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Investigating the role of macrophages in the suppression of NK cell functions in mouse models of metastatic breast cancer

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Abstract

Breast cancer is the leading cause of cancer-related death in females worldwide. Although 5-year survival of breast cancer patients in early stages is 89-100%, that of patients with metastatic tumours is reduced to just 21%, suggesting the requirement of effective therapies for metastatic breast cancer (MBC). MBC is primarily treated with chemotherapeutics however the efficacy of such treatments is limited due to resistance. As an alternative approach, NK cell-based immunotherapy (i.e., adoptive transfer of NK cells to patients) has been focused on since it shows significant therapeutic effects on haematopoietic tumours. Nevertheless, its efficacy is limited in MBC probably due to an immune suppressive tumour microenvironment (TME).

Tumour-associated macrophages (TAMs) are an abundant cell type within the TME of breast cancer and promote the metastatic process such as cancer cell egress from the primary tumour in mouse models. In mouse models of breast cancer, a distinct population of TAMs in the metastatic site called metastasis-associated macrophages (MAMs) can promote tumour cell seeding, survival and growth. Moreover, we have recently shown that MAMs and their progenitor cells can suppress cytotoxicity of $CD8^+$ T cells *in vitro*. Interestingly, a recent study suggests that TAMs isolated from the 'primary' mammary tumour in mice can suppress tumour killing ability of NK cells *in vitro*, whereas the effects of MAMs on NK functions in the 'metastatic' tumour is largely unknown. We hypothesise that MAMs in the metastatic site suppress NK cell function, and that the depletion of these MAMs can improve NK cell immunotherapy efficacy for MBC.

To investigate this hypothesis, we first established an *in vitro* NK cell cytotoxicity assay whereby mouse breast cancer cells were co-cultured with splenic NK cells, and the resultant tumour cell apoptosis was determined by quantitative fluorescence

microscopy. Using this assay, we found that the NK cell-induced tumour cell apoptosis was significantly reduced in the presence of MAMs isolated from metastatic tumours in the lung of tumour cell injected mice. We also found that bone marrow-derived macrophages cultured with M-CSF (M- BMMs) that resemble MAMs also reduced NK cell cytotoxicity in a cell-to-cell contact dependent manner. In contrast, BMMs cultured with GM-CSF that represent pro-inflammatory macrophages did not suppress NK cell cytotoxicity. We further identified by flow cytometry that MAMs and M-BMMs expressed high levels of NK cell inhibitory ligands such as H2-Kb and H2-Db, and NK cells in the metastatic lung expressed high levels of their receptors. However, blockade of H2-Kb or H2-Db did not prevent macrophage mediated NK cell suppression in our assay. Alternatively, we found that M-BMMs expressed higher levels of membrane bound TGF-β than GM-BMMs and blocking TGF-β rescued the macrophage-mediated NK cell suppression, although these data must be confirmed. Using a mouse model of breast cancer metastasis, we further demonstrated that depletion of MAMs promoted maturation of NK cells in the metastatic lung as well as recruitment of NK cells towards the metastatic site. Importantly, the MAM depletion in this model significantly increased the efficacy of transferred NK cells in reducing metastatic tumour burden whereas NK cell transfer on its own did not suppress metastatic tumour growth.

Collectively, our data suggest that MAMs in metastatic tumours can suppress NK cell cytotoxicity towards breast cancer cells by direct contact with NK cells that transmit suppressive signals via membrane bound TGF-β as well as by suppressing NK cell maturation and recruitment in the metastatic site. Our data also indicate that the depletion of MAMs can alter the immune suppressive TME and thereby improve the efficacy of NK cell infusion therapy efficacy. Further investigation of the mechanisms

behind MAM-mediated NK suppression would lead to the increased success of NK cell-based immunotherapy for MBC.

Abbreviations List

APC	Antigen Presenting Cell
BMM	Bone Marrow Derived Macrophage
CAR	Chimeric Antigen Receptor
CLP	Common Lymphoid Progenitor
CSF1	Colony Stimulating Factor 1
CTLA-4	Cytotoxic T Lymphocyte Associated Antigen-4
DC	Dendritic Cell
DCIS	Ductal Cell Carcinoma *In Situ*
Dox	Doxycycline
Tu:NK	Tumour:NK
ER	Estrogen Receptor
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HER2	Human Epidermal Growth Factor Receptor 2
hESC	Human Embryonic Stem Cell
HGF	Hepatocyte Growth Factor
IFN	Interferon
iPSC	Induced Pluripotent Stem Cell
ITAM	Immunoreceptor Tyrosine based Activation Motif
ITIM	Immunoreceptor Tyrosine based Inhibition Motif
KIR	Killer-cell Immunoglobulin-like Receptor
LLC	Lewis Lung Carcinoma
M-CSF	Macrophage Colony Stimulating Factor
MAM	Metastasis Associated Macrophage
MAMPC	Metastasis Associated Macrophage Progenitor cell
MBC	Metastatic Breast Cancer
MDSC	Myeloid-Derived Suppressor Cell
NCR	Natural Cytotoxicity Receptor
NK	Natural Killer
PD-1	Programmed Death Receptor-1
PD-L1/2	Programmed Death Ligand 1/2
PR	Progesterone Receptor
PyMT	Polyoma Middle T
RMAC	Resident Macrophage
TAM	Tumour Associated Macrophage
TAN	Tumour Associated Neutrophil
TCR	T cell Receptor
TGF- β	Transforming Growth Factor-β

TME	Tumour Microenvironment
TMEM	Tumour Microenvironment of Metastasis
TNBC	Triple-Negative Breast Cancer
TNF-α	Tumour Necrosis Factor-α
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
Treg	T Regulatory Cell
VEGF	Vascular Endothelial Growth Factor
Veh	Vehicle

