of the primers (synthesised

at Geneworks) used for PCR amplification are shown in Table 2.1. The reaction was carried out in a final volume of 25µl and contained 5µl 5′ primer (5µM), 5µl 3′ primer (5µM), 2.5µl 10x Mg^{2+}-free EXT buffer and 1.25µl $MgCl_2$ (50mM), 1µl dNTPs (10mM), 0.25µl DyNAzyme EXT polymerase and 1.25µl of template cDNA. The reaction was carried out on a Programmable Thermal Controller under the following conditions: 94°C for 2 minutes, then 35 cycles of 94°C for 30 seconds, 52°C for 45 seconds and 72°C for 1 minute, followed by a final extension step of 72°C for 10 minutes. Fragments were visualized by agarose gel electrophoresis on a 2% agarose gel.

2.4.3 Sandwich ELISA for detection of chemokines

Capture and detection antibodies were purchased from R & D Systems (Minneapolis, MN, USA; Table 2.2). Anti-CXCL12 and anti-CXCL16 capture antibodies were used at 2µg/ml and 1µg/ml, respectively. Biotinylated anti-CXCL12 and biotinylated anti-CXCL16 detection antibodies were used at 200ng/ml and 25ng/ml, respectively. Cell culture supernatants were collected from 2.5 x 10^6 cells grown in 500µl complete Alpha MEM. Protease inhibitor cocktail (Sigma) was added to the supernatants at a 1:200 dilution to prevent protein degradation. The wells of a 96-well ELISA plate (Costar) were coated overnight at room temperature with 100µl/well of capture antibody prepared in $NaHCO_3$ (0.1M) coating buffer. Wells were rinsed 3 times with PBS containing 0.05% Tween20 (Sigma), then blocked with 300µl/well of PBS containing 1% BSA and 5% sucrose or PBS containing 3% BSA for 1-2 hours at room temperature. Wells were rinsed again 3 times with PBS/Tween. One hundred µl per well of standard dilutions of recombinant mouse chemokine/cytokine prepared in diluent (0.1% in PBS) and culture supernatants were added to the tray, which was then incubated, shaking, for 2 hours at room temperature. Wells were rinsed 3 times with PBS/Tween, then 100µl/well of detection antibody prepared in diluent was added. The tray was incubated, shaking, for 2 hours at room temperature, then wells were washed 3 times with PBS/Tween. Streptavidin-conjugated horseradish peroxidase (HRP; Table 2.3) was diluted 1:20000 in diluent and 100µl was added to each well. The tray was incubated for 30 minutes, shaking, at room temperature. OPD solution was prepared from a kit (Sigma) as per the manufacturer's instructions, and 200µl was added to each well. The tray was incubated in the dark at room temperature for 20 minutes while colour developed, then 50µl 3M HCl was added to each well to stop the

reaction. The tray was read at 490nm on a Biotrak II Plate Reader (GE Healthcare, NSW, Australia).

2.4.4 Calcium mobilisation

4T1.2 cells were grown to confluence in 10cm tissue culture dishes (approximately 2 x 10^7 cells) and harvested using a cell scraper. Approximately 2 x 10^7 300.19 cells were collected from tissue culture flasks. Cells were resuspended in 2ml pre-warmed calcium buffer (Section 2.6.2.4) and incubated at 37°C in 5% CO_2 with 1μg/ml FURA-2AM (Invitrogen Molecular Probes) for 30 minutes. Cells were washed once and resuspended in 4.3ml in warmed calcium buffer. Two ml of cell suspension was added to a 10mm x 10mm x 48mm cuvette (Sarstedt, Germany) with a magnetic stirrer and analysed in an SLM AMINCO Bowman Series 2 Luminescence Spectrophotometer. Cells were stimulated with control $CCL2_{(9\text{-}76)(4Ala)}$ ($CCL2_{(Ala)}$) peptide, 300ng/ml synthetic human CXCL12 (both kindly provided by Professor Ian Clark-Lewis, Biomedical Research Centre, University of British Columbia, Vancouver, Canada) or 300ng/ml recombinant mouse CXCL16 (R & D Systems) at the indicated times over a monitored period of 100 seconds. Maximum and minimum calcium mobilisation values were obtained by lysing the cells with 10% Triton X-100 (Sigma), then sequestering Ca^{2+} ions with 100mM EGTA and 2M NaOH, respectively.

2.4.5 In vitro *proliferation assay*

Cells were washed twice in serum-free Alpha MEM (Section 2.6.1.1) and resuspended at 1 x 10^5 cells/ml. Alpha MEM was prepared containing either 0.1%, 1% or 10% FCS. One millilitre of the appropriate medium and 25μl of cell suspension, or 1ml medium alone to serve as a blank, were added to each well of a 12-well tray. Cells were allowed to proliferate at 37°C in 5% CO_2 for up to 7 days. At days 0, 2, 5 and 7, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate (XTT; 1mg/ml; Sigma) was combined with N-methyl dibenzopyrazine methyl sulfate (PMS; 1.25mM; Sigma) in a 50:1 ratio. Two hundred microlitres were added per well and colour was allowed to develop at 37°C in 5% CO_2 for 2 hours. One hundred microlitres of culture supernatant from each well was transferred to a 96-well tray, which was then read at 490 nm (ref 650nm) on a Biotrak II Plate Reader.

2.4.6 Soft agar assay

Complete 2x Iscove's Modified Dulbecco's Medium (IMDM; Section 2.6.1.3) was prepared and warmed in a 40°C water bath. A 1.4% Bacto Agar (BD) solution was prepared in milliQ H_2O and boiled until dissolved. Complete 2x IMDM and 1.4% Bacto Agar were combined in a 1:1 ratio, then 1ml aliquots were used to coat the wells of a 6-well tissue culture plate (BD) and allowed to set. A 0.6% Bacto Agar solution was prepared and combined with complete 2x IMDM as described above, and then placed in a 40°C water bath to equilibrate. Cells were prepared at 1 x 10^4 cells/ml in complete Alpha MEM, and 100µl aliquots were mixed with 1ml of the 0.3% Bacto Agar in IMDM. The agar-cell mix was overlaid onto the 0.7% agar in IMDM in the 6-well trays and allowed to solidify at room temperature for half an hour. Trays were incubated at 37°C in 5% CO_2 for 10 days and colonies formed were counted under a dissecting microscope.

2.4.7 Haemoglobin assay

Tumours were harvested and snap frozen in liquid nitrogen. Tumours were then homogenized to a fine powder using a liquid nitrogen cooled stainless steel homogenizer (custom-made by the Peter MacCallum Cancer Centre). The powdered tumour was weighed and resuspended in 500µl deionised water. The homogenate was sonicated for approximately 30 seconds and debris was removed by centrifugation at 14000rpm for 30 minutes at 4˚C. The resulting supernatant was then diluted 1:3 and used for the haemoglobin assay as follows. Drabkin's reagent was prepared containing Brij 35 Solution (both from Sigma) as per the manufacturer's instructions. Fifty microlitres per well of tumour supernatants were transferred to a 96-well tray and 200µl/well of Drabkin's solution was added. The plate was incubated for 15 minutes at room temperature to allow colour to develop and then read at 562nm on a Biotrak II Plate Reader. A standard curve was generated using haemoglobin from bovine blood (Sigma) dissolved in deionised water.

2.4.8 Immunohistochemical staining

Tissues were embedded in Tissue-Tek O.C.T. embedding medium (Miles, Eckhart, IN, USA), snap-frozen in liquid nitrogen and stored at –80˚C. Six-micron cryostat sections on

gelatin-coated slides were fixed in 60%acetone/ 40%methanol for 10 minutes. Slides were rehydrated in PBS, and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 10 minutes. Slides were washed through 3 changes of PBS, then incubated with 100μg/ml mouse gamma globulin for 30 minutes. After 3 washes in PBS, slides were incubated with primary antibody for 2 hours in a humidified chamber at room temperature. After 3 washes in PBS, slides were incubated with HRP-conjugated secondary antibody (for unconjugated primary antibodies) or HRP-conjugated streptavidin (for biotinylated primary antibodies) for 1 hour. Slides were washed 3 times in PBS and binding of antibodies was revealed by incubation with 3,3′-diaminobenzidine (DAB) substrate (DAKO, NSW, Australia). Sections were counterstained with haematoxylin (Section 2.6.2.6), mounted and examined by routine light microscopy.

2.4.9 In vitro *cytotoxicity assay*

Cytotoxicity assay with effector cells from late stage tumours: Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad with 1×10^5 tumour cells. After approximately 5 weeks, spleens and tumour-draining (brachial) LN for each mouse were pooled and macerated through a 70μm nylon sieve to generate single cell suspensions of effector cells. Effector cells were resuspended in 7ml MRCRB and incubated for 3 minutes in a 37°C water bath to lyse red blood cells. Effector cells were then prepared in minimal complete RPMI (Section 2.6.1.5) at the appropriate concentrations to give the indicated effector:target ratios. Target 4T1.2 cells were prepared in the same medium containing 0.2μM calcein.AM (Invitrogen Molecular Probes) and incubated for 30 minutes at 37°C in 5% CO_2. Target cells were washed twice with PBS, then prepared in minimal complete RPMI and plated at 2.5×10^4 cells/well (100μl/well) in a 96-well flat-bottomed tray. Targets were allowed to adhere approximately 3 hours at 37°C in 5% CO_2. Effector cell suspensions were plated at 100μl/well and the plates were incubated at 37°C in 5% CO_2. Four days later, wells were washed 3 times with 200μl/well PBS to remove non-adherent cells, then 100μl/well PBS was added for reading of the plate. Fluorescence of the remaining adherent cells was measured on a Molecular Imager FX using Quantity One software version 4.6 (BioRad) and percentage cytotoxicity was calculated as 100-(fluorescence of target cells/fluorescence of target cells only x 100).

Cytotoxicity assay with effector cells from early stage tumours: Female 6-8 week old Balb/c mice were injected in both hind foot pads with 1 x 10^6 cells per foot pad. Seven days later, tumour-draining popliteal LN were obtained and macerated through a 70µm nylon sieve to generate single cell suspensions of effector cells. Effector cells were prepared in minimal complete RPMI at the appropriate concentrations to give the indicated effector:target ratios. Target 4T1.2 cells were prepared in the same medium and plated at 1 x 10^4 cells/well (100µl/well) in a 96-well flat-bottomed tray. Targets were allowed to adhere approximately 3 hours at 37°C in 5% CO_2. Effector cell suspensions were plated at 100µl/well and the plates were incubated at 37°C in 5% CO_2. After 3 days, non-adherent cells were removed and the wells were washed 3 times with 200µl/well of incomplete RPMI (Section 2.6.1.4). Remaining adherent target cells were labelled with 1µM calcein.AM in incomplete RPMI for 25 minutes at 37°C in 5% CO_2. Wells were washed 3 times with 200µl/well PBS, then 100µl/well PBS was added for reading of the plate. Fluorescence was measured on a Molecular Imager FX using Quantity One software version 4.6 and percentage cytotoxicity was calculated as 100-(fluorescence of target cells/fluorescence of target cells only x 100).

2.4.10 IL-17 ELISPOT assay

Female 6-8 week old Balb/c mice received 1 x 10^6 tumour cells in 25µl PBS in each hind foot pad. Seven days later, tumour-draining popliteal LN were obtained and macerated through a 70µm nylon sieve to generate single cell suspensions. A mouse IL-17 ELISPOT kit (R & D Systems) was used to assess IL-17 production by the LN cells as per the manufacturer's instructions. Briefly, the wells of the supplied 96-well filter plate pre-coated with anti-mouse IL-17 antibody were pre-wet with 200µl/well complete RPMI for 20 minutes at room temperature. Cells were prepared in complete RPMI containing 20ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma) and 1µM ionomycin (Invitrogen Molecular Probes) and aliquoted at 1.2 x 10^5 cells/well (100µl/well) into the wells of the filter plate. Recombinant mouse IL-17 at 0.25ng/ml was used as a positive control and medium alone was used as a negative control. Cells were incubated overnight at 37°C. The following day, cells were flicked off and the plate washed four times with the wash buffer supplied. Bound IL-17 was detected by incubation of plate with 100µl/well biotinylated anti-mouse IL-17 overnight at 4°C. The plate was washed four times with wash buffer,

then incubated for 2 hours at room temperature with 100µl/well HRP-conjugated streptavidin. The plate was washed again and 100µl/well 5-bromo-4-chloro-3′ indolylphosphate p-toluidine salt/Nitro Blue tetrazolium (BCIP/NBT) chromogenic substrate was added to visualise spots. The plate was incubated 1h at room temperature in the dark before being washed and allowed to dry. Spots representing individual IL-17-producing cells were counted under a dissecting microscope.

2.4.11 Intracellular cytokine staining

Female 6-8 week old Balb/c mice received 1 x 10^6 tumour cells in 25µl PBS in each hind foot pad. Seven days later, tumour-draining popliteal LN or pooled popliteal, inguinal, brachial and axillary LN from two naïve mice were obtained and macerated through a 70µm nylon sieve to generate single cell suspensions. Cells were prepared in complete RPMI and aliquoted at 200µl/well into the wells of a 96-well U-bottom plate. Cells were incubated overnight at 37°C and stimulated with 20ng/ml PMA and 1µM ionomycin in the presence of GolgiStop™ (BD Biosciences) at a 1:100 dilution. Cells were fixed, permeabilised (using the BD Biosciences Cytofix/Cytoperm kit) and stained with fluorochrome-conjugated antibodies to mouse CD4, CD8, IFN-γ, IL-4 and IL-17 (Table 2.2) and analysed on an LSRII flow cytometer.

2.4.12 Cytokine bead array

Female 6-8 week old Balb/c mice received 1 x 10^5 tumour cells in 10µl PBS in the fourth mammary fat pad on each flank. Six days later, tumour-draining inguinal LN were obtained and macerated through a 70µm nylon sieve to generate single cell suspensions. Cells were prepared at 5 x 10^6 cells/ml in complete RPMI containing 20ng/ml PMA and 1µM ionomycin and aliquoted at 500µl/well into the wells of a 24-well plate. The plate was incubated overnight at 37°C. The following day, culture supernatants were taken and filtered through a 0.2µm filter in preparation for cytokine bead array analysis of IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, IFN-γ, TNF-α, and GM-CSF production. The mouse Th1/Th2 10plex FlowCytomix kit (Bender MedSystems, Vienna, Austria) was used according to the manufacturer's instructions with minor modifications. Briefly, standards were prepared in the assay buffer supplied. The wells of the 96-well filter plate supplied

were pre-wet with 50µl/well of assay buffer. Standards and samples were added at 25µl/well to the filter plate. Bead mixture and biotinylated antibody mixture were prepared at a 1:10 dilution of the suggested concentration of each bead set and antibody, except for the IL-10 bead set and antibody, which were prepared at a 1:4 dilution. The bead mixture and the antibody mixture were added at 25µl/well and 50µl/well, respectively, to the plate, which was covered and incubated for 2 hours in the dark at room temperature on a microplate shaker. Wells were emptied using a vacuum filtration manifold (Millipore, Billerica, MA, USA) and washed twice with 100µl/well of assay buffer. Assay buffer was added at 100µl/well and streptavidin-PE conjugate, prepared at a 1:31.25 dilution as directed, was added at 50µl/well. The plate was covered and incubated for 1 hour in the dark at room temperature on a microplate shaker. Wells were washed as before, then 200ml/well assay buffer was added to the plate. The contents of each well were resuspended and transferred to FACS tubes (BD) for analysis on a BD FACSCanto flow cytometer (BD Biosciences). Data was analysed using the Bender MedSystems FlowCytomix Pro v2.1 software.

2.5 Statistical analysis

Statistical analysis was performed using GraphPad Instat version 3.05 and GraphPad Prism version 5 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

2.6 Solutions and buffers

2.6.1 Media

2.6.1.1 Alpha Medium (serum-free)

Alpha MEM (Invitrogen Life Technologies, Grand Island, NY, USA) was supplemented with 1% penicillin/gentamycin stock solution (IMVS).

2.6.1.2 Alpha Medium (complete)

Alpha MEM was supplemented with 10% foetal calf serum (FCS; JRH Biosciences, Lenexa, KS, USA) and 1% penicillin/gentamycin stock solution.

2.6.1.3 2x Iscove's Modified Dulbecco's Medium (complete)

Powdered incomplete IMDM (Invitrogen Life Technologies) was made up to 2x concentration according to the manufacturer's instructions. Twenty ml of complete 2x IMDM was prepared by adding 4ml FCS, 400µl penicillin/gentamycin stock and 400µl Fungizone (Invitrogen Life Technologies) to 15ml incomplete 2x IMDM.

2.6.1.4 RPMI (incomplete)

RPMI 1640 medium (IMVS) was supplemented with 0.1% FCS, 1% penicillin/gentamycin stock solution and 0.1M HEPES (IMVS).

2.6.1.5 RPMI (minimal complete)

RPMI 1640 medium was supplemented with 10% FCS and 1% penicillin/gentamycin stock solution.

2.6.1.6 RPMI (complete)

RPMI 1640 medium was supplemented with 10% FCS, 1% penicillin/gentamycin stock solution, 100mM sodium pyruvate (Sigma), 10mM non-essential amino acids (Invitrogen Life Technologies) and 30mM β2-mercaptoethanol (Sigma).

2.6.2 Other solutions and buffers

2.6.2.1 Buffer P1

0.61g Tris base and 0.37g EDTA.H_2O were dissolved in 80ml milliQ H_2O and the pH was adjusted to 8.0 with HCl. The volume was made up to 100ml with H_2O and the solution autoclaved. DNase-free RNase A (Sigma) was added to a final concentration of 1µg/ml.

2.6.2.2 Buffer P2

0.8g NaOH and 1g sodium dodecyl sulfate (SDS) were dissolved in 100ml milliQ H_2O and the solution autoclaved.

2.6.2.3 Buffer P3

29.5g potassium acetate was dissolved in 50ml milliQ H_2O and the pH was adjusted to 5.5 with glacial acetic acid. The volume was made up to 100ml with milliQ H_2O and the solution autoclaved.

2.6.2.4 Calcium Buffer

10ml 10x Hank's Balanced Salt Solution (Section 2.6.2.7), 1ml HEPES (1M) and 1ml $CaCl_2$ (0.16M) were added to 88ml milliQ H_2O. The pH was adjusted to 7.4 with 2M NaOH.

2.6.2.5 Collagenase Solution

Collagenase Type I-A (Sigma) was prepared at 5mg/ml in incomplete RPMI (Section 2.6.1.4) containing 1M $CaCl_2$ and 1U/ml DNAse (Promega).

2.6.2.6 Gill's Haematoxylin

250ml ethylene glycol, 2g Gurr Haematoxylin 75290, 0.2g sodium periodate ($NaIO_4$), 17.6g aluminium sulphate ($Al_2(SO_4)_3.18H_2O$), and 20ml glacial acetic acid were added in that order to 730ml distilled H_2O wile stirring. The solution was stirred 1 hour at room temperature until dissolved.

2.6.2.7 10x Hank's Balanced Salt Solution

80g NaCl, 4g KCl, 0.32g Na_2HPO_4, 0.6g KH_2PO_4 and 10g D-glucose were dissolved in 1L milliQ H_2O and the solution filter sterilized.

2.6.2.8 Mouse Red Cell Removal Buffer (MRCRB)

To make Solution A, 8.3g NH_4Cl was dissolved in 1L MQ H_2O. To make Solution B, 20.594g Tris base was dissolved in 1L MQ H_2O and the pH adjusted to 7.65 with HCl. Solution A and Solution B were combined in a 9:1 ratio to make 1L. The pH was adjusted to 7.2 with HCl and then the solution was filter sterilized and stored at 4°C.

2.6.2.9 PBS/azide (PA)

2ml 20% sodium azide (final concentration 0.04%) was added to 1L PBS.

2.6.2.10 PBS/BSA/azide (PBA)

10g bovine serum albumin (BSA; Sigma; final concentration 1%) was dissolved in 1L PBS and 2ml 20% sodium azide (final concentration 0.04%) was added.

Table 2.1Primers used in this study.

Name	Sequence (5′-3′)	Purpose
mCXCL12(His)-F	GGAATTCGCCACCATGGACGCCAAGGTCGTCG	Cloning; RT-PCR
mCXCL12(His)-R	GACTAGTTCAGTGATGGTGATGGTGGTGCATCTTGAGCCTC	Cloning; RT-PCR
mCXCL16FLH-F	CGAGAATTCATGAGGCGGGGCTTTGCACCC	Cloning
mCXCL16FLH-R	CGAGATATCCTAGTGATGGTGATGGTGGTGGGGTCTTGGTTC	Cloning
mCXCL16SP-R	CGATCACAAGAACAGCCATCGCCTGGCAGGG	Overlap extension PCR
mCXCL16A-F	TGTTCTTGTGATCGTACCATTTCTTCTGG	Overlap extension PCR
pEF-F	CCACTCCCAGTTCAATTACAGCTCTTAAGGCTAG	Sequencing
pEF-R	GGGAGAGGGGCGGAATTGGGC	Sequencing
mGAPDH-F	TCCTTGGAGGCCATGTAGGCCAT	RT-PCR
mGAPDH-R	TGATGACATCAAGAAGGTGGTGAAG	RT-PCR
mCXCL16RT-F	TCTTCTGGCACCCAGATACC	RT-PCR
mCXCL16RT-R	CGAGATATCTTAAGGCAAATGTTTTTGGTG	RT-PCR
mCXCR4-F	CCGGTACCTCGCCATTGTC	RT-PCR
mCXCR4-R	ACCATTATATGCTGGAATTGAAACAC	RT-PCR
mCXCR7-F	GTCAGCTGGAGAATGTGCTCTTTA	RT-PCR
mCXCR7-R	CTCTGTCTCTGACACTCTGGAGGC	RT-PCR
mCXCR6-F	GCTAGCATGGATGATGGGCATCAAGAGTCA	RT-PCR
mCXCR6-R	CAGACAAACACCAGGTCAGCCAGGGGC	RT-PCR

All primers were synthesised at Geneworks (Adelaide, SA, Australia).

Table 2.2Antibodies used in this study.

Antigen	Clone	Isotype	Conjugation[a]	Vendor	Application[b]
control	A95-1	rat IgG2b	biotin	BD Biosciences	FC
control	54447	rat IgG2a	none	R & D Systems	IHC-Fr
control	A19-3	Armenian hamster IgG1	biotin	BD Biosciences	IHC-Fr
control	n/a	polyclonal rabbit IgG	none	lab preparation	FC
control	R35-95	rat IgG2a	APC-Cy7, FITC, PE-Cy7	BD Biosciences	FC
control	R4-22	rat IgM	FITC	BD Biosciences	FC
control	GL113	rat IgG1	FITC	lab preparation	FC
control	R3-34	rat IgG1	PE	BD Biosciences	FC
huCXCL12	n/a	goat polyclonal	biotin	R & D Systems	ELISA
hu/muCXCL12	79018	mouse IgG1	none	R & D Systems	ELISA
muCD4	H129.19	rat IgG2a	biotin; PE-Cy7	BD Biosciences	IHC-Fr; FC
muCD4	GK1.5	rat IgG2b	FITC	BD Biosciences	FC
muCD8	53-6.7	rat IgG2a	biotin; APC-Cy7, FITC, PE-Cy7	BD Biosciences	IHC-Fr; FC
muCD11c	HL3	Armenian hamster IgG1	biotin	BD Biosciences	IHC-Fr
muCD31	MEC13.3	rat IgG2a	none	BD Biosciences	IHC-Fr
muCXCL16	142417	rat IgG2a	none	R & D Systems	ELISA
muCXCL16	n./a	goat polyclonal	biotin	R & D Systems	ELISA
muCXCL16	n/a	rabbit polyclonal	none	lab preparation	FC
muCXCR4	2B11	rat IgG2b	biotin	BD Biosciences	FC
muCXCR6	n/a	rabbit polyclonal	none	lab preparation	FC
muIFN-γ	XMG1.2	rat IgG1	FITC, PE	BD Biosciences	FC
muIL-4	BVD4-1D11	rat IgG2b	PE	BD Biosciences	FC
muIL-17	TC11-18H10.1	rat IgG1	PE	BD Biosciences	FC
muLYVE-1	223322	rat IgG2a	none	R & D Systems	IHC-Fr
rabbit IgG	n/a	goat polyclonal	Alexa Fluor 488	Invitrogen	FC
rabbit IgG	n/a	goat polyclonal	biotin	DAKO	FC
rat IgG	n/a	donkey polyclonal	HRP	Rockland	IHC-Fr

a, *APC-Cy7*, allophycocyanin-cytochrome 7; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; *PE-Cy7*, phycoerythrin-cytochrome 7; *HRP*, horseradish peroxidase.
b, *ELISA*, enzyme-linked immunosorbent assay; *FC*, flow cytometry; *IHC-Fr*, immunohistochemistry on frozen sections.

Table 2.3Other reagents used in this study.

Reagent[a]	Vendor	Application[b]
hydroxystilbamidine nucleic acid stain	Invitrogen	FC
mouse gamma globulin	Rockland	FC, IHC-Fr
recombinant mouse CXCL12	Peprotech[c]	ELISA
mouse CXCL16	donation (Prof. Ian Clark-Lewis, Vancouver, Canada)	ELISA
streptavidin-conjugated HRP	Rockland	ELISA, IHC-Fr
streptavidin-conjugated PE	Rockland	FC

a. PE, phycoerythrin; *HRP*, horseradish peroxidase.

b. ELISA, enzyme-linked immunosorbent assay; *FC*, flow cytometry; *IHC-Fr*, immunohistochemistry on frozen sections.

c. Peprotech, Rocky Hill, NJ, USA.

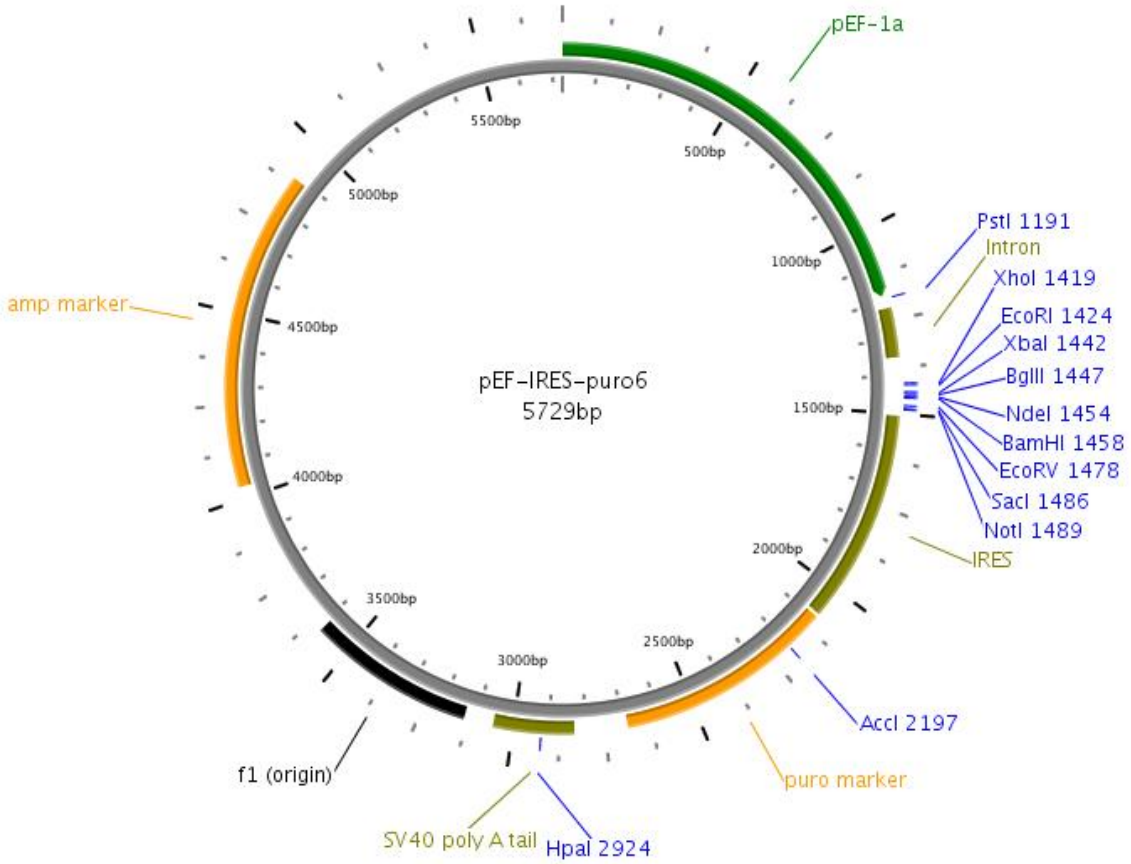


Figure 2.1 *The pEF-IRES-puro6 (pEF) mammalian expression vector.*

The pEF-IRES-puro6 (pEF) plasmid contains the puromycin-resistance gene (puro marker) downstream of an internal ribosome entry site (IRES) and a multiple cloning site (MCS). Expression of cloned DNA inserts is driven by the strong, constitutive human Elongation Factor-1α promoter (pEF-1a). In addition, the vector carries the β-lactamase gene (amp marker) for selection during manipulations in bacteria. LEGEND: *black*, origin of replication; *green*, promoter; *khaki*, regulatory sequence; *orange*, selectable marker; *blue*, unique restriction site.

CHAPTER 3

Generation of Chemokine Constructs & Cell Lines

CHAPTER 3: GENERATION OF CHEMOKINE CONSTRUCTS & CELL LINES

3.1 Overview

The initial aims of this project were to clone the coding sequences for CXCL12 and CXCL16 and their antagonists into a mammalian expression vector and to transfect the resulting DNA constructs into 4T1.2 cells. The 4T1.2 model was selected for investigation of the influence of these chemokines on breast cancer progression as it recapitulates many of the aspects of the human disease and represents perhaps the most complete orthotopic model of breast cancer available for study. It was thus necessary to characterise 4T1.2 cells in terms of their endogenous CXCL12 and CXCL16 expression, as well as to determine the expression levels of the receptors for these chemokines. Following transfection of 4T1.2 cells with the DNA constructs, chemokine and antagonist expression, as well as *in vitro* growth characteristics of the transfected cell lines, were assessed.

3.2 Generation of chemokine constructs

3.2.1 Chemokine constructs used in this study

To study the role of CXCL12 in the progression of breast cancer, a DNA construct encoding wild-type murine CXCL12 was generated, as well as a construct encoding an antagonist of CXCL12 function, $CXCL12_{(P2G)}$ (Table 3.1). This CXCL12 antagonist is identical in peptide sequence to wild-type CXCL12 except that the second proline residue of the mature peptide is mutated to a glycine residue. $CXCL12_{(P2G)}$ has only 3-fold lower binding affinity for CXCR4 than wild-type CXCL12, however the amino acid substitution results in a complete loss of activity, as shown by the inability of the analogue to induce calcium mobilization in CEM cells (a human T cell line). Moreover, $CXCL12_{(P2G)}$ is a potent antagonist of CXCL12 activity capable of inhibiting chemotaxis of CEM cells towards CXCL12[131]. Sequence alignments of the CXCL12 constructs are shown in Figure 3.1A.

For investigation of the role of CXCL16 in breast cancer, a construct encoding murine wild-type membrane-bound CXCL16 and a construct encoding $CXCL16_{(9-220)}$, an N-terminally truncated variant of CXCL16, were produced (Table 3.1 and Figure 3.1B).

The $CXCL16_{(9-220)}$ construct lacked the coding sequence for the first eight amino acids of the mature CXCL16 protein (but retained the signal sequence) to generate a protein that would antagonise wild-type CXCL16 function by binding to the CXCR6 receptor without inducing signalling. Previous work in our laboratory has shown the $CXCL16_{(9-220)}$ protein to inhibit the chemotaxis of CXCR6-expressing 300.19 cells towards CXCL16 (Scott Townley, unpublished results).

The $CXCL12_{(Ala)}$ construct was designed as a control for non-specific effects of chemokine overexpression that were independent of chemokine activity. $CXCL12_{(Ala)}$ was a variation of wild-type murine CXCL12 in which the first two conserved cysteines (Cys9 and Cys11) of the mature peptide were replaced with alanine residues (Table 3.1 and Figure 3.1A). The putative effect of these amino acid substitutions was destruction of the tertiary structure of CXCL12, and hence its biological function, as has been reported for other chemokines in which the conserved cysteine residues are mutated[275]. Since $CXCL12_{(Ala)}$ lacked any specific biological activity, it was deemed suitable as a control for overexpression of both the CXCL12 and CXCL16 constructs. All five chemokine constructs used in this study were designed to encode six histidine residues at the carboxyl-terminus of the protein so that the engineered constructs could be distinguished from endogenous protein *in vivo*.

3.2.2 Cloning of chemokine constructs

The coding sequences for the CXCL12, $CXCL12_{(P2G)}$ and $CXCL12_{(Ala)}$ constructs, including the C-terminal histidine tag, had been cloned previously into the cloning vector pCR2.1-TOPO in this laboratory and were engineered such that they were flanked by a 5′ EcoRI restriction site and a 3′ SpeI site. To subclone the His-tagged CXCL12 and $CXCL12_{(P2G)}$ constructs, the CXCL12 cDNA cassettes in pCR2.1-TOPO were excised using EcoRI and SpeI restriction enzymes. The CXCL12 cDNA cassettes were separated from the pCR2.1-TOPO vector backbone by agarose gel electrophoresis and then purified. They were then ligated into the pEF-IRES-puro6 (pEF) vector backbone, which had been similarly digested and purified (Figure 3.2A).

Direct subcloning of the $CXCL12_{(Ala)}$ construct from pCR2.1-TOPO to pEF was not possible as a KDEL motif for protein retention in the endoplasmic reticulum had been

previously engineered, C-terminal to the His tag. This had to be removed for the present study. Primers complementary to the $CXCL12_{(Ala)}$ sequence, including the histidine tag, were used to amplify the $CXCL12_{(Ala)}$ cassette without the nucleotides encoding the KDEL motif. These primers also incorporated EcoRI and SpeI restriction sites which were used to clone the PCR product into pEF (Figure 3.2B). All three CXCL12 constructs were sequenced to confirm the absence of mutations and, in the case of $CXCL12_{(Ala)}$, the successful removal of the twelve nucleotides encoding the KDEL motif.

The coding sequence of full-length wild-type mouse CXCL16 had been cloned previously into the pEF vector in this laboratory. However, to distinguish engineered expression of full-length CXCL16 from endogenous expression, PCR primers were designed to add a nucleotide sequence encoding a six histidine tag at the 3′ end of the chemokine coding sequence. This tag could not be used to distinguish soluble CXCL16 derived from engineered and endogenous sources, as the tag is retained intracellularly when the chemokine domain of CXCL16 is cleaved. Since the N-terminus of chemokines is a crucial determinant of receptor binding, the histidine tag could not be engineered 5′ of the CXCL16 coding sequence. Should it become necessary to distinguish engineered versus endogenous expression of secreted CXCL16, a construct encoding only the chemokine domain (amino acid residues 1 to 88) with a C-terminal histidine tag could be used. In addition to the histidine tag, the CXCL16 primers were designed to incorporate EcoRI and EcoRV restriction sites to facilitate cloning of the PCR product into the pEF vector. CXCL16-His was amplified using these primers from the CXCL16::pEF template generated previously in the laboratory. The resulting PCR product was digested with EcoRI and EcoRV and ligated into pEF that had been similarly digested (Figure 3.2C). The new construct was then sequenced to confirm that no mutations had been introduced into the coding sequence and that the histidine tag had been successfully appended during the cloning process.

To generate the $CXCL16_{(9\text{-}220)}$ construct, an overlap extension PCR approach was taken (Figure 3.3). To this end, PCR primers were designed and used to amplify the signal sequence of CXCL16 and the nucleotide sequence encoding amino acid residues 9 to 220. Sequence analysis software (SignalP v3.0; http://www.cbs.dtu.dk/services/SignalP/[273, 274]) was employed to verify that deletion of the eight N-terminal amino acids of the CXCL16 protein would not alter the cleavage site of the signal peptide. The primers for the 3′ end of

the signal sequence and the 5′ end of the antagonist portion were designed such that they included a complementary region that would facilitate annealing of the two PCR products when combined in one reaction. The combined PCR products were then subjected to a second round of PCR with the primers used to amplify wild-type CXCL16 above. This produced the $CXCL16_{(9\text{-}220)}$ construct complete with signal sequence and C-terminal histidine tag and flanked by EcoRI and EcoRV restriction sites. This construct was then digested and cloned into pEF as described for the wild-type CXCL16 construct above (Figure 3.2D). The construct was sequenced to confirm that the correct sequence had been cloned.

Detailed vector maps of the chemokine constructs generated for this study are shown in Figure 3.4.

3.3 The 4T1.2 model system

To study the role of CXCL12 and CXCL16 in the progression of breast cancer, the 4T1.2 mouse model of breast cancer was chosen[276]. The 4T1.2 cell line was derived by single cell cloning from the 4T1 cell line, which originated from a spontaneously arising mammary carcinoma in a Balb/c mouse[277]. The 4T1.2 cell line was selected for its propensity to metastasise to lung, bone and other sites, a pattern of metastatic spread that closely resembles that of human breast cancer[278]. It is a particularly good model of spontaneously metastatic mammary carcinoma because unlike some metastatic breast cancer models, in which metastases are forced by injecting tumour cells intravenously, metastases in this model form by spontaneous migration of cells from the primary tumour. Primary mammary tumours can be generated by an orthotopic injection of 4T1.2 cells into the fat pad of syngeneic Balb/c mice.

Preliminary experiments were conducted to characterise the endogenous expression of CXCL12, CXCR4, CXCR7, CXCL16 and CXCR6 in the 4T1.2 model. There was no detectable expression of CXCL12 messenger RNA in total RNA extracted from the cell line or from 4T1.2 tumours (Figure 3.5A). Similar assessment of CXCL16 expression by RT-PCR produced a faint but distinct band for both the cell line and tumour samples (Figure 3.6A). The results of the RT-PCR were confirmed at the protein level by ELISA analysis of supernatants from cultured 4T1.2 cells. CXCL12 production by the 4T1.2 cell

line was below the lower limit of detection of the assay (Figure 3.5B). A low level of endogenous CXCL16 shed by 4T1.2 cells into culture supernatants was detectable by ELISA, however expression of membrane-bound CXCL16 could not be detected by flow cytometry (Figure 3.6B and C). Thus it seems that the expression level of endogenous CXCL16 is too low for chemokine retained on the cell surface to be detectable.

The expression of the CXCL12 receptor CXCR4 in the 4T1.2 cell line was examined by RT-PCR and flow cytometry. CXCR4 mRNA was expressed in 4T1.2 tumours, however no CXCR4 message was detected in the 4T1.2 cell line. Despite this, flow cytometric analysis indicated that the receptor was present on the surface of cells. It may be that very low levels of mRNA expression are sufficient for translation of the CXCR4 protein detected on the cell surface, and that the RT-PCR approach used here was not sensitive enough to detect such low levels of CXCR4 transcripts. Indeed, CXCR4 expression in 4T1.2 cells has been detected by semi-quantitative real-time PCR (Robin Anderson, personal communication). No functional response was detected when cells were stimulated with CXCL12 in calcium mobilisation assays. Due to the adherent nature of 4T1.2 cells, some small spikes in fluorescence were observed at random intervals during the test period due to the propensity of 4T1.2 cells to form clumps when in suspension. Thus greater variation in baseline fluorescence was observed for these cells than for the control 300.19 cells, which grow naturally in suspension.

Another receptor for CXCL12, CXCR7, has been identified recently, a receptor that does not induce mobilisation of intracellular calcium stores upon ligation by CXCL12. Expression of this receptor by 4T1.2 cells was analysed by RT-PCR, as no antibodies to mouse CXCR7 suitable for flow cytometric analysis are currently commercially available. Low levels of CXCR7 mRNA were detected in both the 4T1.2 cell line and 4T1.2 tumour samples (Figure 3.7A). This may explain the absence of calcium mobilisation in response to CXCL12 stimulation: CXCL12 may bind preferentially to CXCR7 rather than CXCR4 on these cells.

To determine if 4T1.2 cells expressed CXCR6, which could form a potential autocrine loop with endogenously produced CXCL16, the mRNA content of the cells was examined by RT-PCR. A faint band corresponding to a portion of the CXCR6 sequence could be detected (Figure 3.8A). However, flow cytometric analysis with an anti-CXCR6 antibody

demonstrated the absence of surface CXCR6 protein (Figure 3.8B). This was confirmed by a calcium mobilisation assay to detect receptor function: no mobilisation of intracellular calcium stores was seen in response to stimulation with recombinant CXCL16 protein, in contrast to positive control 300.19 cells transfected with a construct expressing CXCR6 (BCX6 cells; Figure 3.8C). Endogenous CXCL16 secreted by 4T1.2 cells may be sufficient to bind and induce internalisation of CXCR6, hence the apparent absence of CXCR6 surface expression and function, despite the presence of CXCR6 mRNA.

In addition to the *in vitro* analyses described above, expression of all three chemokine receptors in 4T1.2 tumours was analysed by RT-PCR, and a specific band for each receptor was produced (Figure 3.7A and Figure 3.8A). Whether this represents expression of the receptors by the tumour cells, by tumour-infiltrating cells, or both could not be determined.

3.4 *In vitro* generation and characterisation of chemokine construct-expressing cell lines

3.4.1 Generation of chemokine construct-expressing cell lines

The chemokine constructs generated as described in Section 3.1 above were transfected into 4T1.2 cells by electroporation. Stably transfected populations were screened by ELISA for expression of the protein of interest (Figure 3.9). Clones were isolated by limiting dilution from those populations expressing the highest amount of the desired protein. These clones were then screened individually by ELISA for protein production (Figure 3.10 and Figure 3.11). Five clones from each transfected population were selected and pooled to produce cell lines expressing the highest levels of protein. The exception to this was the selection of five clones from the 4TX12 2 population expressing levels of CXCL12 similar to those of the 4TX12 1 clones. This was to facilitate comparisons between the two 4TX12 cell lines generated. Cell lines were derived from pooled clones because formation of stable transfectants involves insertion of the transfected construct into the genome of the cell, with insertion occurring at a random point. Thus pooling multiple clones also minimised the chance that any changes in the general characteristics of the cell line were due to the specific effects of the chemokine expressed, and not to the genomic insertion point of the expression construct in any particular clone.