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Introduction

1.1. Metastatic Breast Cancer

1.1.1. Statistics of Breast Cancer

Breast cancer is currently the most commonly diagnosed cancer in women in the UK. It is reported that around 1 in 8 women will develop breast cancer over the course of their lifetime, with over 55,000 new diagnoses each year (Breast Cancer Now: breastcancernow.org). It is also estimated that breast cancer will account for a colossal 30% of new cancer diagnoses and 15% of cancer-related deaths in women in the US in 2019[1]. Due in part to improvements in early detection and treatment methods, survival of breast cancer patients has improved significantly over the last 50 years (e.g., a 40% reduction in mortality rate from 1989 to 2016), and 5-year survival of patients with primary breast cancer is now around 99%. However, when patients develop distant metastases, their survival ratio markedly drops down to just 21%[1]. It is reported that around 30% of patients diagnosed with non-metastatic breast cancer will eventually develop metastases, and that around 6% of breast cancer patients will present with distant metastases at diagnosis[2]. The most common organs for breast cancer metastasis are the brain, bone and lung, and such distant metastasis accounts for over 90% of breast cancer related mortality[3]. Despite advances in treatments for breast cancer patients, the overall survival of those with metastatic breast cancer (MBC) has not improved over the last 30 years[4]. These statistics emphasize the urgent clinical need for effective therapies to treat patients with metastatic breast cancer (MBC).

1.1.2. Classification of Breast Cancer

Breast cancer is the general name given to the aberrant cell mass within the breast. It is actually a heterogenous disease that develops in distinct areas of the mammary tissue such as epithelial cells lining the lobules or terminal ducts of the breast[5]. Ductal

carcinoma *in situ* (DCIS) is a cancer that starts within and stays within the normal ducts or lobules. While the formation of this type of cancer is not life threatening (10-year survival estimates of over 98%), development of DCIS increases the risk of developing a more invasive form of breast cancer by approximately 11 times[6]. In contrast to DCIS, invasive breast cancers penetrate into the surrounding breast tissue and have the potential to metastasise to sentinel lymph nodes and/or distant organs. It is estimated that 90-95% of breast cancer cases are invasive breast cancers[5], and around 30% of breast cancer patients eventually develop distant metastases[2].

Given their differences in terms of malignancy, breast cancers are divided into several stages by a scoring system called the TNM classification (**Table 1.1**). 'T' indicates the size of primary tumour and its invasiveness. 'N' indicates whether or how many lymph

Table 1.1 TNM staging of breast cancer

Stage	Primary tumour (T)	Node involvement (N)	Metastasis (M)
0	Carcinoma in situ	None	None
IA	1 tumour <20mm	None	None
IB	No evidence of primary tumour/ 1 tumour <20mm	Local lymph node	None
IIA	No evidence of primary tumour/ 1 tumour <20mm/ 2 tumours >20 but <50mm	Local lymph node/ None	None
IIB	2 tumours >20 but <50mm/ 3 tumours >50mm	Local lymph node/ None	None
IIIA	No evidence of primary tumour/ 1 tumour <20mm/ 2 tumours >20 but <50mm/ 3 tumours >50mm	2x Local lymph node	None
IIIB	4 tumours of any size with extension to the chest wall or under the skin	None/ up to 2 local lymph nodes	None
IIIC	Any size	3 local lymph nodes	None
IV	Any size	None/ any involvement	Yes, anywhere, larger than 0.2mm

nodes contain disseminated tumour cells. 'M' describes whether distant metastases have occurred[7].

In addition to the classifications based on tumour size and localisation (TNM staging), breast cancer can be divided into different subtypes based on hormone receptor expression determined by immunohistochemistry and/or gene expression profiling. Currently, at least 5 subtypes are proposed (**Table 1.2**)[8].

Table 1.2. Breast cancer subtype classification (adapted from[8])

Subtype name	Receptor expression	General characteristics
Luminal A	ER+, PR+/–, HER2–	Usually chemotherapy/ endocrine therapy responsive
Luminal B	ER+, PR+/–, HER2+	Usually chemotherapy/ endocrine therapy responsive. HER2+ responsive to anti-HER2 Ab therapy
HER2+	ER–, PR–, HER2+	Chemotherapy and anti- HER2 Ab responsive
Triple negative (Basal)	ER–, PR–, HER2–	Usually chemotherapy responsive
Triple negative (Claudin Low)	ER–, PR–, HER2–	Intermediate response to chemotherapy

Breast cancers classified into the Luminal A type express the estrogen receptor (ER) with or without the progesterone receptor (PR) and do not express epidermal growth factor receptor 2 (HER2)[8]. This type of breast cancer grows more slowly than other types and is generally treated with endocrine therapy. Luminal B breast cancer is characterised as ER positive, PR positive (or negative) and HER2 positive. This type of breast cancer grows faster than Luminal A and is usually treated with chemotherapy and/or endocrine therapy[9]. Breast cancer classified into the $HER2^+$ subtype also expresses HER2 but is negative for ER and PR. Treatment for this type of cancer depends on staging but involves chemotherapy and HER2 targeted

therapy[9]. Triple negative (TN) breast cancer does not express any of the above-mentioned receptors, which makes them harder to treat by endocrine therapy or HER2 targeted therapy. Furthermore, this type of breast cancer is more aggressive and results in a poorer prognosis compared to luminal and $HER2^+$ types. TN breast cancer can be further divided into 2 groups; basal and claudin low. Around 50-75% of TN breast cancers are classified as the basal subtype. The name 'basal' came from the original finding that these types of cancer cells express cytokeratins associated with basal epithelium. Basal TN breast cancers are highly proliferative and usually have a p53 mutation[10]. As its name suggests, Claudin low TN tumours have a low expression of claudin genes that are involved in cell-cell junctions. These tumours usually show a high immune cell infiltrate, stem cell properties and evidence of epithelial-mesenchymal transition[10]. Both of these subtypes are usually treated with chemotherapy.

1.1.3. Current Therapeutic Modalities for Breast Cancer

1.1.3.1. Radiotherapy

Since highly proliferative cells (i.e., tumour cells) are susceptible to X-ray that causes DNA damage leading to cell death, the application of radiation to the tumour area can eliminate cancer cells relatively selectively[11]. Radiation is thus often given to breast cancer patients after surgical removal of breast tumours to treat any remaining diseased tissue and has been shown to significantly improve rates of recurrence and mortality compared to surgery alone[12]. However, cancer cells including breast cancer cells can become resistant to irradiation[13].