Six cell lines were generated: 4TX12 1 and 4TX12 2 (expressing the wild-type CXCL12 construct), 4T12P2G (expressing the $CXCL12_{(P2G)}$ construct), 4T16 (expressing the wild-type CXCL16 construct), 4TΔ16 (expressing the $CXCL16_{(9-220)}$ construct) and 4T12Ala (expressing the control $CXCL12_{(Ala)}$ construct). All six were screened for expression of their respective constructs by RT-PCR and ELISA (Figure 3.12 and Figure 3.13). All the cell lines expressed their respective constructs significantly over and above the endogenous levels of chemokine, as determined by ELISA, with the exception of the 4T12Ala line. The disruption of the tertiary structure of the $CXCL12_{(Ala)}$ protein is likely to render its detection by ELISA less efficient. It may also more readily target the protein for destruction, resulting in reduced secretion. However, the RT-PCR data clearly indicate that the $CXCL12_{(Ala)}$ construct is robustly expressed at the mRNA level, similar to the other constructs.

In addition to the RT-PCR and ELISA analyses, the 4T16 and 4TΔ16 lines were assessed for surface expression of their respective CXCL16 constructs by flow cytometry (Figure 3.13C). Small but clear increases in fluorescence intensity were observed for both cell lines. Together with the ELISA analysis, these data indicate that both the membrane-bound and secreted forms of CXCL16 are produced by these cells.

3.4.2 In vitro characterisation of chemokine construct-expressing cell lines

The *in vitro* growth characteristics of the newly generated cell lines were assessed in two ways: assessment of proliferation on plastic and assessment of colony formation in semi-solid agar. The first assay compared the proliferation rates of the eight cell lines with the parental 4T1.2 line, while the second was used to compare the anchorage-independent growth (a measure of tumorigenicity) of the cells.

The proliferative capacity was assessed by measuring the reduction of XTT by metabolically active cells growing in medium containing 10%, 1% or 0.1% FCS. No significant differences in cell growth properties were observed between the parental and CXCL12 or CXCL16 construct-expressing cell lines (Figure 3.14 and Figure 3.15). Similarly, there were no significant differences in the number of colonies formed by the cells in soft agar (Figure 3.16). These data suggested that neither the process involved in

generating stable transfectants, nor the production of CXCL12 and CXCL16, had a direct effect on the growth of 4T1.2 cells, at least in an *in vitro* setting.

3.5 Summary of findings and preliminary discussion

In summary, no endogenous CXCL12 expression was detected in 4T1.2 cells. Although no CXCR4 mRNA was detected, surface CXCR4 was clearly present on 4T1.2 cells as determined by flow cytometry. A low level of CXCR7 mRNA was revealed by RT-PCR, but this could not be confirmed at the protein level due to the lack of reagents available for detecting CXCR7 protein. Neither the expression of CXCR4 nor CXCR7 correlated with a functional response to CXCL12 stimulation. It should be noted that only mobilisation of intracellular calcium stores was assessed as an indicator of receptor signalling, thus CXCL12-induced signalling through CXCR7 would not be detected using this method. In the case of CXCR4, it was hypothesised that the lack of calcium mobilisation in response to CXCL12 was due to competition of CXCR7 with CXCR4 for CXCL12 binding, as CXCR7 seems to have a higher affinity for CXCL12 than CXCR4[129]. Since CXCR7 also binds the CXCR3 ligand CXCL11[129], one way to determine whether or not CXCL12 binds to CXCR7 on 4T1.2 cells is to conduct competitive binding studies. Displacement of CXCL12 by CXCL11 would indicate that CXCL12 binds CXCR7 rather than CXCR4 on 4T1.2 cells.

A low level of CXCL16 was detected in 4T1.2 cells using both RT-PCR and ELISA. A very low level of CXCR6 mRNA expression was observed, but this did not correlate with detectable surface protein expression. The lack of calcium mobilisation by 4T1.2 cells in response to CXCL16 was not surprising given the absence of surface-expressed CXCR6. It may be that the lack of surface CXCR6 was a consequence of the low level of mRNA expression, though clearly CXCR4 protein was detectable despite very low transcript levels. Alternatively, internalisation of CXCR6 may be induced by endogenous CXCL16, perhaps forming an autocrine signalling loop. Intracellular staining for CXCR6 could be employed to resolve this question.

Chemokine and chemokine antagonist production was clearly detectable in the transfected cell lines. In an attempt to confirm the functionality of the expressed proteins, culture supernatants from the transfectants were collected and used as stimuli in calcium

mobilisation and chemotaxis assays with CXCR4- or CXCR6-expressing 300.19 cells. However, chemokine concentrations achieved in the supernatants were too low for responses to be observed *in vitro* (data not shown). High background due to the presence of serum in the culture medium could not be avoided due to the sensitivity of 4T1.2 cells to serum-starvation. Specially formulated serum-free medium may be required for generation of culture supernatants with minimal background effects. It may be also necessary to purify and concentrate the secreted chemokines using the histidine tag prior to testing. In the case of the CXCL16 constructs, where the histidine tag is lost when the chemokine is cleaved from the cell surface, culture supernatants from cell lines expressing the soluble chemokine domain with a C-terminal histidine tag could be used for functional assays.

Five clones from each transfected cell population were pooled to produce cell lines for further study. It may seem counterproductive to clone out a cell line only to pool clones together again. However, as discussed above, multiple clones were pooled to obtain high-level chemokine expression, as well as to ensure that any changes in the general characteristics of the cell line were due to the specific effects of the chemokine expressed. Ideally, a panel of individual clones would be selected for *in vivo* study, but this is a very labour intensive approach. Cultured cell lines were discarded and fresh aliquots defrosted on a regular basis to prevent a preponderance of any one particular clone developing and altering the properties of the cell line as a whole.

No differences in the *in vitro* growth characteristics of the transfected cell lines and the 4T1.2 cell line were observed. This suggests that chemokine or antagonist expression does not directly affect tumour cell growth properties. Additional tests *in vitro*, for instance migration and invasion assays, could be employed to further characterise the effect of chemokine expression on tumour cells. However, a more complete picture of the effect of chemokine expression on tumour progression could be gained in an *in vivo* system where the influence of host components can be studied as well. The experiments described in the following chapter examine the effects of chemokine expression on tumour growth *in vivo*.

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Table 3.1 Chemokine constructs and their functions.

Construct	Mutation	Function
CXCL12	None	Wild-type chemokine
$CXCL12_{(P2G)}$	Pro2 → Gly	CXCL12 antagonist
CXCL16	None	Wild-type chemokine
$CXCL16_{(9-220)}$	Deletion of residues 1-8	CXCL16 antagonist
$CXCL12_{(Ala)}$	Cys9 → Ala; Cys11→ Ala	Biologically inactive control

Figure 3.1 *Amino acid sequences of chemokine constructs.*

The amino acid sequences of the chemokine constructs used in this study are shown in alignment. The sequences shown are those of the precursor proteins. **(A)** CXCL12 constructs. The first 21 amino acids *(blue and underlined)* form the signal peptide that targets the protein for secretion; these are cleaved from the mature protein. Each construct carries a six histidine residue tag *(green italics)* at its C-terminus. The glycine substitution in the $CXCL12_{(P2G)}$ antagonist is highlighted in red, as are the alanine substitutions in the biologically inactive $CXCL12_{(Ala)}$ control construct. **(B)** CXCL16 constructs. The first 26 amino acids *(blue and underlined)* form the signal peptide that targets the protein to the cell membrane; these are cleaved from the mature protein. Each construct carries a six histidine residue tag *(green italics)* at its C-terminus. The eight amino acids deleted in the $CXCL16_{(9\text{-}220)}$ antagonist are represented by red dots.

A

```
CXCL12          1   MDAKVVAVLALVLAALCISDGKPVSLSYRCPCRFFESHIARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLK
CXCL12(P2G)     1   MDAKVVAVLALVLAALCISDGKGVSLSYRCPCRFFESHIARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLK
CXCL12(Ala)     1   MDAKVVAVLALVLAALCISDGKPVSLSYRAPARFFESHIARANVKHLKILNTPNCALQIVARLKNNNRQVCIDPKLK

CXCL12         78 WIQEYLEKALNKRLKMHHHHHH
CXCL12(P2G)    78 WIQEYLEKALNKRLKMHHHHHH
CXCL12(Ala)    78 WIQEYLEKALNKRLKMHHHHHH
```

B

```
CXCL16          1   MRRGFGPLSLAFFLFLLALLTLPGDGNQGSVAGSCSCDRTISSGTQIPQGTLDHIRKYLKAFHRCPFFIRFQLQSKS
CXCL16(9-220)     1 MRRGFGPLSLAFFLFLLALLTLPGDG·····CSCDRTISSGTQIPQGTLDHIRKYLKAFHRCPFFIRFQLQSKS

CXCL16         78VCGGSQDQWVRELVDCFER KECGTGHGKSFHHQKHLPQASTQTPEAAEGTPSDTSTPAHSQSTQHSTLPSGALSLNK
CXCL16(9-220)  78  VCGGSQDQWVRELVDCFERKECGTGHGKSFHHQKHLPQASTQTPEAAEGTPSDTSTPAHSQSTQHSTLPSGALSLNK

CXCL16         155 EHTQPWEMTTLPSGYGLEARPEAEANEKQQDDRQQEAPGAGASTPAWVPVLSLLAIVFFLTAAMAYVLCNRRATQQN
CXCL16(9-220)  155 EHTQPWEMTTLPSGYGLEARPEAEANEKQQDDRQQEAPGAGASTPAWVPVLSLLAIVFFLTAAMAYVLCNRRATQQN

CXCL16         232SAGLQLWYTPVEPRP HHHHHH
CXCL16(9-220)  232SAGLQLWYTPVEPRP HHHHHH
```

Figure 3.2 *Cloning of chemokine constructs.*

(A) The CXCL12 and $CXCL12_{(P2G)}$ coding sequences were excised from the pCR2.1-TOPO vector with the restriction enzymes EcoRI and SpeI. The resulting fragments were separated by agarose gel electrophoresis and the desired bands were excised and purified. The purified chemokine DNA was then ligated into pEF-IRES-puro6 which had been similarly digested and purified. **(B)** The $CXCL12_{(Ala)}$ coding sequence was amplified by PCR to eliminate the KDEL motif previously engineered at the 3′ end of the template. The PCR product was digested with EcoRI and SpeI and ligated into pEF-IRES-puro6. **(C)** The CXCL16 coding sequence was amplified by PCR with primers to append nucleotides encoding a six histidine tag at the 3′ end. The PCR product was digested with EcoRI and EcoRV and ligated into pEF-IRES-puro6 which had been similarly digested. **(D)** The $CXCL16_{(9\text{-}220)}$ construct was generated using overlap extension PCR as outlined in Figure 3.3 below. The final PCR product was digested with EcoRI and EcoRV and ligated into pEF-IRES-puro6.

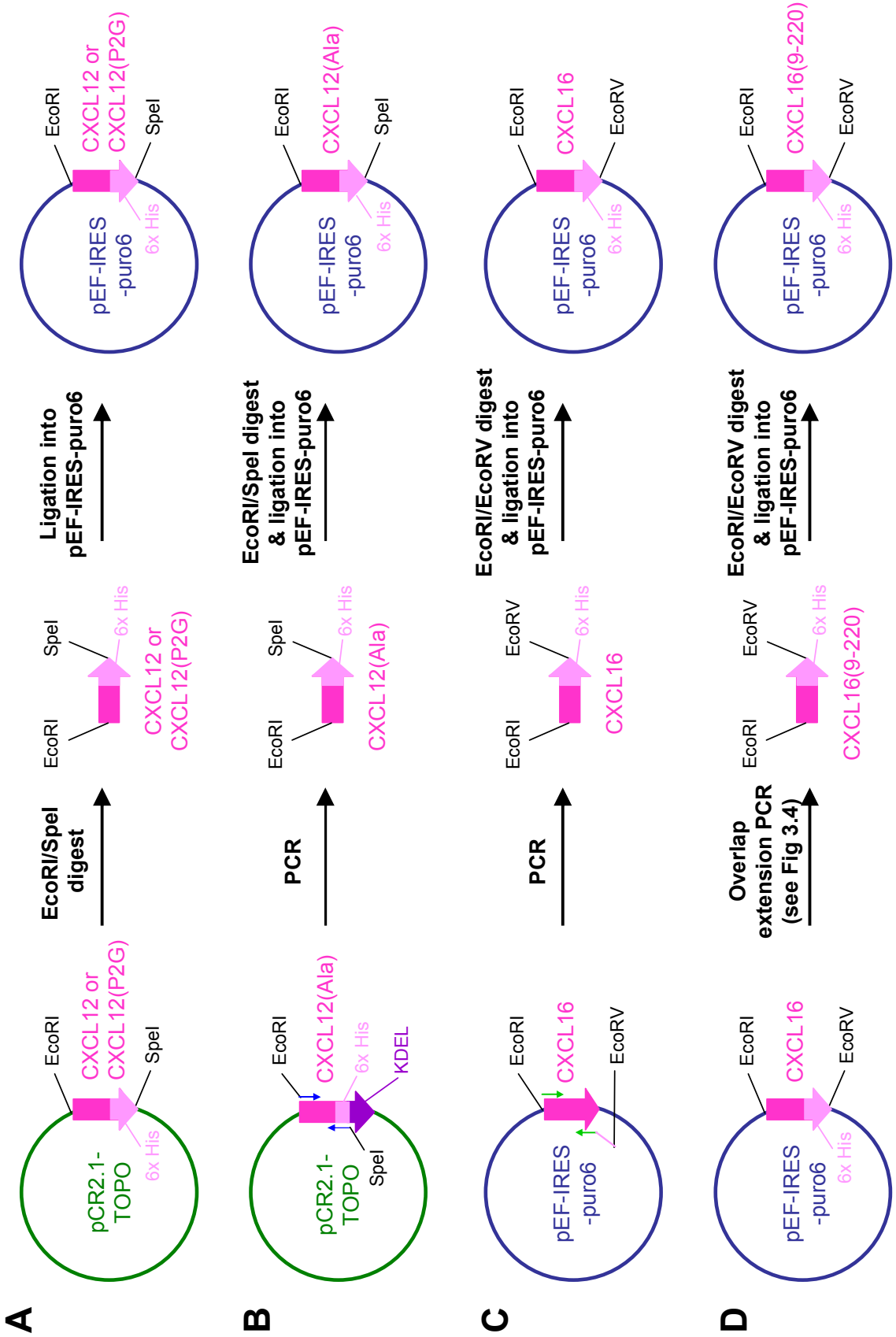
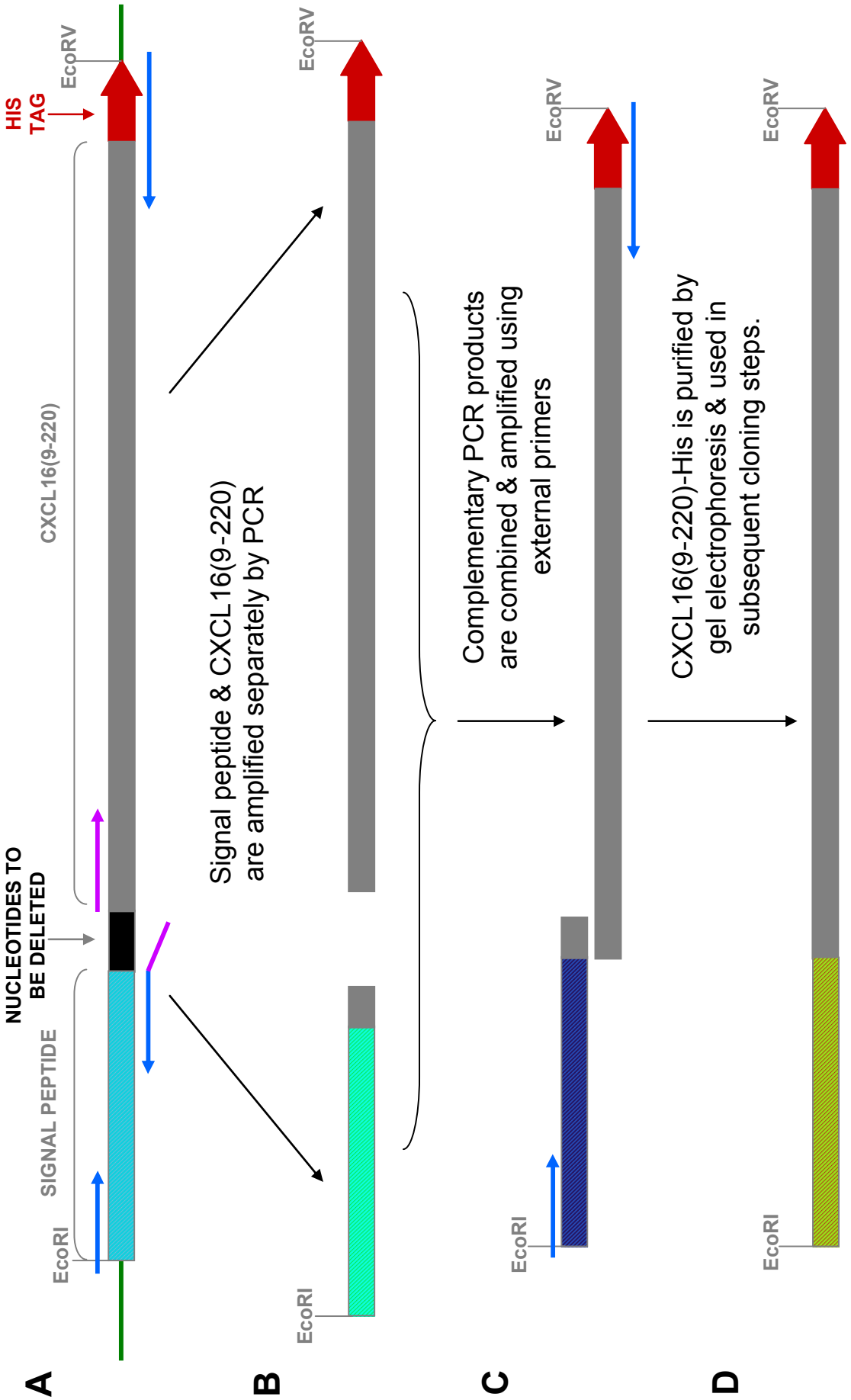

A
pCR2.1-TOPO
EcoRI
CXCL12 or CXCL12(P2G)
SpeI
6x His
EcoRI/SpeI digest
EcoRI
SpeI
6x His
CXCL12 or CXCL12(P2G)
Ligation into pEF-IRES-puro6
pEF-IRES -puro6
EcoRI
CXCL12 or CXCL12(P2G)
SpeI
6x His
B
pCR2.1-TOPO
EcoRI
CXCL12(Ala)
6x His
KDEL
SpeI
PCR
EcoRI
SpeI
6x His
CXCL12(Ala)
EcoRI/SpeI digest & ligation into pEF-IRES-puro6
pEF-IRES -puro6
EcoRI
CXCL12(Ala)
SpeI
6x His
C
pEF-IRES -puro6
EcoRI
CXCL16
EcoRV
PCR
EcoRI
EcoRV
6x His
CXCL16
EcoRI/EcoRV digest & ligation into pEF-IRES-puro6
pEF-IRES -puro6
EcoRI
CXCL16
EcoRV
6x His
D
pEF-IRES -puro6
EcoRI
CXCL16
EcoRV
6x His
Overlap extension PCR (see Fig 3.4)
EcoRI
EcoRV
6x His
CXCL16(9-220)
EcoRI/EcoRV digest & ligation into pEF-IRES-puro6
pEF-IRES -puro6
EcoRI
CXCL16(9-220)
EcoRV
6x His

Figure 3.3 *Generation of the CXCL16$_{(9-220)}$-His construct by overlap extension PCR.*

The CXCL16$_{(9-220)}$ construct was amplified from wild-type CXCL16 template using overlap extension PCR. **(A)** PCR primers *(blue and purple arrows)* complementary to the signal sequence and primers complementary to the nucleotide sequence encoding amino acid residues 9 to 220 of CXCL16 were designed. The primers for the 3′ end of the signal sequence and the 5′ end of the antagonist portion were designed such that they included a complementary region *(purple)*. **(B)** The CXCL16 signal sequence and antagonist portion were amplified in separate polymerase chain reactions. **(C)** The PCR products from *(B)* were combined in a third polymerase chain reaction. The complementary region appended to the signal sequence in the first round of PCR enabled the two PCR products to anneal to each other. Thus they formed a template from which a single product could be amplified by the external PCR primers used in the first round of PCR. The external primers contained EcoRI and EcoRV restriction sites to facilitate cloning of the final product into the pEF-IRES-puro6 vector. **(D)** CXCL16$_{(9-220)}$-His was purified by gel electrophoresis. The purified product was then digested with EcoRI and EcoRV and cloned into pEF-IRES-puro6. The construct was sequenced to confirm that the overlap extension PCR had produced the desired sequence.


A
B
C
D
HIS TAG
EcoRV
CXCL16(9-220)
NUCLEOTIDES TO BE DELETED
SIGNAL PEPTIDE
EcoRI
Signal peptide & CXCL16(9-220) are amplified separately by PCR
Complementary PCR products are combined & amplified using external primers
CXCL16(9-220)-His is purified by gel electrophoresis & used in subsequent cloning steps.

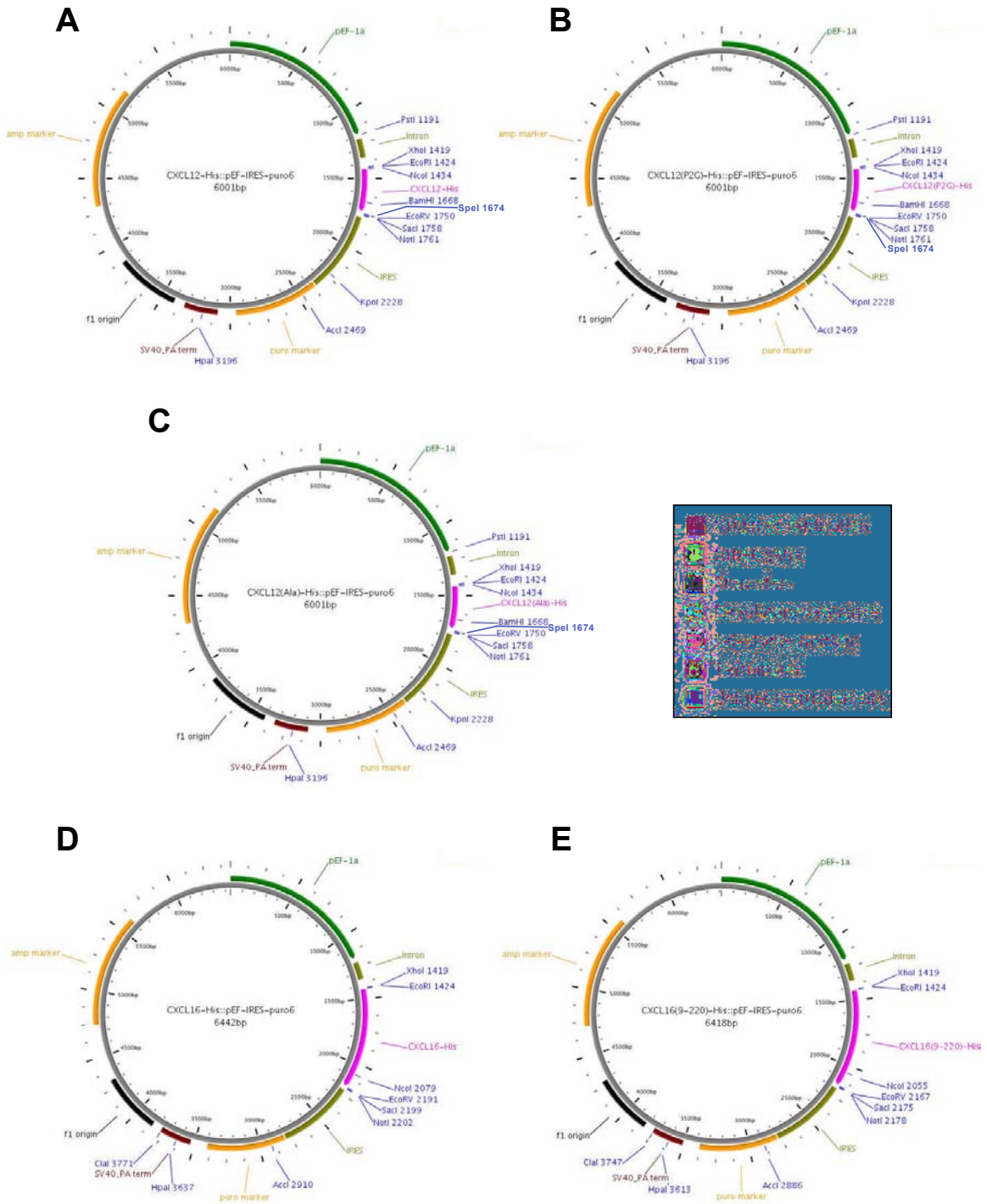


Figure 3.4 *Vector maps of chemokine constructs.*

(A) CXCL12-His::pEF-IRES-puro6. **(B)** CXCL12$_{(P2G)}$-His::pEF-IRES-puro6. **(C)** CXCL12$_{(Ala)}$-His::pEF-IRES-puro6. **(D)** CXCL16FL-His::pEF-IRES-puro6. **(E)** CXCL16$_{(9-220)}$-His::pEF-IRES-puro6. The nature and function of the various elements of each construct are indicated in the legend.

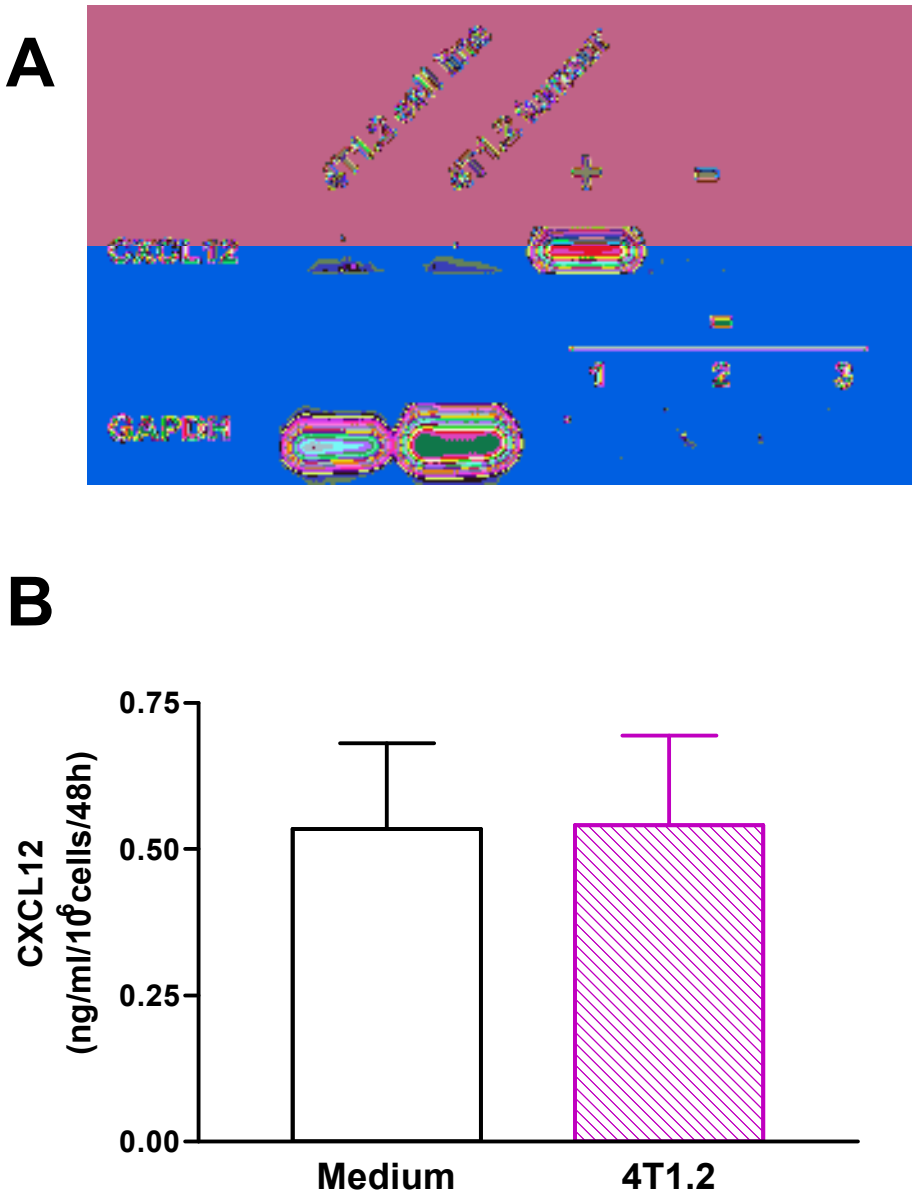


Figure 3.5 *Expression of CXCL12 by 4T1.2 cells.*

(A) Total RNA was extracted from 4T1.2 cells (5 x 10^6) or excised 4T1.2 tumours using TRIzol® reagent according to the manufacturer's instructions. Five micrograms of total RNA were subjected to DNase treatment and reverse-transcribed as described in Section 2.4.2. The resulting cDNA was subjected to 35 cycles of PCR with mouse CXCL12 primers or glutaraldehyde 3-phosphate dehydrogenase (GAPDH) primers as a control. +, positive control; -, negative control. For CXCL12 primers, H_2O was added to the negative control instead of template. For GAPDH negative controls: *1,* reverse transcription control with no reverse transcriptase added; *2,* reverse transcription control with no template added; *3,* PCR control with no template added. **(B)** 4T1.2 cells were cultured for 48 hours in complete medium. Culture supernatant was harvested, passed through a 2µm filter and protease inhibitor cocktail was added at a 1:100 dilution. The supernatant was then assayed for CXCL12 content by ELISA as described in Section 2.4.3. Culture medium alone served as a background control for comparison.

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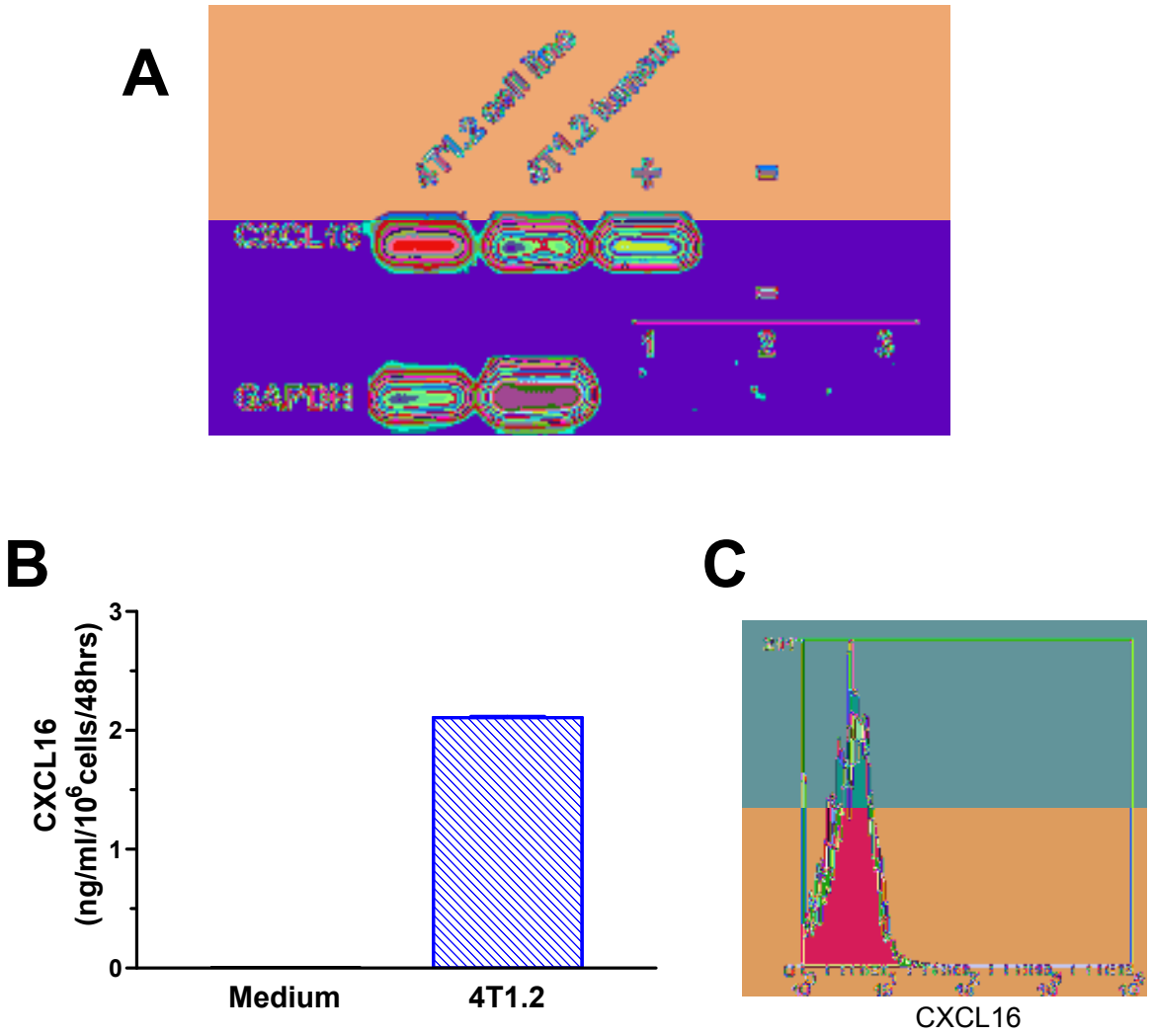


Figure 3.6 *Expression of CXCL16 by 4T1.2 cells.*

(A) Total RNA was extracted from 4T1.2 cells (5 x 10^6) or excised 4T1.2 tumours using TRIzol® reagent according to the manufacturer's instructions. Five micrograms of total RNA were subjected to DNase treatment and reverse-transcribed as described in Section 2.4.2. The resulting cDNA was subjected to 35 cycles of PCR with mCXCL16 primers or GAPDH primers as a control. +, positive control; -, negative control. For CXCL16 primers, H_2O was added to the negative control instead of template. For GAPDH negative controls: *1,* reverse transcription control with no reverse transcriptase added; *2,* reverse transcription control with no template added; *3,* PCR control with no template added. **(B)** 4T1.2 cells were cultured for 48 hours in complete medium. Culture supernatant was harvested, passed through a 2µm filter and protease inhibitor cocktail was added at a 1:100 dilution. The supernatant was then assayed for CXCL16 content by ELISA as described in Section 2.4.3. Culture medium alone served as a background control for comparison. **(C)** 4T1.2 cells were stained with a biotinylated anti-mouse CXCL16 antibody or isotype-matched control followed by streptavidin-conjugated phycoerythrin as described in Section 2.4.1. Fluorescing cells were quantified on the same day using an LSRII flow cytometer. *Shaded region,* anti-CXCL16 antibody. *Solid line,* isotype-matched control antibody.

Figure 3.7 *Expression of CXCR4 and CXCR7 by 4T1.2 cells.*

(A) CXCR4 and CXCR7 mRNA expression in 4T1.2 cells. Total RNA was extracted from 10^7 cells using TRIzol® reagent according to the manufacturer's instructions. Five micrograms of total RNA were subjected to DNase treatment and reverse-transcribed as described in Section 2.4.2. The resulting cDNA was subjected to 35 cycles of PCR with mCXCR4 or mCXCR7 primers, or GAPDH primers as a control. +, positive control; -, negative control. For CXCR4 and CXCR7 primers, H_2O was added to the negative control instead of template. For GAPDH negative controls: *1,* reverse transcription control with no reverse transcriptase added; *2,* reverse transcription control with no template added; *3,* PCR control with no template added. **(B)** Cell surface expression of CXCR4. Cells were fixed in 4% formaldehyde in PBS for 10 minutes prior to staining (300.19 cells) or left unfixed (4T1.2 cells) and incubated with a monoclonal rat anti-mouse CXCR4 antibody or isotype-matched control followed by streptavidin-conjugated phycoerythrin as described in Section 2.4.1. Fluorescing cells were quantified on the same day using a FACScan flow cytometer. Histograms are shown for 4T1.2 (25.72% of cells CXCR4 positive after subtracting background determined from isotype control) and positive control 300.19 cells (22.49% of cells CXCR4 positive after subtracting background determined from isotype control). *Shaded region,* anti-CXCR4 antibody. *Solid line,* isotype-matched control antibody. Representative data from one experiment are shown. (C) Intracellular calcium mobilisation in response to CXCL12 stimulation of 4T1.2 cells. Approximately 10^7 cells were incubated with 2μM FURA-2AM for 30 minutes at 37°C in 5% CO_2. Cells were washed once in calcium buffer (Section 2.6.2.4) and then stimulated at the indicated time points with buffer alone, control peptide ($CCL2_{(Ala)}$ at 300ng/ml) or mouse CXCL12 (300ng/ml). 300.19 cells were used as a positive control. Data are normalized to background fluorescence readings for each sample.