1.1.3.2. Endocrine Therapy

Hormones in women, such as estrogen and progesterone are known to promote proliferation and dissemination of certain types (i.e., luminal A/B) of breast cancer

cells. Endocrine therapy aims to block the pro-tumour signals via ER or PR. Tamoxifen is the most common drug used in this therapy and prevents binding of estrogen to the ER. Aromatase inhibitors are another class of drugs that prevent the conversion of androgens to estrogen, thereby reducing estrogen availability to the tumours. Although endocrine therapy using these drugs dramatically improved survival of patients with Luminal A breast cancer, tumours in more advanced stages acquire resistance to this type of therapy[14].

1.1.3.3. Chemotherapy

Cytotoxic drugs that cause DNA damage or impaired cytoskelexston rearrangement can suppress highly proliferative cells, including cancer cells. Currently, treatment with these chemicals (chemotherapy) is the only systemic therapy for triple negative and metastatic breast cancers. Chemotherapy is also applied to aid other types of therapies such as endocrine therapy for other breast cancer subtypes[14]. Anthracyclines such as doxorubicin, causes DNA intercalation, interacts with DNA-topoisomerase II and generates free radicals, which causes DNA damage. This leads to the induction of DNA repair or causes the cell to go through apoptosis[15]. This type of drug is usually given to patients with triple negative breast cancer or those with lymph node metastasis[16]. Anthracyclines however have been shown to induce cardiotoxicity, which limits the dose that can be given. Moreover, clinical signs do not become obvious in many cases until well after completion of the treatment[17]. Taxanes are another class of chemotherapeutics that interfere with microtubule assembly, a vital process for cell division and cellular transport[18]. Although this type of drug is commonly used as a standard regimen, serious side effects including peripheral neuropathies are reported[19].

Platinum drugs are a third class of chemotherapeutics often used in the treatment of breast cancer. These drugs cause the inappropriate cross-linking of DNA causing 'lesions'. These lesions are detected by the cell and are either repaired, or apoptosis is induced. Problems associated with these drugs include kidney toxicities and peripheral neuropathies. As well as this toxicity it has been shown that some tumours are resistant from the out-set, while others develop resistance over time[20,21].

1.1.3.4. Targeted Therapies

As described above, Luminal B and $HER2^+$ types of breast cancer express high levels of HER2 that promotes cancer cell proliferation. These types of cancers can be treated with monoclonal antibodies against HER2 such as trastuzumab[22]. Treatment with trastuzumab along with a standard chemotherapy regimen were introduced after a large-scale clinical study in 2001[23]. Although this therapy improved disease-free and overall survival of patients with certain types of breast cancer, metastasis still occurs in patients receiving this therapy. For example, 34% of patients develop central nervous system metastases of which 50% will die from this progression[24]. Moreover, this type of therapy is not applicable for breast cancers lacking HER2 expression, such as TN breast cancers.

Another example of targeted therapy for breast cancer is treatment with small molecule inhibitors against PI3K/AKT/mTOR pathways, such as everolimus that targets the mTOR pathway and prevents cell cycle progression[25].Since ER overexpressing breast cancer cells depend on the PI3K/AKT/mTOR pathways for their survival and proliferation, inhibitors against these pathways are used for ER expressing breast cancers that have become resistant to endocrine therapy as well as metastatic breast cancers that cannot be treated by other therapeutic modalities. PARP is an enzyme that repairs DNA breaks in proliferating cells and can be another

target for triple negative breast cancers. However, up until now these inhibitors have only been efficacious in TNBC patients with specific mutations (BRCA1/2)[26].

Although several different therapies exist for different breast cancer subtypes, chemotherapy is currently the main option to treat patients with metastatic disease. However, clinical studies have demonstrated that treatment with chemotherapy cannot improve outcome of metastatic breast cancer patients. Since a certain population of breast cancer patients develop metastasis at diagnosis and almost 1/3 of patients will develop MBC over their disease course, it is clear that novel and more effective therapeutic strategies are needed to overcome MBC.

1.1.4. Mouse Models of Metastatic Breast Cancer

Clinical data has illustrated the requirement of novel therapeutic strategies to prevent metastasis formation. For this to be possible, mouse models of MBC are essential in order to define mechanisms of progression and to test therapies. Currently, there are largely two types of models available, i.e., spontaneous metastasis models using genetically engineered mice and experimental metastasis models using cancer cell injection.

1.1.4.1. Spontaneous Models of Metastasis

Two well-known models of spontaneous metastasis are the PyMT model and the Neu model. The PyMT model utilizes transgenic mice in which the polyoma middle T (PyMT) oncogene is expressed under the control of the mouse mammary tumour virus (MMTV) promoter, which leads to the growth of tumours within the mammary glands in a way that recapitulates the progression of human breast cancer; proliferation of normal epithelial cells (hyperplasia) that progresses to adenoma, then early and late carcinoma. In this model, tumour cells lose hormone receptor expression during the course of progression and lung metastasis with high incidence[27,28]. The Neu model uses genetically modified mice that express the ErbB2

(also known as HER2) gene under the control of MMTV promoter, which resembles HER2 gene amplification in the $HER2^+$ type of human breast cancers. The MMTV-Neu mice also develop mammary carcinomas however the latency period is longer and lung metastasis formation is less frequent compared to those in the MMTV-PyMT mice[27]. These models recapitulate characteristics of human breast cancer and thus are suitable to investigate therapeutic efficacy of novel treatments. In contrast to the human disease, however, these models establish multiple tumours with different stages in the same individual as mice have ten mammary glands. Moreover, it is difficult to control the frequency and timing of metastasis in these models. It is important to note here that many other genetically engineered mouse models of breast cancer have been developed by Jos Jonkers and others which have a range of different common mutations to human breast cancers and mimic a range of different breast cancer subtypes[29].

1.1.4.2. Experimental Models of Metastasis

Orthotopic injection of tumour cells into the mammary fat pad of mice provides another model of breast cancer that mimics spontaneous metastasis from the primary site to the distant organ (predominantly the bone and lung). On the other hand, it is difficult to distinguish the effects of treatments on the late step of metastasis (metastatic tumour expansion) from those of the early metastatic steps (egress of tumour cells from the primary tumours) in this model. Alternatively, direct injection of cancer cells into the circulation can represent systemic dissemination of cancer cells and enables controllable metastasis formation in the distinct organs depending on the injection route. For example, intravenous injection of tumour cells allows micro-metastasis formation in the lung within a few days that grows into lethal metastatic tumours around days 10-14 after injection[30,31]. Cancer cells injected into the heart or caudal arteries develop bone metastasis[32], and cells injected into the portal vein establish liver metastasis[33]. Although these models do not recapitulate the changes that cancer

cells may face during the journey from the primary site to the secondary site, studies have shown that tumours established in the orthotopic injection model and those in the intravenous injection model are comparable [34]. Not to mention the controllable metastasis formation, the experimental metastasis models enable infusion of genetically manipulated cancer cells as well as use of human cancer cells. Ideally, using a combination of all the different models is preferable.

1.2. Tumour-infiltrating Immune Cells in the Tumour Microenvironment (TME)

1.2.1. Pro-tumour Immune Cells in the TME

Studies in mouse models of MBC have shown that there are a number of immune cells within the TME that can promote metastasis. Regulatory T (T_{reg}) cells are one cell type with this ability. For example, it has been shown that T_{regs} are recruited to tumours, which promotes $CD8^{+}$T cell death and bone metastasis in models utilizing the mammary fat pad injection of breast tumour cells[35]. In the mammary fat pad model of breast cancer, T_{reg} can also induce NK cell apoptosis that correlates with an increase in lung metastasis[36]. Tumour-associated neutrophils (TANs) are another pro-tumour cell type within the TME. TANs in breast cancer promote production of VEGF and CCL2 which promote progression of the cancer as well as resistance to chemotherapy[37]. It is also reported that high infiltration of TANs correlates with lower overall survival in lung adenocarcinoma and highly correlates with adverse outcomes in both breast cancer and lung adenoma[38]. In addition to T_{reg} cells and TANs, myeloid derived suppressor cells (MDSCs) have been reported to be recruited to the primary tumour site where they increase the invasiveness of murine models of metastatic cancer and promote spontaneous metastasis from mammary fat pad injection models of MBC[39]. It has also been shown that MDSCs accumulate within the pre-metastatic lung and correlate with metastatic tumour burden in breast cancer models utilizing

orthotopic injection of breast cancer cells into the mammary fat pad of mice[40].Another important pro-tumoural cell type abundant within the breast cancer TME is the macrophage (see below).

1.2.2. Macrophages in the 'Primary' TME

Macrophages are derived from yolk sac progenitors, the fetal liver or classical monocytes. Classical monocytes (characterized as $CD11b^{+}Ly6C^{+}$ in mice and $CD14^{hi}$ $CD16^{-}$ in human) are released from the bone marrow into the blood and then migrate into tissues in response to tissue injury, tumourigenesis and inflammation where they differentiate into macrophages[41]. Differentiation from bone marrow monocytes to mature macrophages requires the growth factor called macrophage-colony stimulating factor (M-CSF also known as CSF1). In addition to differentiation, CSF1 receptor (CSF1R) signaling also regulates the growth and survival of macrophages[41]. On the other hand, certain populations of tissue-resident macrophages including alveolar macrophages are long-lived cells that differentiate from fetal monocytes in response to GM-CSF during the first week of life[42].

Macrophages are involved in a wide range of processes within the body. They can identify, phagocytose, process and present antigens to T lymphocytes[43]. They also regulate tissue growth and homeostasis by clearing dead or dying cells as well as toxic substances from tissues and are important mediators in tissue repair after damage[44]. It is also well known that macrophages are able to change their phenotype and functions in response to their microenvironment. For example, macrophage stimulation via IFNs or pathogen recognition receptor engagement causes differentiation of macrophages into a more pro-inflammatory phenotype (referred to as 'classically activated macrophages') which help promote an immune response. Stimulation with IL-4 or IL-13 however causes differentiation into an 'alternative macrophage' which has a more anti-inflammatory/pro-repair-like phenotype. This

plasticity is well documented in different biological settings, including cancer as well as other pathologies[45].

Importantly, macrophages represent a significant part of the immune cells infiltrating solid tumours and can account for up to 50% of the tumour mass[46]. Given their pro-inflammatory roles, these tumour-infiltrating macrophages were initially thought to suppress tumour development. However, several studies have demonstrated that higher macrophage infiltration correlates with poor prognosis in various malignant solid tumours including breast cancer[47,48]. Moreover, depletion of a certain macrophage population in tumours by *Csf1r* gene deletion suppresses tumour progression of mammary tumours in mice[49] suggesting that tumour-infiltrating macrophages promote rather than suppress the establishment of malignant mammary tumours. Following this pioneering study, several studies have demonstrated that tumour-associated macrophages (TAMs) promote cancer cell egress from the primary site in mouse models of breast cancer. For example, TAMs within the TME secrete VEGF in response to hypoxic stress which correlates with the formation of blood vessels to support tumour growth[50]. It was also shown that TAMs regulated the 'angiogenic switch' (formation of a dense blood vessel network) within tumours and promoted progression to malignancy in the PyMT breast carcinoma model[51]. Tie-2 expressing monocytes (TEMs) (pre-cursors of macrophages) also promote angiogenesis in murine mammary tumours[52]. In addition to angiogenesis it has also been shown that VEGF along with CCL18 increases the invasion of breast tumour cells into the surrounding area[53]. Invasion of tumour cells is significantly increased by TAMs via the production of matrix metalloproteases that degrade the surrounding tissue and allow the invasion of tumour cells[54]. Furthermore, the invasion of tumours into the surrounding tissue as well as into blood vessels has been shown to be dependent on paracrine signalling between TAMs and tumour cells. In these studies, it was shown that macrophages produced EGF which binds to the EGF

receptor on tumour cells and tumour cells produce CSF1 that binds to the CSF1 receptor on macrophages. Blocking either CSF1 or EGF was sufficient to prevent both macrophage and tumour cell migration and invasion[55,56]. This co-localisation of TAMs and tumour cells and the paracrine signalling that accompanies it is known as the tumour environment of metastasis or TMEM[56-58]. Together, these studies clearly indicate that TAMs are important cells in breast cancer progression, especially the initial steps of metastasis.