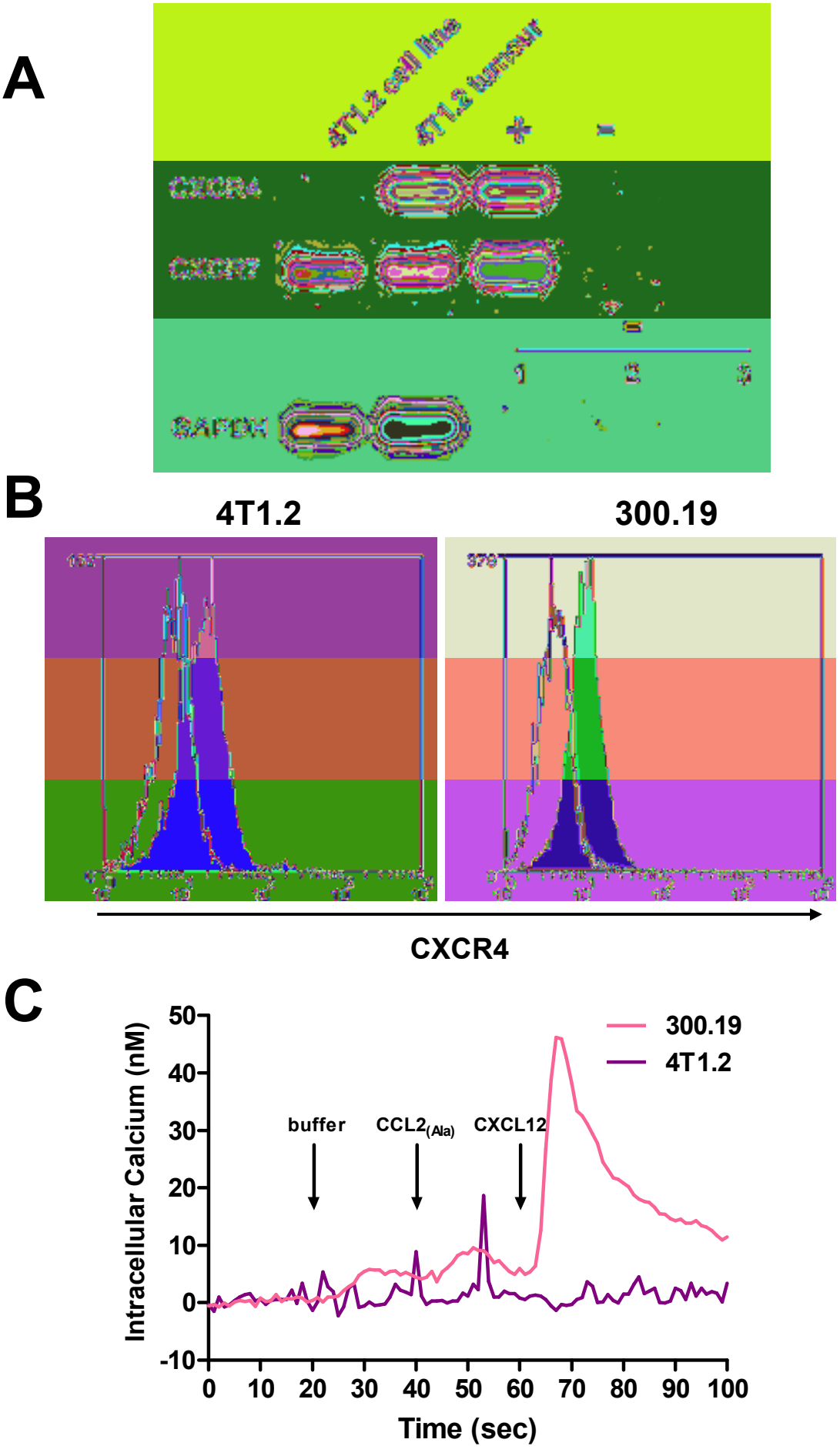
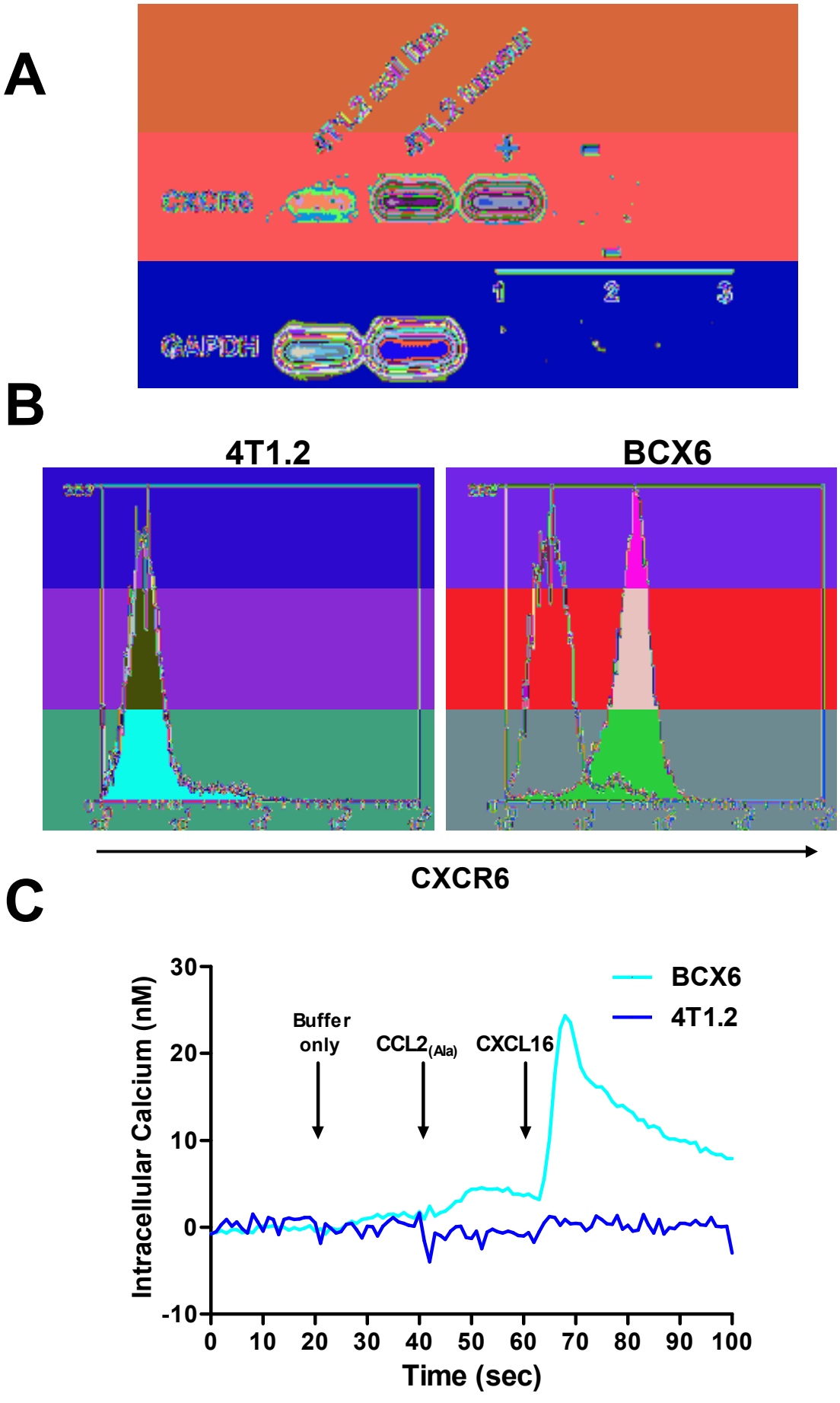

A
4T1.2 cell line
4T1.2 supernat
+
-
CXCR4
CXCR7
GAPDH
1
2
3
B
4T1.2
300.19
CXCR4
C
Intracellular Calcium (nM)
50
40
30
20
10
0
-10
buffer
CCL2(Ala)
CXCL12
300.19
4T1.2
0
10
20
30
40
50
60
70
80
90
100
Time (sec)

Figure 3.8 *Expression of CXCR6 by 4T1.2 cells.*

(A) CXCR6 mRNA expression in 4T1.2 cells. Total RNA was extracted from 10^7 cells using TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. Five micrograms of total RNA were subjected to DNase treatment and reverse-transcribed as described in Chapter 2: Materials & Methods. The resulting cDNA was subjected to 35 cycles of PCR with mCXCR6 primers. +, positive control; -, negative control. For CXCR6 primers, H_2O was added to the negative control instead of template. For GAPDH negative controls: *1,* reverse transcription control with no reverse transcriptase added; *2,* reverse transcription control with no template added; *3,* PCR control with no template added. **(B)** Cell surface expression of CXCR6. Cells were incubated with a polyclonal rabbit anti-mouse CXCR6 antibody or isotype-matched control followed by streptavidin-conjugated phycoerythrin as described in Section 2.4.1. Fluorescing cells were quantified on the same day using a FACScan flow cytometer. Histograms are shown for 4T1.2 (-2.94% of cells CXCR6 positive after subtracting background determined from isotype control) and positive control CXCR6-expressing 300.19 ('BCX6') cells (84.88% of cells CXCR6 positive after subtracting background determined from isotype control). *Shaded region,* anti-CXCR6 antibody. *Solid line,* isotype-matched control antibody. Representative data from one experiment are shown. **(C)** Intracellular calcium mobilisation in response to CXCL16 stimulation of 4T1.2 cells. Approximately 10^7 cells were incubated with 2μM FURA-2AM for 30 minutes at 37°C in 5% CO_2. Cells were washed once in calcium buffer (Section 2.6.2.4) and then stimulated at the indicated time points with buffer alone, control peptide ($CCL2_{(Ala)}$ at 300ng/ml) or recombinant mouse CXCL16 (300ng/ml). BCX6 cells were used as a positive control. Data are normalized to background fluorescence readings for each sample.


A
4T1.2 wild type
4T1.2 hCXCR6
+
-
CXCR6
-
1
2
3
GAPDH
B
4T1.2
BCX6
CXCR6
C
Intracellular Calcium (nM)
30
20
10
0
-10
Buffer only
CCL2(Ala)
CXCL16
BCX6
4T1.2
0
10
20
30
40
50
60
70
80
90
100
Time (sec)

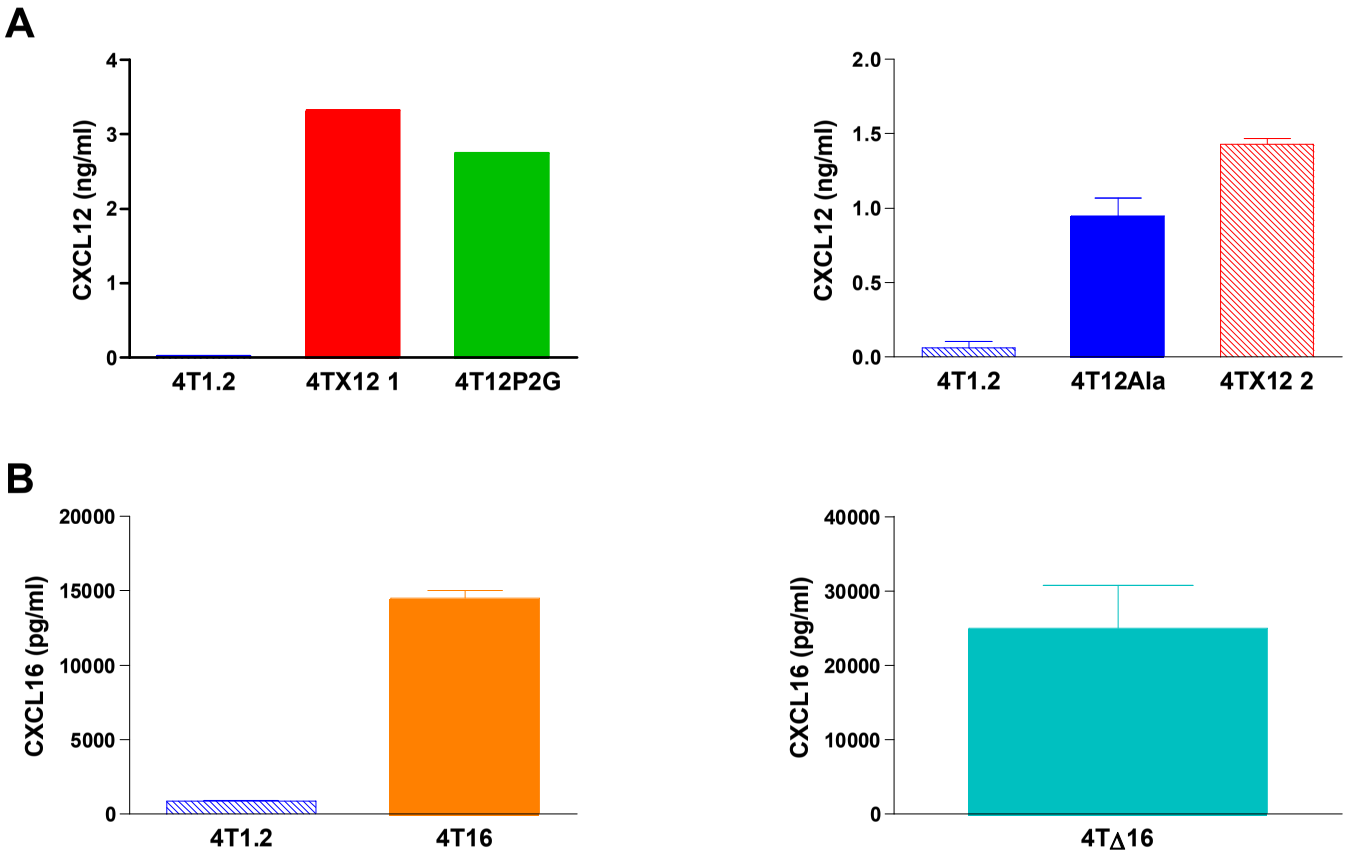


Figure 3.9*Expression of chemokine constructs by transfected 4T1.2 cell populations.*

Transfected populations of 4T1.2 cells were cultured in complete medium. Culture supernatant was harvested, passed through a 2µm filter and protease inhibitor cocktail was added at a 1:100 dilution. The supernatant was then assayed for CXCL12 or CXCL16 content by ELISA as described in Section 2.4.3. Culture medium alone or non-transfected 4T1.2 culture supernatant served as background controls for comparison. **(A)** Bars represent a single determination. **(B)** Bars represent the mean ± SD of quadruplicate determinations. **(C)** Bars represent the mean ± SD of duplicate determinations. **(D)** Bar represents the mean ± SD of triplicate determinations.

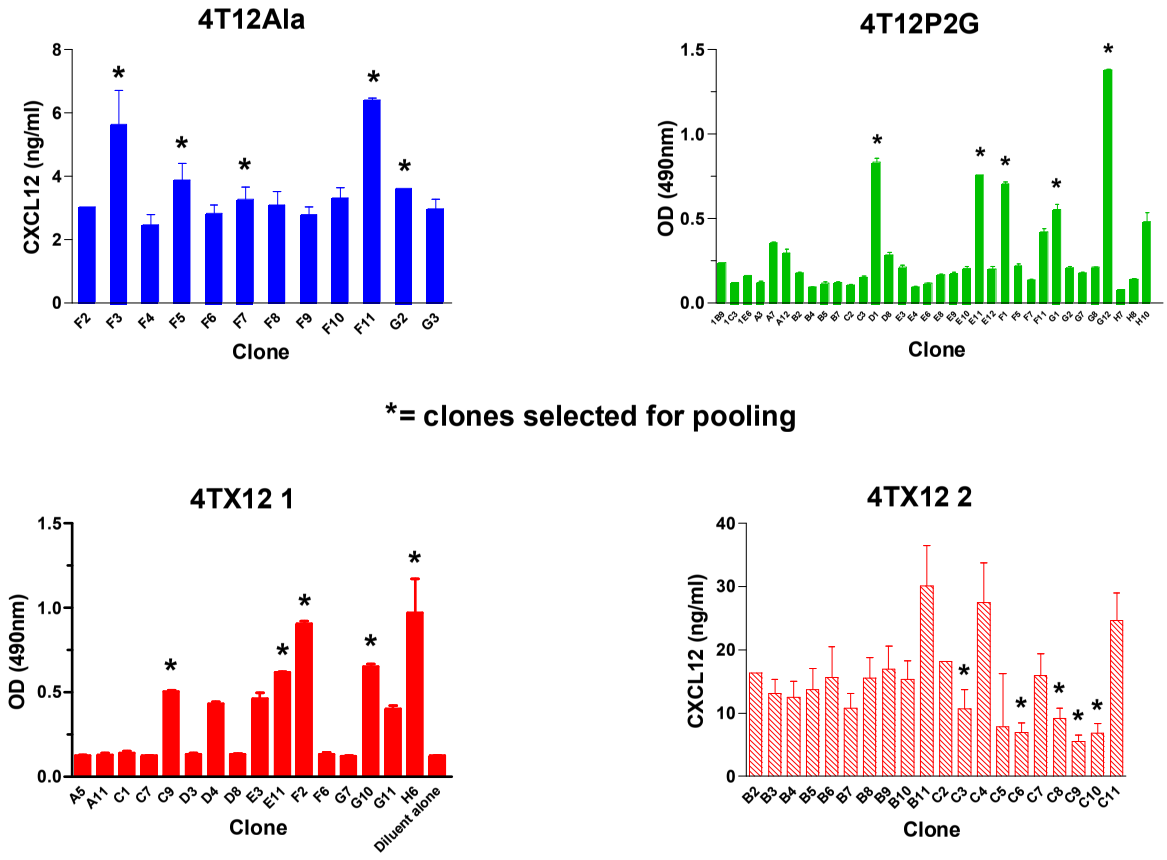


Figure 3.10*Level of CXCL12 construct production by individual clones from transfected 4T1.2 populations.*

Individual clones from each of the CXCL12-producing transfected populations in Figure 3.9 were isolated by limiting dilution and plated in 96-well trays. Supernatants from wells containing individual colonies were harvested and assayed for CXCL12 content by ELISA as described in Section 2.4.3. The five highest CXCL12 construct-expressing clones from each transfected population *(indicated by asterisks)*were selected and pooled to generate high-expressing cell lines.For the 4TX12 2 population, the five lowest CXCL12-expressingclones *(indicated by asterisks)* were selected to best match the CXCL12 expression level of the 4TX12 1 clones.

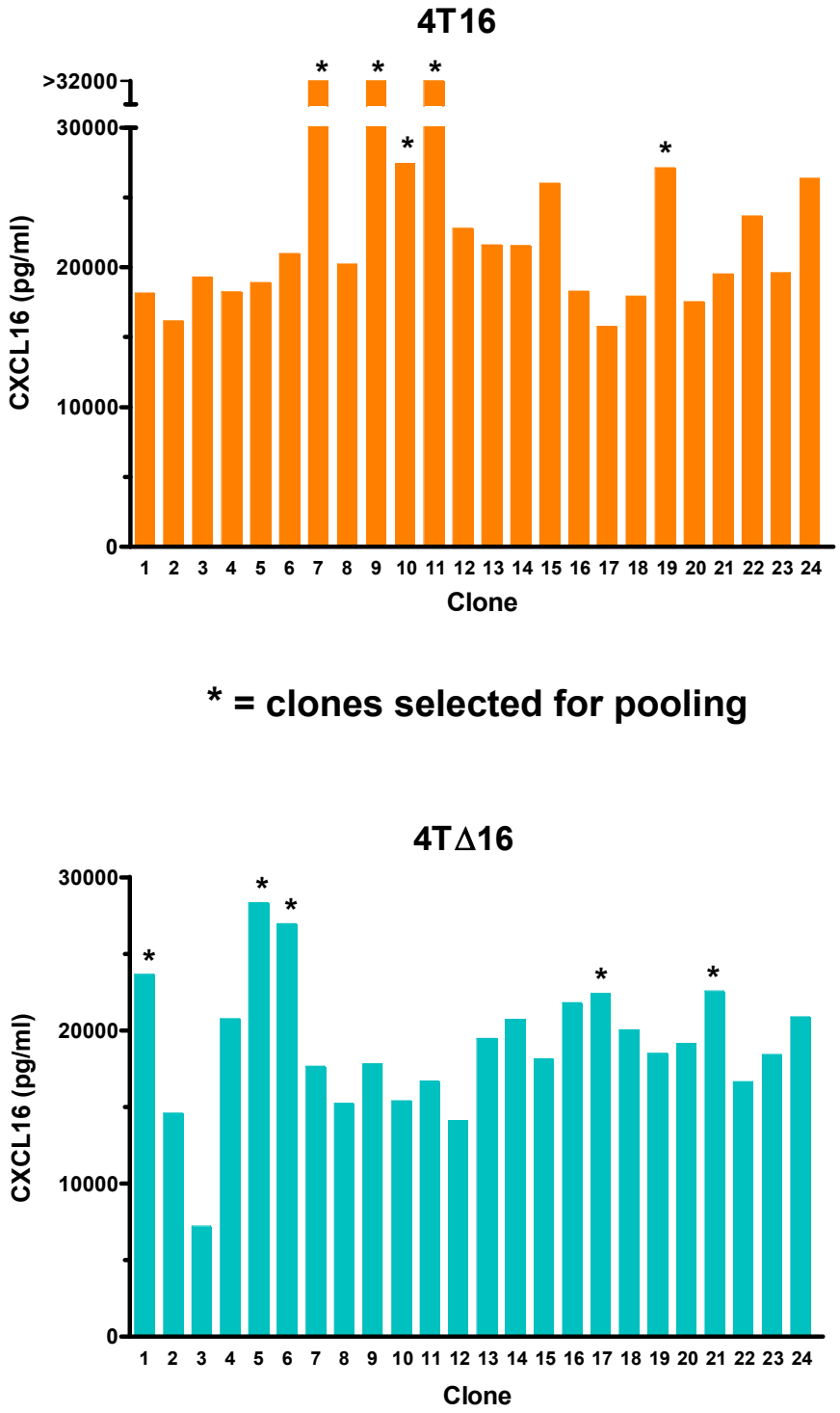


Figure 3.11 *Level of CXCL16 construct production by individual clones from transfected 4T1.2 populations.*

Individual clones from each of the CXCL16-producing transfected populations in Figure 3.9 were isolated by limiting dilution and plated in 96-well trays. Supernatants from wells containing individual colonies were harvested and assayed for CXCL16 content by ELISA as described in Section 2.4.3. The five highest CXCL16 construct-expressing clones from each transfected population *(indicated by asterisks)* were selected and pooled to generate high-expressing cell lines.

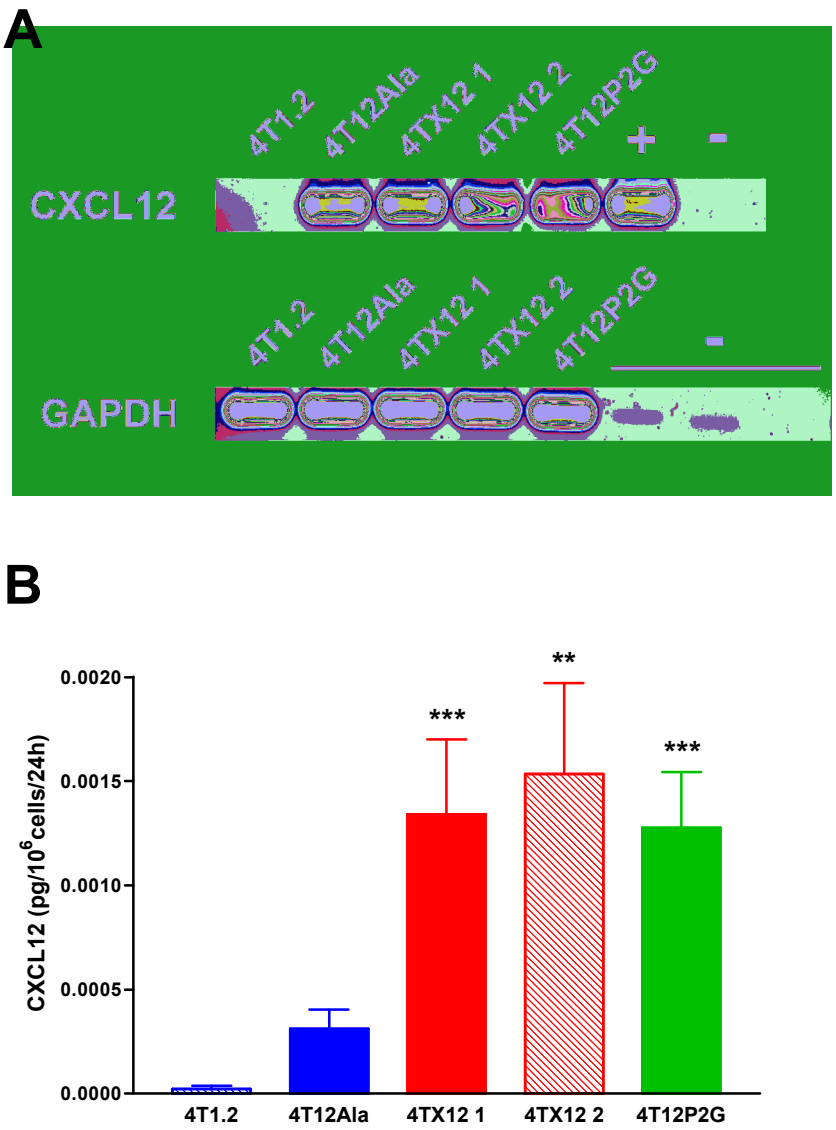
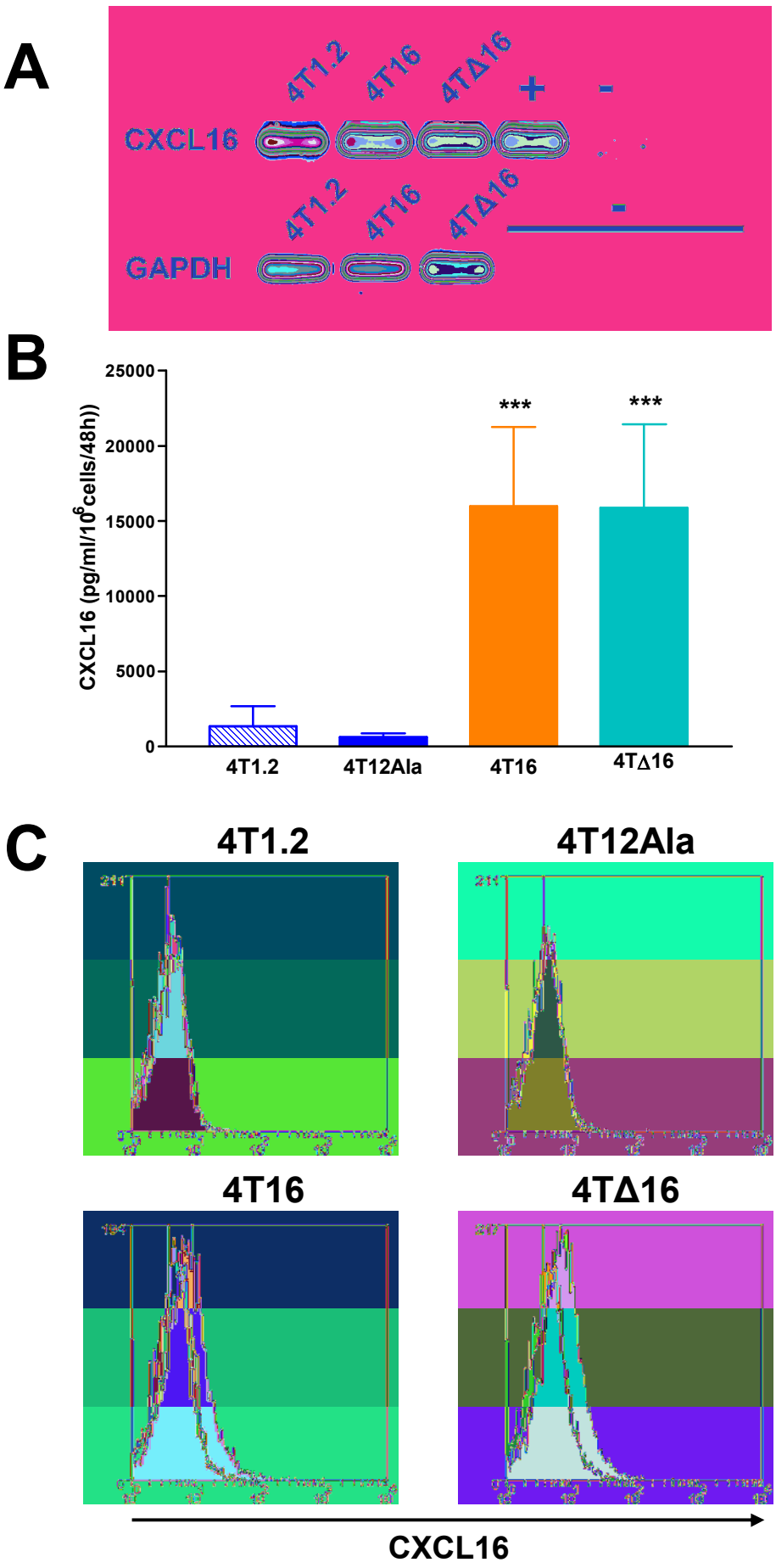


Figure 3.12 *CXCL12 construct expression by cell lines derived from pooled clones.*

(A) Total RNA was extracted from 5 x 10^6 cells from each of the cell lines derived from the pooled clones in Figure 3.10 using TRIzol® reagent according to the manufacturer's instructions. Five micrograms of total RNA were subjected to DNase treatment and reverse-transcribed as described in Section 2.4.2. The resulting cDNA was subjected to 35 cycles of PCR with mCXCL12 primers or GAPDH primers as a control. +, positive control CXCL12::pEF-IRES-puro6 plasmid; -, negative control. For CXCL12 primers, H_2O was added to the negative control instead of template. For GAPDH primers, the negative controls were, from left to right: reverse transcription control with no reverse transcriptase added, reverse transcription control with no template added, and PCR control with no template added. **(B)** Cell lines derived from the pooled clones in Figure 3.10 were cultured in complete medium. Culture supernatant was harvested, passed through a 2µm filter and protease inhibitor cocktail was added at a 1:100 dilution. The supernatant was then assayed for CXCL12 content by ELISA as described in Section 2.4.3. Culture supernatants from the parental 4T1.2 line served as a background control for comparison *(upper graph)*. ***, $p<0.001$; **, $p<0.01$, significantly different from control 4T1.2 culture supernatant, Kruskal-Wallis non-parametric ANOVA.

Figure 3.13 *CXCL16 construct expression by cell lines derived from pooled clones.*

(A) Total RNA was extracted from 5 x 10^6 cells from each of the lines derived from the pooled clones in Figure 3.11 using TRIzol® reagent according to the manufacturer's instructions. Five micrograms of total RNA were subjected to DNase treatment and reverse-transcribed as described in Section 2.4.2. The resulting cDNA was subjected to 35 cycles of PCR with mCXCL16 primers or GAPDH primers as a control. +, positive control CXCL16::pEF-IRES-puro6 plasmid; -, negative control. For CXCL16 primers, H_2O was added to the negative control instead of template. For GAPDH primers, the negative controls were, from left to right: reverse transcription control with no reverse transcriptase added, reverse transcription control with no template added, and PCR control with no template added. **(B)** Cell lines derived from the pooled clones in Figure 3.11 were cultured in complete medium. Culture supernatant was harvested, passed through a 2µm filter and protease inhibitor cocktail was added at a 1:100 dilution. The supernatant was then assayed for CXCL16 content by ELISA as described in Section 2.4.3. Culture supernatants from the parental 4T1.2 line or control 4T12Ala line served as background controls for comparison. ***, significantly different from control 4T1.2 and 4T12Ala culture supernatants, $p<0.001$, One Way ANOVA. **(C)** To detect surface-bound CXCL16, cells from each of the lines derived from the pooled clones in Figure 3.11 were stained with a biotinylated anti-mouse CXCL16 antibody or isotype-matched control followed by streptavidin-conjugated phycoerythrin as described in Section 2.4.1. Fluorescing cells were quantified on the same day using an LSRII flow cytometer. *Shaded region,* anti-CXCL16 antibody. *Solid line,* isotype-matched control antibody.


A
4T1.2
4T16
4TΔ16
+
-
CXCL16
4T1.2
4T16
4TΔ16
-
GAPDH
B
CXCL16 (pg/ml/10^6cells/48h))
25000
20000
15000
10000
5000
0
***
***
4T1.2
4T12Ala
4T16
4TΔ16
C
4T1.2
4T12Ala
4T16
4TΔ16
CXCL16

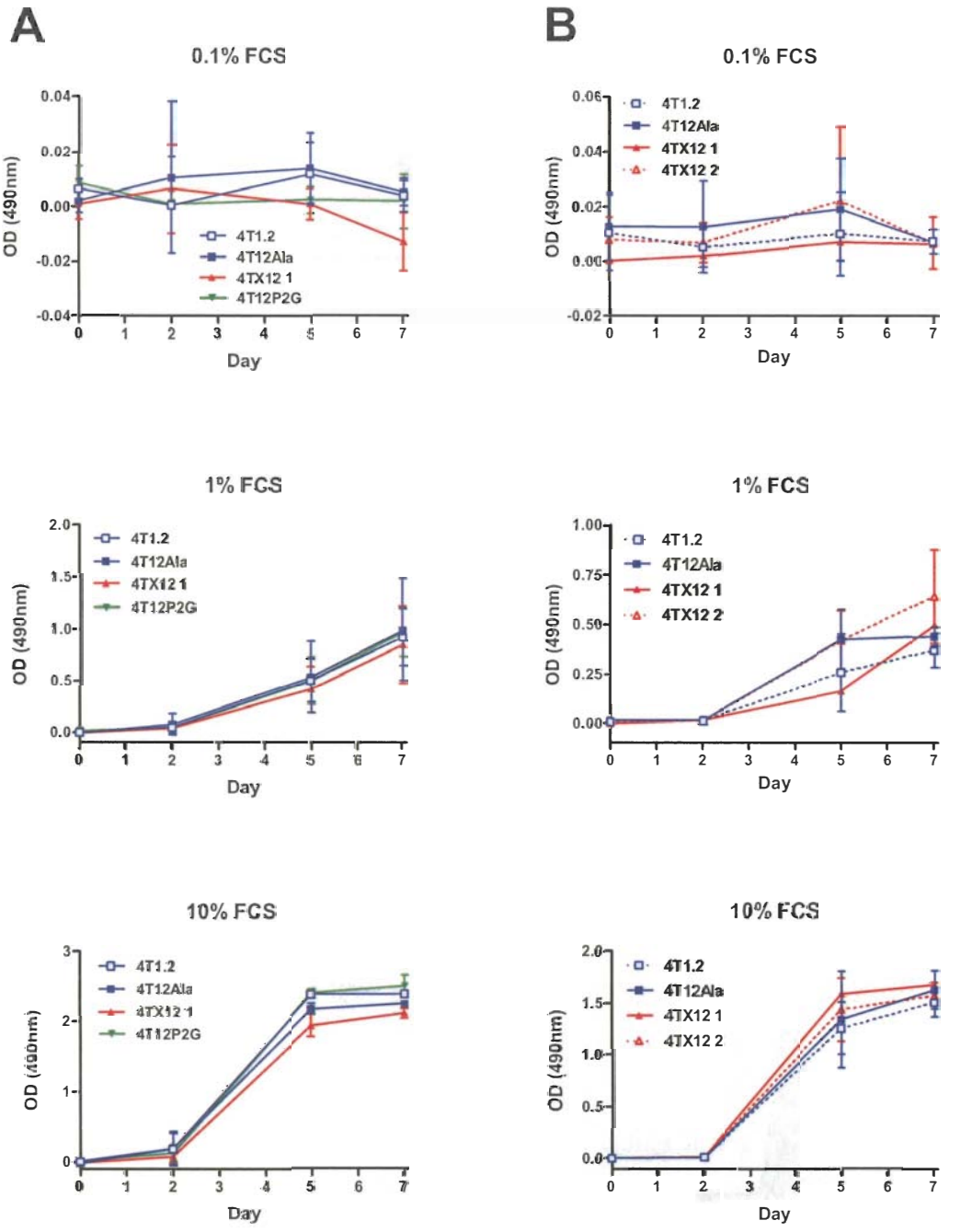


Figure 3.14 In vitro *proliferation rates of CXCL12-expressing 4T1.2 cell lines.*

In vitro proliferation rate of CXCL12-expressing cell lines. Cells were prepared at 10^5 cell/ml in serum-free medium and aliquots of 25µl were added to wells containing medium with 0.1%, 1% or 10% FCS. Cells were then allowed to proliferate at 37°C in 5% CO_2. After the indicated time points, the number of viable cells in each well was assessed by reduction of XTT (1mg/ml) added in a 50:1 ratio with PMS (1.25mM) to each well. Absorbance at 490nm (reference 650nm) was determined using a microplate reader, and background XTT reduction (determined from wells containing no cells) was subtracted. **(A)** Comparison of 4T1.2, 4T12Ala, 4TX12 1 and 4T12P2G lines. **(B)** Comparison of 4T1.2, 4T12Ala, 4TX12 1 and 4TX12 2 lines. Data points represent mean ± SD of 2 (A) or 3 (B) independent experiments. Differences between data points were not significant.

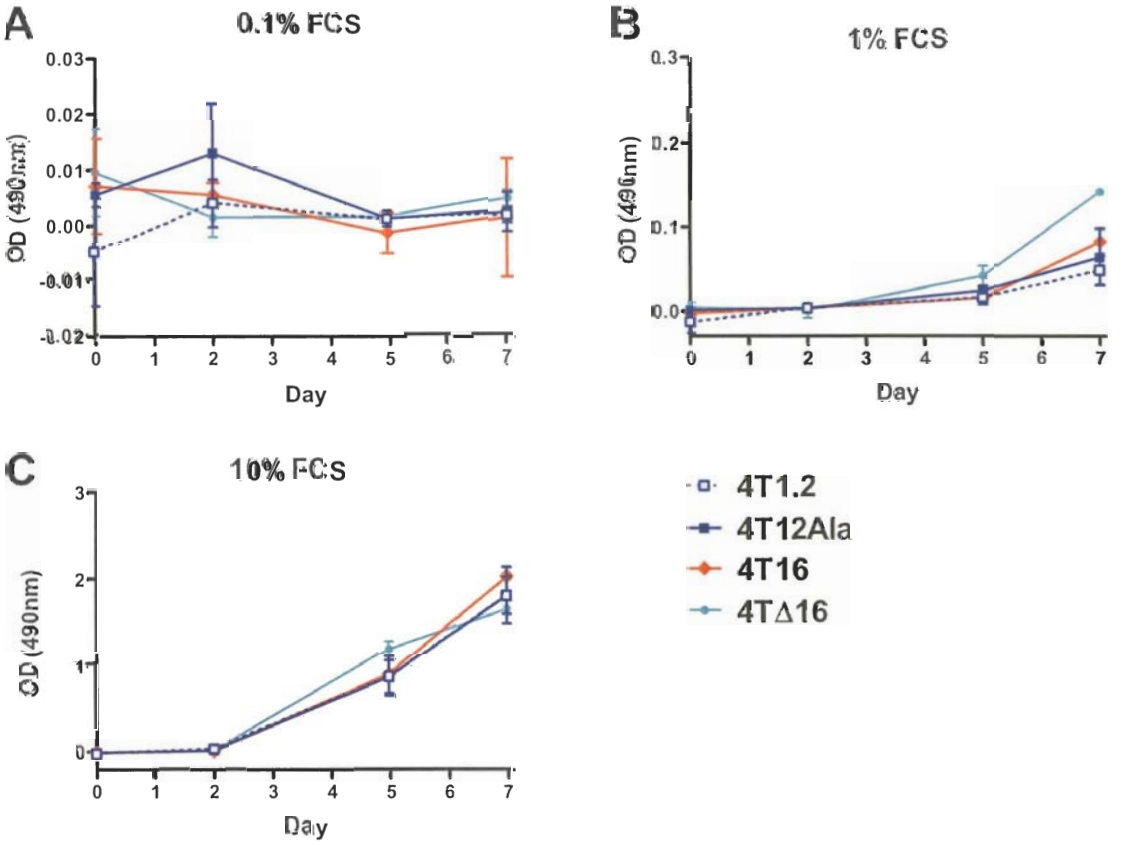


Figure 3.15 In vitro *proliferation rates of CXCL16-expressing 4T1.2 cell lines.*

In vitro proliferation rate of CXCL16-expressing cell lines. Cells were prepared at 10^5 cell/ml in serum-free medium and aliquots of 25µl were added to wells containing medium with **(A)** 0.1%, **(B)** 1% or **(C)** 10% FCS. Cells were then allowed to proliferate at 37°C in 5% CO_2. After the indicated time points, the number of viable cells in each well was assessed by reduction of XTT (1mg/ml) added in a 50:1 ratio with PMS (1.25mM) to each well. Absorbance at 490nm (reference 650nm) was determined using a microplate reader, and background XTT reduction (determined from wells containing no cells) was subtracted. Data points represent mean ± SEM of 2 independent experiments. Differences between data points were not significant.

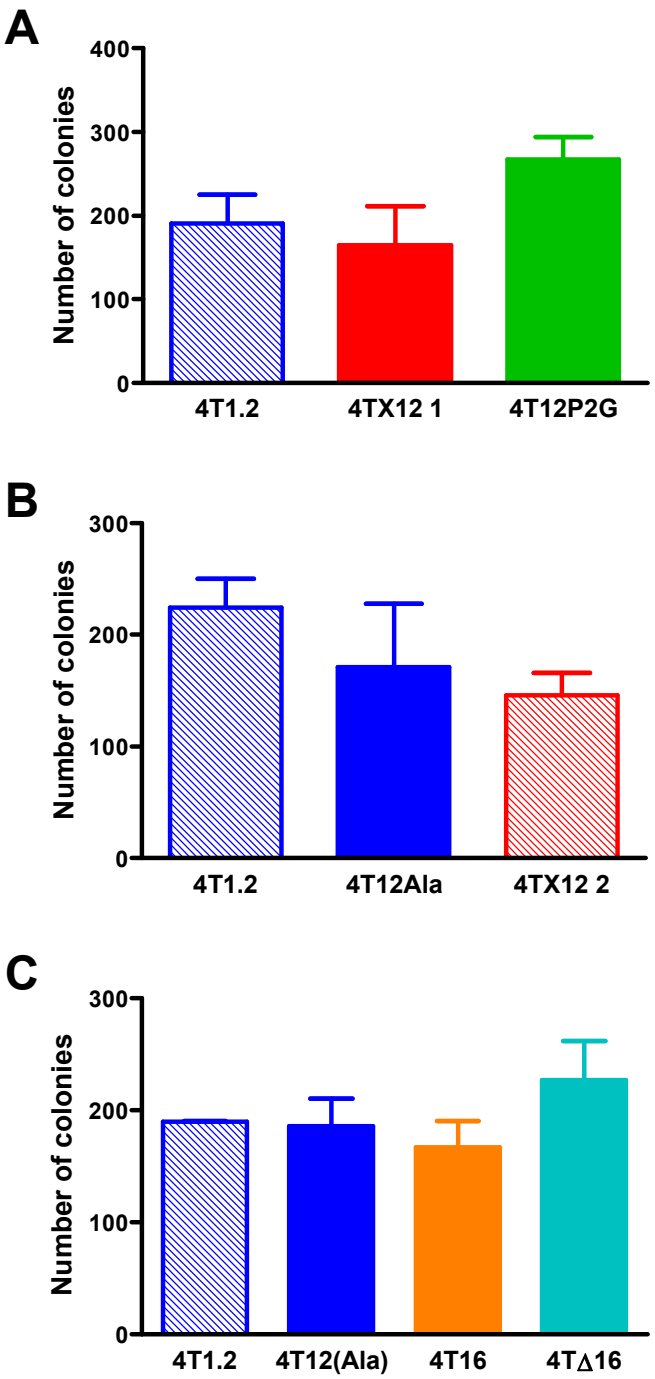


Figure 3.16 In vitro *tumorigenicity of chemokine construct-expressing cell lines.*

The wells of 6-well trays were coated with 0.7% agar made up in complete IMDM (Section 2.6.1.3), which was allowed to set. A layer of 0.3% agar containing 500 cells was then overlaid and allowed to solidify. Cells were incubated at 37°C in 5% CO_2 for 10 days. The number of colonies per well was then counted under a dissecting microscope. **(A & B)** CXCL12 construct-expressing cell lines. Bars represent mean ± SEM of 3-4 independent experiments with duplicate or triplicate determinations. Differences between columns were not significant, P=0.2059 (A) and P=0.3218 (B), One-Way ANOVA. **(C)** CXCL16 construct-expressing cell lines. Bars represent mean ± SEM of 2 independent experiments with duplicate determinations. Differences between columns were not significant, P=0.2824, One-Way ANOVA.

CHAPTER 4

The Effect of chemokine expression on breast cancer *in vivo*

CHAPTER 4: THE EFFECT OF CHEMOKINE EXPRESSION ON BREAST CANCER *IN VIVO*

4.1 Overview

The ultimate aim of this project was to investigate the effects of CXCL12, CXCL16 and their antagonists on the progression of breast cancer *in vivo*. Thus growth and metastasis of tumours in syngeneic mice was examined to determine the ability of CXCL12 and CXCL16 to potentiate or inhibit these processes. These experiments provided a holistic picture of the effect of chemokine expression on tumour cells, stromal cells and infiltrating cells present in tumours *in situ*.

4.2 Effects of exogenous chemokine expression on primary tumour growth

To investigate the effect of CXCL12, CXCL16 and their antagonists on tumour growth, construct-expressing 4T1.2 cells and parental or control 4T12Ala cells were injected into the mammary fat pads of female Balb/c mice. Tumour growth was monitored for approximately one month, during which time tumour diameter was measured every two days in two perpendicular directions using electronic callipers. Tumour volume was calculated as width^2 x length/2, where width was the smaller diameter and length the larger. Tumours were also weighed at the end of the experiment to confirm the data generated from the calliper measurements.