In addition to these pro-metastatic functions, recent studies have demonstrated that TAMs can suppress anti-tumour immune reactions. For instance, TAMs have been shown to suppress cytotoxic T cells in mouse models of breast cancer as they reduce T cell proliferation and affect their viability significantly[59]. Furthermore, several studies have indicated that depletion of TAMs can enhance efficacy of chemotherapeutics in mouse models of breast cancer[60]. These results suggest that TAMs also play important roles in immune suppression and chemoresistance in primary breast cancer.

1.2.3. Macrophages in the 'Metastatic' TME

TAMs have been well studied in primary breast cancer and have been shown to promote the progression of breast cancer as well as the formation of the pre-metastatic niche. However, macrophages are also paramount in the promotion of metastatic tumour formation, growth and progression. In mouse models of breast cancer metastasis, there are at least two macrophage populations within the metastatic lung, i.e., resident macrophages (RMAC) characterised as $F4/80^{+}CD11b^{low}CD11c^{high}$ and metastasis associated macrophages (MAMs) characterised as $F4/80^{+}CD11b^{high}CD11c^{low}$ [61]. The major function of alveolar RMACs is to survey and protect the lung from respiratory pathogens. They phagocytose foreign material and secrete a range of factors to destroy and limit pathogen spread[62]

although their contribution to tumour metastasis seems to be minor[61]. In contrast, MAMs play pivotal roles in breast cancer metastasis as evidenced by a significant reduction in metastasis formation by depletion of $CD11b^{high}$ macrophages in mouse models[61].

It has been reported that MAMs originate from inflammatory monocytes that have been recruited to the metastatic niche by tumour-derived chemokine CCL2 [63]. Using experimental models of breast cancer lung metastasis, we recently reported that classical monocytes ($CD11b^{+}Ly6C^{+}$) recruited to the metastatic lung develop into a distinct population defined by increased expression of CD11b and Ly6C ($CD11b^{high}Ly6C^{high}$) that give rise to $CD11b^{high}Ly6C^{low}$ population that represent MAMs. We have also shown that accumulation of these monocyte-derived MAM progenitors (called MAMPCs) associates with metastatic tumour outgrowth, and that both MAMs and their progenitor MAMPCs significantly reduce apoptosis of mammary tumour cells induced by pre-activated $CD8^{+}$ T cells. Given their unique characteristics, MAMPCs represent monocytic-MDSCs[31]. This study thus identified that classical monocytes recruited to the metastatic site differentiate into MDSCs and mature into MAMs.

After differentiation, MAMs secrete the chemokine CCL3 which binds in an autocrine manner to CCR1. This CCR1 stimulation promotes the retention of MAMs within the metastatic lung by enhancing the attachment of MAMs via $\alpha 4$ to VCAM-1 on tumour cells. This in turn enhances extravasation and consequently metastasis[30]. We also showed in our most recent study that MAMs promote metastatic growth through the secretion of hepatocyte growth factor (HGF). HGF from MAMs binds to its receptor, Met, which is more highly expressed on highly metastatic E0771 tumour cells (HML2) compared to parental cells from which HML2 were derived. This HGF derived Met stimulation was shown to increase the invasiveness of E0771 both *in vitro*

and in vivo and caused increased lung metastasis in a mouse model of human breast cancer. Together with other mouse models of MBC, Met/HGF signaling suppressed NK cell mediated tumour cell apoptosis[64].

Collectively these studies suggest that targeting MAMs is an attractive strategy to prevent metastatic tumour expansion and immune suppression in metastatic breast tumours.

1.3. Tumour-infiltrating Immune Cells that can Suppress Metastasis

1.3.1. Cytotoxic $CD8^+$ T cells

1.3.1.1. Involvement of $CD8^+$ T Cells in Tumour Metastasis

$CD8^+$ T cells are cytotoxic lymphocytes capable of killing target cells by triggering apoptosis. They express a large and highly diverse repertoire of antigen recognition receptors made possible through T cell receptor gene rearrangement. $CD8^+$ T cells can only recognise antigens when presented by MHC-I. Once they recognise their cognate antigen T cells must also receive costimulatory signals as well as IL-2 stimulation before they kill target cells via the release of perforin and granzyme (discussed further on) leading to target cell apoptosis[65].

It has been reported that solid tumours including breast cancer contain not only the above-mentioned immune cells but also cytotoxic lymphocytes such as $CD8^+$ T cells that have the capabilities to eliminate immunogenic cancer cells[66]. A recent report has demonstrated that the presence of $CD8^+$ T cells within the tumour associates with longer survival of patients with ER negative breast cancer[67]. Furthermore, another study has shown that EMT6 mammary tumour cells introduced into the circulation are eradicated in syngeneic mice with EMT6 tumours in the mammary fat pad, whereas depletion of $CD8^+$ T cells enhanced the outgrowth of the disseminated tumor cells[68]. In another mouse model of metastatic breast cancer using 4T1 mammary tumour cells, pulmonary metastasis of tumour cells from the primary

tumour is suppressed by IL-1β blocking antibodies or toll-like receptor 7 antagonist, whereas such metastasis suppression was not found in mice in which $CD8^+$ T cells are depleted[69,70]. These results suggest that $CD8^+$ T cells can suppress metastasis of certain types of breast cancer cells, whereas their anti-tumour function is restricted in the tumour microenvironment.

The infiltration of $CD3^+$ cells including $CD8^+$ T cells has also been used to 'immunoscore' patient samples. This is based on the density of $CD3^+$ tumour infiltrating lymphocytes within the centre of the tumour (CT) and the invasive margin (IM)[71]. Several studies have shown the positive correlation between a high $CD8^+$ T cell infiltrate with better prognosis in cancers that are less aggressive. Incidentally, TAMs can also be used to immunoscore patients and TAM infiltration correlates with poorer prognosis[72].

1.3.1.2. Development, Activation, and Suppression of $CD8^+$ T Cells

Progenitors of T cells originate in the bone marrow and move to the thymus. In the thymus, T cell progenitors (i.e., thymocytes) that bind to major histocompatibility complex class I (MHC-I) proteins (positive selection) but do not bind to self-antigen (negative selection) can differentiate into self-tolerant thymocytes expressing CD8 that further differentiate into mature cytotoxic T cells in the lymphatic tissues. However, these mature but naïve $CD8^+$ T cells require further steps to exert killing abilities against infected or tumour cells. As a first step, naïve $CD8^+$ T cells in the lymph node receive activation signals through their T cell receptor (TCR) that binds to a specific antigen on MHC-I expressed by antigen-presenting cells (APCs) such as dendritic cells. In a second step, the primed and activated $CD8^+$ T cells that recognize specific antigen clonally expand in the lymph node and migrate into the site of infection or tumour development. In a final step, the effector $CD8^+$ T cells recognize the

antigenic peptides presented on MHC-I expressed on infected or tumour cells, and transmit apoptotic signals into the target cells[73].

Although mature T cells are selected not to destroy self-cells, cytotoxicity of effector $CD8^+$ T cells is also regulated by several inhibitory receptors on $CD8^+$ T cells such as programmed cell death-1 (PD-1) and cytotoxic T lymphocyte-associated protein-4 (CTLA-4). Upon binding with their ligands, these receptors transmit suppressive signals to $CD8^+$ T cells and inhibit their cytotoxic functions. Thus, these receptors are called inhibitory checkpoint receptors. In many cases, cancer cells express PD-1 ligands (PD-L1 and PD-L2) and/or CTLA-4 ligands (CD80 and CD86), and thereby suppress tumour killing activities of $CD8^+$ T cells. Interestingly, macrophages including TAMs have also been shown to express checkpoint ligands[74]. Another mechanism by which solid tumours (including breast cancers) evade the immune response is by the expression and/or secretion of immune suppressive cytokines such as TGF-β which correlates with increased tumour progression and metastasis[75]. Although TGF-β is important to maintain self-tolerance by regulating the proliferation, differentiation and survival of immune cells, this suppressive mechanism can be hijacked by cancer cells. For example, TGF-β in the tumour microenvironment blocks the production of IL-2[76], an essential cytokine for $CD8^+$ T cell survival and function[77]. IL-10 is another cytokine expressed in breast cancer[78] that can inhibit cytotoxic cell proliferation and function as well as enhance tumour cell survival, proliferation and metastasis. As described above, distinct types of immune cells (e.g., macrophages) also contribute to the suppression of $CD8^+$ T cells in the tumour microenvironment. For example, macrophages were shown to produce IL-10 that indirectly suppressed $CD8^+$T cells and therefore chemotherapy efficacy by reducing IL-12 expression by dendritic cells in the PyMT model[79].

1.3.2. Natural killer (NK) Cells

1.3.2.1. Origin and Development of Functional NK cells

Natural killer (NK) cells are cytotoxic cells defined as $CD3^{-}CD56^{+}$ cells in humans or $CD3^{-}$ $NK1.1^{+}$/$NKp46^{+}$ cells in mice[80]. They are usually found in the blood, spleen and lung[40] and are most well known for their roles in cancer cell elimination, transplant rejection and viral immunity[81] and comprise around 5-10% of peripheral blood lymphocytes. They develop in the bone marrow from a common lymphoid progenitor (CLP) from which B and T cells also derive[42]. Factors such as IL-7, IL-15, stem cell factor and FLT3 ligand are required for NK differentiation from the CLP. Of these, IL-15 has been shown to be critical as the absence of IL-15 or the IL-15 receptor alpha chain leads to a significant reduction in NK numbers[82]. Although derived from the same CLP, NK cells are considered innate for a number of reasons. One reason is that they do not require prior sensitisation or antigen presentation via MHC restriction to exhibit their full cytotoxic capacity[75]. This allows them to kill target cells quickly.

1.3.2.2. Effector Functions of NK Cells

Mature NK cells can eliminate aberrant cells from the body through several mechanisms, i.e., secretion of cytolytic granules, expression of ligands for death receptors, and release of inflammatory cytokines.

Following activation, NK cells produce high levels of perforin and granzymes. The activated NK cells also form stable cell-to-cell contacts with target cells (a structure called the immune synapse) via the reorganization of the actin cytoskeleton[83,84]. Lytic granules including perforin and granzymes then move to the microtubule organizing centre and polarize towards the newly formed immune

synapse where they are released[85]. The released perforin forms small pores within the target cell membrane, which enables granzymes to enter the target cells[86]. The transferred granzymes activate apoptotic signals by cleaving pro-apoptotic enzyme caspases and thereby kill the target cells[87]. The pore can be established in as little as 30 seconds, and initiation of apoptosis is evident within 2 minutes after membrane permeabilization[88]. This whole process is incredibly quick compared to other effector mechanisms (see below), apoptosis induction via perforin and granzymes is the first and main pathway for NK cells to eliminate their targets including cancer cells[89]. However, it has been reported that subsets of human breast carcinoma express a granzyme B inhibitor (PI-9), and that mouse leukemia cells expressing SPI-6 (a murine homolog of PI-9) are less susceptible to cytotoxic cell killing[90]. It is also reported that human breast cancer cells under hypoxia become less susceptible to NK-mediated lysis via the activation of autophagy in tumour cells that leads to the degradation of granzyme B[91]. It is thus possible that certain types of breast cancer cells can evade apoptosis through the lytic granule dependent mechanism.