Initial experiments compared the growth of CXCL12 construct-expressing tumours with that of control parental 4T1.2 tumours (Figure 4.1) or control 4T12Ala tumours (Figure 4.2). Growth of CXCL12-expressing tumours was significantly reduced compared with that of the parental 4T1.2 and control 4T12Ala tumours. This tumour-inhibitory effect of CXCL12 was confirmed in a subsequent experiment in which the growth of a second independently generated CXCL12-expressing cell line (4TX12 2) was compared with that of the control and the original 4TX12 1 line (Figure 4.3). The 4TX12 2 tumours exhibited reduced growth compared with control 4T12Ala tumours, similar to 4TX12 1 tumours.

In contrast to the CXCL12-expressing tumours, growth of the $CXCL12_{(P2G)}$-expressing tumours was similar to controls (Figure 4.1 and Figure 4.2). This was apparent in terms of both the rate of tumour growth and final tumour weight, and was confirmed in subsequent experiments. A representative experiment is shown in Figure 4.4. These data raised two possibilities: one, that CXCL12 was not directly involved in primary tumour growth; or two, that the 4T12P2G cell line did not express $CXCL12_{(P2G)}$ at sufficiently high levels to inhibit the effects of endogenous CXCL12 *in vivo*. The results obtained from study of metastasis in this model indicate that the former was the case (see below).

Growth of CXCL16-expressing and $CXCL16_{(9\text{-}220)}$-expressing tumours was also compared with that of control 4T12Ala tumours. Initial experiments suggested a slight trend for growth of 4T16 tumours to be enhanced and 4TΔ16 tumours to be reduced compared with controls (not shown). However, further experiments conducted to increase statistical power showed no significant differences in the rate of primary tumour growth between the control and 4T16 and 4TΔ16 lines (Figure 4.5). This was confirmed by both the calliper measurements and comparison of the final tumour weights at the end of the experiment.

4.3 Effects of CXCL12 construct expression on metastasis

To determine if expression of any of the constructs affected metastasis of 4T1.2 tumours, the lungs of tumour-bearing mice were harvested after approximately one month. To determine the frequency of metastasis, lungs were fixed and examined under the dissecting microscope for the presence of surface metastases.

Enumeration of surface metastatic nodules revealed that 4TX12 1 tumours metastasised spontaneously to the lungs significantly less frequently than control tumours (Figure 4.6), a finding that was not surprising given the inhibitory effect of CXCL12 on primary tumour growth. Unexpectedly, 4T12P2G tumours also spontaneously metastasised to the lungs less frequently than control tumours, despite the fact that $CXCL12_{(P2G)}$ had no discernible effect on primary tumour growth. These results also confirmed that $CXCL12_{(P2G)}$ was being expressed at sufficient levels to exert a biological effect in mice.

CXCL12 and CXCR4 are pro-angiogenic, and both have been shown to be expressed in EC[111, 117]. It was therefore postulated that expression of $CXCL12_{(P2G)}$ might inhibit

angiogenesis within the tumour, thus reducing the opportunity for tumour cells to enter the bloodstream and metastasise to distant sites. Hence, angiogenesis was examined in 4T1.2 tumours to determine if this was a potential mechanism through which $CXCL12_{(P2G)}$ might be acting to inhibit metastasis. To quantify the degree of vascularisation of CXCL12-expressing tumours, the haemoglobin content of primary tumour homogenates was compared. $CXCL12_{(P2G)}$-expressing tumours contained similar levels of haemoglobin to control tumours (Figure 4.7). Immunohistochemical analysis of CD31 expression (a marker of EC) was also employed to assess vascularisation of 4T1.2 tumours. Considerable variability in the vascular density between tumours within the same group was observed. However, the blood vessel density was not found to be obviously decreased in $CXCL12_{(P2G)}$-expressing tumours compared with controls (Figure 4.8), in agreement with the haemoglobin results. Interestingly, overexpression of CXCL12 induced an increase in the haemoglobin content of tumour tissues; however, this increase was not statistically significant. Moreover, the vascular density of CXCL12-expressing tumours appeared reduced compared with control tumours (Figure 4.8). The implications of these apparently contradictory results will be discussed in Section 4.5 below and in Section 6.2.

Since $CXCL12_{(P2G)}$ did not seem to affect the haematogenous route of tumour metastasis, investigative efforts were directed towards the lymphatic route. The effect of $CXCL12_{(P2G)}$ expression on lymphangiogenesis in the primary tumour was assessed using immunohistochemical staining for LYVE-1, a marker of lymphatic endothelium. Again, considerable variability in the lymphatic vascular density between tumours within the same group was observed. However, no general reduction in the lymphatic vessel density of $CXCL12_{(P2G)}$-expressing tumours compared with control tumours was apparent (Figure 4.9). Similarly, overexpression of CXCL12 did not affect lymphangiogenesis within primary tumours (Figure 4.9). Together, these data indicate that $CXCL12_{(P2G)}$-mediated inhibition of 4T1.2 tumour metastasis is not a result of reduced vascularisation or lymphangiogenesis within the primary tumour.

Since there are multiple stages in the metastatic process at which $CXCL12_{(P2G)}$ could exert its inhibitory effect, experiments were undertaken to broadly ascertain when and where $CXCL12_{(P2G)}$ was acting. 4T12P2G cells, or control 4T12Ala cells for comparison, were injected intravenously into Balb/c mice. This bypassed the need for the cells to form a primary tumour and then escape from the tumour mass to metastasise. Similar to the results

of the spontaneous metastasis experiments, $CXCL12_{(P2G)}$ expression resulted in the formation of fewer lung metastases than observed in controls as determined by enumeration of surface lung nodules (Figure 4.10). Given that $CXCL12_{(P2G)}$ had no effect on primary tumour growth, it seems most plausible that $CXCL12_{(P2G)}$ inhibits survival of metastatic cells in the circulation and/or homing to and/or extravasation at secondary sites. It is important to note that these data do not rule out a role for $CXCL12_{(P2G)}$ in escape of cells from the primary tumour. Of note, intravenously injected 4TX12 1 cells also produced fewer lung metastases than control tumour cells, indicating that the anti-tumour properties of CXCL12 are still in effect once metastatic cells leave the primary tumour. The implications of these findings will be discussed in more detail in Chapter 6.

4.4 Effects of CXCL16 construct expression on metastasis

When spontaneous metastasis to the lungs of mice bearing control, 4T16 and 4TΔ16 tumours was compared, no statistically significant differences in the incidence of lung metastasis was observed (Figure 4.11A). Interestingly, upon intravenous injection, the 4TΔ16 cell line formed significantly fewer lung metastases than the control 4T12Ala line (Figure 4.11B). In light of this, it is surprising that $CXCL16_{(9-220)}$ expression had no effect on spontaneous metastasis. A possible explanation for this apparent paradox is discussed in Section 6.4. It should be noted that due to time constraints, the metastasis assays were performed once only. Additional experiments would provide more statistical power and confirm the observed effect of $CXCL16_{(9-220)}$ on metastasis.

4.5 Summary and preliminary discussion

In summary, these experiments show that CXCL12 overexpression has a profound suppressive effect on primary tumour growth and metastasis in the 4T1.2 model. Given the lack of effect of CXCL12 on growth of 4T1.2 cells *in vitro*, it seems probable that CXCL12 exerts its effect through host components, rather than through direct effects on tumour cells themselves. However, the possibility that tumour cells become sensitive to CXCL12 signalling under growth conditions encountered *in vivo* cannot be ruled out. Analysis of the effect of CXCL12 on tumour angiogenesis indicated that there was an increase, though not statistically significant, in the haemoglobin content of CXCL12-expressing tumours. This result made a stark contrast with the apparent reduction in the

vascular density of these same tumours. Possible explanations for these divergent results are discussed further in Section 6.2, but it seems likely that any effects of CXCL12 on the tumour vasculature play a minor role, if any, in this model. The precise mechanisms by which CXCL12 influences the host are further investigated in the next chapter.

Expression of the CXCL12 antagonist, $CXCL12_{(P2G)}$, inhibited metastasis of tumour cells to the lungs of tumour-bearing mice, despite having no overt inhibitory effect on primary tumour growth, angiogenesis or lymphangiogenesis. However, the potential for $CXCL12_{(P2G)}$ to influence angiogenesis and lymphangiogenesis should not be discounted, for reasons to be discussed further in Section 6.3. Of particular interest, $CXCL12_{(P2G)}$ was able to inhibit lung metastasis of tumour cells injected directly into the circulation, indicating the importance of endogenous CXCL12 in metastatic steps following detachment of cells from the primary tumour and intravasation. Blockade of CXCL12 signalling through CXCR4 may be responsible for the effects observed, but it should not be forgotten that 4T1.2 cells also express CXCR7. The ability of $CXCL12_{(P2G)}$ to bind CXCR7 and to block CXCR7-associated functions (e.g. enhancement of adhesion to EC) needs to be further investigated to fully dissect the mechanism behind $CXCL12_{(P2G)}$ inhibition of metastasis.

Neither CXCL16 nor its antagonist had any discernible effect on primary tumour growth or spontaneous metastasis. Surprisingly, 4TΔ16 cells metastasised significantly less frequently to the lungs when injected intravenously, despite the expression of $CXCL16_{(9\text{-}220)}$ having no effect on spontaneous metastasis. As these metastasis experiments were conducted only once, further experiments to increase statistical power are required before any solid conclusions can be drawn. However, a hypothesis that could explain the reduced metastasis of intravenously injected 4TΔ16 cells is put forward in Section 6.4.

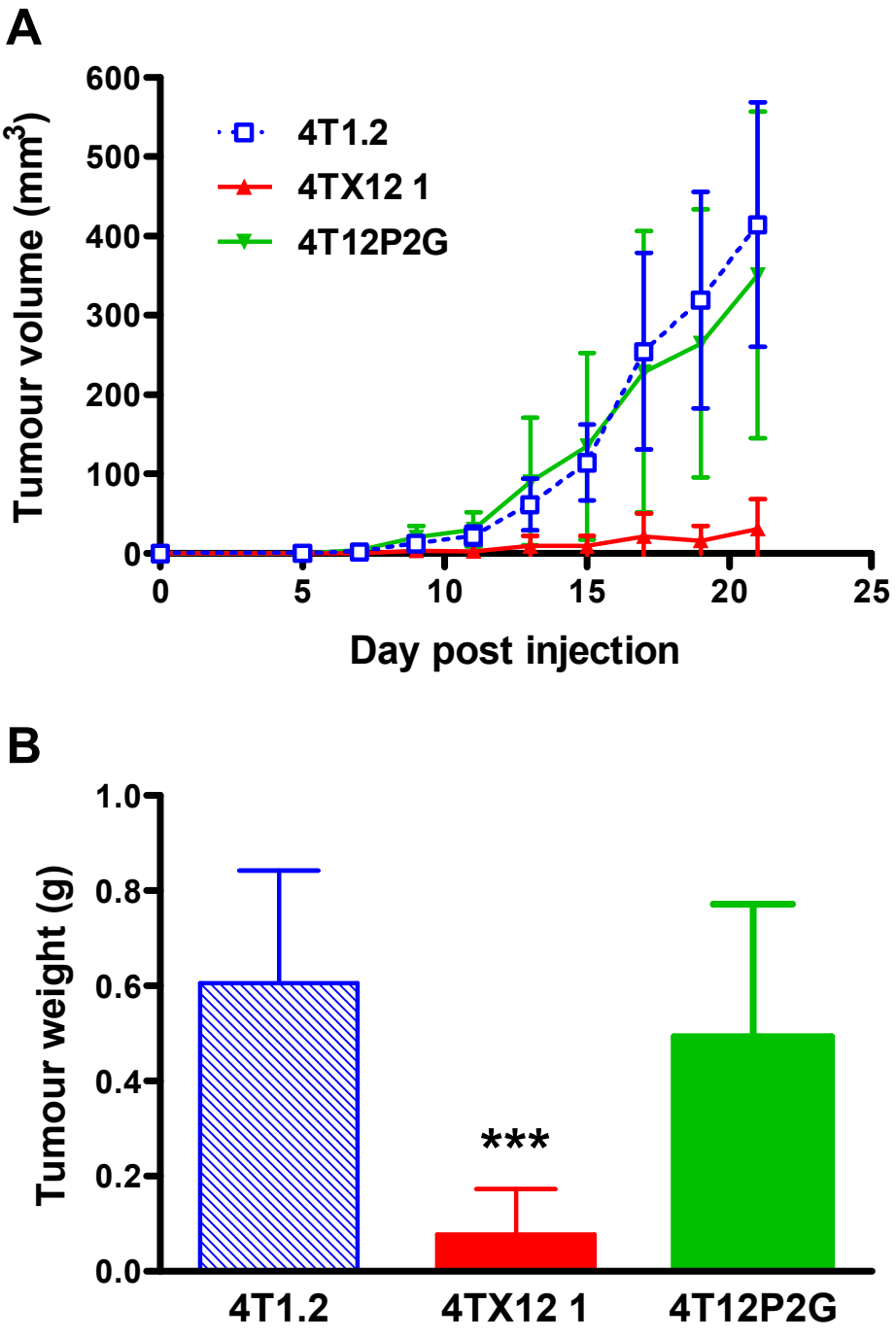


Figure 4.1 *Comparison of the growth of CXCL12 construct-expressing 4T1.2 mammary tumours and parental 4T1.2 tumours* in vivo.

Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad with 1 x 10^5 cells. **(A)** Tumour diameter was measured every 2 days once tumours became palpable. Tumour volume in mm^3 was calculated according to the equation $width^2$ x length/2. Data points represent the mean tumour volume ± SD from one representative experiment of two performed (4T1.2, *n*=24; 4TX12 1 and 4T12P2G, *n*=15). **(B)** Final weights of tumours were determined at the termination of the experiment. Bars represent the mean final tumour weight ± SD from one representative experiment of two performed (4T1.2, *n*=24; 4TX12 1 and 4T12P2G, *n*=15). ***, P<0.0001 (Kruskal-Wallis), significantly different compared with control 4T1.2 and 4T12P2G tumours.

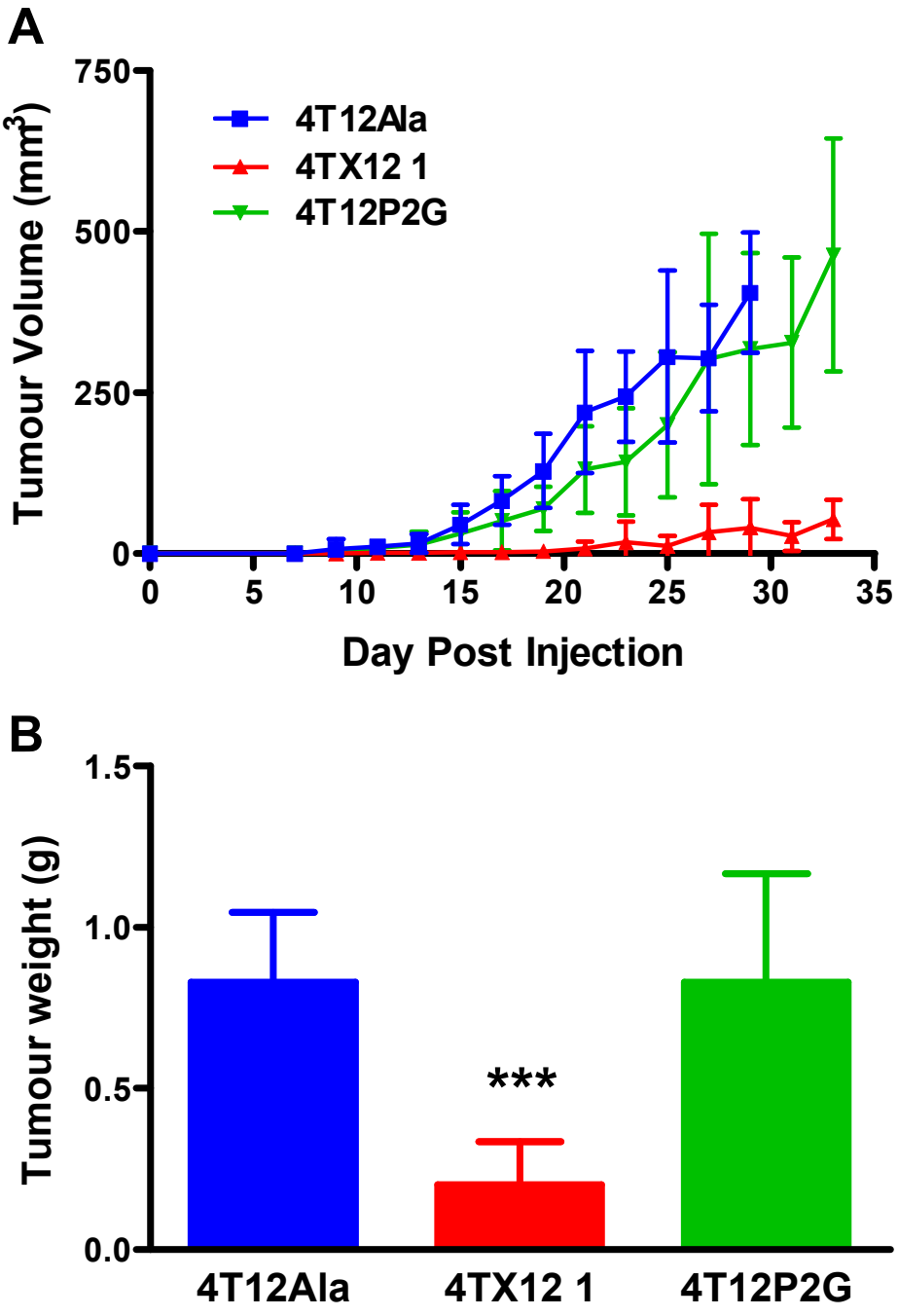


Figure 4.2 *Comparison of the growth of CXCL12 construct-expressing 4T1.2 mammary tumours and control 4T12Ala tumours* in vivo.

Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad with 1 x 10^5 cells. **(A)** Tumour diameter was measured every 2 days once tumours became palpable. Tumour volume in mm^3 was calculated according to the equation $width^2$ x length/2. Data points represent the mean tumour volume ± SD from one representative experiment of two performed (*n*=12 mice per group). **(B)** Final weights of tumours were determined at the termination of the experiment. Bars represent the mean final tumour weight ± SD from one representative experiment of two performed (*n*=12 mice per group). ***, P<0.0001 (Kruskal-Wallis), significantly different compared with control 4T12Ala and 4T12P2G tumours.

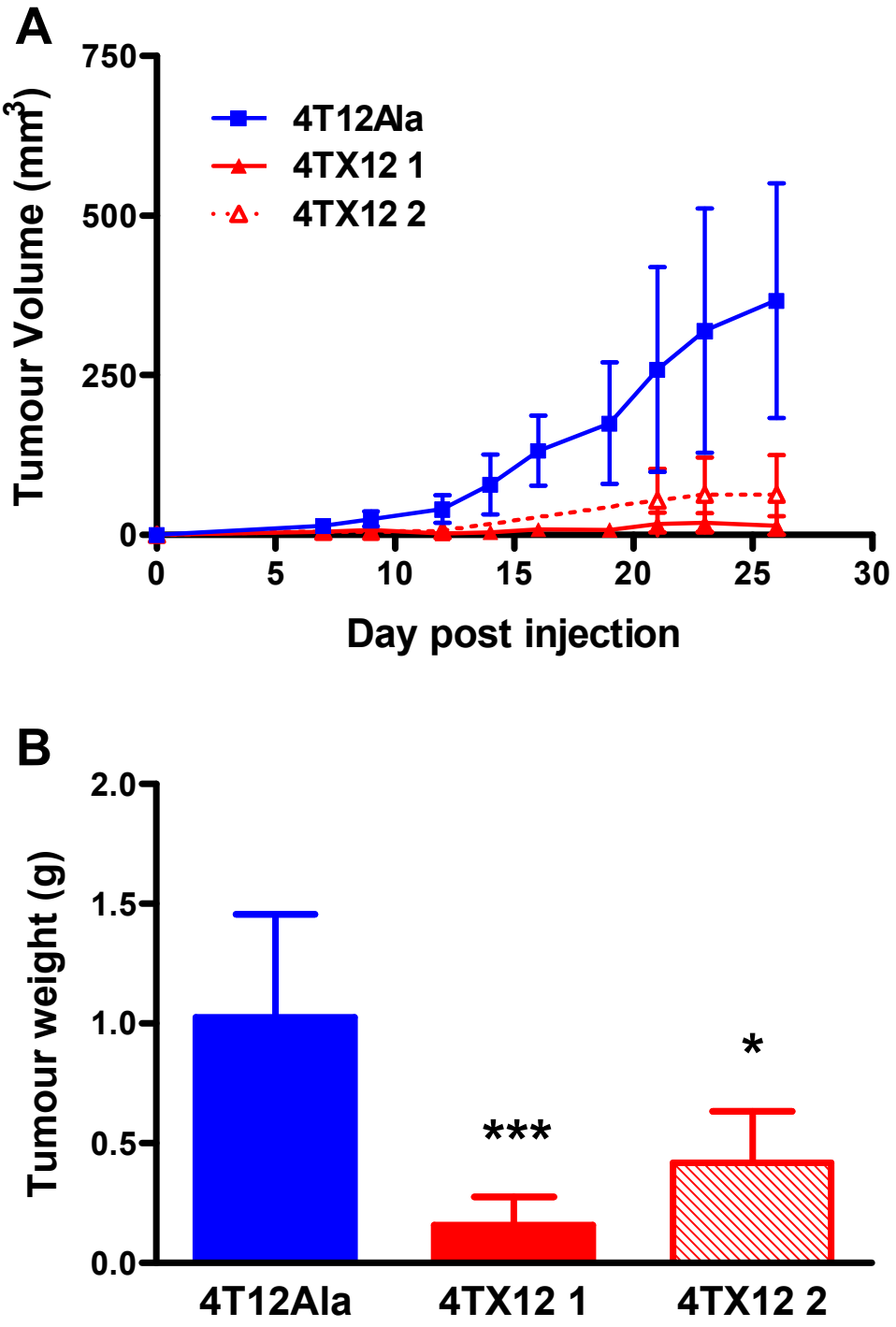


Figure 4.3 *Growth of CXCL12-expressing 4T1.2 mammary tumours* in vivo.

Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad with 1 x 10^5 cells. **(A)** Tumour diameter was measured every 2 days once tumours became palpable. Tumour volume in mm^3 was calculated according to the equation $width^2$ x length/2. Data points represent the mean tumour volume ± SD (*n*=12 mice per group). **(B)** Final weights of tumours were determined at the termination of the experiment. Bars represent the mean final tumour weight ± SD (*n*=12 mice per group). Final tumour weights were significantly different, P<0.0001 (Kruskal-Wallis). ***, P<0.001 and *, P<0.05 significantly different compared with control 4T12Ala tumours (Dunn's post test).

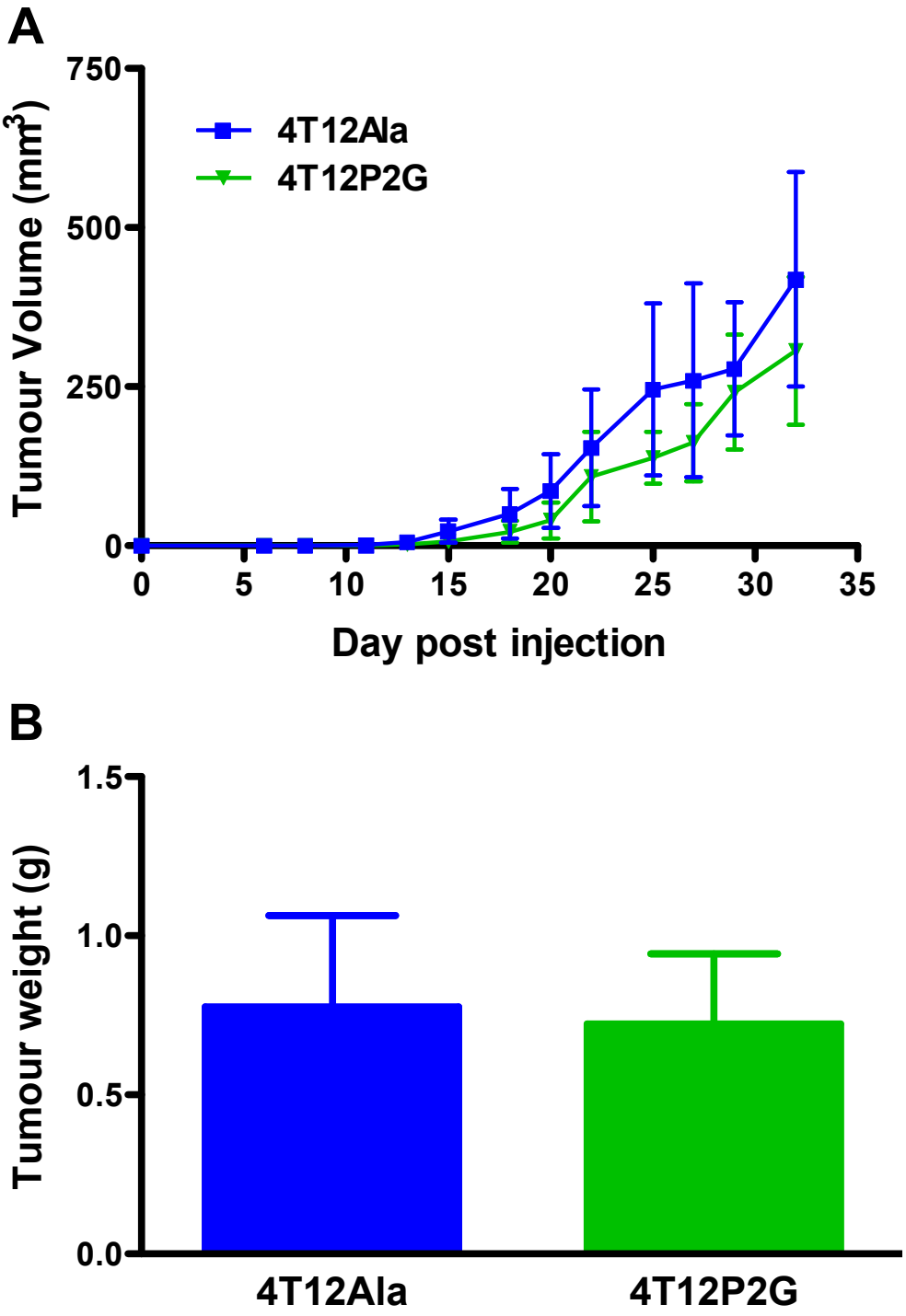


Figure 4.4 *Growth of CXCL12$_{(P2G)}$-expressing 4T1.2 mammary tumours* in vivo.

Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad with 1 x 10^5 cells. **(A)** Tumour diameter was measured every 2 days once tumours became palpable. Tumour volume in mm^3 was calculated according to the equation width^2 x length/2. Data points represent the mean tumour volume ± SD (*n*=12 mice per group). **(B)** Final weights of tumours were determined at the termination of the experiment. Bars represent the mean final tumour weight ± SD (*n*=12 mice per group). Final tumour weights were not significantly different (P=0.7125, Mann-Whitney U test).

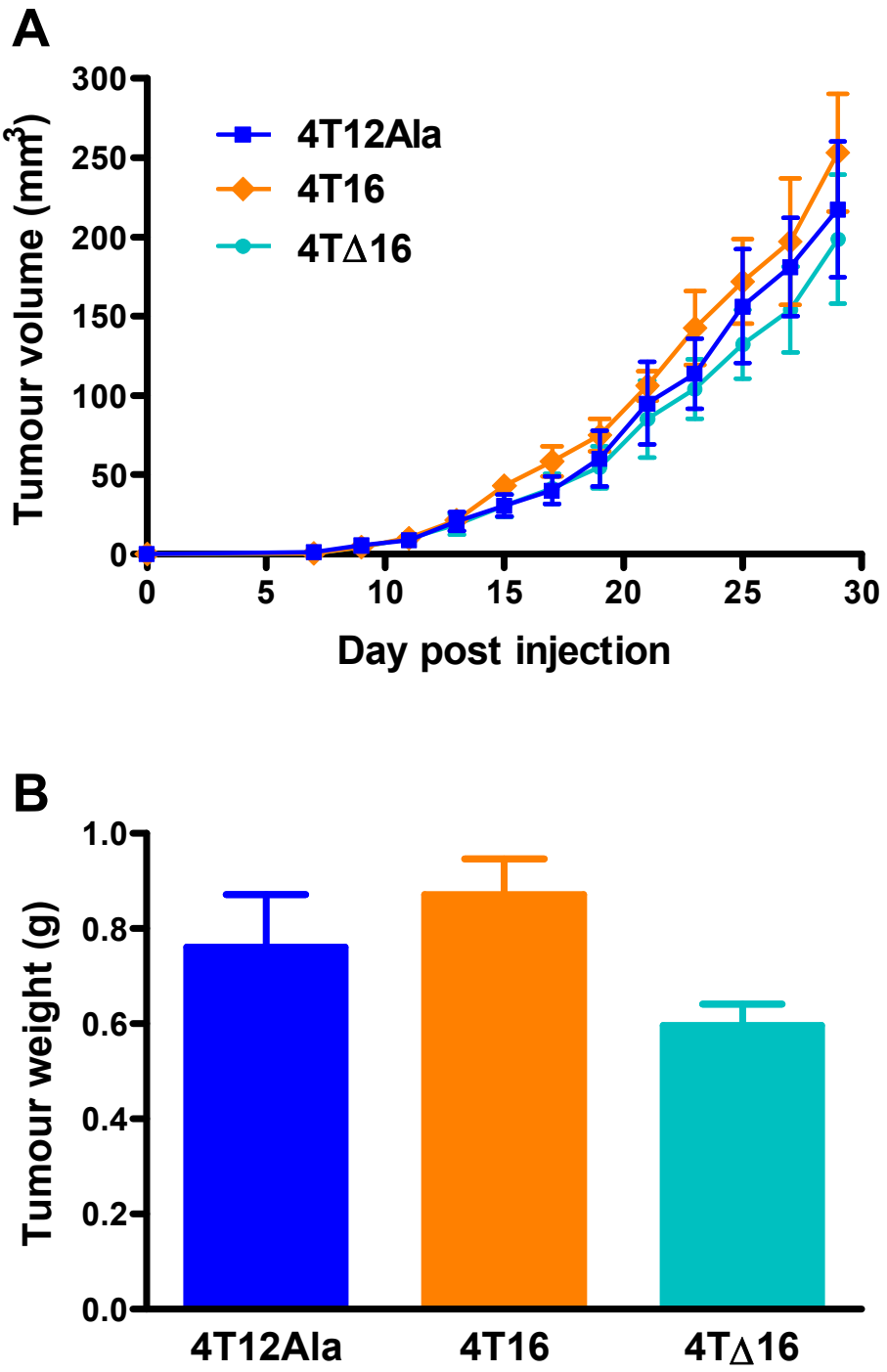


Figure 4.5 *Growth of CXCL16-expressing 4T1.2 mammary tumours* in vivo.

Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad with 1 x 10^5 cells. **(A)** Tumour diameter was measured every 2 days once tumours became palpable. Tumour volume in mm^3 was calculated according to the equation $width^2$ x length/2. Data points represent the mean tumour volume ± SEM from 4 independent experiments (*n*=6, 10, 6 and 12, respectively). **(B)** Final weights of tumours were determined at the termination of the experiment. Bars represent the mean final tumour weight ± SEM from 4 independent experiments (*n*=6, 10, 6 and 12, respectively). Final tumour weights were not significantly different (P=0.1183, Kruskal-Wallis).

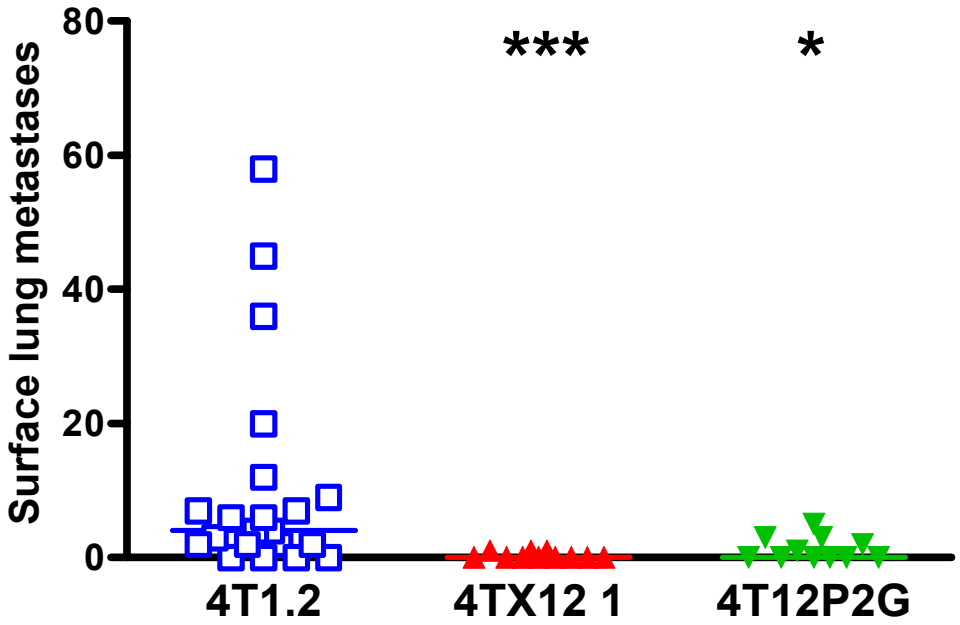


Figure 4.6 *Spontaneous metastasis to the lungs of 4TX12 1 and 4T12P2G tumour-bearing mice.*

Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad with 1×10^5 cells. After 3-4 weeks, lungs were harvested and fixed overnight in 4% formaldehyde in PBS. Lungs were washed twice for at least two hours in 30% sucrose solution and were stored in 70% ethanol at 4°C until analysis. The five lobes of each lung were separated and examined under a dissecting microscope for surface metastatic nodules. Data points represent the number of surface metastatic nodules in the lungs for individual mice from two independent experiments. Bars represent the median. Medians were significantly different, P=0.0003 (Kruskal-Wallis). ***, P<0.001; *, P<0.05 (Dunn's multiple comparisons post test), compared with control 4T1.2 tumour-bearing mice.

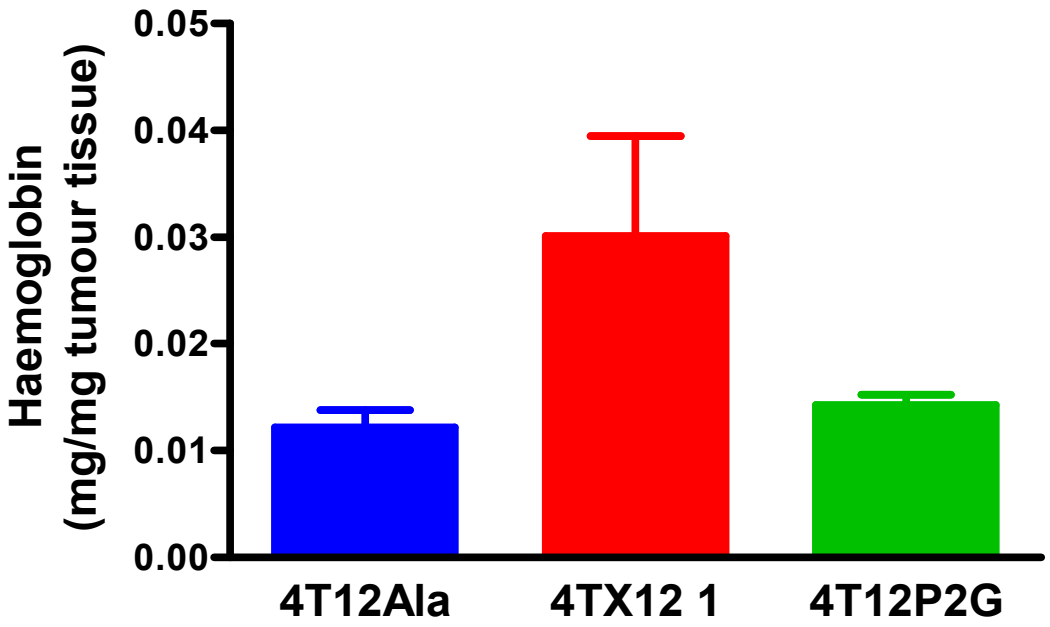


Figure 4.7 *Haemoglobin content of CXCL12-construct expressing tumours.*

Tumours were snap frozen in liquid nitrogen and homogenized to a fine powder. The powdered tumour was weighed before being resuspended in 500µl deionised water and sonicated for approximately 30 seconds. Debris was removed by centrifugation and the resulting supernatant was then used for the haemoglobin assay. Drabkin's reagent was prepared containing Brij 35 Solution as per the manufacturer's instructions. Fifty microlitres per well of tumour supernatants were transferred to a 96-well tray and 200µl/well of Drabkin's solution was added. The plate was incubated for 15 minutes at room temperature to allow colour to develop and then the absorbance at 562nm was determined. A standard curve was generated using haemoglobin from bovine blood dissolved in deionised water.

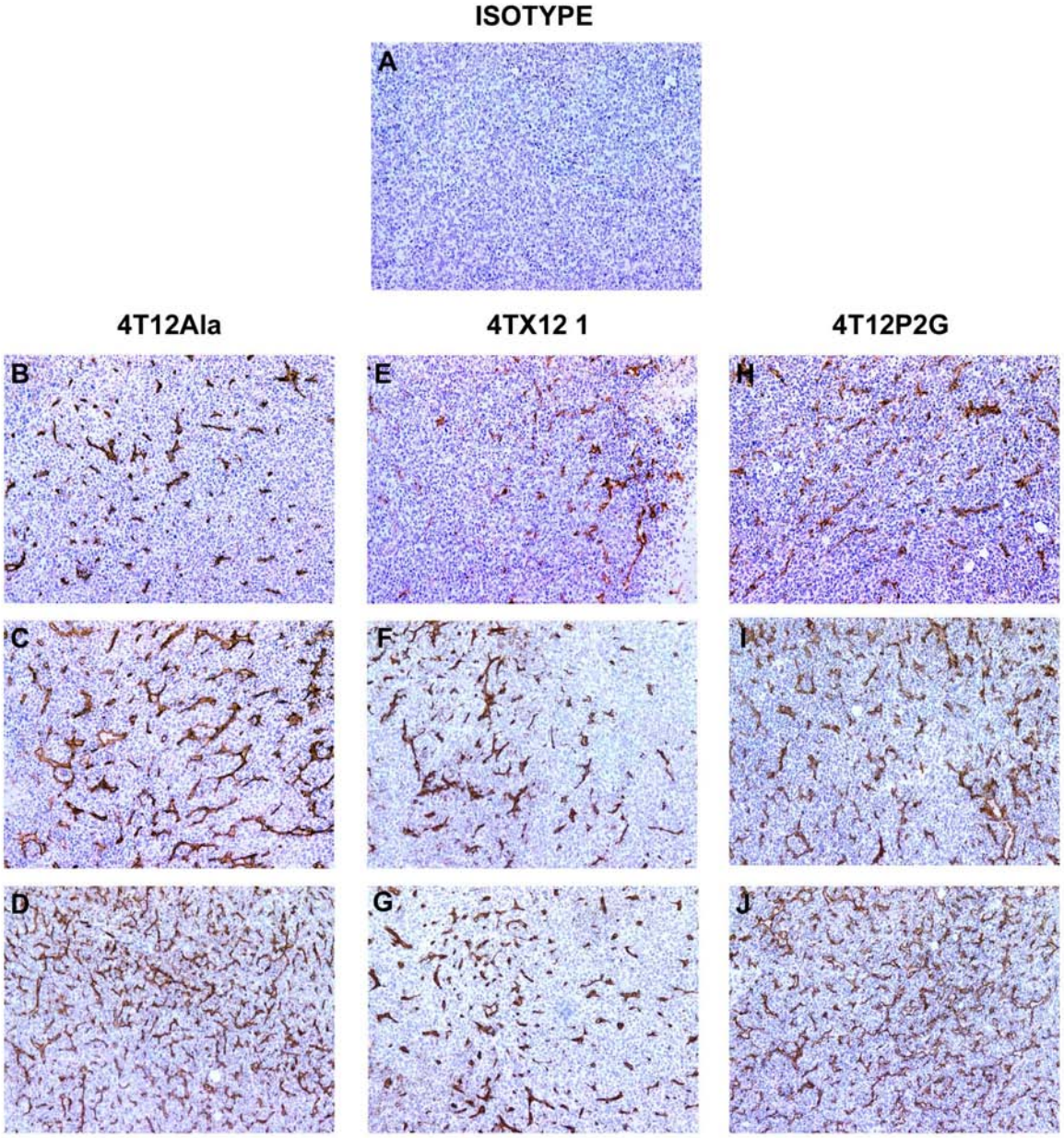


Figure 4.8 *Vascularisation of CXCL12 construct-expressing tumours.*

CD31 staining of EC in sections of CXCL12 construct-expressing 4T1.2 tumours. Sections were stained with rat anti-mouse CD31 antibody followed by HRP-conjugated anti-rat IgG antibody. Binding of antibodies was revealed by incubation with DAB substrate and cell nuclei were counterstained with haematoxylin. Sections were mounted and examined by light microscopy. Representative micrographs showing areas of greatest vascular density of individual tumours are shown. Magnification: 100x. **(A)** Isotype control. **(B-D)** 4T12Ala tumours. **(E-G)** 4TX12 1 tumours. **(H-J)** 4T12P2G tumours.