It has been shown that the activation of NK cells leads to an increase in expression of Fas ligand (FasL)[92] that binds to its receptor Fas (also known as CD95) on target cells and triggers the caspase-mediated apoptosis cascade[93]. It is also reported that NK cells can increase Fas expression on target cells (e.g., murine T cell lymphoma) via secretion of cytokines[94]. It has been shown that 49% of triple-negative breast cancers and 16-20% of ER^+ breast cancer express Fas, and Fas expression can be an independent prognostic marker for recurrence free survival in human breast cancer[95]. NK cells can also cause apoptosis of target cells via expression of tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) that binds to death receptors DR4 or DR5. However, human breast cancer cell lines have been shown to be resistant to this pathway since the receptors for TRAIL can be endocytosed in

these cells[96]. Human breast cancer cell lines have also been shown to avoid FasL mediated killing by shedding FasL as a decoy[97].

In addition to direct contact with target cells, NK cells can kill target cells by releasing pro-inflammatory cytokines. NK cells possess the ability to rapidly translate interferon (IFN)-γ into protein and release it upon activation[75,98]. NK cell IFN-γ release has been reported to induce target cell cytolysis[99]. Furthermore, IFN-γ can recruit immune cells such as neutrophils and T cells[100], increase the cytotoxic capabilities of $CD8^+$T cells[101], and promote Th1 differentiation of $CD4^+$ T cells[102-104]. It can also educate macrophages to be directly cytotoxic (phagocytic) cells[104]. Therefore, NK cells can eliminate target cells indirectly by inducing anti-tumour immune responses via the release of IFN-γ. Activated NK cells also release TNF-α[93] that binds to TNF receptor 1 (TNFR1)[105-107]. TNFR1 is widely expressed and induces death via a range of signaling pathways that end in either apoptosis or necrosis of the target cell[99,107].

As such, NK cells can eliminate tumour cells through several mechanisms. In contrast to $CD8^+$ T cells, however, NK cells do not require antigen presentation or clonal expansion in order to exert these cytotoxic activities (the name 'natural killer' originates from this feature). Instead, activities of NK cells are tightly regulated by regulatory receptors and cytokines (see below- 'missing-self' hypothesis).

1.3.2.3. Activation of NK Cell Functions via Activating Receptors

NK cells recognise tumour or infected cells via a range of activating receptors that can be grouped into families based on their structural similarity (**Table 1.3**). The natural cytotoxicity receptor (NCR) family consists of 3 members, i.e., NKp46, NKp44 and NKp30. NKp46 and NKp30 are expressed on resting NK cells[108] whereas NKp44 is upregulated upon activation[109]. The absence of NKp46 has been linked to the impairment of tumour cell clearance in mouse models of melanoma and Lewis lung

carcinoma (LLC)[110]. In mouse models of primary breast cancer, reduced expression of NKp46 within the tumour microenvironment correlates with immaturity and reduced efficacy of NK cells[111]. However, ligands for NCRs especially those expressed by cancer cells are still unknown, although hemagglutinin has been identified as an NCR ligand[110,112].

The NKG2 family has seven members, i.e., NKG2A, B, C, D, E, F and H that are expressed both in humans and mice. All members have a high degree of sequence homology apart from NKG2D which is the most individual of this family. NKG2C, D, E, F and H are activating while A and B are inhibitory (discussed later)[113]. Among these receptors NKG2D has been described as 'the master activating NK cell receptor' and can over-ride signals from NK inhibitory receptors. Human NKG2D ligands such as MIC-A, MIC-B and ULBP1-6 are known to be upregulated in infected or cancerous cells, which allow NK cells to recognise these aberrant cells and be activated[114]. It has been shown that a downregulation of NKG2D in tumour infiltrating NK cells correlates with breast cancer progression[115]. Shedding of NKG2D ligands has also been reported in human breast cancer cell lines *in vitro*, which prevents NK cell mediated cytolysis[114]. Murine ligands for NKG2D include histocompatibility antigen 60 (H60), retinoic acid induced early transcript (Rae-1) and murine ULBP-like transcript 1 (Mult1)[116]. Rae-1 expression has been shown to activate NK cells and promote NK cell mediated tumour rejection in murine models of melanoma and lymphoma[117].

In human, there is another group of NK cell regulatory receptors called the Killer cell Immunoglobulin-like Receptor (KIR) family. These receptors are functional homologs of murine Ly49 family receptors as these receptors bind to MHC-I molecules. KIRs have two or three IgG-like domains and short (S) or long (L) cytoplasmic tails that determines whether the receptor activates or inhibits NK cell

functions. The activating receptors in this family (KIR2DS1-4) have short intracellular domains that interact with DAP12 and other intracellular proteins containing immunoreceptor tyrosine activation motifs (ITAMs) and thereby transmit the activation signal[118]. Another human NK cell activating receptor is DNAM-1 that binds to CD155 (poliovirus receptor) and CD112 (Nectin-2)[119]. Nectin-2 has been shown to be

Table 1.3. NK cell regulatory Receptors

Family	Members	Activating/ Inhibitory	Ligands
NCR (Hu and Mu)	NKp46	Activating	Tumour (?), Hemagglutinin
	NKp30	Activating	Uknown
	NKp44	Activating	Tumour (?), Hemagglutinin
NKG2 (Hu and Mu)	NKG2C	Activating	HLA-E (Hu), $Qa1^b$ (Mu)
	NKG2D	Activating	ULBPs, MICA&B (Hu), Rae-1, H-60 (Mu)
	NKG2A	Inhibitory	HLA-E (Hu), $Qa1^b$ (Mu)
Ly49 (Mu only)	Ly49A	Inhibitory	H2-D, H2-K
	Ly49C	Inhibitory	H2-D
	LY49H	Activating	m157
	Ly49I	Inhibitory	H2-B
KIR (Hu only)	KIR2DS1-4	Activating	HLA-C
	KIR2DS5	Activating	Unknown
	KIR3DS1	Inhibitory	HLA-B
	KIR2DL1-3	Inhibitory	HLA-C
	KIR2DL4	Inhibitory	HLA-G
	KIR3DL1	Inhibitory	HLA-A/B
	KIR3DL2	Inhibitory	HLA-A
	KIR3DL3	Inhibitory	Unknown
	KIR3DL5	Inhibitory	Unknown
Ig family	DNAM-1	Activating	CD155 and CD112

overexpressed in human breast cancer tissues and therefore may help determine which patients would respond to NK cell therapies[120]. A recent report has shown that treatment of breast cancer cells with Adriamycin increases expression of CD155, therefore rendering these cells more responsive to apoptosis[121]. It is also reported that human breast cancer cells with different subtypes commonly express ligands for DNAM-1 and NKG2D, which is required for NK cell mediated killing of these cancer cells[115].