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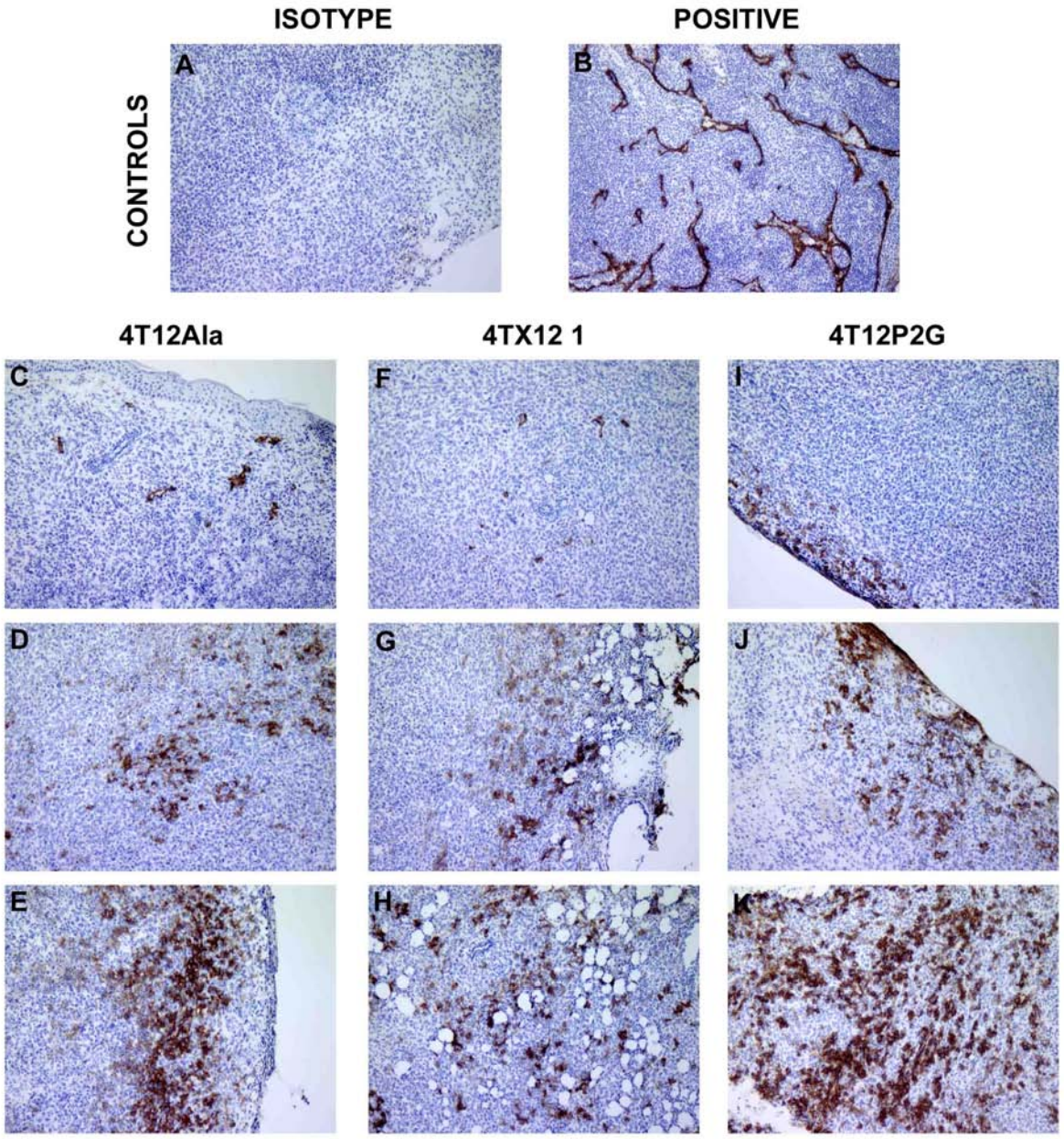

Figure 4.9 *Lymphangiogenesis within CXCL12 construct-expressing tumours.*

LYVE-1 staining of lymphatic vasculature in sections of CXCL12 construct-expressing 4T1.2 tumours. Sections were stained with rat anti-mouse LYVE-1 antibody followed by HRP-conjugated anti-rat IgG antibody. Binding of antibodies was revealed by incubation with DAB substrate and cell nuclei were counterstained with haematoxylin. Sections were mounted and examined by light microscopy. Representative micrographs showing areas of greatest lymphatic vascular density of individual tumours are shown. Magnification: 100x. **(A)** Isotype control. **(B)** Positive control staining of a LN. **(C-E)** 4T12Ala tumours. **(F-H)** 4TX12 1 tumours. **(I-K)** 4T12P2G tumours.

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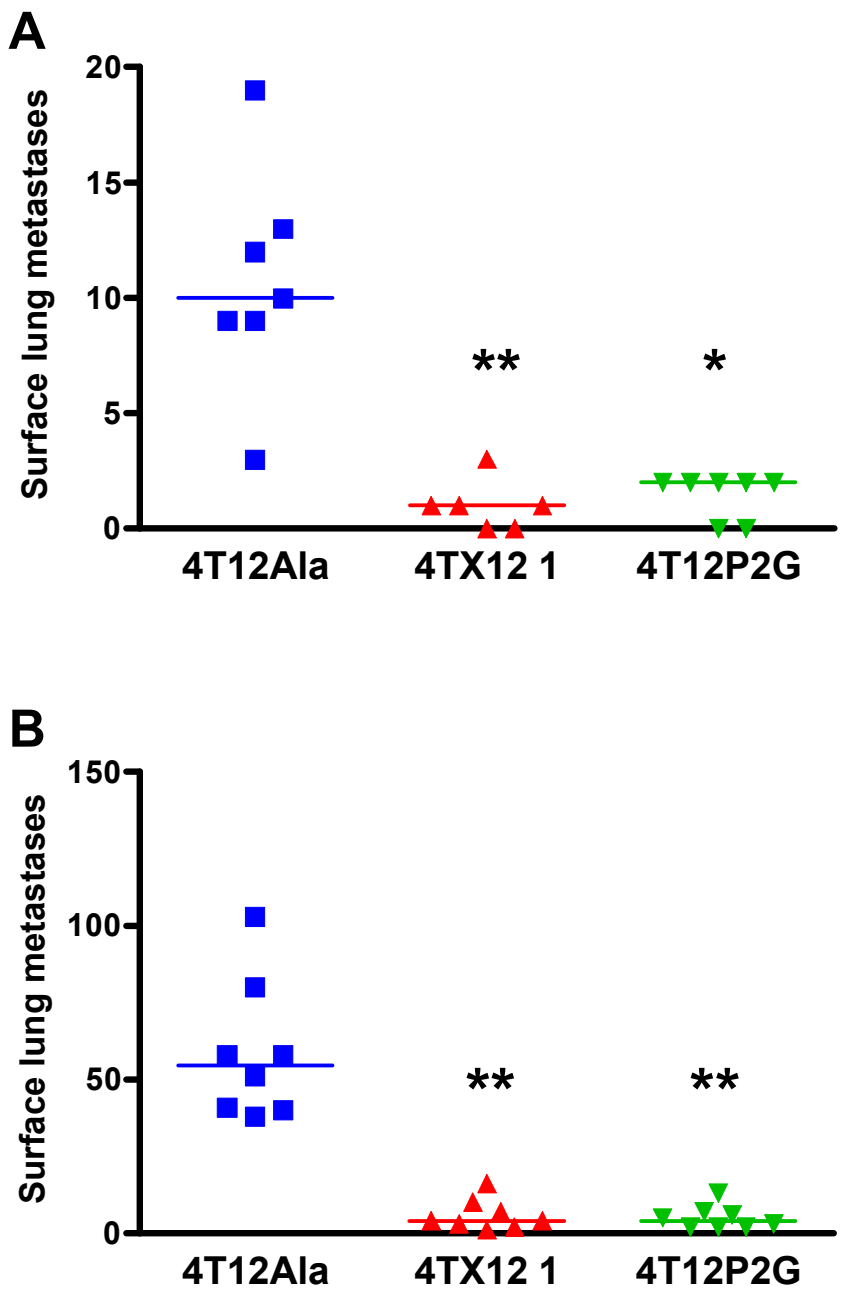

Figure 4.10 *Experimental metastasis of CXCL12-expressing tumour cell lines to the lungs of Balb/c mice.*

Female 6-8 week old Balb/c mice were injected in the tail vein with 5 x 10^5 cells **(A)** or 7.5 x 10^5 cells **(B)** in PBS. After approximately 2 weeks, lungs were harvested and fixed overnight in 4% formaldehyde in PBS. Lungs were washed twice for at least two hours in 30% sucrose solution and were stored in 70% ethanol at 4°C until analysis. The five lobes of each lung were separated and examined under a dissecting microscope for surface metastatic nodules. Data points represent the number of surface metastatic nodules in the lungs of individual mice. Bars represent the median. **(A)** Medians were significantly different, P=0.0013 (Kruskal-Wallis). **, P<0.01; *, P<0.05 (Dunn's multiple comparisons post test), compared with mice injected with control 4T12Ala cells. **(B)** Medians were significantly different, P=0.0004 (Kruskal-Wallis). **, P<0.01 (Dunn's multiple comparisons post test), compared with mice injected with control 4T12Ala cells.

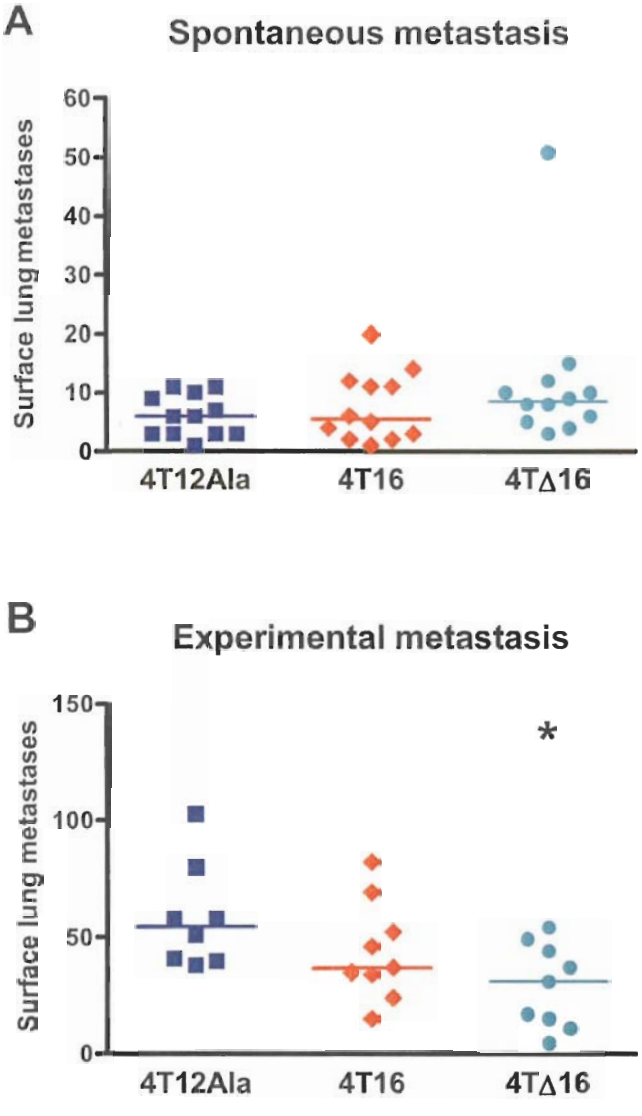

Figure 4.11 *Metastases in the lungs of 4T16 and 4TΔ16 tumour-bearing mice.*

(A) Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad with 1 x 10^5 cells. After 31 days, lungs were harvested and fixed overnight in 4% formaldehyde in PBS. Lungs were washed twice for at least two hours in 30% sucrose solution and were stored in 70% ethanol at 4°C until analysis. The five lobes of each lung were separated and examined under a dissecting microscope for surface metastatic nodules. Data points represent the number of surface metastatic nodules in the lungs of individual mice. Bars represent the median. Medians were not significantly different, P=0.3231 (Kruskal-Wallis). **(B)** Female 6-8 week old Balb/c mice were injected in the tail vein with 7.5 x 10^5 cells in PBS. After approximately 2 weeks, lungs were harvested and fixed overnight in 4% formaldehyde in PBS. Lungs were washed twice for at least two hours in 30% sucrose solution and were stored in 70% ethanol at 4°C until analysis. The five lobes of each lung were separated and examined under a dissecting microscope for surface metastatic nodules. Data points represent the number of surface metastatic nodules in the lungs of individual mice. Bars represent the median. Medians were significantly different, P=0.0318 (Kruskal-Wallis). *, P<0.05 (Dunn's multiple comparisons post test), compared with mice injected with control 4T12Ala cells.

CHAPTER 5

The Effect of CXCL12 expression on the anti-tumour immune response

CHAPTER 5: THE EFFECT OF CXCL12 EXPRESSION ON THE ANTI-TUMOUR IMMUNE RESPONSE

5.1 Overview

As described in the previous chapter, CXCL12 expression had a striking inhibitory effect on the growth of 4T1.2 tumours. Since CXCL12 did not have any discernible effect on 4T1.2 cell growth *in vitro*, it was concluded that CXCL12 was most likely to be exerting its inhibitory effects by influencing host cells, rather than tumour cells. CXCL12 is pro-angiogenic, but the data presented in the previous chapter indicated that CXCL12 was unlikely to be influencing vascularisation in this model. Given that CXCL12 induces responses in a variety of leukocytes, it seemed most likely that tumour growth was being inhibited by a CXCL12-induced immune response. In this chapter, experiments to identify leukocyte subsets involved in the CXCL12-induced anti-tumour response, as well as the mechanisms by which they mediate their effects, are presented.

5.2 Identification of leukocyte subsets important for the anti-tumour effect of CXCL12

5.2.1 The role of T cells

The data presented in Section 4.2 suggested that the inhibitory effects of CXCL12 were not a consequence of its direct action on tumour cells. Rather, it seemed likely that it was exerting its activity on the tumour stroma and accessory cells, in particular, the cells of the immune system. To elucidate the mechanism by which growth of CXCL12-expressing tumours was inhibited, the CXCL12 construct-expressing cell lines were injected into the mammary fat pads of SCID mice, which lack both functional T and B cells. Growth of CXCL12-expressing tumours in these mice was comparable to controls, implying that a functional adaptive immune response is required for the tumour-inhibitory effects of CXCL12 (Figure 5.1).

To further clarify the role of particular lymphocyte subsets in the tumour inhibitory effect of CXCL12, Balb/c mice carrying the nu/nu mutation (nude mice) and lacking functional T cells were injected with control $CXCL12_{(Ala)}$-expressing or CXCL12-expressing tumour cells. There was a significant inhibition in the growth rate of CXCL12-expressing tumours

compared with control tumours in these mice (Figure 5.2). However, the reduction in 4TX12 1 tumour growth was less pronounced in nude mice compared with that observed in wild-type mice (Table 5.1). This partial abrogation of tumour growth inhibition indicates that T cells are involved in mediating the anti-tumour effect of CXCL12. However, the incomplete loss of the anti-tumour response suggests that other cell subsets are also important for CXCL12-mediated tumour inhibition.

Further support for the role of T cells in the anti-tumour response came from correlative analysis of final tumour weight and the percentages and absolute numbers of both $CD4^+$ and $CD8^+$ splenocytes (Figure 5.3 and Table 5.2). For both 4T12Ala and 4TX12 1 tumours, the total number of splenocytes positively correlated with tumour weight (correlation coefficients 0.5874 and 0.8231, respectively). Additionally, a significant negative correlation of the percentage $CD4^+$ splenocytes and tumour weight was observed (i.e. smaller tumour weight corresponded with a higher percentage of $CD4^+$ cells in the spleen). However, this correlation was much stronger for 4TX12 1 tumours (correlation coefficient = -0.8462) than for control tumours (correlation coefficient = -0.5849). In addition, a slight but significant positive correlation (correlation coefficient = 0.6503) of the absolute number of $CD8^+$ splenocytes and tumour weight was observed for 4TX12 1 tumours. Why an increased number of $CD8^+$ splenocytes should be associated with enhanced tumour growth remains unclear.

T cells from tumour-DLN and tumours were isolated to characterise their surface marker phenotype (Figure 5.4). DLN and tumours were taken 7 days post-injection of tumour cells and single-cell suspensions were prepared for flow cytometric analysis. T cells were identified as CD4 or CD8 positive, and the expression of the markers CD25 and CD62L was examined. CD25 is the high affinity IL-2 receptor α-chain, which is up-regulated by T cells upon activation, although when expressed together with CD4, it also defines a population of T_{reg} cells. CD62L or L-selectin, is an adhesion molecule expressed on LN-homing cells, and is generally down-regulated upon activation. On the whole, no obvious differences in CD25 or CD62L expression were observed on either CD4 or CD8 cells isolated from LN or tumours. The exception to this was the dramatic reduction in $CD4^+CD25^-$ and $CD4^+CD62L^-$ cells seen in DLN compared with naïve mice. This reduction in the proportion of $CD4^+$ cells did not seem to be specific to a particular tumour

group; rather, it appeared to be a phenomenon common to all tumour-bearing mice. A reduction in $CD8^+$ cells in the DLN of tumour-bearing mice was also noted; however, the difference was not as dramatic or as consistent as that observed for $CD4^+$ cells.

In another approach, sections of primary tumours were examined for infiltration of $CD4^+$ and $CD8^+$ T cells to assess the relative contribution of these T cell subsets to the reduction of 4TX12 1 tumour growth. However, no difference in the level of T cell infiltration into CXCL12-expressing and control tumours was apparent at either early (Figure 5.5) or late stages of tumour growth (Figure 5.6 and Figure 5.7).

5.2.2 The role of dendritic cells

The data outlined above suggested that CXCL12 was not acting by altering T cell infiltration of tumours, and this led to the hypothesis that CXCL12 was acting to enhance the activation state of those cells that enter the tumour. DC are potent antigen-presenting cells and are responsible for the activation of T cells during the initiation of an immune response. CD11c is the α_x chain of the α_x-β_2 integrin, and is expressed predominantly on conventional DC. To determine if an increase in DC priming and hence activation of T cells was occurring, immunohistochemistry to detect CD11c in tumour-DLN and tumours was undertaken. Tumour-DLN (Figure 5.8) from 4TX12 1 tumour-bearing mice, but not the tumours (Figure 5.9), were found to harbour an increased proportion of $CD11c^+$ cells compared with controls by day 6 post-injection of tumour cells. This suggests that CXCL12 overexpression by the tumour enhances DC accumulation in the DLN, which in turn may promote the activation of T cells.

5.2.3 The role of NKT cells

As nude mice do not lack natural killer T (NKT) cells[279, 280], while SCID mice do[281], and since these cells have documented tumour cytotoxic activity[279, 280], the role of NKT cells in the CXCL12-induced anti-tumour effect was investigated. The CXCL12 construct-expressing cell lines were injected into the mammary fat pads of $J\alpha18^{-/-}$ mice. These mice lack Type I NKT cells, which express an invariant TCR containing the Vα14-Jα18 α-chain, however Type II CD1d-dependent Vα14-Jα18-independent NKT cells are still present[282]. There was a statistically significant inhibition of the growth of primary CXCL12-

expressing tumours in Jα18$^{-/-}$ mice, similar to wild-type mice (Figure 5.10). This suggests that Type I NKT cells are not required for the anti-tumour effects of CXCL12 overexpression.

5.3 The role of cell-mediated immunity

It was postulated that increased activation of T cells in 4TX12 1 tumour-bearing mice could lead to an enhanced cell-mediated anti-tumour immune response. Since T cells are capable of inducing cytolysis directly, the quality of the cell-mediated cytotoxic response against control and CXCL12-expressing tumours was compared using an *in vitro* cytotoxicity assay (Figure 5.11). Lymphocytes were isolated from naïve mice or mice bearing control 4T12Ala or 4TX12 1 tumours for use as effectors. Wild-type 4T1.2 cells were used as targets. In the initial experiment (Figure 5.11A), effector cells were obtained from mice bearing late-stage tumours (30 days post injection) by pooling lymphocytes isolated from spleens and tumour-draining (i.e. inguinal and brachial) LN. In general, the level of cytotoxicity towards 4T1.2 targets was relatively low. However, at a 10:1 effector-to-target ratio, effector cells from 4TX12 1 tumour-bearing mice exerted significantly enhanced cytolytic activity compared with naïve effector cells and effector cells derived from 4T12Ala tumour-bearing mice. At lower effector-to-target cell ratios, no significant differences in cytotoxicity were observed between effector cells derived from the two tumour groups. Interestingly, at a 30:1 effector-to-target ratio, effector cells from 4T12Ala tumour-bearing mice exerted significantly less cytotoxicity than did effectors from 4TX12 1 tumour-bearing mice and naïve mice, suggesting that the basal level of cytotoxicity of effector cells from control tumour-bearing mice is suppressed.

At such a late stage of tumour growth, it was possible that the relatively weak cytotoxicity observed was a result of tumour-mediated immunosuppression mechanisms, and indeed the systemic immunosuppressive properties of 4T1 tumours have been documented[283]. Additionally, CXCL12-expressing tumours grew more slowly than controls from the outset, suggesting that the immune response to the tumour was becoming engaged early in tumour progression. Hence, effector cells from mice with early-stage tumours were tested for cytolytic function against 4T1.2 cells *in vitro* (Figure 5.11B). Considerable variability in the cytolytic activity of effector cells from individual mice was observed in these experiments. This was to be expected because at this early time point, the inherent

variability of the burgeoning immune response in individual mice is more apparent than at later stages when the immune response has become established. However, it was clear that the cytotoxic response of lymphocytes from mice with early-stage tumours was enhanced compared with that of lymphocytes from naïve mice. Moreover, there was a trend, though not significant, for effector cells from 4TX12 1 tumour-bearing mice to exhibit greater cytotoxicity than those from 4T12Ala tumour-bearing mice. Attempts were made to enhance the cytotoxic response of effector cells *in vitro* by re-stimulating them with mitomycin C-treated tumour cells prior to their addition to the cytotoxicity assay. However, the cytotoxic response induced by the conditions used was too strong for differences between effectors from the two tumour groups to be observed (data not shown). Further optimisation of the re-stimulation conditions (e.g. method of inactivating tumour cells, number of stimulators, length of re-stimulation time) may better highlight differences in the cytotoxic capabilities of effector cells from mice bearing 4T12Ala and 4TX12 1 tumours.

Further experiments were conducted to elucidate the mechanism by which effector cells from 4TX12 1 tumour-bearing mice were mediating their cytotoxic effects. Lymphocyte-mediated cytolysis is generally thought to proceed through two main pathways: the perforin/granzyme pathway or via members of the TNF death receptor family. Perforin and granzymes are major components of the cytotoxic granules of CTL and NK cells. On their release during granule-mediated killing, perforin prompts uptake of granzymes by target cells, which then induce cell death by initiating apoptotic signalling pathways[284]. Of the TNF family members, TRAIL is thought to have evolved to induce cell death in virally-infected cells whilst sparing normal cells, and is thought to be the main contributor to the elimination of transformed cells[285].

Perforin (pfp)-deficient mice were obtained to determine the contribution of the perforin/granzyme pathway to CXCL12-mediated tumour growth inhibition, while TRAIL-deficient mice were obtained to determine the contribution of the TNF death receptor pathway (Figure 5.12). As in wild-type mice, there was a trend for 4TX12 1 tumour growth to be slower compared with 4T12Ala tumours in $TRAIL^{-/-}$ mice. Though differences in final tumour weight were not statistically significant, differences in tumour volume were significant at most late time points. Two mice from the 4T12Ala group had to be culled earlier than the other mice in that group as they were displaying overt signs of

morbidity, which may have affected the statistical analysis. In $pfp^{-/-}$ mice, while control 4T12Ala tumour growth was considerably reduced compared with that observed in TRAIL knockout and wild-type mice, the growth rate and final weight of these tumours was still significantly greater than that of 4TX12 1 tumours. Interestingly, for both $pfp^{-/-}$ and $TRAIL^{-/-}$ mice, the reduction in 4TX12 1 tumour growth was less pronounced compared with that observed in wild-type mice (Table 5.1). This partial abrogation of tumour inhibition indicates that perforin and TRAIL are involved in mediating the anti-tumour effect of CXCL12. However, the incomplete loss of the anti-tumour response suggests that any anti-tumour cytotoxic activity against CXCL12-expressing tumours is not solely dependent on either of these two cell death pathways alone. Deficiency in both cell death pathways may lead to complete abrogation of the CXCL12-mediated anti-tumour effect. Alternatively, other cell death pathways not tested here may be utilised in the response against the tumour, and should not be discounted.

5.4 The role of cytokines

While T cells may mediate the cytotoxic response against CXCL12-expressing tumours directly, they may also exert anti-tumour functions indirectly through production of cytokines that mobilise other important effector cell types, e.g. macrophages and NK cells. The signature cytokine produced in a cell-mediated immune response is interferon-γ (IFN-γ), which plays a critical role in the effector function of T cells, as well as NK cells and NKT cells[34]. It exerts numerous effects particularly pertinent to anti-tumour immunity including activating the cytotoxic and phagocytic activity of macrophages[286] and increasing the cytotoxic activity of NK cells[34]. As shown in Figure 5.12, the cytotoxic activity of LN cells from 4TX12 1 tumour-bearing mice *in vitro* was relatively low, which suggested that other cells, such as innate immune cells, were responsible for cytotoxic killing of tumour cells. For this reason, it was postulated that T cells in the LN were exerting their major function through production of cytokines and in particular, IFN-γ. To determine whether IFN-γ was important for the anti-tumour effect of CXCL12, 4T12Ala and 4TX12 1 cells were injected into IFN-$\gamma^{-/-}$ mice and the development of tumours was observed (Figure 5.13). The pattern of 4T12Ala and 4TX12 1 tumour growth was similar in IFN-$\gamma^{-/-}$ mice to that in wild-type mice, in that the rate of tumour growth was slower for CXCL12-expressing tumours than for controls. However, tumours grew to be generally

larger in size compared with tumours grown in wild-type mice. Additionally, while CXCL12-expressing tumours were smaller compared with control tumours, this difference was not statistically significant. Furthermore, the ratio of 4TX12 1 tumour weight to 4T12Ala tumour weight was higher in IFN-$\gamma^{-/-}$ mice than in wild-type mice (Table 5.1), indicating that the tumour inhibitory effect of CXCL12 was partly lost in the absence of IFN-γ. However, the fact that 4TX12 1 tumours continued to grow more slowly than control 4T12Ala tumours demonstrates that IFN-γ is clearly not the sole contributor to the anti-tumour effect of CXCL12.

A recent report has described the importance of IL-23 in the promotion of tumour growth[287]. IL-23 plays a major role in the maintenance of Th17 cells which, as the name suggests, are major producers of the pro-inflammatory cytokine IL-17[288]. Inflammation is thought to be pro-tumorigenic[289] and the requirement for IL-23 in tumour promotion implies a role for inflammatory IL-17-producing cells in this process. To investigate the hypothesis that CXCL12 may block the generation of IL-17^{+} cells, an ELISPOT assay for IL-17-producing cells was performed (Figure 5.14). Mice were injected with either control or CXCL12-expressing tumour cells and seven days later the tumour-DLN were removed. Lymphocytes were extracted by mechanical disruption of the LN, and the lymphocytes obtained were cultured overnight in the presence of PMA and ionomycin in an anti-IL-17-coated filter plate. The following day, cells were washed from the plate and IL-17 secreted by the cells was detected with a secondary antibody and chromogenic substrate. 'Spots' of colour that developed in the bottom of the wells represented individual IL-17-producing cells. Enumeration of the spots in wells containing cells from 4T12Ala and 4TX12 1 tumour-bearing mice revealed that fewer IL-17-producing cells were present in the DLN of 4TX12 1 tumour-bearing mice.

The requirement of IFN-γ for the CXCL12-induced anti-tumour response and the reduction of IL-17-producing cells in mice bearing CXCL12-expressing tumours suggested that CXCL12 might serve to bias effector T cell differentiation away from an inflammatory IL-17-producing lineage to an IFN-γ-producing Type I lineage able to induce anti-tumour cell-mediated cytotoxicity. Differentiation of Type I IFN-γ-producing T cells also inhibits differentiation of Type II IL-4-producing T cells which promote humoral immunity and resistance to multicellular parasites[288]. To analyse the balance of .IFN-γ, IL-17 and IL-4

cytokine production in the 4T1.2 model, intracellular cytokine staining and flow cytometric analysis was employed (Figure 5.15). Cells were prepared from tumour-bearing mice as described for the ELISPOT assay above and cultured overnight with PMA and ionomycin to enhance cytokine production. The following day, cells were stained for cytokine production and analysed by flow cytometry. $CD4^+$ and $CD8^+$ cells were gated, and the expression of the aforementioned cytokines was assessed in each of these cell subsets. For $CD4^+$ cells, there was a general reduction in the percentage of cells expressing each cytokine in mice bearing 4TX12 1 tumours compared with those bearing control tumours, though this only reached statistical significance for IL-4. A similar trend was observed within the $CD8^+$ cell subset, although this did not reach statistical significance for any of the cytokines tested. Interestingly, a significant reduction in the proportion of IFN-γ^+ LN cells in general was observed in mice bearing 4TX12 1 tumours compared with those bearing control tumours (Figure 5.15C). The intensity of cytokine staining was also assessed to determine if cytokine-producing cells from different tumour groups were producing different amounts of cytokine. However, no significant differences between the groups were found.

The general reduction in cytokine-producing cells observed in DLN of 4TX12 1 tumours compared with the DLN of 4T12Ala tumours as described above was unexpected. Thus an alternative approach using a cytokine bead array to quantify cytokine secretion by DLN cells was employed. The range of cytokines analysed was expanded to include other effector cytokines, as well as anti-inflammatory cytokines, to provide a more complete picture of the cytokine milieu induced during the anti-tumour immune response. A panel of ten cytokines was assessed, including IFN-γ, IL-17 and IL-4, as well as TNFα, GM-CSF, IL-1α, IL-2, IL-5, IL-6 and IL-10. Lymphocytes were extracted from the DLN of tumours 7 days post injection and cultured overnight in the presence of PMA and ionomycin to enhance cytokine secretion. Culture supernatants were then analysed using the cytokine bead array kit. Of the ten cytokines tested, the most notable differences in cytokine expression between tumour groups were observed for TNF-α and IL-4 (Figure 5.16). TNF-α secretion by DLN cells from 4TX12 1 tumour-bearing mice was elevated compared with that secreted by cells from control tumour-DLN, however the difference did not reach statistical significance. Production of IL-4 was increased in cells from

4T12Ala DLN compared with cells from naïve LN and 4TX12 1 DLN, but again, the difference was not statistically significant.

5.5 Summary of findings and preliminary discussion

In this chapter, it was established that CXCL12-mediated tumour growth inhibition was absent in SCID mice, and partly abrogated in nude mice. These experiments highlighted the importance of the adaptive immune response, and particularly T cells, for the effect of CXCL12. It should be pointed out that the extent of metastasis was not formally assessed in these mice, although there was an indication in nude mice that the number of lung metastases was reduced in the 4TX12 1 tumour-bearing group, in parallel with the reduction of primary tumour size. It will be interesting in future to determine if metastasis in SCID mice similarly parallels primary tumour growth. Such experiments would give an indication of the contribution of both immune- and non-immune-mediated mechanisms to the decreased metastatic capability of 4TX12 1 tumours.

Correlative analysis further supported the importance of T cells for CXCL12-induced anti-tumour immunity. This was apparent in the correlation of reduced tumour weight with an increased proportion of splenic $CD4^+$ cells, particularly in mice bearing 4TX12 1 tumours. Curiously, the total number of splenic $CD8^+$ cells in 4TX12 1 tumour-bearing mice correlated positively with tumour weight. Perhaps this is indicative of a failure of $CD8^+$ T cells, which have been successfully activated, to leave the spleen and migrate to the tumour site. The lack of a similar accumulation of $CD8^+$ cells in 4T12Ala spleens may reflect a relative absence of T cell activation and subsequent proliferation. Further study is required, however, to confirm this hypothesis and to elucidate the reason why $CD8^+$ cells are retained in the spleens of some 4TX12 1 tumour-bearing mice.

Apart from implicating T cells in the CXCL12-induced anti-tumour immune response, the studies of tumour growth in immunocompromised mice indicated that other cell subsets present in nude mice, but not in SCID mice, were required. These were not Type I NKT cells, as determined from an experiment in $Ja18^{-/-}$ mice lacking these cells. This by no means rules out a role for NKT cells; Type II NKT cells, which are CD1d-dependent but do not express the invariant Vα14-Jα18 chain, are still present in $Ja18^{-/-}$ mice. It may be that Type II, rather than Type I, NKT cells play a part in CXCL12-induced tumour

growth inhibition. As B cells are also lacking in SCID, but not nude, mice, it is possible that they too are involved in the anti-tumour response. However, time constraints prevented investigation of their input in this study. The potential contribution of NKT and B cells to the anti-tumour effect of CXCL12 is discussed further in Section 6.2.

Tumour expression of CXCL12 was found to increase the number of $CD11c^+$ cells in tumour-DLN, suggesting that there was an increase of DC infiltrating the DLN to prime T cells. This discovery led to the hypothesis that T cell activation was enhanced in mice bearing CXCL12-expressing tumours. In line with this, cell-mediated cytotoxicity was found to be enhanced in mice bearing 4TX12 1 tumours, as demonstrated by the partial requirement for the cytotoxic mediators perforin and TRAIL and by *in vitro* cytotoxicity assays.

IFN-γ was also shown to be required for CXCL12-induced anti-tumour immunity through the partial loss of CXCL12-mediated tumour growth inhibition in IFN-γ-deficient mice. Further analysis of cytokine expression within tumour-DLN in wild-type mice indicated that IL-17^+ and IL-4^+ cells were reduced in 4TX12 1 tumour-DLN compared to 4T12Ala tumour-DLN. Together, these findings suggest that 4T1.2 tumour growth is promoted through bias of the immune system away from a more productive cell-mediated anti-tumour response, and that expression of CXCL12 may serve to tip the scales back in favour of this form of immunity. Paradoxically, the proportion of IFN-γ^+ cells in 4TX12 1 DLN was reduced as assessed by intracellular cytokine staining, and cytokine bead array analysis did not reveal any difference in the level of IFN-γ production by DLN cells from the two tumour groups. Possible explanations for these discrepancies are discussed in Section 6.2, but may reflect limitation of the analysis to the DLN. Assessment of systemic cytokine levels or cytokine levels within the tumour may give a more complete picture of their role in the immune response to 4TX12 1 tumours.

As presented in this chapter, intriguing insights into the anti-tumour immune response induced by CXCL12 have been gained. However, there remain many questions to be answered about the cells involved and the mechanisms by which they exert their effects. Much information might be gained by study of cells and cytokines within tumours, and this represents an avenue of investigation to be followed in future. Attempts were made to

further characterise T cells and $CD11c^+$ cells infiltrating tumours, as well as to identify differences in the levels of innate immune cells within tumours (Figure 5.4B and data not shown). However, time constraints prevented sufficient optimisation of the protocols used and further experiments are required for meaningful conclusions to be drawn. The contribution of innate immune cells, and particularly macrophages, to the anti-tumour response is of particular interest, as is the nature of immunosuppressive mechanisms induced by the tumour. Study of these aspects of the immune response will provide valuable insight into the mechanism of CXCL12-induced anti-tumour immunity and build on the work presented in this thesis.

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Table 5.1 Comparison of tumour growth inhibition by CXCL12 in wild-type and nude mice.

Strain	Experiment	Mean tumour weight (g)		4TX12 1 tumour weight as % of control	% inhibition of 4TX12 1 tumour growth
		4T12Ala	4TX12 1		
Wild-type	1	0.83	0.20	24.2	75.8
	2	0.90	0.21	22.9	77.1
Nude	1	0.81	0.66	81.1	18.9
	2	0.91	0.48	52.7	43.3
$pfp^{-/-}$	1	0.79	0.49	61.4	38.6
$TRAIL^{-/-}$	1	0.78	0.44	57.1	42.9
IFN-$\gamma^{-/-}$	1	1.21	0.64	52.9	47.1

Table 5.2 Correlation of splenic T cell populations with final tumour weight.

	Spearman ρ	P value	Significance
4T12Ala			
Total splenocytes	0.5874	0.0446	*
% $CD4^+$ splenocytes	-0.5849	0.0457	*
Number of $CD4^+$ splenocytes	0.4755	0.1182	ns
% $CD8^+$ splenocytes	-0.3298	0.2951	ns
Number of $CD8^+$ splenocytes	0.4266	0.1667	ns
4TX12 1			
Total splenocytes	0.8231	0.0010	**
% $CD4^+$ splenocytes	-0.8462	0.0005	***
Number of $CD4^+$ splenocytes	0.07692	0.8122	ns
% $CD8^+$ splenocytes	-0.5464	0.0660	ns
Number of $CD8^+$ splenocytes	0.6503	0.0220	*

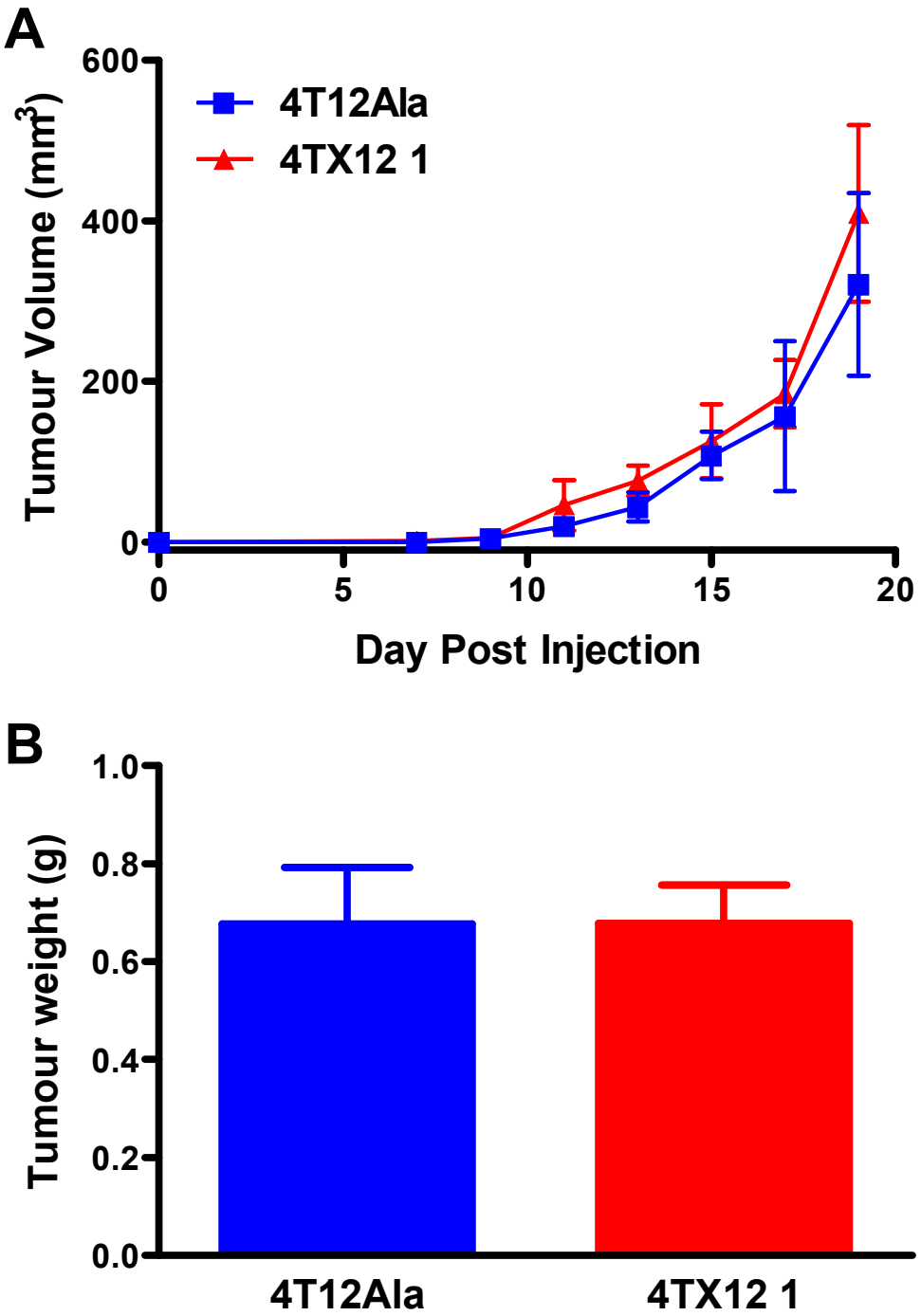


Figure 5.1 *Growth of CXCL12-expressing tumours in SCID mice.*

Female 6-8 week old SCID mice were injected in the fourth mammary fat pad with 1 x 10^5 tumour cells. **(A)** Tumour diameter was measured every 2 days once tumours became palpable. Tumour volume in mm^3 was calculated according to the equation $width^2$ x length/2. Data points represent the mean tumour volume ± SD (*n*=5 mice per group). One representative experiment of two is shown. No significant differences between groups (Mann-Whitney U test). **(B)** Final weights of tumours were determined at the termination of the experiment. Bars represent the mean final tumour weight ± SEM (*n*=10 mice per group from 2 independent experiments). No significant differences between groups. (P=0.9985, Student's t test.)

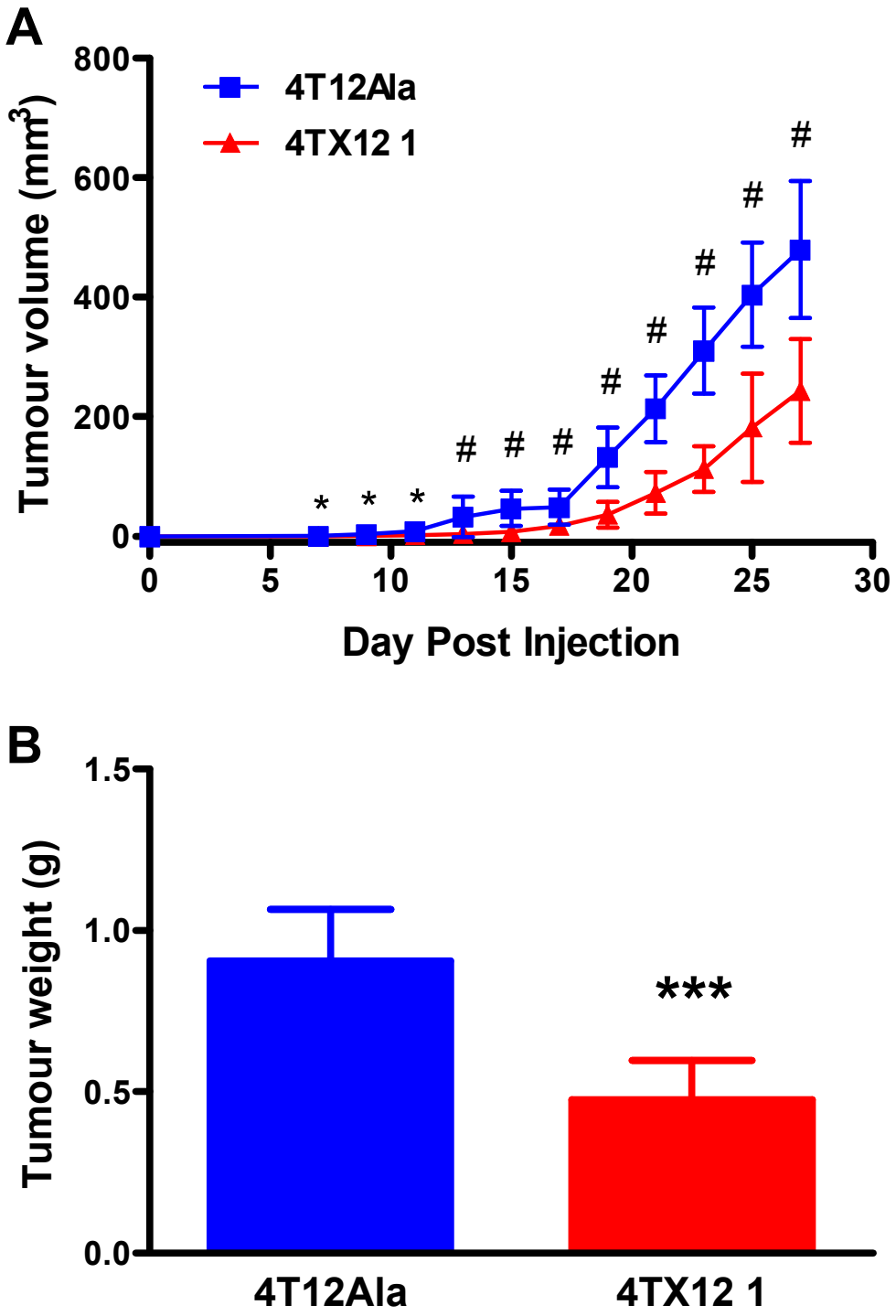
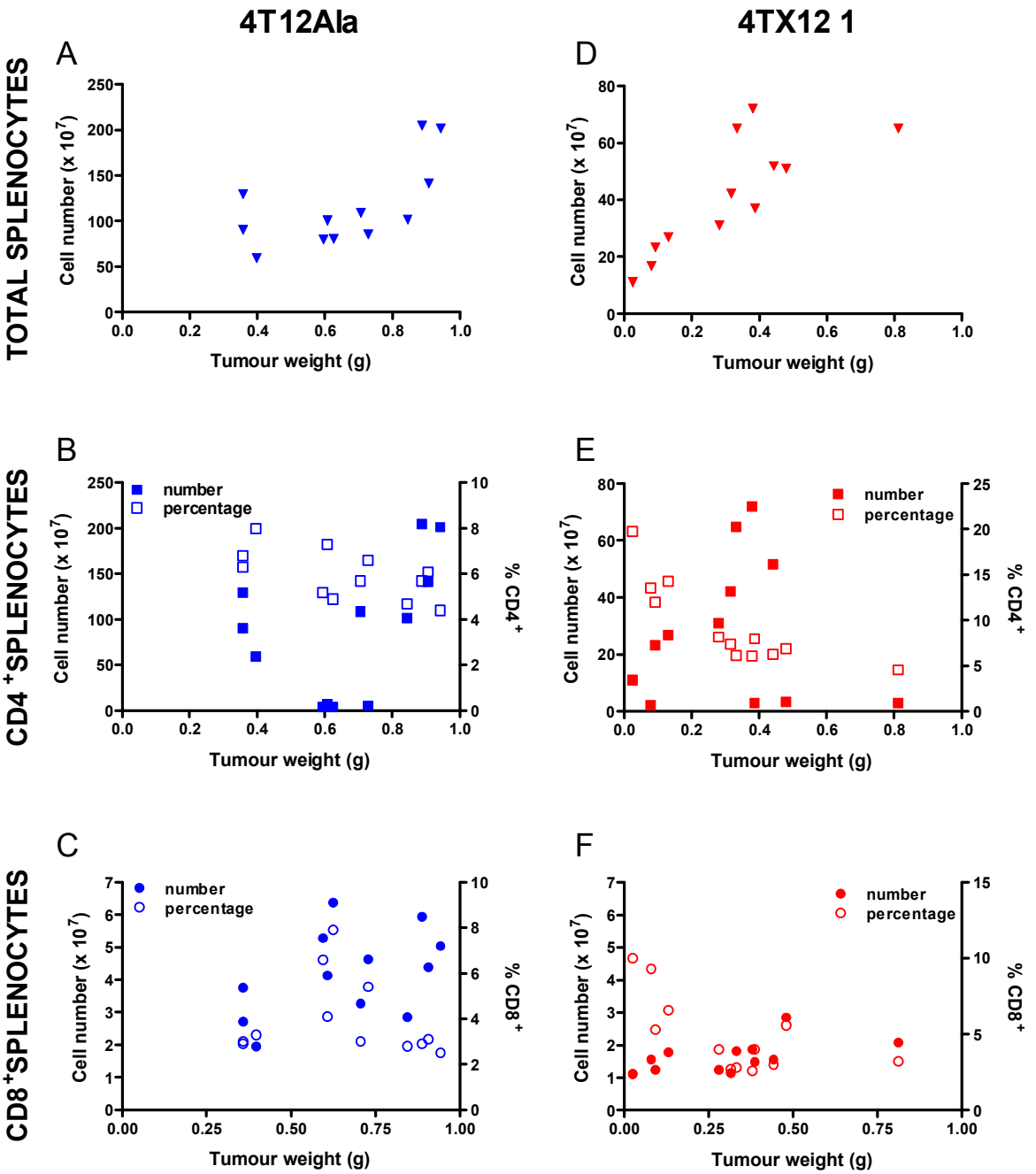


Figure 5.2 *Growth of CXCL12-expressing tumours cells in nude mice.*

Female 6-8 week old Balb/c mice carrying the nude mutation were injected in the fourth mammary fat pad with 1 x 10^5 tumour cells. **(A)** Tumour diameter was measured every 2 days once tumours became palpable. Tumour volume in mm^3 was calculated according to the equation $width^2$ x length/2. Data points represent the mean tumour volume ± SD. *, P<0.01; #, P<0.001, Mann-Whitney U test, *n*=11-12 mice per group. **(B)** Final weights of tumours were determined at the termination of the experiment. Bars represent the mean final tumour weight ± SD. ***, P<0.0001, Mann-Whitney U test, *n*=11-12 mice per group.

Figure 5.3 *Correlation of the number and proportion of splenic T cells subsets with tumour weight.*

Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad with 1 x 10^5 tumour cells. Final weights of tumours were determined at the termination of the experiment 29 days later. Spleens were removed and single cell suspensions were prepared for flow cytometric labelling with phycoerythrin-conjugated rat anti-mouse CD4, CD8, or matched isotype control. Fluorescing cells were quantified on the same day using an LSRII flow cytometer. Percentages and absolute numbers of $CD4^+$, $CD8^+$ and total splenocytes were determined and correlated with final tumour weight for each mouse. Data points represent the measurements for individual mice from two independent experiments. **(A-C)** 4T12Ala tumour-bearing mice. **(D-F)** 4TX12 1 tumour-bearing mice. **(A & D)** Correlation of tumour weight with total number of splenocytes. **(B & E)** Correlation of tumour weight with total number (filled squares) and percentage (open squares) of $CD4^+$ splenocytes. **(C & F)** Correlation of tumour weight with total number (filled circles) and percentage (open circles) of $CD8^+$ splenocytes. For a summary of the Spearman's ρ correlation coefficients and P values, refer to Table 5.2.


4T12Ala
4TX12 1
A
D
B
E
C
F
TOTAL SPLENOCYTES
CD4+SPLENOCYTES
CD8+SPLENOCYTES
Cell number (x 10^7)
Tumour weight (g)
number
percentage
% CD4+
% CD8+

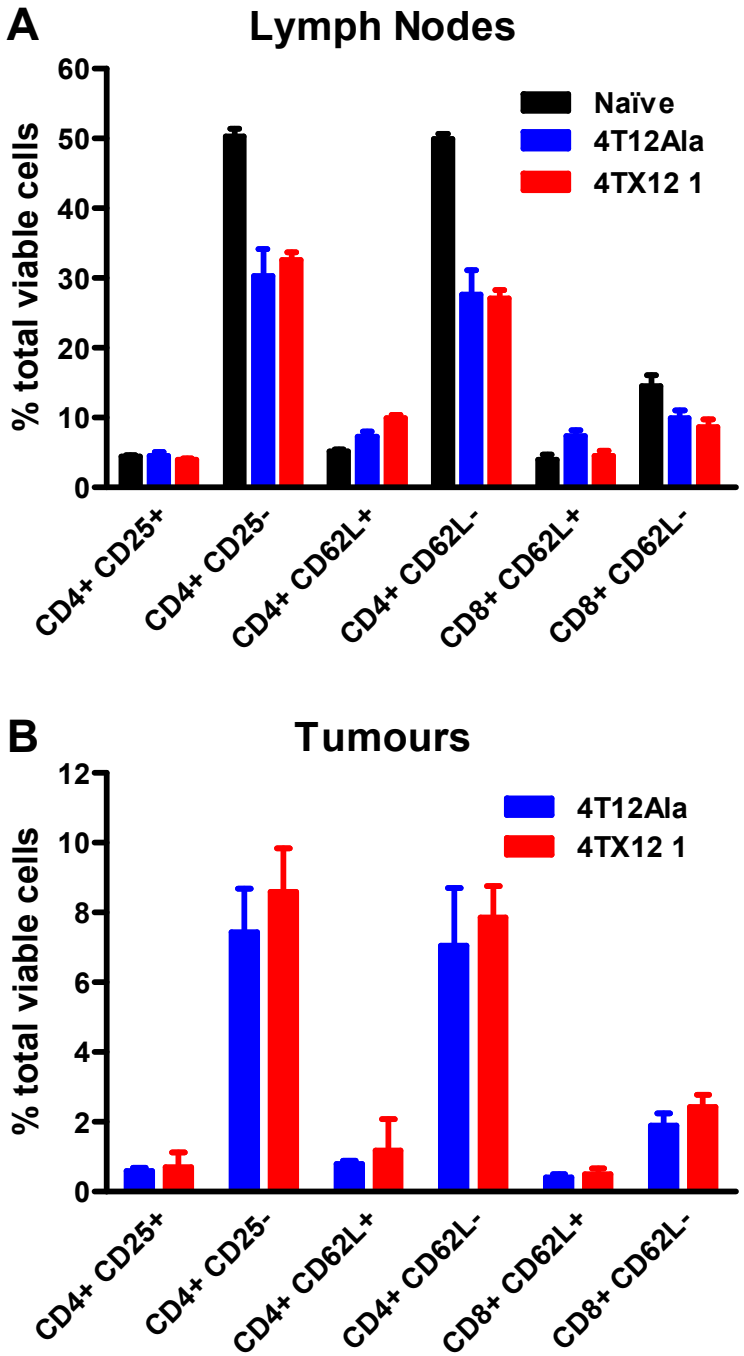


Figure 5.4 *Characterisation of T cell subsets from early stage tumours and tumour draining lymph nodes.*

Female 6-8 week old Balb/c mice were injected in the hind foot pad with 1 x 10^6 tumour cells or PBS (naïve controls). Seven days later, draining (popliteal) LN and tumours were removed and single cell suspensions were prepared for flow cytometric labelling. Cells were stained with fluorochrome-conjugated rat anti-mouse CD4, CD8, CD25 and CD62L antibodies or matched isotype controls. Fluorescing cells were quantified on the same day using an LSRII flow cytometer. **(A)** Quantitation of the proportion of cells obtained from tumour-DLN expressing the indicated markers. **(B)** Quantitation of the proportion of cells obtained from tumours expressing the indicated markers. Bars represent the mean ± SD (*n*=3-4 mice per group) from one representative experiment of three independent experiments.

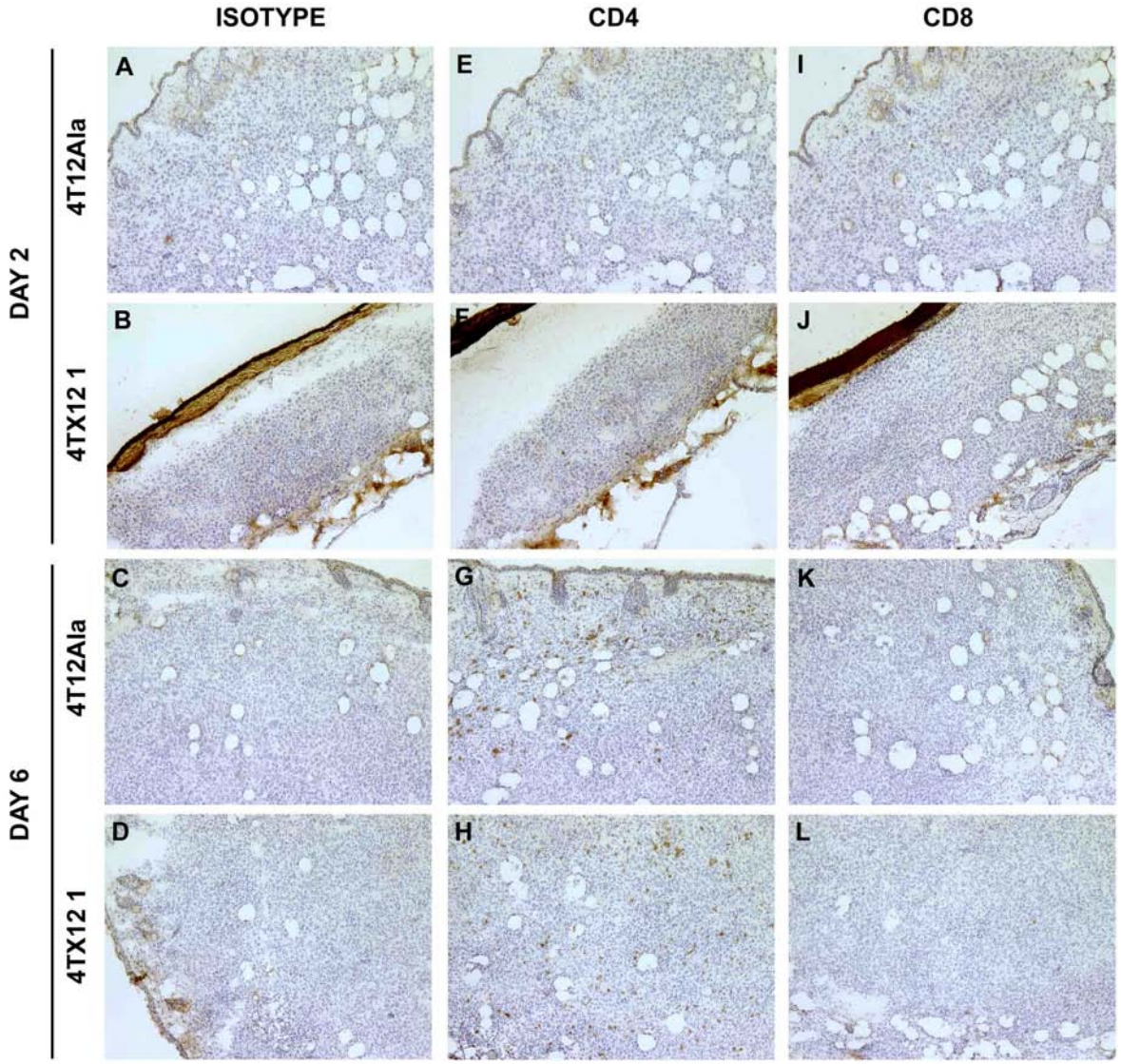


Figure 5.5 *T cell infiltration of CXCL12-expressing 4T1.2 tumours at early time points.*

CD4 and CD8 staining of infiltrating T cells in sections of early stage CXCL12 construct-expressing 4T1.2 tumours. Sections of tumours obtained either two **(A, B, E, F, I, J)** or six **(C, D, G, H, K, L)** days post tumour cell injection were stained with biotinylated rat anti-mouse CD4 or CD8 antibody followed by streptavidin-conjugated HRP. Binding of antibodies was revealed by incubation with DAB substrate and cell nuclei were counterstained with haematoxylin. Sections were mounted and examined by light microscopy. Representative micrographs of individual tumours are shown. Magnification: 100x. **(A, C, E, G, I, K)** Control 4T12Ala tumours. **(B, D, F, H, J, L)** 4TX12 1 tumours. **(A-D)** Isotype control staining. **(E-H)** CD4 staining. **(I-L)** CD8 staining.

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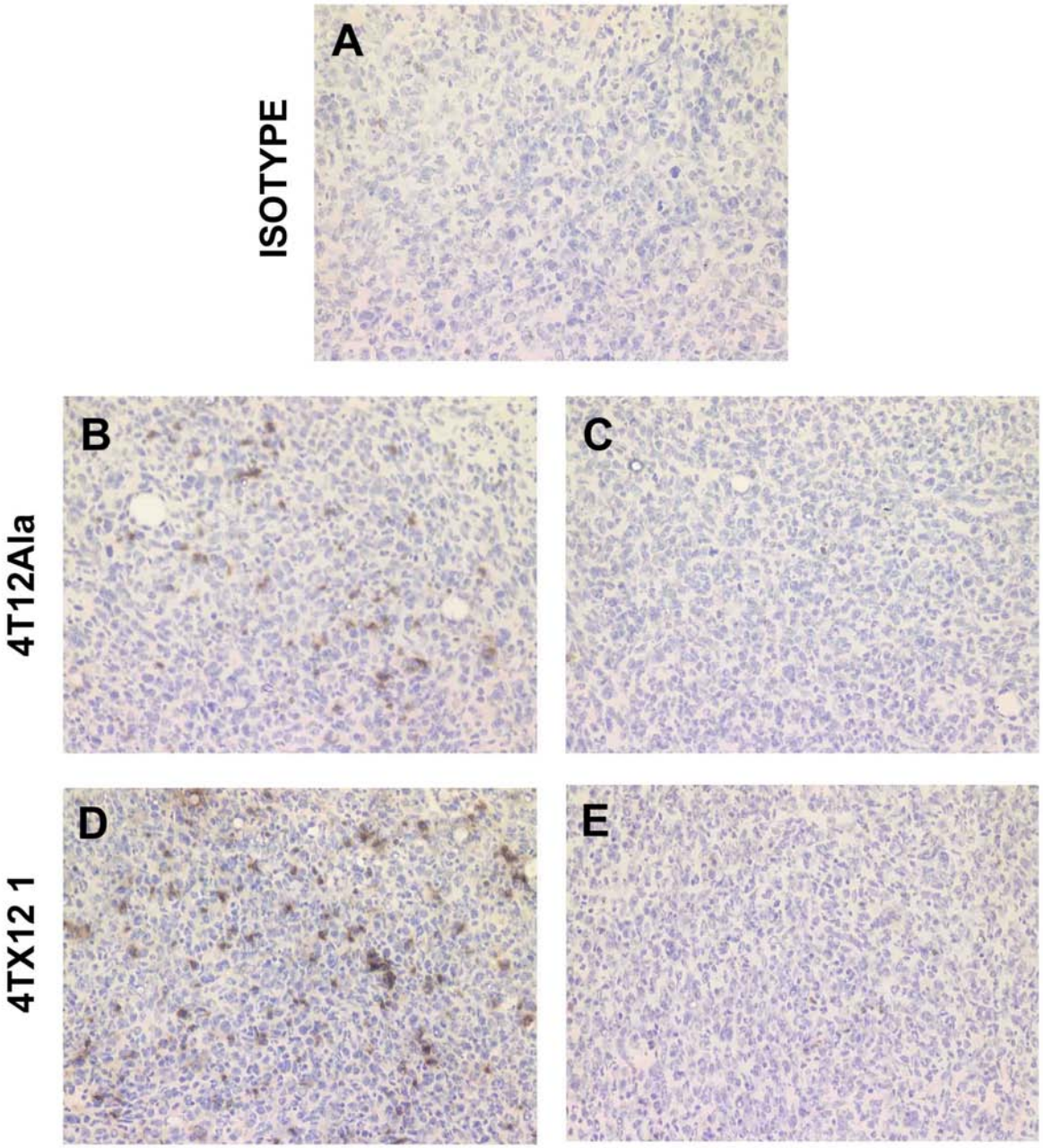

Figure 5.6 *$CD4^+$ T cell infiltration of late stage CXCL12-expressing 4T1.2 tumours.*

CD4 staining of infiltrating T cells in sections of late stage CXCL12 construct-expressing 4T1.2 tumours. Sections of tumours obtained approximately 4 weeks post tumour cell injection were stained with rat anti-mouse CD4 antibody followed by streptavidin-conjugated HRP. Binding of the antibody was revealed by incubation with DAB substrate and cell nuclei were counterstained with haematoxylin. Sections were mounted and examined by light microscopy. Representative micrographs of two individual tumours from each group are shown. Magnification: 200x. **(A)** Isotype control staining. **(B & C)** Control 4T12Ala tumours. **(D & E)** 4TX12 1 tumours.

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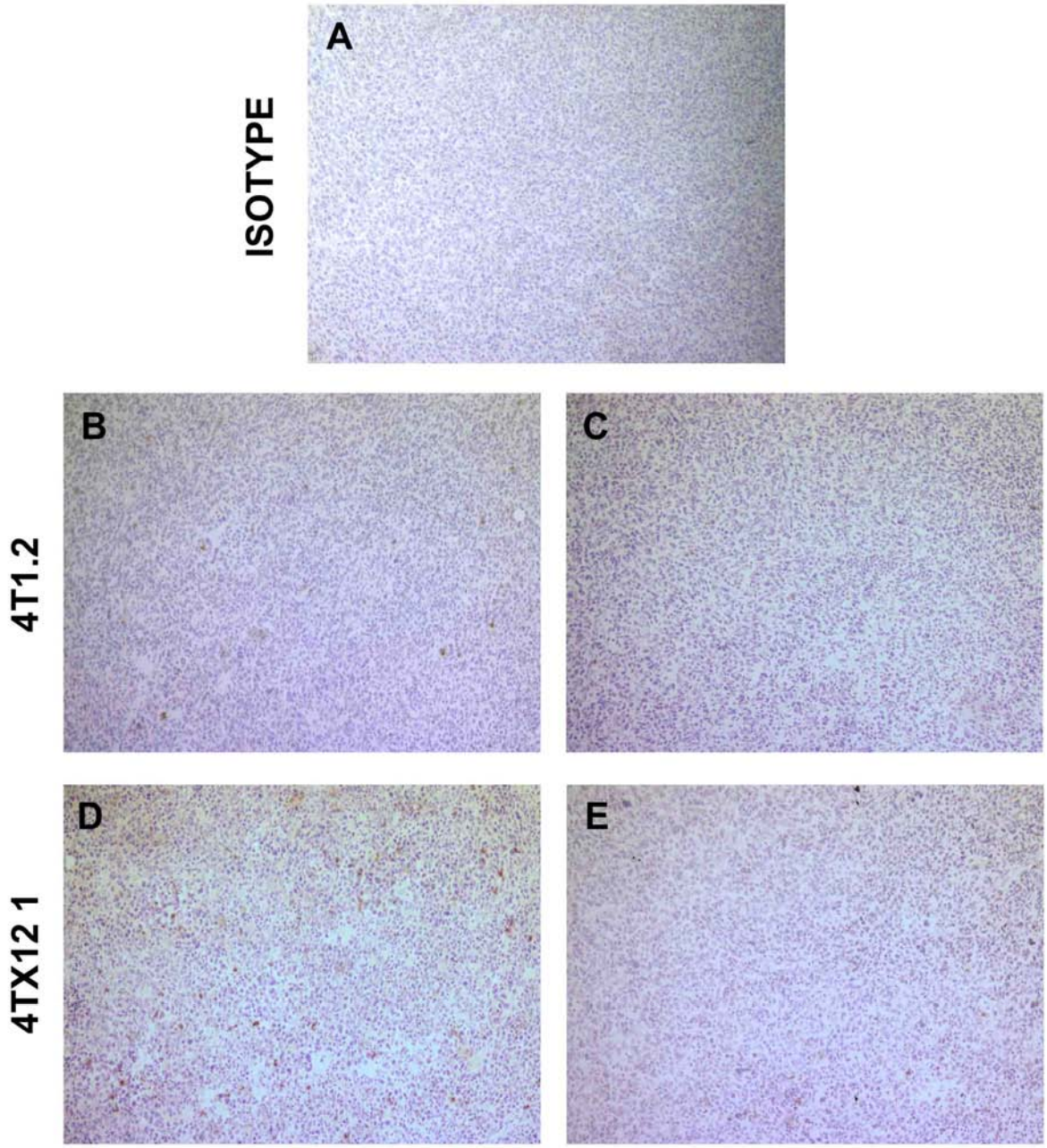

Figure 5.7 *$CD8^+$ T cell infiltration of late stage CXCL12-expressing 4T1.2 tumours.*

CD8 staining of infiltrating T cells in sections of late stage CXCL12 construct-expressing 4T1.2 tumours. Sections of tumours obtained approximately 4 weeks post tumour cell injection were stained with rat anti-mouse CD8 antibody followed by streptavidin-conjugated HRP. Binding of the antibody was revealed by incubation with DAB substrate and cell nuclei were counterstained with haematoxylin. Sections were mounted and examined by light microscopy. Representative micrographs of two individual tumours from each group are shown. Magnification: 100x. **(A)** Isotype control staining. **(B & C)** Control 4T1.2 tumours. **(D &E)** 4TX12 1 tumours.

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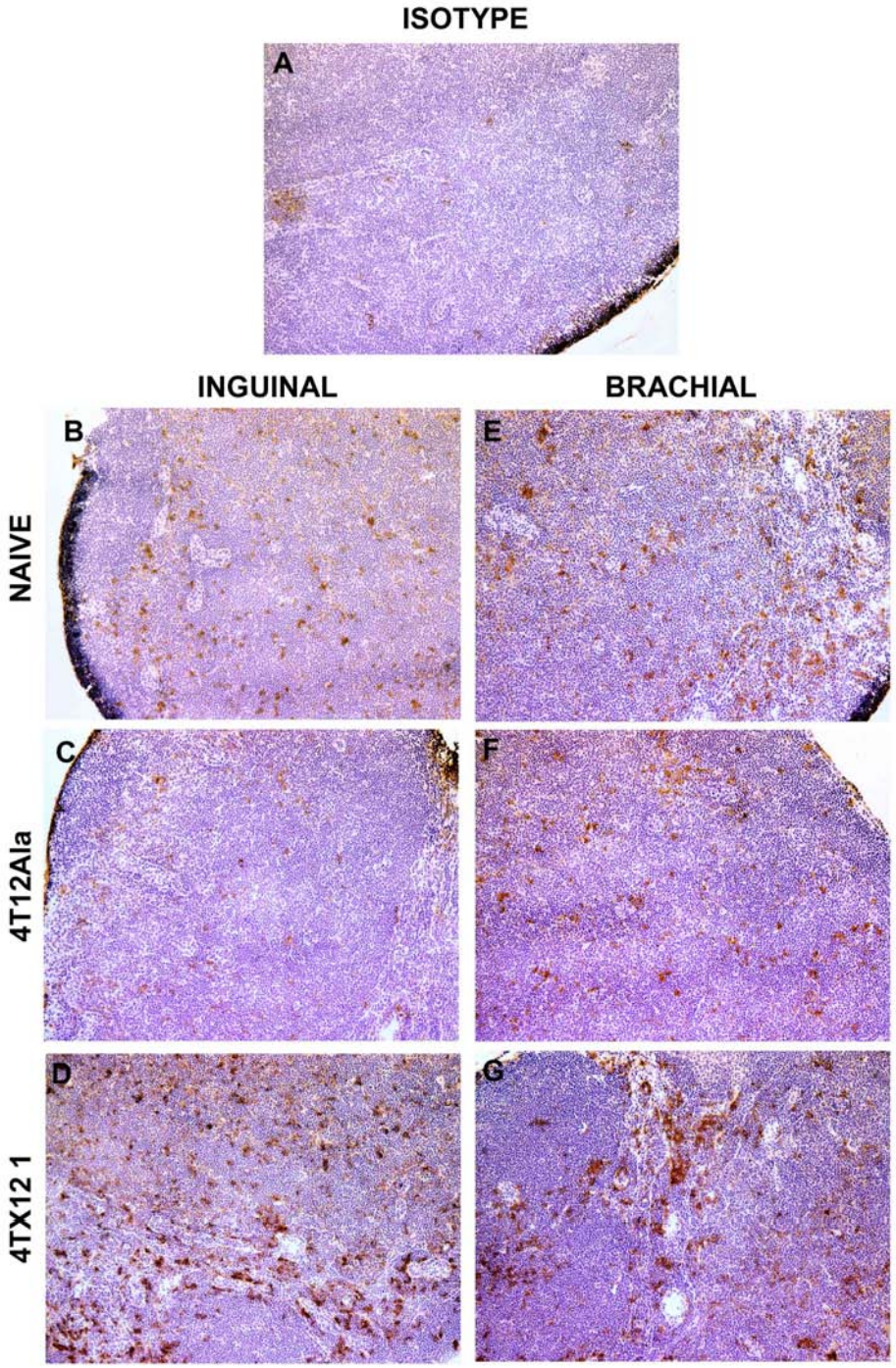

Figure 5.8 *Accumulation of CD11c$^+$ cells in the tumour-draining lymph nodes.*

CD11c$^+$ cells in sections of 4T1.2 tumour-DLN. Sections of tumour-draining (inguinal **[B-D]** and brachial **[E-G]**) LN were obtained six days post tumour cell injection. Sections were stained with biotinylated hamster anti-mouse CD11c antibody followed by streptavidin-conjugated HRP. Binding of antibody was revealed by incubation with DAB substrate and cell nuclei were counterstained with haematoxylin. Sections were mounted and examined by light microscopy. Representative micrographs of individual LN are shown. Magnification: 100x. **(A)** Isotype control. **(B & E)** LN from naïve mice. **(C & F)** DLN from control 4T12Ala tumour-bearing mice. **(D & G)** DLN from 4TX12 1 tumour-bearing mice.

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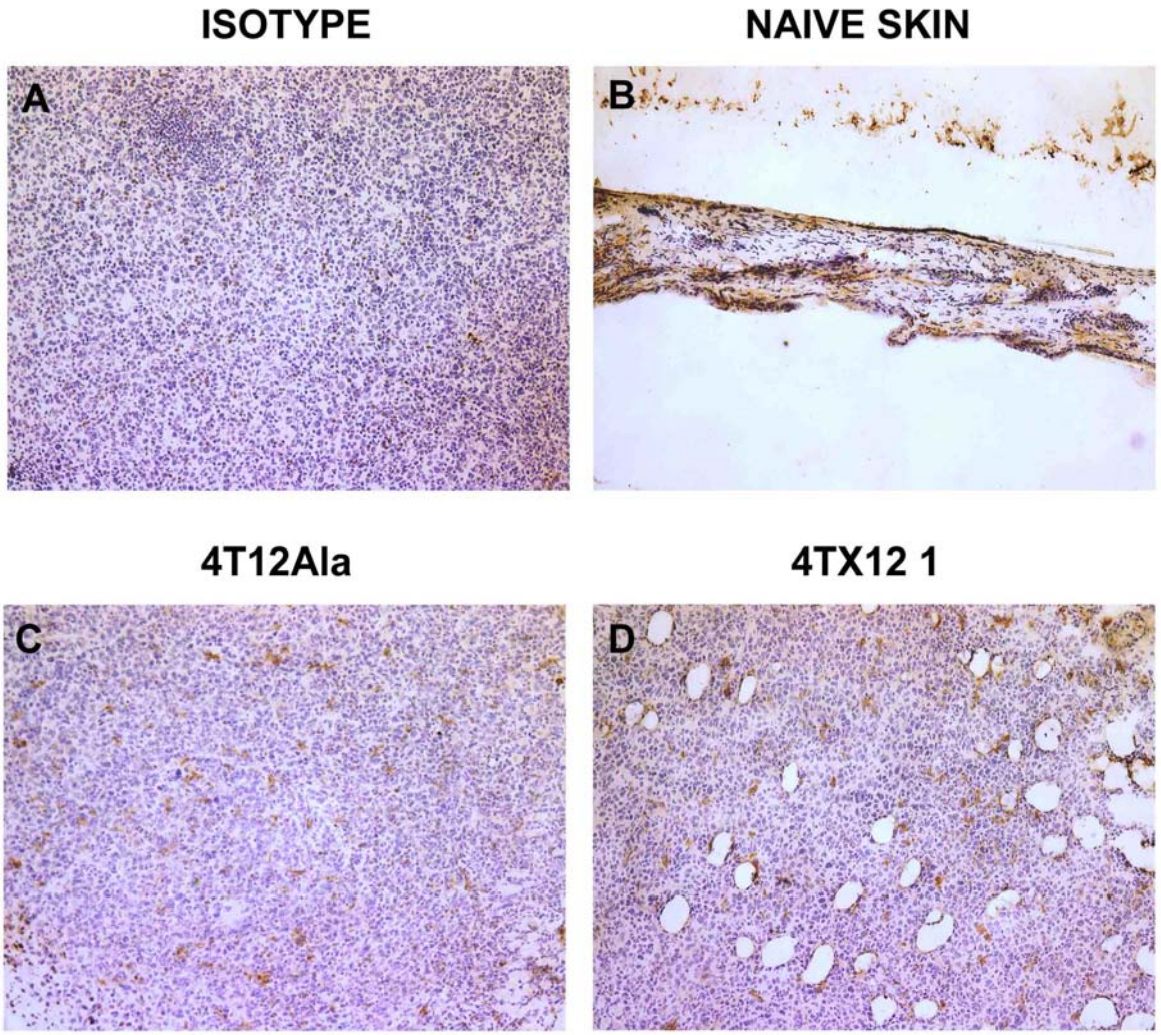

Figure 5.9 *$CD11c^+$ cells within CXCL12-expressing tumours.*

$CD11c^+$ cells in sections of CXCL12 construct-expressing 4T1.2 tumours. Sections of tumours obtained six days post tumour cell injection were stained with biotinylated hamster anti-mouse CD11c antibody followed by streptavidin-conjugated HRP. Binding of antibody was revealed by incubation with DAB substrate and cell nuclei were counterstained with haematoxylin. Sections were mounted and examined by light microscopy. Representative micrographs of individual tumours are shown. Magnification: 100x. **(A)** Isotype control. **(B)** Naïve skin. **(C)** Control 4T12Ala tumour. **(D)** 4TX12 1 tumour.

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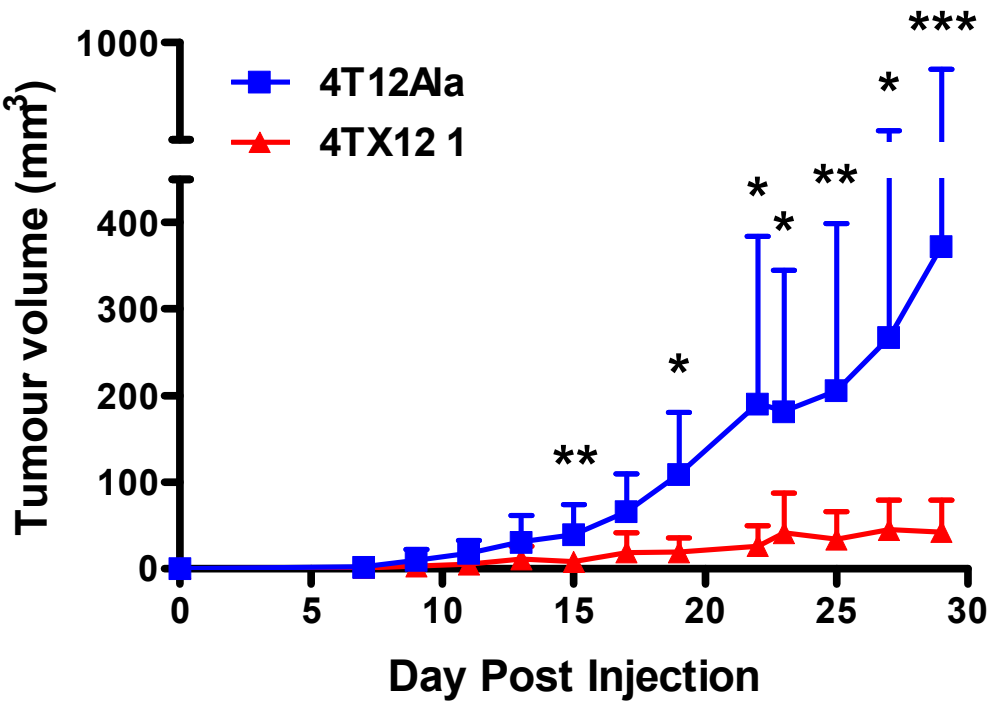


Figure 5.10 *Growth of CXCL12-expressing tumours in invariant NKT cell-deficient mice.*

Female 6-8 week old Jα18$^{-/-}$ mice were injected in the fourth mammary fat pad with 1 x 10^5 tumour cells. Tumour diameter was measured every 2 days once tumours became palpable. Tumour volume in mm^3 was calculated according to the equation width^2 x length/2. Data points represent the mean tumour volume ± SD (*n*=7 mice per group). *, P<0.05; **, P<0.01; ***, P<0.001, Mann-Whitney U test.

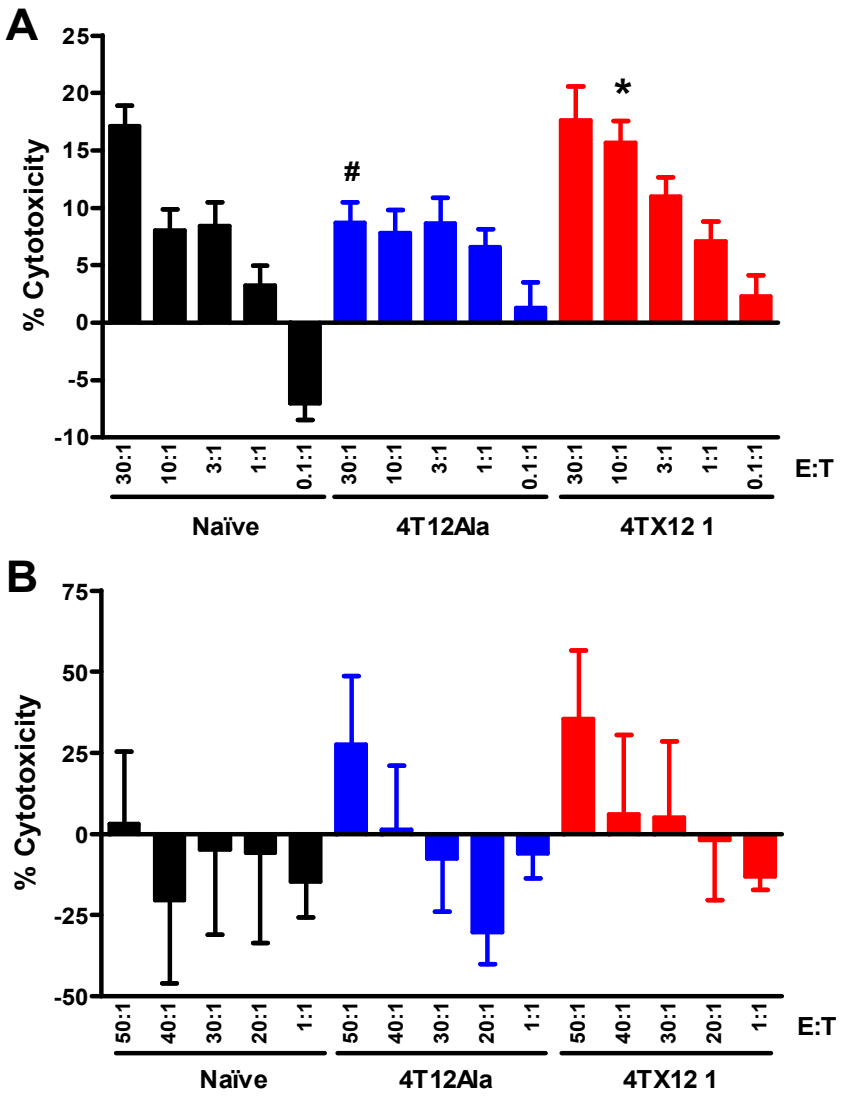


Figure 5.11 *Cytotoxic activity of lymphocytes against 4T1.2 tumour cells* in vitro.

(A) Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad with 1 x 10^5 tumour cells. After approximately 5 weeks, single cell suspensions were prepared from the pooled spleen and tumour-draining (brachial) LN for each mouse and used as effector cells in a cytotoxicity assay as described in Section 2.4.9. Target 4T1.2 cells were labelled with calcein, then plated at 2.5 x 10^4 cells/well in 96-well trays and allowed to adhere prior to the addition of effector cells. Four days later, non-adherent cells were washed away, and fluorescence of remaining target cells was measured on a Molecular Imager FX. Percentage cytotoxicity was calculated as 100-(fluorescence of target cells/fluorescence of target cells only x 100). *, $P<0.05$, significantly different from naïve effectors and effectors from 4T12Ala tumour-bearing mice (Kruskal-Wallis). #, $P<0.05$, significantly different from naïve effectors and effectors from 4TX12 1 tumour-bearing mice (Kruskal-Wallis). **(B)** Female 6-8 week old Balb/c mice were injected in both hind foot pads with 1 x 10^6 cells per foot pad. Seven days later, lymphocytes were isolated from the draining popliteal LN and used as effector cells in a cytotoxicity assay. 4T1.2 cells plated at 1 x 10^4 cells/well were used as targets. After 3 days, non-adherent cells were washed away, and remaining target cells were labelled with calcein. Fluorescence was measured on a Molecular Imager FX and percentage cytotoxicity was calculated as 100-(fluorescence of target cells/fluorescence of target cells only x 100).

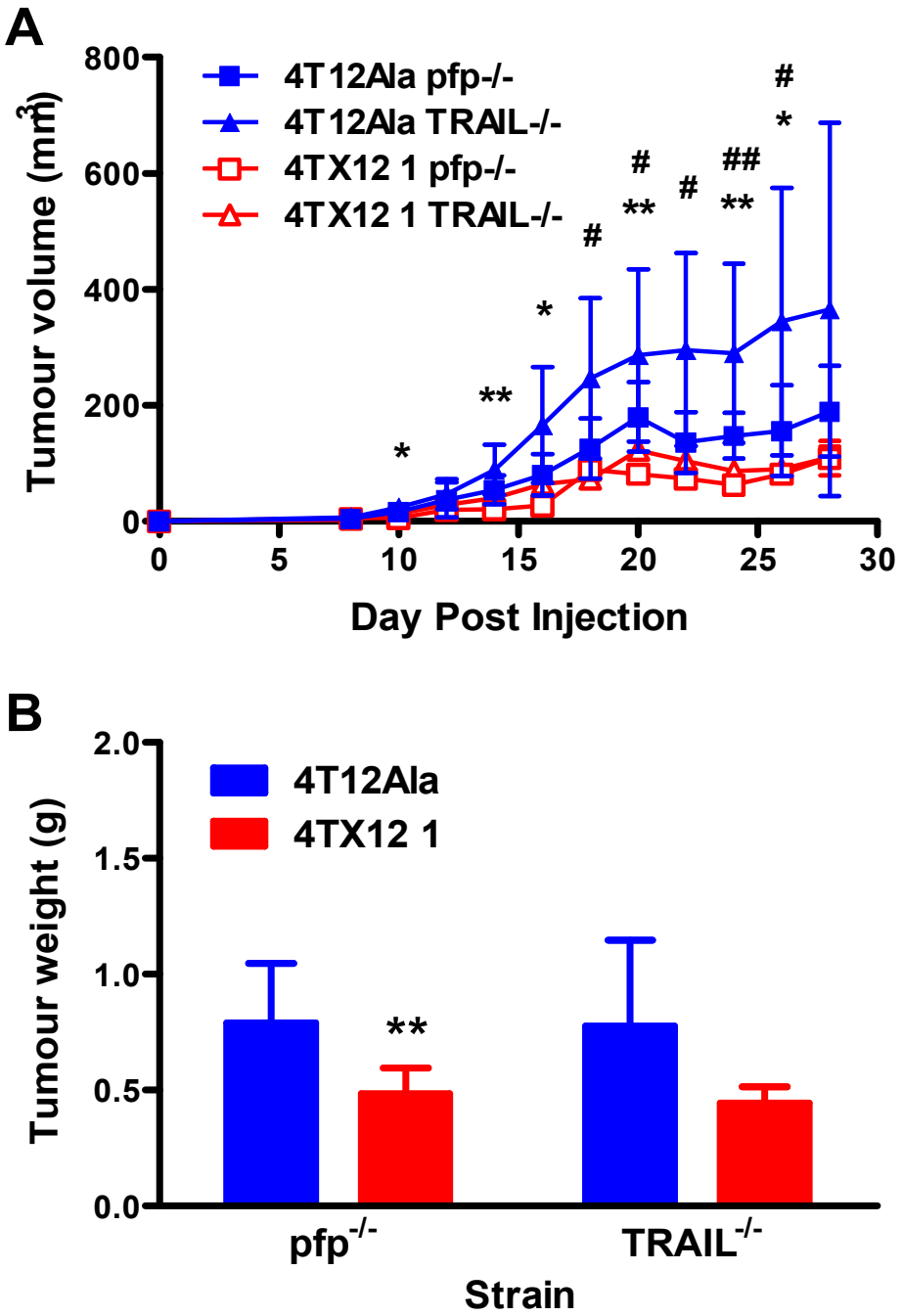


Figure 5.12 *Growth of CXCL12-expressing tumours in perforin- and TRAIL-deficient mice.*

Female 6-8 week old pfp$^{-/-}$ or TRAIL$^{-/-}$ mice were injected in the fourth mammary fat pad with 1 x 10^5 tumour cells. **(A)** Tumour diameter was measured every 2 days once tumours became palpable. Tumour volume in mm^3 was calculated according to the equation width^2 x length/2. Data points represent the mean tumour volume ± SD (n=5-7 mice per group). For pfp$^{-/-}$ mice, *, P<0.05; **, P<0.01, Mann-Whitney U test. For TRAIL$^{-/-}$ mice, #, P<0.05; ##, P<0.01, Mann-Whitney U test. **(B)** Final weights of tumours were determined at the termination of the experiment. Bars represent the mean final tumour weight ± SD (n=5-7 mice per group). **, significantly different from control 4T12Ala tumour weight, P=0.0022, Mann-Whitney U test.

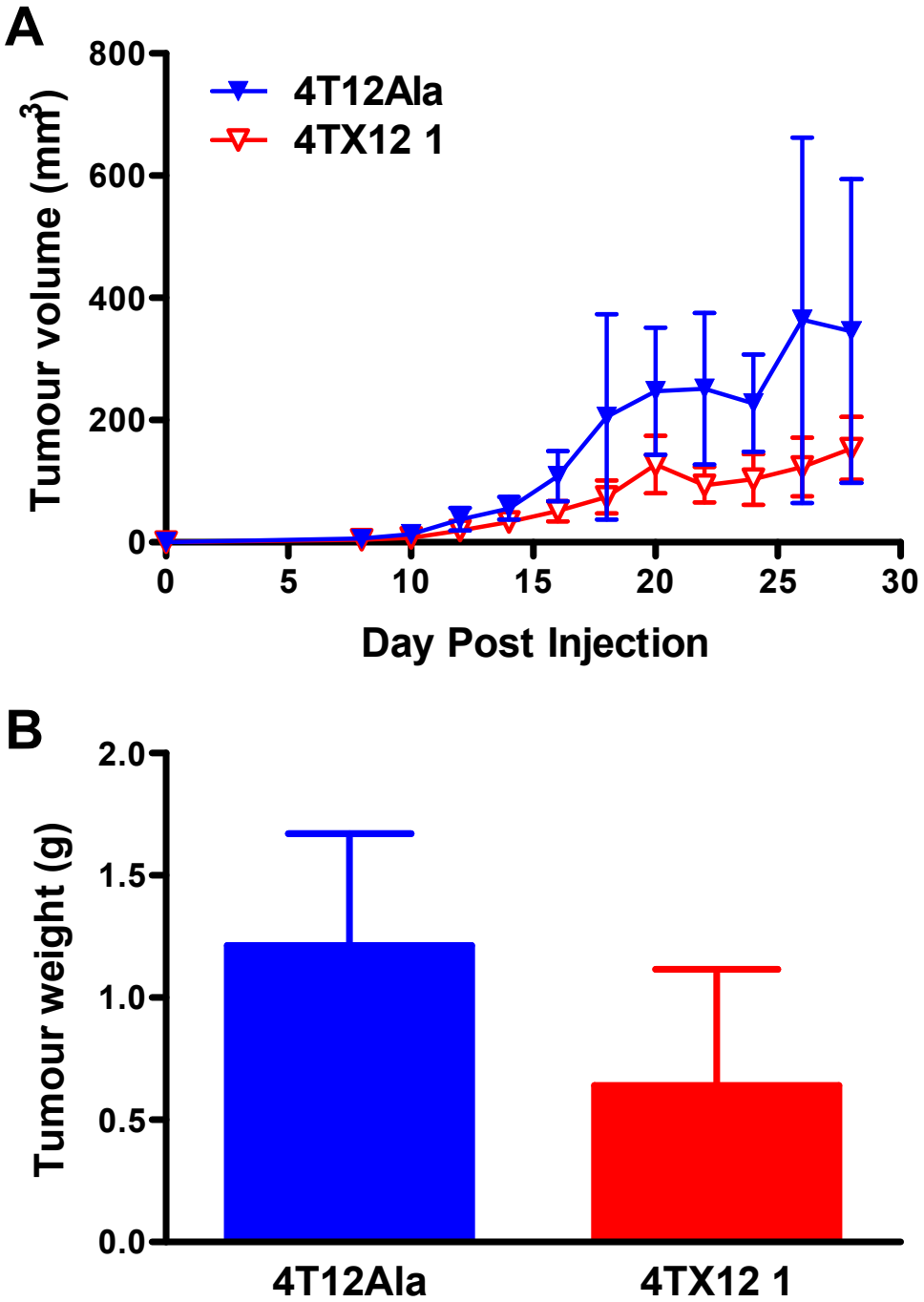


Figure 5.13 *Growth of CXCL12-expressing tumours in IFN-γ knockout mice.*

Female 6-8 week old IFN-$\gamma^{-/-}$ mice were injected in the fourth mammary fat pad with 1 x 10^5 tumour cells. **(A)** Tumour diameter was measured every 2 days once tumours became palpable. Tumour volume in mm^3 was calculated according to the equation width^2 x length/2. Data points represent the mean tumour volume ± SD (*n*=7 mice per group). No significant differences between groups (Mann-Whitney U test). **(B)** Final weights of tumours were determined at the termination of the experiment. Bars represent the mean final tumour weight ± SD (*n*=7 mice per group). No significant difference between groups (P=0.0530, Mann-Whitney U test).

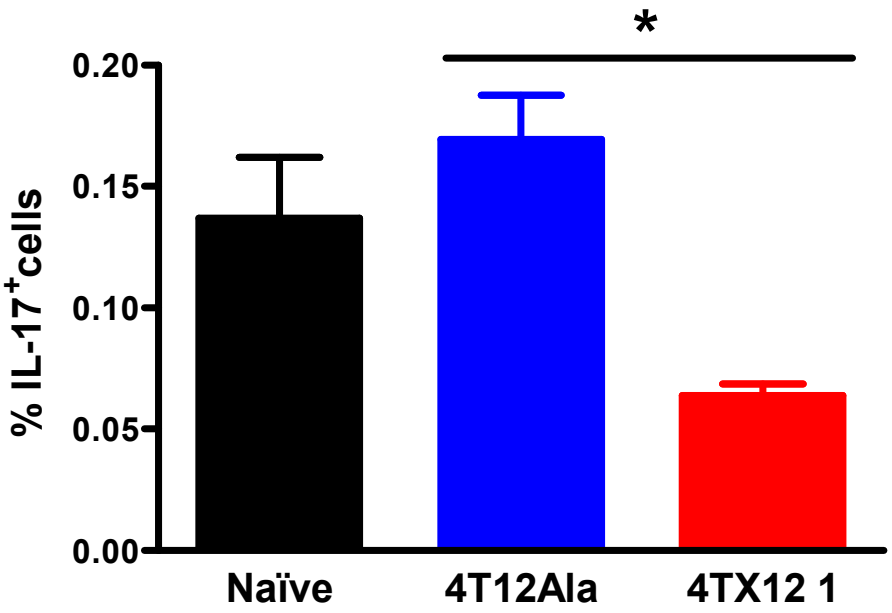


Figure 5.14 *Quantitation of IL-17-producing cells in tumour-draining lymph nodes by ELISPOT assay.*

Female 6-8 week old Balb/c mice were injected in both hind foot pads with 1 x 10^6 tumour cells each. Seven days later, tumour-draining popliteal LN were obtained and macerated through a 70μm nylon sieve to generate single cell suspensions. Cells were prepared in complete medium containing PMA and ionomycin and aliquoted at 1.2 x 10^5 cells/well into the wells of a 96-well filter plate coated with anti-mouse IL-17 antibody. Cells were then incubated overnight at 37°C. The following day, cells were washed away and bound IL-17 was detected with biotinylated anti-mouse IL-17 followed by HRP-conjugated streptavidin. BCIP/NBT chromogenic substrate was used to visualise spots, and then plate was washed and allowed to dry. Spots representing individual IL-17-producing cells were counted under a dissecting microscope. Bars represent mean (± SD) percentage of LN cells expressing IL-17 (*n*=3 mice per group). *, $P<0.05$, Kruskal-Wallis test.

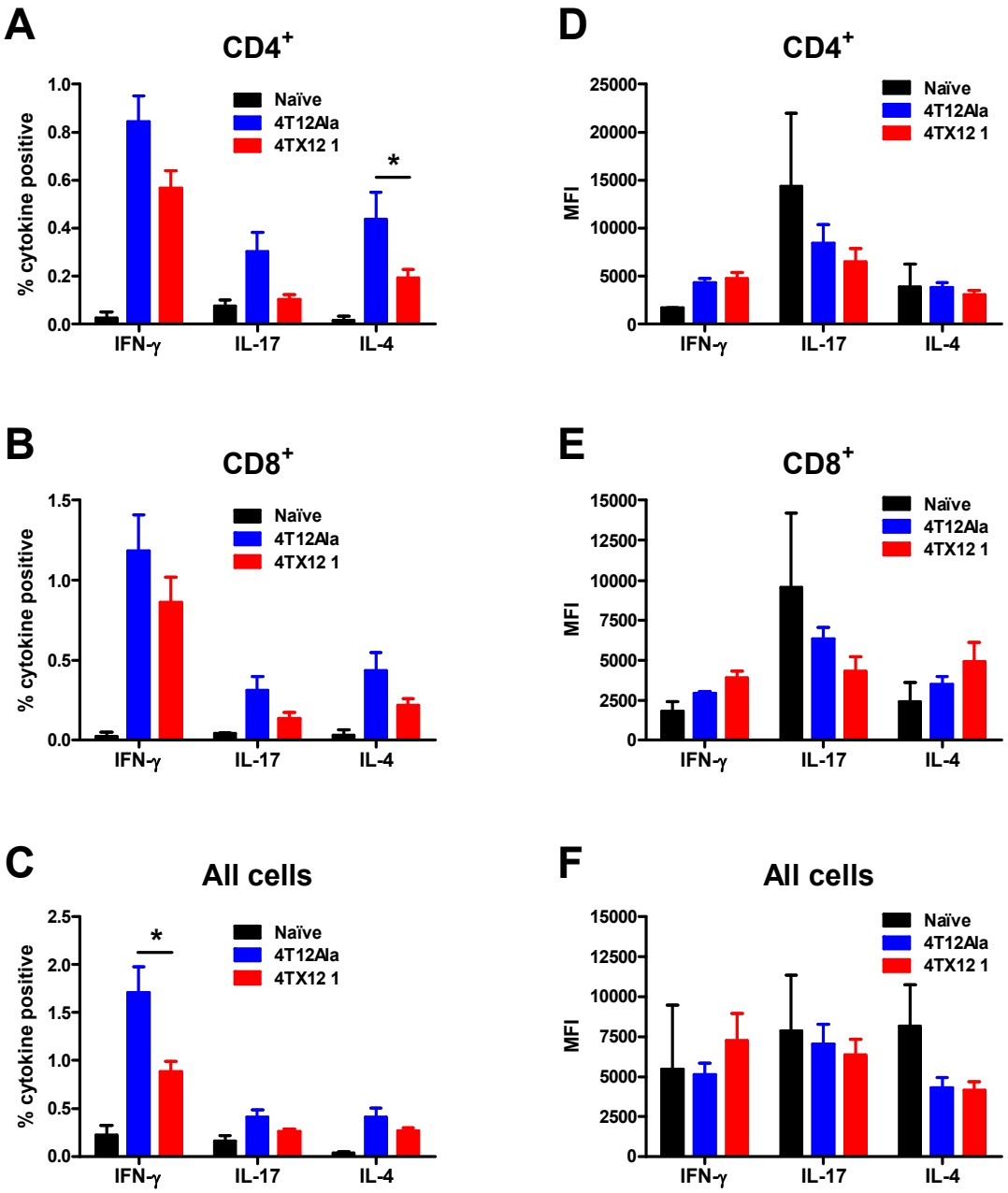


Figure 5.15 *Intracellular cytokine staining of lymphocytes isolated from tumour-draining lymph nodes.*

Female 6-8 week old Balb/c mice were injected in both hind foot pads with 1 x 10^6 tumour cells each. Seven days later, draining popliteal LN were removed and single cell suspensions were prepared. Cells were incubated overnight with PMA and ionomycin at 37°C. Five hours prior to the end of the stimulation, GolgiStop™ was added to the cells to arrest cytokine secretion. Cells were then fixed, permeabilised and stained with fluorochrome-conjugated rat anti-mouse CD4 or CD8, and anti-mouse IFN-γ, IL-17 or IL-4. Fluorescing cells were quantified using an LSRII flow cytometer. Bars represent the mean ± SEM (n=10 mice per group from 2 independent experiments). **(A-C)** Percentage of cells positive for cytokine expression. **(D-F)** Mean fluorescence index of cytokine positive cells. **(A & D)** Cytokine positive $CD4^+$ cells. **(B & E)** Cytokine positive $CD8^+$ cells. **(C & F)** Total cytokine positive cells. *, 4TX12 1 significantly different from control 4T12Ala group, P<0.05, Mann-Whitney U test.

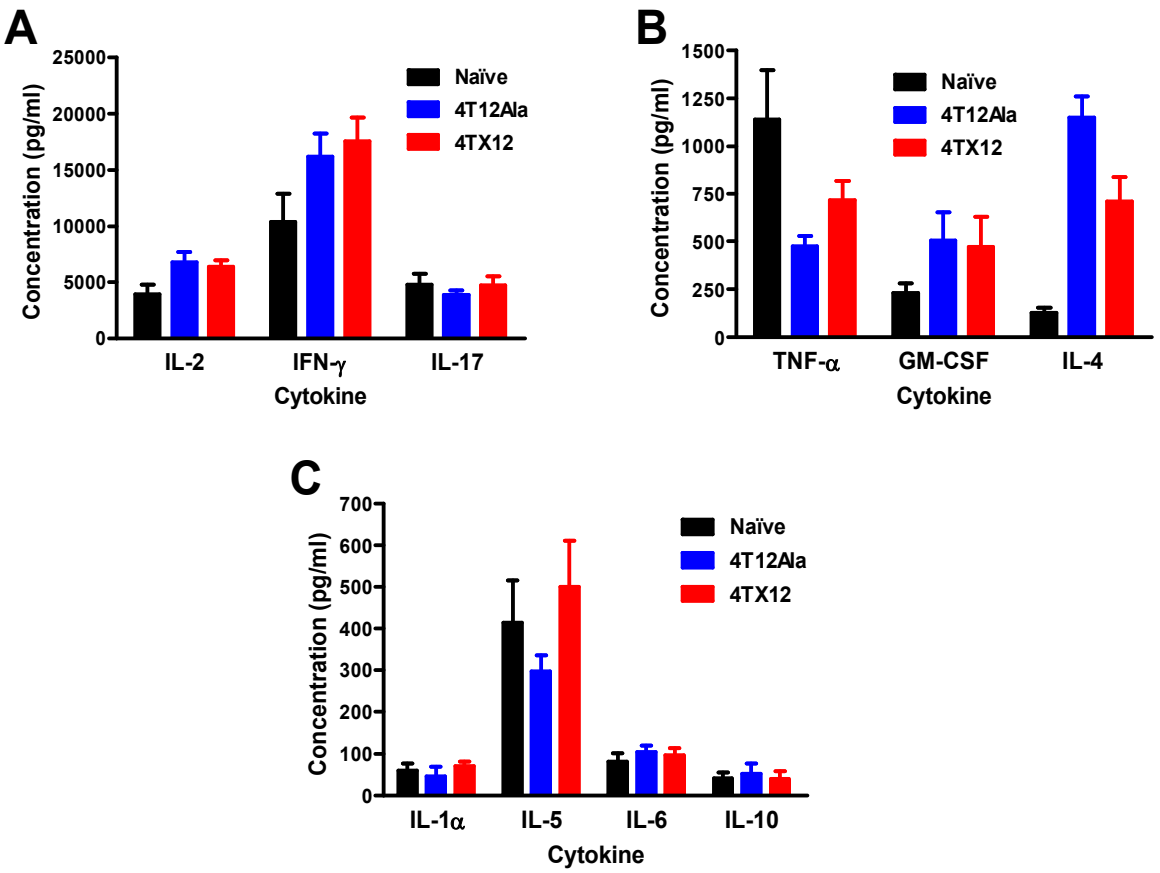


Figure 5.16 *Cytokine production by cells isolated from tumour-draining lymph nodes.*

Female 6-8 week old Balb/c mice were injected in the fourth mammary fat pad (both flanks) with 1 x 10^5 tumour cells. Six days later, draining (inguinal and brachial) LN were removed and single cell suspensions were prepared. Cells were incubated overnight with PMA and ionomycin at 37°C. The following day, culture supernatants were harvested and filtered through a 0.2µm filter and analysed by cytokine bead array as described in Section 2.4.12. Samples were acquired within 24 hours on a FACSCanto flow cytometer and analysed using the Bender MedSystems FlowCytomix software. Bars represent mean ± SEM (n=7 mice per group from 2 independent experiments). No significant differences between groups. **(A)** Concentration of IL-2, IFN-γ, and IL-17 in culture supernatants from cells derived from tumour-DLN. **(B)** Concentration of TNF-α, GM-CSF and IL-4 in culture supernatants from cells derived from tumour-DLN. **(C)** Concentration of IL-1α, IL-5, IL-6 and IL-10 in culture supernatants from cells derived from tumour-DLN.

CHAPTER 6

Discussion

CHAPTER 6: DISCUSSION

6.1 Introduction

The importance of CXCL12 and CXCR4 in cancer has become increasingly apparent over the last few years. However, while CXCR4 expression has been correlated with tumour malignancy and metastatic disease in multiple cancer types[6, 127, 178-195], the precise mechanisms by which it promotes cancer progression are still poorly understood. Much attention has been focused on immunotherapy as a strategy for treating cancer, and chemokines, with their ability to modulate leukocyte migration and activation, hold much promise as potential therapeutics. A handful of studies have reported anti-tumour activity of CXCL12 when overexpressed in fibrosarcoma[215], melanoma, lung carcinoma[218] and leukaemia[216], but at the time this research was conducted, the anti-tumour effects of CXCL12 in breast cancer had not been investigated.

The relatively newly identified chemokine CXCL16, which acts as a chemoattractant for activated polarised Type I T cells and other effectors of cell-based immunity, is a possible candidate for enhancing anti-tumour immune responses. Very little has been published regarding the role of this chemokine in cancer, and the few studies that have been recorded have documented differing effects. As yet, no reports about the potential use of CXCL16 as a therapeutic agent in cancer have been published.

For the reasons outlined above, this study was conducted to assess the ability of CXCL12 and CXCL16 and their respective antagonists, $CXCL12_{(P2G)}$ and $CXCL16_{(9\text{-}220)}$, to influence breast tumour growth and metastasis. A number of significant findings were made, specifically that (1) overexpression of CXCL12 induced a significant anti-tumour immune response that was in part dependent on T cells and the accumulation of $CD11c^{+}$ cells within the tumour-DLN, (2) expression of the antagonist $CXCL12_{(P2G)}$ did not affect primary tumour growth but inhibited both spontaneous and experimental metastasis, and (3) overexpression of CXCL16 did not affect tumour growth or metastasis, but expression of the $CXCL16_{(9\text{-}220)}$ antagonist inhibited experimental metastasis. Attempts were made to elucidate the mechanisms behind these key observations; however, time constraints prevented detailed characterisation of the mode of action of the chemokines and their antagonists in this model. Therefore, the following discussion will address the major

findings of this work, but also will include speculation as to the mechanistic details of chemokine/antagonist-mediated inhibition of tumour growth and metastasis. In addition, suggestions as to how these mechanistic details might be investigated in future are discussed.

6.2 The effects of CXCL12 overexpression on breast tumour progression

As demonstrated in the preceding chapters, CXCL12 had a profound suppressive effect on 4T1.2 tumour growth *in vivo*. This was despite any apparent effect on 4T1.2 cell growth *in vitro*. Subsequent experiments to elucidate the mechanism of CXCL12-mediated tumour inhibition showed that (1) the inhibitory effect was completely abrogated in SCID mice, partly abrogated in nude mice and intact in Ja18$^{-/-}$ mice, (2) the inhibitory effect was partly dependent on IFN-γ, perforin and TRAIL, and (3) CXCL12 overexpression in the tumour increased the proportion of CD11c^{+} cells, but reduced the proportion of IFN-γ^{+}, IL-17^{+} and IL-4^{+} cells in the tumour-DLN.

6.2.1 The effects of CXCL12 on tumour cells and the tumour vasculature

The fact that CXCL12 could inhibit tumour growth *in vivo*, without affecting the *in vitro* growth characteristics of 4T1.2 cells, strongly suggested that CXCL12 was mediating its effects through modulation of host components, rather than acting directly on the tumour. This does not rule out the possibility that CXCL12 is able to regulate tumour cell processes that are required only for growth *in vivo*, and that are dispensable for growth *in vitro*. However, direct stimulation of cells with CXCL12 generally activates functions that would be expected to promote tumour growth, such as secretion of degradative enzymes[207] (which could assist the growing tumour to invade surrounding tissue as well as supply growth factors and nutrients freed from the ECM) and activation of integrins[290] (which could enhance anchorage of tumour cells). In light of the reduction of tumour growth seen in this model, it seems unlikely that direct action of CXCL12 on tumour cells plays a significant role here.

From reports of the functions of CXCL12 in the literature, two of the major ways in which CXCL12 could influence the host are through induction of tumour angiogenesis[117, 196, 210], and through augmentation of the anti-tumour immune response[215-218]. Generally,

neovascularisation is thought to be a tumour-promoting process, providing oxygen and nutrients to the rapidly growing tumour mass. CXCL12 is a pro-angiogenic chemokine[117], and as such, it might be expected that its expression would enhance tumour angiogenesis. However, the data obtained in this study indicated that the vascularity of CXCL12-expressing tumours was reduced compared with control tumours. Thus it seems likely that the decrease in vascular density was not a direct result of overexpressing CXCL12, but rather an indirect consequence of the inhibition of tumour growth. Despite the reduced vascularity of CXCL12-expressing tumours, there was a trend for the haemoglobin concentration within these tumours to be higher. It is possible for vascular permeability to be increased without any change in blood vessel density, as has been reported in a model of lung carcinoma[291], and it may be that CXCL12 directly affects only this aspect of the vasculature in the 4T1.2 model. However, it seems unlikely that increasing vascular permeability and instability is a mechanism by which CXCL12 inhibits tumour progression as it has been shown that inducing maturation and stabilisation of tumour blood vessels blocks tumour expansion[292].

6.2.2 The importance of T cells and dendritic cells

The actions of CXCL12 on the tumour vasculature and on tumour cells directly would be expected to have pro-tumour rather than anti-tumour effects, as discussed above. Thus it seemed most likely that CXCL12 was exerting its anti-tumour function by influencing the immune response to 4T1.2 tumours. For this reason, analysis of the effect of CXCL12 on the anti-tumour immune response was a significant focus of this work. Experiments with strains of mice lacking various leukocyte subsets were carried out to clarify which cell populations could be mediating tumour inhibition by CXCL12. SCID mice have a defective *Prkdc* gene and therefore cannot repair the DNA double strand breaks required for antigen receptor gene rearrangement. These mice lack T, B and NKT cells[293]. Nude mice have a mutation in the *Foxn1* gene, which encodes a transcription factor essential for development of the thymus. These mice are deficient in T cells that are dependent on the thymus for their development, though NKT cells and some thymus-independent T cells are still present[294, 295].

The complete loss of CXCL12-mediated tumour inhibition in SCID mice indicated that T cells, B cells and/or NKT cells were required for this effect. The experiments in nude

mice confirmed a role for T cells in CXCL12-mediated tumour inhibition. This is in agreement with other studies that demonstrated the importance of T cells in CXCL12-mediated growth inhibition of fibrosarcoma[215], melanoma, colon carcinoma, lung carcinoma[218] and leukaemia[216]. Further support for the role of T cells in this model came from correlative analysis in the present study, which indicated that the greater the proportion of $CD4^+$ splenic T cells, the smaller the tumour, particularly when the tumours expressed CXCL12.

Another significant finding of this work was that CXCL12 expression by 4T1.2 tumours resulted in increased numbers of $CD11c^+$ cells in the tumour-DLN. CD11c is the α_x chain of the α_x-β_2 integrin, and is expressed predominantly on conventional DC. It can also be expressed on activated T cells[296]. It is plausible that the CD11c detected by immunohistochemistry in the DLN was expressed by cells from either or both populations. However, no obvious differences in $CD11c^+$ cell infiltration of tumours from the different groups was apparent. In an effective immune response, DC activated at the tumour site mature and migrate to the tumour-DLN. There, they prime naïve T cells, which become activated and migrate back to the tumour site to mediate their effector functions[297, 298]. Given the increase of $CD11c^+$ cells within the DLN, but not the tumours, it seems likely that these cells represent DC rather than activated T cells. This is in agreement with a previous report that CXCL12 overexpression in colon carcinoma increased the numbers of $CD11c^+$ DC in the tumour-DLN[218].

Based on the observations discussed above, it seems likely that CXCL12 acts to promote T cell activation by increasing the proportion of DC in the tumour-DLN. There, DC can prime naïve T cells which then exert anti-tumour effector functions. One way in which CXCL12 may promote migration of DC to the tumour-DLN is through induction of their maturation. CXCL12 may enhance infiltration of the tumour by innate inflammatory cells, which could then provide the requisite 'danger signals' to induce maturation of immunostimulatory DC. Interestingly, responsiveness to CXCL12 increases during the maturation process of DC[150], and a very recent publication has documented the role of CXCR4 in directly promoting survival of DC, particularly those of a mature phenotype[299]. The ability of CXCL12 to enhance the survival of mature DC may be critical to the

generation of sufficient mature DC for priming of anti-tumour T cells and may represent a key aspect of the anti-tumour response in this model.

Upon maturation, DC up-regulate CCR7 expression[300], increasing their responsiveness to CCL21 and CCL19 expressed in lymphatic vessels and LN and facilitating their migration to LN[301, 302]. Though the chemotactic responsiveness of mature DC to CXCL12 is also increased[150], it may be that signals from CCL21 and CCL19 are sufficient to overcome CXCL12 signalling, or the high levels of CXCL12 within the tumour desensitise CXCR4 so that DC are free to respond to chemotactic signalling by CCL21 and CCL19. Alternatively, since chemokines are readily transported through the lymphatics and accumulate in DLN[303], it is possible that CXCL12 expressed by the tumour drains into surrounding LN. Together with other LN chemokines, CXCL12 may facilitate directly the migration of activated DC into the DLN to initiate the anti-tumour immune response. CXCL12 filtering into the tumour-DLN may also serve an additional function. Co-stimulation of T cells by CXCL12 enhances activation of cell signalling pathways commonly associated with cell survival and promotes secretion of IL-2[81]. It may be that in this model, CXCL12 enhances survival and proliferation of T cells receiving signals from incoming DC in the DLN, thus facilitating their optimum activation for anti-tumour activity.

A logical next step would be to further narrow down the T cell subsets important for the CXCL12-mediated anti-tumour response. To this end, experiments were conducted in which growth of control and CXCL12-expressing tumours was monitored in mice depleted of $CD4^+$ and $CD8^+$ lymphocytes using specific antibodies. Unfortunately, the preparation of anti-CD8 antibody did not efficiently deplete $CD8^+$ cells, and time constraints prevented the production of sufficient fresh antibody for further experiments. The data from mice treated with anti-CD4 antibody, however, indicated that $CD4^+$ cells were not required for CXCL12-mediated tumour inhibition. Growth of CXCL12-expressing tumours was inhibited to the same extent in mice depleted of $CD4^+$ cells as in mice treated with control IgG (data not shown). These findings strongly suggest that $CD8^+$ T cells are the mediators responsible for the anti-tumour effects of CXCL12, and it is expected that this will be confirmed in future experiments with an effective anti-CD8 depleting antibody.

If $CD8^+$ T cells are confirmed to be the main effector population in this model, then it is most likely that they are activated by direct cross-presentation of tumour cell antigens by mature DC in the tumour-DLN. This hypothesis fits with the observed increase of $CD11c^+$ cells in tumour-DLN and the finding that $CD4^+$ T cells are dispensable for the CXCL12-induced anti-tumour response. DC that acquire tumour antigens at the tumour site are able to process these molecules and present them on MHC class I molecules to $CD8^+$ T cells. It has been shown that cross-presentation of tumour antigens to $CD8^+$ T cells can occur efficiently in the absence of $CD4^+$ T cell help[304]. However, in mice lacking tumour-DLN due to ablation of peripheral LN, $CD4^+$ T cell help was crucial for efficient $CD8^+$ T cell activation[304]. It was postulated by the authors that $CD4^+$ T cell help became necessary only when antigens were limiting or DC were unable to interact efficiently with $CD8^+$ T cells, such as may occur at sites outside of the DLN. This could explain why $CD4^+$ T cells do not seem to be required for the CXCL12-induced anti-tumour response described in this thesis, as DLN are intact in this model. It also provides an explanation for the correlation of reduced tumour weight with an increased proportion of splenic $CD4^+$ cells. Yu et al. showed that while dispensable for priming in the DLN, $CD4^+$ T cell help was required for efficient priming of $CD8^+$ T cells in the spleen. It is possible that in the 4T1.2 model, $CD4^+$ cell-dependent priming of effector cells occurs in the spleen in response to tumour CXCL12 expression, but the predominant site of activation is the DLN.

6.2.3 The role of cell-mediated cytotoxicity

Despite the evidence for the involvement of T cells in CXCL12-induced tumour inhibition, increased infiltration of CXCL12-expressing tumours with either $CD4^+$ or $CD8^+$ cells was not apparent. It has been reported that CXCR4 is down-regulated on activated T cells and that this correlates with a decreased migratory response to CXCL12[156, 157]. However, highly activated $CD4^+$ T cells, which become hypersensitive to apoptotic signals, were found to be partially protected from apoptosis by CXCL12[305]. Thus expression of CXCL12 within the tumour microenvironment, while not enhancing T cell infiltration, may in fact enhance survival of activated T cells within the tumour mass.

Another potential explanation for the enhanced immune response to CXCL12-expressing tumours, despite unchanged T cell infiltration, is that T cells entering the tumour were in an enhanced activation state. However, flow cytometric analysis of $CD4^+$ and $CD8^+$ cells

isolated from tumours did not reveal any significant differences in the activation marker profile between tumour groups. As an alternative to analysis of phenotypic markers of T cell activation, functional assessment of the activation state of T cells within the different tumour groups was employed. *In vitro* cytotoxicity assays suggested that the cytotoxic activity towards 4T1.2 targets of LN cells from 4TX12 1 tumour-bearing mice was enhanced compared with LN cells from control tumour-bearing mice. The cytotoxic activity observed was relatively low (around 20-30%), in keeping with previous reports that 4T1 tumours (from which 4T1.2 cells are derived[276]) are very poorly immunogenic[283]. It should be considered also that the source of effector cells (i.e. the secondary lymphoid organs) may have yielded cells that were not yet fully activated for cytotoxic function; activated lymphocytes may not realise their full cytotoxic capabilities until they reach the tumour site. Another point for consideration is that the method of obtaining effector cells (i.e. mechanical disruption of tissues) favours the isolation of non-adherent cells, such as lymphocytes, rather than more adherent cells, such as macrophages. It is possible that the main function of T effector cells is not direct cytotoxic activity against tumour cells, but stimulation of the cytotoxic activity of innate immune cells, such as macrophages and NK cells, through production of cytokines such as IFN-γ. Indeed, it was found in the current study that CXCL12-mediated inhibition of tumour growth was partially lost in IFN-γ-deficient mice (discussed further below). Interestingly, a recent study demonstrated that IFN-γ required for tumour immunity can be produced by innate immune cells, but only if they are provided with help, most likely mediated by IL-2, from T cells[306]. Such a scenario may be the case in the 4T1.2 model studied here. $CD8^+$ Tc1 cells would be the most likely source of IL-2[307], since depletion of $CD4^+$ cells in the present work did not seem to affect CXCL12-mediated tumour growth inhibition (discussed in Section 6.2.2 above). If innate immune cells are the direct effectors of cytotoxicity in this model, they are more likely to be found in the tumour than in the secondary lymphoid organs. Thus the true extent of the cytotoxic response to 4T1.2 tumours might be better demonstrated if effector cells collected from the tumour, rather than the LN and spleen, were to be used in the *in vitro* assay.

Mice deficient in common mediators of $CD8^+$ T cell cytotoxic function were used to provide insight into the mechanism by which T cells were exerting their effects. The inhibitory effect of CXCL12 was partially lost in both perforin-, and TRAIL-deficient

mice. This suggests that both the perforin/granzyme cytolytic pathway and the TRAIL-dependent pathway are important for the CTL response against 4TX12 1 tumours and provides further evidence for the role of $CD8^+$ T cells in this model. However, it is important to note that both perforin[308] and TRAIL[309] are important for the cytolytic properties of NK cells. NK cells also express CXCR4 and migrate in response to CXCL12[153], and may be preferentially recruited to tumours where they can be activated by IFN-γ-producing cells to exert their cytotoxic functions. It has been shown previously that the cytotoxicity of liver and spleen mononuclear cells towards 4T1 cells *in vitro* is due solely to the activity of NK cells[310]. Moreover, that cytotoxic activity was dependent on both perforin and TRAIL. Comparison of the growth of 4T1 tumours in wild-type and $TRAIL^{-/-}$ mice in that study revealed that tumour growth was enhanced in the absence of TRAIL. Importantly, however, enhanced 4T1 tumour growth in $TRAIL^{-/-}$ mice was only observed when the tumour burden was small (i.e. an inoculum size of 1×10^4 cells or less), presumably because at larger tumour cell doses, the NK cell response was overwhelmed. In the experiments conducted in the present study, a tumour cell inoculum size of 1×10^5 cells was used, ten times greater than that used when TRAIL deficiency was observed to have an effect on 4T1 tumour growth. Interestingly, in mice inoculated with 2.5×10^4 4T1 cells and treated with α-galactosylceramide, tumour growth was inhibited in the presence of TRAIL[310]. This indicates that further activation of NK cells can be induced to effectively overcome larger tumour cell doses. Since IFN-γ is another factor that can enhance the cytotoxic activity of NK cells[34], and since experiments in IFN-γ-deficient mice in the present study revealed that the anti-tumour effect of CXCL12 is partly dependent on IFN-γ, it is possible that NK cells augment the cytotoxicity of CTL towards 4TX12 1 tumours observed here. It is also interesting to note that NK-like cytolytic functions have been attributed to DC, and that these cells use TRAIL to kill their targets[311, 312]. DC also produce large amounts of IFN-γ. Following activation and initial cytotoxic activity, these 'interferon-producing killer dendritic cells (IKDC)' diminish their cytolytic capacity and up-regulate antigen presentation functions[313]. An intriguing possibility is that the DC accumulating in the DLN in this model might have been derived from such an IKDC population. This could be further investigated by flow cytometric analysis of $CD11c^+$ cells isolated from the DLN to determine if these cells express the characteristic $CD11c^{int}B220^+Gr1^-CD49b^+$ IKDC surface marker phenotype.

6.2.4 The role of cytokines

Clearly, the production of IFN-γ is important for the inhibition of 4T1.2 tumour growth mediated by CXCL12, as demonstrated by the experiments in IFN-γ-deficient mice. IFN-γ production is a major function of effector $CD8^+$ T cells[314], which suggests that T cells in the 4T1.2 model may exert not only direct cytotoxic function against tumour cells, but also indirect effects via IFN-γ-mediated activation of other effector cells. Following the observation that CXCL12-induced tumour inhibition was partly lost in IFN-$\gamma^{-/-}$ mice, it was somewhat surprising to find that the proportion of IFN-γ^+ cells within 4TX12 1 tumour-DLN was reduced compared with control tumour-DLN. Indeed, the percentage of cytokine-positive cells in general within 4TX12 1 tumour-DLN tended to be reduced compared with that in controls. Furthermore, no significant difference in the amount of cytokine secreted by LN cells from either tumour group was apparent. Again, however, it should be noted that only cytokine-secreting cells from DLN were assessed, and the proportion of cytokine-secreting cells within the tumours themselves may be quite different. This being said, it would be expected that a reduction in the proportion of IL-17^+ and IL-4^+ cells within the DLN would reflect enhanced anti-tumour immunity. IL-23 (and by extension IL-17) is thought to be important for the promotion of tumour progression[287], presumably through induction of inflammation, which can be pro-tumorigenic[315]. IL-17 also inhibits the generation of a Th1-type immune response, which is thought to be the most effective anti-tumour response. Similarly, IL-4 inhibits Th1 responses and instead promotes Th2 humoral immunity, which by itself is generally ineffective against most tumours. Thus a reduction in the numbers of cells positive for these cytokines in mice bearing 4TX12 1 tumours would not be surprising. On the other hand, the reduction of IFN-γ^+ cells in 4TX12 1 DLN is perhaps unexpected. However, if the majority of effector cells produce IFN-γ and migrate from the DLN back to the tumour, then a reduction in IFN-γ^+ cells in the DLN is not so surprising. An increase in IFN-γ^+ cells in DLN from 4TX12 1 tumour-bearing mice might be observed if the LN are taken at earlier time points, prior to the egress of effector cells. In addition, it should be considered that not only effector $CD8^+$ T cells, but also innate immune cells present in the tumour, could be sources of IFN-γ. Analysis of cytokine levels in the tumour or systemic levels in the blood may give a better indication of the contribution of IFN-γ, and indeed other cytokines, to the immune response against 4TX12 1 tumours.

6.2.5 The potential effects of CXCL12 on suppressor cells

Another finding from the analysis in this thesis was that increased total splenocytes correlated with tumour weight. It has been shown previously that mice bearing 4T1 tumours have elevated levels of splenic MSC, which are potent inhibitors of $CD4^+$ and $CD8^+$ T cell effector functions[316]. In that study it was found that in $Stat6^{-/-}$ mice, which are unable to transduce signals induced by IL-4, reduction of MSC and resistance to metastatic disease occurred following removal of the primary tumour. Consistent with observations in the present study, $CD8^+$, but not $CD4^+$, cells were required for this effect, which was also IFN-γ-dependent[316]. Low doses of IFN-γ combined with TNF-α were found to reduce MSC numbers in a model of lung cancer by forcing their differentiation into mature macrophages[317]. These data may have implications for the 4T1.2 model described in this thesis. Here, IFN-γ was found to be required in part for the CXCL12-mediated inhibition of tumour growth, and secretion of TNF-α by DLN cells was elevated in mice bearing CXCL12-expressing tumours. CXCL12 stimulation can induce TNF-α production by ovarian cancer cells[318] and astrocytes[319, 320], and it is possible that CXCL12 can induce TNF-α production by cells within 4T1.2 tumours and DLN in a similar manner. Thus reduction in 4TX12 1 tumour growth may be the result of CXCL12-induced IFN-γ and TNF-α forcing maturation of MSC and abrogating their immunosuppressive effects.

Another observation from the studies of Sinha et al. was that cytotoxic nitric oxide (NO)-producing M1 macrophages were required for resistance to metastatic disease[316]. Macrophage infiltration of 4TX12 1 tumours and 4T12Ala tumours was examined by immunohistochemical staining of tumour sections for the macrophage marker F4/80, but no differences in macrophage infiltration between tumour groups were evident (data not shown). However, the phenotype of tumour-associated macrophages was not assessed, and it may be that there are differences in the bias of cytotoxic NO-producing M1 macrophages versus immunosuppressive arginase-producing M2 macrophages in 4TX12 1 and 4T12Ala tumours. Differentiation of macrophages towards an M1 phenotype is thought to be dependent on IFN-γ, as $STAT6^{-/-}$ mice produce M1 macrophages, whereas wild-type IL-4-responsive Balb/c mice produce M2 macrophages[321]. Thus IFN-γ production in the 4T1.2 model may have a dual role in the anti-tumour response by reducing MSC numbers and inducing their maturation towards a cytotoxic M1 macrophage phenotype. It is also interesting to note that gp120 stimulation of CXCR4-expressing macrophages induces their

activation and local inflammation[322]. Whether CXCL12 exerts a similar function in the context of the 4T1.2 tumour, to induce macrophage activation and cytotoxic function directly, remains to be determined.

Aside from MSC, Treg cells represent another immunosuppressive cell type that has the potential to inhibit anti-tumour immunity. Elevated levels of T_{reg} cells have been documented in the peripheral blood, tumours and regional LN of breast cancer patients[46] and depletion of these cells enhances anti-tumour immunity in mouse models of the disease[47, 48]. Previous work has shown that 4T1 tumours produce large amounts of TGF-β[42], a major inducer of regulatory T cell (T_{reg}) differentiation[38]. As yet, however, there have been no studies published documenting increased T_{reg} cell numbers in 4T1 tumours. Two reports that Treg migrate in response to CXCL12 have been published[323, 324], but it seems unlikely that CXCL12 overexpression in 4T1.2 tumours is acting in this capacity in the present work. If this were the case, it would be expected that tumour growth would be enhanced, rather than inhibited as observed here. Moreover in the current study, tumour growth in mice depleted of $CD4^+$ cells (including, presumably, $CD4^+CD25^+$ T_{reg} cells) was not inhibited compared with tumour growth in mock-depleted mice (data not shown). This suggests that T_{reg} cells are not a major contributor to tumour-induced immunosuppression in this model.

6.2.6 Other potential mediators of the effects of CXCL12

As discussed above, the experiments in SCID and nude mice confirmed a role for T cells in CXCL12-mediated anti-tumour immunity. The complexity of the response to the tumour became apparent following the experiments in nude mice, which revealed that cell types other than T cells must be involved as well, because some growth retardation of CXCL12-expressing tumours was still observed. NKT cells and B cells, being present in nude mice but not in SCID mice, were potential co-mediators of tumour inhibition. Much attention has focused on the potential anti-tumour activity of NKT cells, in particular those expressing Vα14-Jα18 and responding to α-galactosylceramide[325]. $J\alpha18^{-/-}$ mice lack the Jα18 joining segment of the TCRα chain and are unable to produce Type I NKT cells, which require expression of the invariant Vα14-Jα18 TCRα chain for their development[282, 326]. However, in the current study, the growth of CXCL12-expressing

4T1.2 cells in Jα18$^{-/-}$ mice was similarly inhibited as in wild-type mice, indicating that deficiency of Type I NKT cells did not affect the CXCL12-mediated suppression of tumour growth. It is possible that Type II NKT cells, which are still present in Jα18$^{-/-}$ mice, could mediate the anti-tumour effects of CXCL12 to some extent. However, recent work suggests that this subtype of NKT cells exerts an immunosuppressive effect that promotes tumour growth[327]. Thus it seems unlikely that NKT cells are the co-mediators of tumour inhibition observed here.

The role of B cells in this model must also be considered. The work of recent years has highlighted the importance of cell-mediated immunity in the eradication of tumours, and the potential of humoral immunity to contribute to the anti-cancer response has slipped from notice somewhat. However, a few studies have shown that immunotherapy of patients can induce effector cell populations that can mediate antibody-dependent cell-mediated cytotoxicity (ADCC)[328-331]. Interestingly, plasma cells become increasingly sensitive to the chemoattractive properties of CXCL12 as they mature[332], a process that facilitates their migration into the bone marrow. It is possible that in the 4T1.2 model, CXCL12 overexpression by tumour cells may recruit mature plasma cells producing tumour-specific antibodies into the tumour itself, thus enhancing the antibody supply for ADCC at the tumour site. However, the absence of a requirement for CD4$^+$ T cells in the anti-tumour immunity towards 4TX12 1 tumours has implications for the potential for B cells to contribute to this response. Since efficient class-switching of antibodies requires the help of CD4$^+$ T cells, it would be expected that only anti-tumour antibodies of the IgM isotype would be produced in this system. A murine Fcα/μ receptor has been identified on macrophages and B cells[333], but the ability of this receptor to contribute to ADCC has not been determined. IgM is a potent activator of complement, though whether 4T1.2 tumour cells are sensitive to complement-mediated destruction has not been determined.

Another possibility to be considered is that CXCL12-induced anti-tumour functions of NKT or B cells only come to the fore in the absence of T cells. When T cells are present, they may be sufficient to mediate CXCL12-induced tumour growth inhibition, and the role of NKT and/or B cells becomes redundant. When T cells are lacking, as is the case in nude mice, then the anti-tumour effects of NKT and/or B cells then become evident. A role for

these cell subsets in the anti-tumour response may be revealed only upon concomitant depletion of T cells and NKT cells or of T cells and B cells.

Finally, it should not be forgotten that the nude mutation exhibits some 'leakiness'. Populations of T cells that develop independently of the thymus have been identified in the intestine, appendix, liver and epidermis[334-339]. Moreover, it has been shown that intraepithelial γδ T cells are important for protection of mice from skin carcinogenesis[340]. It is thought that such cells may play a role in immunosurveillance of epithelial tissues, eliminating newly transformed cells and preventing tumour development[24]. Whether such cells can contribute significantly to the anti-tumour response once tumours have become well established, as in this model, remains uncertain.

6.2.7 Summary and future directions

Work still needs to be done to clarify the precise components and mechanisms of the anti-tumour immune response induced by CXCL12. However, the data obtained thus far have given rise to the working model of the anti-tumour response shown in Figure 6.1. The model incorporates the major findings of this study, as well as some speculation on processes that remain to be elucidated. The role of mature DC and T cells, IFN-γ and perforin- and TRAIL-dependent cytotoxicity (as mediated by T cells and NK cells) is presented. In addition, the model emphasises the importance of the contribution of innate immune cells to the anti-tumour response, particularly NK cells and macrophages. The potential roles of B cells, antibodies and thymus-independent T cells in the immune response are not included because as yet, no direct evidence for their participation has been found. Similarly, anti-tumour responses generated in the spleen have not been included as they may not follow the same pattern as those occurring in the DLN.

As well as providing the basis for the model of CXCL12 action in Figure 6.1, the findings of this study raise interesting questions regarding additional details of the anti-tumour response induced by CXCL12. Confirmation of the role of $CD8^+$ T cells should be the next step in the investigation of T cell involvement. Examination of the effector functions of T cells isolated from tumours will clarify whether these cells exert anti-tumour activity through direct cytotoxicity, IFN-γ production or both. It will be interesting also to further

characterise the DC infiltrating tumour-DLN. Flow cytometry could be employed for analysis of phenotypic markers of DC activation, such as MHC class II, CD80, CD86, CD83 and CCR7. Assessment of the antigen-presenting function of these DC could prove informative as well. This could be achieved by testing the ability of DC isolated from DLN of 4T12Ala or 4TX12 1 tumour-bearing mice to induce proliferation of anti-tumour T cells isolated from 4T1.2 tumour-bearing mice *in vitro*.

Future experiments involving anti-asialoGM1 depletion of NK cells and liposome-mediated depletion of macrophages[341-343] will provide further insight into the role of innate immune cells in anti-tumour immunity. Quantitation of NO within tumours will also give an indication of the importance of macrophage bias (i.e. NO-producing M1 versus arginase-producing M2 macrophages) to CXCL12-mediated tumour inhibition. Another important aspect of this model to be investigated is the contribution of MSC to the growth of 4T1.2 tumours, and what effect CXCL12 overexpression has on this cell population. Thus cell population(s) in the peripheral blood and infiltrating the spleens of tumour-bearing mice should be characterised to determine if these cells are $Gr1^+CD11b^+$ MSC. Comparison of the numbers of MSC in 4T12Ala versus 4TX12 1 tumour-bearing mice would give an indication of whether or not reduction of MSC levels are important for the anti-tumour effect of CXCL12.

In summary, multiple significant effects of CXCL12 overexpression on 4T1.2 tumour growth have been demonstrated in this work. CXCL12 induces a potent anti-tumour immune response, an effect not previously observed in breast cancer. This immune response is dependent on T cells, increased infiltration of tumour-DLN by DC, the cytotoxic effectors perforin and TRAIL and the cytokine IFN-γ. The findings of this study also point to the involvement of additional cell types in the CXCL12-induced anti-tumour response and open up exciting new avenues for future investigation. What is clear from the data obtained thus far is the complexity of the anti-tumour immune response in this model. The ability of CXCL12 to mobilise so many components of the immune system testifies to its potential as an immunotherapeutic agent.

6.3 The effects of $CXCL12_{(P2G)}$ overexpression on breast tumour progression

Although the present study demonstrates that overexpression of CXCL12 enhances the anti-tumour immune response in a model of breast cancer, reports in the literature indicate that endogenous CXCL12 can act in a pro-tumorigenic capacity. *In vitro* and *in vivo* studies suggest that CXCL12 can enhance the survival[202], proliferation[196-199] and invasion[197, 204, 205, 208, 209] of breast tumour cells and promote the vascularisation of breast tumours[196, 210]. In addition, blockade of CXCR4 using a neutralising antibody has been shown to inhibit human breast tumour cell metastasis in a SCID mouse model[6]. More recent work has demonstrated that the novel CXCL12 receptor CXCR7 also has pro-tumorigenic functions[129], though these effects are yet to be described in breast cancer *in vivo*. Despite the data implicating CXCL12 and its receptors in the progression of breast cancer, the details of the mechanism by which they do so remain elusive. Many of the tumour-promoting functions of CXCL12 have been conjectured from the results of *in vitro* studies, and there is very little direct evidence for CXCL12 and its receptors enhancing specific aspects of breast tumorigenesis and metastasis *in vivo*.

In this study, expression of the CXCL12 antagonist, $CXCL12_{(P2G)}$, did not have any obvious effect on the growth of primary 4T1.2 tumours. However, spontaneous metastasis of 4T1.2 cells from the primary tumour to the lungs was significantly inhibited by $CXCL12_{(P2G)}$, in a manner that did not depend on reduced tumour angiogenesis or lymphangiogenesis. Moreover, $CXCL12_{(P2G)}$ was capable of inhibiting metastasis to the lungs even when tumour cells were injected directly into the circulation, obviating the need for cells to form a primary tumour and then escape from the tumour mass. These data highlight the role of CXCL12 in metastatic steps occurring following the entrance of tumour cells into the circulation, though a role for CXCL12 in earlier metastatic steps cannot be ruled out.

Several studies have reported the inhibition of metastasis of human MDA-MB-231 breast cancer cells in SCID mice using anti-CXCR4 antibodies[6, 344], RNAi[197, 205], or peptide antagonists of CXCR4[344, 345]. The data presented in this thesis extend these findings by demonstrating that blockade of CXCR4 inhibits both spontaneous and experimental metastasis in a more biologically relevant syngeneic, orthotopic model of breast cancer.

The results obtained in the 4T1.2 model are in agreement with those obtained in a similar study by Smith et al.[346] in which RNAi-mediated knockdown of CXCR4 inhibited both spontaneous and experimental breast cancer metastasis. However, in that study, CXCR4 knockdown was also found to inhibit primary tumour growth. In contrast, expression of $CXCL12_{(P2G)}$ in the present work did not affect 4T1.2 tumour growth, indicating the metastasis-specific effect of this antagonist and confirming a role for CXCL12 in metastasis.

While the vascular density of primary 4T12P2G tumours was not reduced compared with control tumours, the potential for $CXCL12_{(P2G)}$ to inhibit metastasis by influencing the tumour vasculature should not be discounted. Criscuoli and colleagues found that in $Src^{-/-}$ mice, reduction of spontaneous metastasis was the result of decreased vascular permeability, though microvessel density was unchanged[291]. The permeability of tumour blood vessels was not assessed in the 4T1.2 model, but represents a possible future avenue of investigation. The extravasation of intravenously injected FITC-dextran into tumours has been used to quantify tumour vascular permeability, and may reveal reduced vascular permeability in 4T12P2G tumours. With regard to tumour lymphangiogenesis, it should be noted that only the density of intratumoral lymphatic vessels was assessed in this study. It has been shown that the presence of lymphatic vessels in the tumour margin (defined as <100μm from the tumour edge) may be a more accurate predictive factor for lymphatic metastasis[347]. Examination of the effects of $CXCL12_{(P2G)}$ on lymphangiogenesis within this region may give a better indication of whether or not $CXCL12_{(P2G)}$ affects the lymphatic route of metastasis.

An essential feature of metastatic cells is the ability to evade anoikis, a form of apoptosis induced in the absence of anchorage-dependent survival signals[20, 348]. Data from our laboratory have demonstrated a role for CXCL12 in preventing anoikis of detached breast cancer cells (M. Kotchetkova and S. McColl, manuscript in preparation). Thus $CXCL12_{(P2G)}$ expression in the 4T1.2 model may serve to block the survival of metastatic cells in the circulation. Given that 4T1.2 cells do not express CXCL12, it seems most likely that survival signals would come from host-derived plasma CXCL12, the basal levels of which have been reported to be 2-3ng/ml in Balb/c mice[349]. Further investigation of the potential of $CXCL12_{(P2G)}$ to block 4T1.2 anoikis would require confirmation of the

ability of CXCL12 to enhance survival of detached 4T1.2 cells *in vitro*, followed by experiments to determine if $CXCL12_{(P2G)}$ could block this effect.

Another function of CXCL12 that may be pertinent to the metastasis of malignant cells is its ability to induce adhesion to endothelium and ECM components via activation of integrins. Various studies have demonstrated the activation of the integrin very late antigen (VLA)-4 on leukocytes[350, 351], haematopoietic progenitor cells[352] and multiple myeloma cells[353] by CXCL12. The increased affinity of VLA-4 promotes firm adhesion of cells to EC expressing vascular cell adhesion molecule (VCAM)-1 and ECM components such as fibronectin. Such interactions in the context of 4T1.2 metastasis may facilitate arrest and adhesion of metastatic cells at secondary sites followed by extravasation into the underlying tissue. It is interesting to note that MDA-MB-435 cells (recently redefined as melanoma-derived cells[203]) transfected with a CXCR7-expressing DNA construct exhibited enhanced adhesion to EC[129]. The ability of $CXCL12_{(P2G)}$ to bind and antagonise the function of CXCR7 has not been determined, but it is possible that $CXCL12_{(P2G)}$ inhibits tumour cell adhesion to endothelium through interaction with this receptor.

In a study of genes that mediate breast cancer metastasis to the lungs, MMP2 was identified as a gene that was expressed in highly aggressive lung metastatic cells but was not required for primary breast tumour growth[354]. Interestingly, CXCL12 has been shown to induce MMP2 production by prostate cancer cells[355], rhabdomyosarcoma cells[193] and human $CD34^+$ haematopoietic progenitor cells[356]. Thus it is possible that $CXCL12_{(P2G)}$ expression by 4T1.2 cells blocks production of MMP2 and hinders their ability to invade lung tissue. Some preliminary experiments were conducted to compare the level of MMP2 mRNA expression in 4T12P2G and 4T12Ala cells by real time RT-PCR, but no significant differences were detected between the cell lines. However, MMP2 needs to be post-translationally cleaved to become activated and exert its proteolytic function[357]. Therefore, functional assays such as zymography to detect MMP2 activity may provide a more accurate picture of the role of the enzyme in this system and uncover differences between the control and $CXCL12_{(P2G)}$-expressing cell lines.

Of course, the defining function of CXCL12, as a chemotactic factor, should not be forgotten. Chemokines are major inducers of cell migration into and within tissues. CXCL12 is highly expressed in the lung[6], potentially creating a chemotactic gradient to

attract metastatic tumour cells out of the circulation and into the tissue. Antagonism of CXCL12 by $CXCL12_{(P2G)}$ expression may neutralise the ability of tumour cells to sense and respond to the CXCL12 gradient within the lung tissue, preventing their establishment in a growth-conducive environment. Given that $CXCL12_{(P2G)}$ had no effect on primary tumour growth, it seems unlikely that CXCL12 promotes tumour growth once metastatic cells are established in the lungs. Still, it is possible that tumour cells encounter different physiological conditions in this tissue and require CXCL12 to proliferate and survive in their new environment, as has been shown for colon carcinoma metastases[358].

In summary, the data presented in this thesis show that antagonism of CXCL12 specifically inhibits spontaneous metastasis in a syngeneic, orthotopic model of breast cancer, without affecting primary tumour growth. These data confirm and extend previous findings that blockade or knockdown of CXCR4 inhibits metastasis of human breast cancer cells in xenogeneic mouse models[6, 197, 205, 344, 345]. In addition, the present work demonstrates that CXCL12 is important for metastatic steps that take place following entry of malignant cells into the circulation, as $CXCL12_{(P2G)}$ was able to inhibit metastasis of intravenously injected tumour cells. A working model of the action of $CXCL12_{(P2G)}$ during metastasis is presented in Figure 6.2. The potential role for endogenous CXCL12 signalling to enhance anchorage-independent survival in the circulation and/or arrest, adhesion and extravasation at secondary sites is highlighted. The potential points at which $CXCL12_{(P2G)}$ could block metastasis are also shown. It is possible that CXCL12 contributes to escape of metastatic cells from the primary tumour, and/or assists survival and expansion of tumour cells in the lung environment. However, the experimental evidence obtained thus far has only confirmed a role for CXCL12 in metastatic steps post tumour escape, and the lack of effect of CXCL12 on primary tumour growth suggests that it is unlikely to be required for growth of secondary tumours. It should also be noted that inhibition of metastasis by $CXCL12_{(P2G)}$ could be the result of interaction with CXCR4 or CXCR7 expressed on the tumour cell surface. This raises interesting questions as to the contribution of each receptor to the metastatic process, and presents a future avenue of research.

6.4 The effects of CXCL16 construct overexpression on breast tumour progression

Some of the physiological functions of the atypical chemokine CXCL16 have relevance to the pathology of breast tumour development. CXCL16 is expressed by vascular EC[222, 226] and at low levels by aortic smooth muscle cells[222, 225], and promotes proliferation, chemotaxis and tube formation by HUVEC[249]. Thus it might be expected that CXCL16 facilitates vascularisation of 4T1.2 tumours *in vivo*. Furthermore, CXCL16 can act as a scavenger receptor of oxidised low density lipoprotein (oxLDL)[234, 250]. LDL has been shown to enhance proliferation of various cancer cells, including breast cancer cells[359, 360]. Thus it is possible that CXCL16 expression by tumour cells could serve to promote their uptake of LDL, and hence their proliferation, *in vivo*. On the other hand, CXCR6 is expressed on polarised Type 1 T cells[231] and NK cells[229], therefore it might be expected that CXCL16 would enhance recruitment of such cells to the tumour to mediate cytotoxic activity.

The initial *in vitro* characterisation of the 4T1.2 cell line revealed a low level of endogenous CXCL16 expression, suggesting that production of this chemokine might be required for the growth of 4T1.2 tumours. Overexpression of CXCL16 or the CXCL16 antagonist, however, did not affect primary tumour growth, indicating that endogenous CXCL16 expression is dispensable for this aspect of 4T1.2 tumour development. It might have been expected that expression of CXCL16 would enhance the immune response to 4T1.2 tumours, but clearly, CXCL16 did not exert such a function in this case. It may be that simply increasing CXCL16 levels in the tumour would be sufficient to produce an effective anti-tumour response, but other complicating factors could be in play. $CXCR6^+$ cells may be recruited, but their anti-tumour activity may be suppressed at the tumour site. CXCR6 expression on T cells is induced only once they have been activated[231], so if T cells are not being primed, then CXCL16 cannot recruit them to the tumour. CXCL16 may still be able to recruit $CXCR6^+$ NK cells, but as discussed above, the large dose of tumour cells used in the *in vivo* experiments (1×10^5 cells) may be such as to overwhelm any anti-tumour activity of these cells. It is possible that inhibition of CXCL16-expressing tumour growth would occur when a smaller inoculum of tumour cells (e.g. 5×10^3 cells) is used.

Curiously, $CXCL16_{(9-220)}$ expression was found to significantly reduce metastasis of intravenously injected tumour cells to the lungs. This was despite a lack of effect on spontaneous metastasis of cells from primary tumours. Since intravenously injected cells have to complete the same metastatic steps as spontaneously metastatic cells that have reached the circulation, an explanation for the different outcomes of the two experiments is not immediately apparent. However, there is potential for the quandary to be resolved if the host immune response is taken into account. It has been reported that while T cells are important for the control of tumours and metastases established within tissues, metastatic cells in transit are targeted by NK cells[361, 362]. Moreover, MSC inhibit the cytotoxic activity of NK cells[59]. In light of this, it should be considered that the timeframe of the experimental metastasis assay is such that there is insufficient time for MSC to accumulate before NK cells are able recognise and destroy tumour cells arrested in the vasculature. This in contrast to the spontaneous metastasis setting where MSC accumulate and begin to suppress NK cell function during the initial phase of primary tumour growth, before metastatic cells are released into the circulation. As discussed above, the size of the tumour cell inoculum in the spontaneous metastasis assay was such as to overwhelm NK cell activity, hence the apparent lack of effect of CXCL16 and $CXCL16_{(9-220)}$ in that setting. However, a significantly smaller inoculum of tumour cells was used in the experimental metastasis assay. Moreover, intravenously injected cells would become dispersed by the circulation, and these isolated cells would be more readily targeted by NK cells. This is in contrast to cells injected into the mammary fat pad, which would remain as a bolus and thus have the protection of neighbouring tumour cells, making it easier for them to overwhelm the local NK cell response.

Since NK cells express CXCR6[229], it might be expected that CXCL16-expressing tumour cells would attract NK cells, effectively bringing about their own doom. Thus it seems paradoxical that 4T1.2 cells express endogenous CXCL16 and furthermore, that $CXCL16_{(9-220)}$ expression renders intravenously injected cells more sensitive to destruction. However, *in vitro* cytotoxicity assays performed in this laboratory have shown that 4T1.2 target cells overexpressing CXCL16 are protected to some extent from cytolytic attack (O. Fahy, unpublished data). Thus expression of the antagonist may block the protective effects of endogenously expressed CXCL16 *in vivo*, resulting in the reduction of lung metastasis of intravenously injected 4T1.2 cells. Overexpressing CXCL16 did not enhance lung metastasis, suggesting that the level of endogenous expression is sufficient to protect

metastatic cells from destruction *in vivo*, and that further increasing CXCL16 expression does not augment their resistance to attack. How CXCL16 mediates its protective effects is as yet undetermined and is the subject of ongoing investigation in this laboratory. However, an interesting possibility to consider is that in normal physiology, the interaction of CXCL16 on DC with CXCR6 on $CD8^+$ T cells during priming protects the DC from destruction by the activated T cell.

It should be noted that the metastasis assays with the 4T16 and 4TΔ16 cells could be performed only once due to time constraints, and further experiments to increase statistical power are required to confirm the observations documented in this thesis. However, the data presented here provide a tantalising glimpse of a potentially novel aspect of CXCL16 function in the context of tumour biology, and possibly also in normal physiology during the generation of immune responses. *In vitro* cytotoxicity assays to confirm the modulatory effect of CXCL16 on the cytolytic killing of 4T1.2 cells by NK cells specifically should be the next step in this line of investigation. *In vivo* experiments with a smaller inoculum of 4T16 and 4TΔ16 cells to avoid overwhelming the NK cell response may reveal differences in primary tumour growth characteristics. In addition, depletion of NK cells with anti-asialoGM1 would give an indication of the significance of these cells for the effects of CXCL16 and $CXCL16_{(9\text{-}220)}$ on tumour growth and metastasis *in vivo*.

6.5 Perspectives and concluding remarks

The work presented in this thesis demonstrates that overexpression of a chemokine and its antagonist has profound effects on the progression of breast cancer in an animal model. These findings await further confirmation in other models of cancer and in human disease, but given the high degree of evolutionary conservation of CXCL12 and CXCR4 and the documented expression of CXCR4 in a wide range of cancers, it seems likely that the anti-cancer effects of this chemokine and its antagonist would not be restricted to the breast cancer model studied here.

It is interesting that both CXCL12 and its antagonist have anti-tumour properties; this demonstrates that the level and location of CXCL12 expression can profoundly affect tumorigenesis and metastatic disease. Early high-level expression of CXCL12 at the

tumour site induces anti-tumour responses that appear to depend on the adaptive arm of the immune system. On the other hand, the data obtained through the expression of $CXCL12_{(P2G)}$ suggest that endogenous CXCL12 in the circulation and/or in secondary tissues can promote metastatic spread of malignant cells. The experimental data obtained confirm the findings of previous studies demonstrating a role for CXCL12/CXCR4 in tumour metastasis and extend this work to show that endogenous CXCL12 can specifically influence metastatic steps independently of any effect on primary tumour growth.

The ability of CXCL12 alone to mobilise anti-tumour immunity, especially against such a poorly immunogenic and immunosuppressive tumour as that investigated here, testifies to its ability to co-ordinate multiple necessary components of the immune response. While not able to induce complete tumour regression on its own, CXCL12 may prove to be a potent and effective adjuvant when combined with current immunotherapeutic strategies. First it will be necessary to establish the ability of CXCL12 to induce anti-tumour responses in a more realistic model of tumour therapy, where tumours and metastases have already developed. Though this study has demonstrated anti-metastatic properties of wild-type CXCL12, whether or not these were due to the inhibition of primary tumour growth, or to induction of a systemic anti-tumour response, remains to be addressed. Vaccination of mice with established disease with CXCL12-expressing tumour cells would provide insight into the effectiveness of the CXCL12-induced immune response against established tumours and metastases.

Antagonism of CXCL12 forms another potential anti-metastatic strategy, as demonstrated by the effect of $CXCL12_{(P2G)}$ on breast cancer metastasis. However, whether this effect is due to the action of $CXCL12_{(P2G)}$ on CXCR4, CXCR7 or both requires further investigation. To date, the majority of studies have focused on the role of CXCR4 in metastasis, and the influence of CXCR7 on this process remains a grey area. Studies to determine whether $CXCL12_{(P2G)}$ can bind to CXCR7 are required to determine if there are grounds for further assessing the role of this receptor in metastasis. Should a role for CXCR7 be confirmed, approaches to anti-metastatic therapies targeting CXCR4 will need to be rethought. Currently, strategies being tested specifically target CXCR4 using small molecule inhibitors, antibodies or RNA interference. A more effective approach may be to target both CXCR4 and CXCR7 at the same time. Another factor for consideration is that targeting CXCR4, while inhibiting tumour metastasis, may also hinder anti-tumour

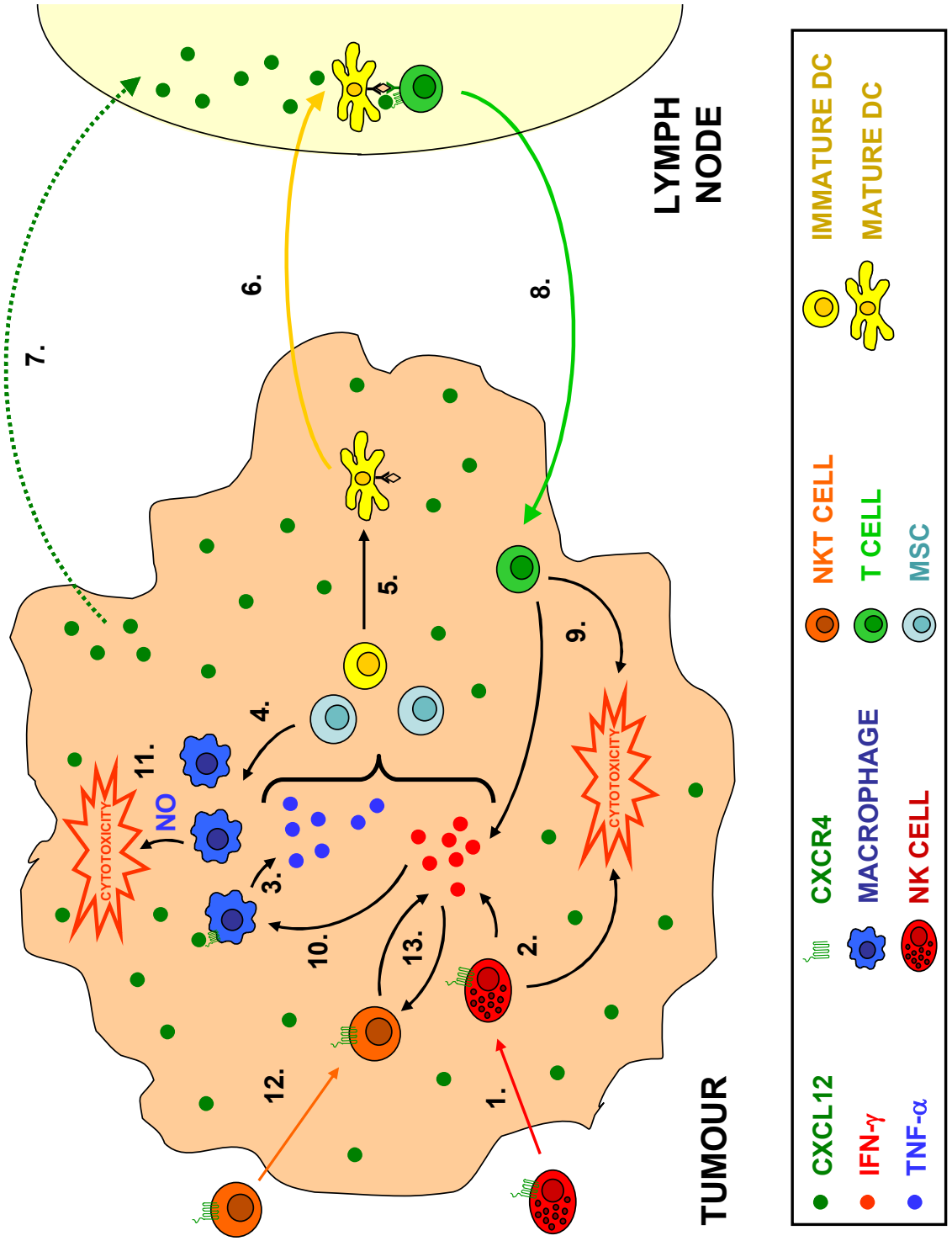
immune responses through blockade of leukocyte recruitment to tumours. As CXCR7 expression seems to be largely restricted to embryonic tissues and transformed cells[129], it possible that specific blockade of this receptor can inhibit tumour metastasis while still leaving the way open for concomitant therapies with CXCL12 to enhance anti-tumour immunity. As specific reagents for the study of CXCR7 become available, experiments to test the viability of CXCR7 inhibition as an anti-metastatic therapeutic strategy will be possible.

The lack of effect of CXCL16 and its antagonist on primary breast tumour growth and spontaneous metastasis was somewhat surprising. More surprising was that despite this, $CXCL16_{(9\text{-}220)}$ significantly inhibited experimental metastasis. As discussed in Section 6.4 above, it may be that the major mediators of this effect are NK cells. Further work is needed confirm the role of these cells in the anti-metastatic effect of $CXCL16_{(9\text{-}220)}$, but the observations outline in this thesis indicate that antagonism of CXCL16 also represents a potential anti-metastatic therapy that can be used as an adjuvant to other anti-cancer therapies. Of course, it must be considered that treatment involving antagonism of CXCL16 may block recruitment to the tumour site of effector T cells generated by concomitant immune priming strategies. Therefore care must be taken to establish precisely how CXCL16 antagonism should be employed to maximise the effectiveness of combination therapies that incorporate this treatment.

In conclusion, this study demonstrates multiple significant roles for the chemokine CXCL12 in breast cancer progression. Endogenous host expression of CXCL12 clearly has pro-metastatic effects, as antagonism of CXCL12 activity inhibits breast cancer metastasis. However, inducing CXCL12 expression at the tumour site can enhance the anti-tumour immune response. In addition, this work has uncovered a potential pro-metastatic role for endogenously expressed CXCL16, which can be inhibited by expression of a CXCL16 antagonist. These findings provide a basis for further investigation of the roles of CXCL12 and CXCL16 in metastatic cancers of the breast, as well as other tissues. This work also highlights the potential of CXCL12, CXCL16 and inhibitors of their receptors as therapeutic agents to treat this complex disease.

Figure 6.1 *Working model of CXCL12 action in primary mammary tumours.*

CXCL12 expressed by the tumour recruits NK cells to the tumour mass *(1)*, where they secrete IFN-γ and exert cytotoxic functions *(2)*. CXCL12 also induces TNF-α production by TAM *(3)* which, together with IFN-γ, induces differentiation of MSC into macrophages *(4)* and immature DC into mature DC *(5)*. Mature DC migrate to the DLN where they cross-present antigen to $CD8^+$ T cells *(6)*. CXCL12 may filter from the tumour to the DLN where it co-stimulates T cell activation and their secretion of IL-2 *(7)*. Activated $CD8^+$ T cells migrate back to the tumour *(8)* where they exert cytotoxic functions and secrete IFN-γ *(9)*. IL-2 produced by these cells may also induce innate immune cells in the tumour to contribute to local IFN-γ production. IFN-γ may facilitate generation of NO-producing M1 macrophages *(10)* that exert cytotoxic functions against tumour cells *(11)*. CXCL12 may also recruit NKT cells to the tumour *(12)* where they receive activation signals that induce their production of IFN-γ *(13)*, though this may be a minor arm of the anti-tumour response which becomes significant only in the absence of T cells. See text for further details. DC, dendritic cell; DLN, draining lymph node; IFN-γ, interferon-γ; MSC, myeloid suppressor cell; NK cell, natural killer cell; NO, nitric oxide; TNF-α, tumour necrosis factor-α.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NKT CELL
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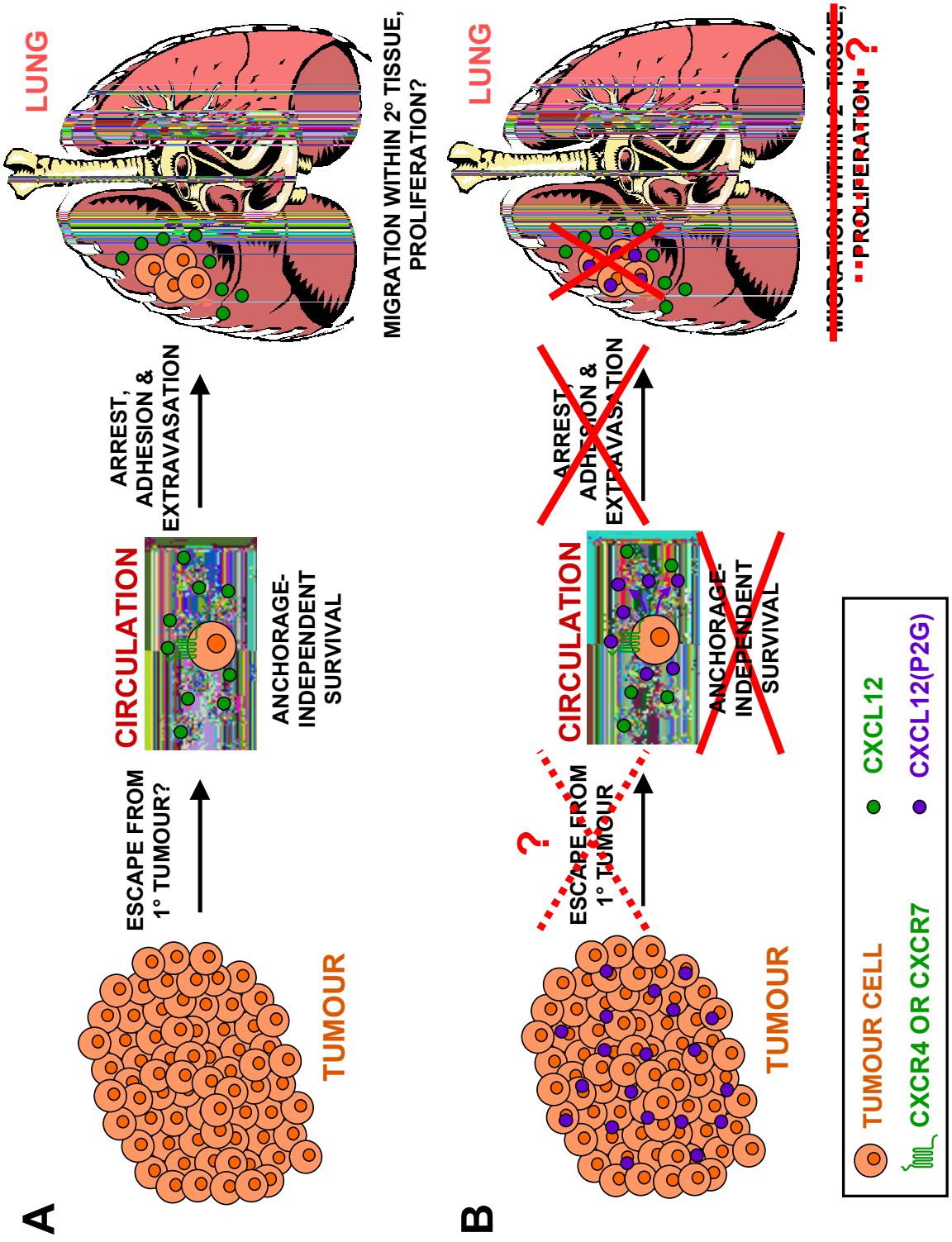
MSC
IMMATURE DC
MATURE DC

Figure 6.2 *Working model of CXCL12$_{(P2G)}$ action in mammary tumour metastasis.*

(A) CXCL12 promotes tumour cell metastasis. Metastatic cells escape from the primary tumour mass and enter the circulation, a process which may or may not require CXCL12 signalling. Circulating CXCL12 provides survival signals to detached tumour cells in the circulation and prevents anoikis. At secondary sites, such as the lungs, tumour cells arrest and adhere to the endothelium in response to CXCL12 produced in the tissues. CXCL12 may induce tumour cell secretion of MMP, thereby facilitating extravasation and invasion of the lung, as well as forming a chemotactic gradient that directs migration of malignant cells into the tissue. It is possible that CXCL12 also provides survival and proliferation signals required for tumour cells to expand in their new environment, though it does not seem to be required for proliferation of the primary tumour. **(B)** CXCL12$_{(P2G)}$ blocks CXCL12-induced metastasis. Tumour cell production of CXCL12$_{(P2G)}$ may block CXCL12 signalling at any or all of the stages of metastasis described in (A). This blockade may the result of interaction with CXCR4, or possibly with CXCR7, expressed on the tumour cell surface. The action of CXCL12$_{(P2G)}$ inhibits metastasis to secondary sites, without affecting the growth of the primary tumour.


A
TUMOUR
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CIRCULATION
ANCHORAGE-INDEPENDENT SURVIVAL
ARREST, ADHESION & EXTRAVASATION
LUNG
MIGRATION WITHIN 2° TISSUE, PROLIFERATION?
B
TUMOUR
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ESCAPE FROM 1° TUMOUR
CIRCULATION
ANCHORAGE-INDEPENDENT SURVIVAL
ARREST, ADHESION & EXTRAVASATION
LUNG
MIGRATION WITHIN 2° TISSUE, PROLIFERATION ?
TUMOUR CELL
CXCL12
CXCR4 OR CXCR7
CXCL12(P2G)

CHAPTER 7

References

CHAPTER 7: REFERENCES

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