In addition to the above-mentioned activating receptors, Fcγ receptors such as CD16 (FcγRIIIA) and CD32 (FcγRIIC) on the cell membrane of NK cells can transduce activating signals via their ITAMs upon binding the Fc region of IgG. Signal activation via Fcγ receptors leads to cytolytic granule secretion, death ligand expression, and cytokine secretion by NK cells[122]. Therefore, NK cells can bind to target cells opsonised by antibodies (IgG) and efficiently kill the target cells. This type of killing mechanism is called antibody-dependent cellular cytotoxicity (ADCC) and is well documented in breast cancer treated with anti-HER2 antibodies and in B cell leukaemia treated with antibodies against CD20[123].

1.3.2.4. NK cell Activation via Cytokines

Cytokines also play important roles in regulating NK cell activity (**Table 1.4**). IL-2 is one of the most important cytokines for activation of NK cells. It has been reported that IL-2 deficient mice show impaired NK cell responses to infection although the number of NK cells are normal[124]. IL-2 binds to the IL-2 receptor complex and stimulates proliferation[54] and release of IFN-γ[125] in NK cells and enhances cytotoxicity through the upregulation of perforin[126]. The vast majority of IL-2 comes from activated T cells but can also be released by dendritic cells (DCs)[127]. As mentioned above, IL-15 is indispensable for development and maintenance of NK cells *in vivo*. This cytokine can also enhance proliferation[82] and cytotoxicity of NK cells via the upregulation of activating receptors[128]. The source of IL-15 has been shown to be monocytes and macrophages as well as DCs[129]. IL-12 promotes activation of NK cells along with IL-15 and IL-2. Although IL-12 is poorly stimulatory in terms of cytokine secretion in NK cells, this cytokine is adequate to stimulate cytotoxicity[130]. IL-12 is produced by macrophages and DCs *in vivo*[131,132]. It contrast to the other cytokines, IL-18 does not increase cytotoxicity or proliferation of NK cells, but instead this

cytokine can promote migration of NK cells towards the area of inflammation[131] and stimulate survival of NK cells[133].

Table 1.4 NK cell regulatory cytokines

Cytokine	Activating/ Inhibitory	Effects on NK
IL-2	Activation	Proliferation, IFN-γ secretion, cytotoxicity
IL-15	Activation	Survival, maintenance, cytotoxicity, proliferation
IL-12	Activation	Cytotoxicity
IL-18	Activation	Migration, survival
IL-21	Activation Inhibition	Cytotoxicity, reverses exhaustion/ reduces IL-15 mediated proliferation
Type I IFN	Activation	Proliferation, cytotoxicity, cytokine release
IL-10	Inhibitory	Promoting tolerance
TGF-β	Inhibitory	Reduction in cytotoxicity and cytokine secretion

The role of IL-21 in NK cell stimulation is complicated. It is secreted by $CD4^+$T cells and NKT cells[134] and has some contrasting effects on NK cells. Namely, IL-21 can enhance the effects of IL-2 and IL-15 on NK cell activation and reverse exhaustion of NK cells[135], whereas it has also been shown to increase the expression of the NK inhibitory receptor NKG2A[66] and block IL-15 induced proliferation[136]. Similarly to IL-21, there are contrasting roles for type-I interferons on NK cell biology. Type-I IFNs have been shown to stimulate proliferation of NK cells, increase NK cell cytotoxicity and release of cytokines[137] as well as up-regulate the expression of perforin and FasL in NK cells[138]. On the other hand, the type-I IFNs have also been shown to decrease the release of IFN-γ from NK cells[139]. The type I interferons such as IFN-α and IFN-β were originally thought to be mostly secreted by plasmacytoid DCs but recent evidence suggests that the cell type which acts as a source of these IFNs changes depending on the type of infection/inflammation. Macrophages, inflammatory monocytes and conventional DCs also produce Type I IFNs[140].

1.3.2.5. NK Cell Suppression via Inhibitory Receptors

NK inhibitory receptors bind to MHC-I molecules on cells so that they can be recognized as 'self'. This is the basis of the 'missing-self' hypothesis which states that NK cells can recognize and kill cells which do not express MHC-I molecules as they are seen as 'non-self'[141,142]. Upon binding to self-MHC, NK inhibitory receptors trigger a signaling event which causes the phosphorylation of intracellular immunoreceptor tyrosine-based inhibitors motifs (ITIMs) leading to the recruitment of and activation of intracellular phosphatases like SHP1 and SHP2. These phosphatases can dephosphorylate a number of proteins that regulate activation of NK cells[143]. This inhibitory signaling can prevent NK cell cytotoxicity as well as adhesion of NK cells to target cells[99]. Inhibitory receptor stimulation eventually leads to the collapse of the actin cytoskeleton and the NK cell therefore backs off from the target cell[100]. Up until now several NK cell inhibitory receptors that bind to specific MHC-I molecules have been reported (**Table 1.3.1**).

As mentioned above, the Ly49 family consists of a number of receptors including both activating and inhibitory receptors. The major family of mouse NK cell inhibitory receptors are Ly49 receptors that are functionally equivalent to theKIR family in humans. Ly49A is a murine inhibitory receptor that binds to MHC-I molecules such as H2-M3 and H2-$D^{d/k}$ on the surface of target cells depending on the genetic background of mice[93,144]. For example, C57Bl/6 mice express the 'b' haplotype and thus cells originating from this strain express H2-D^{b} but not H2-$D^{d/k}$. So far, H2-M3 is the only ligand reported for Ly49A in C57Bl/6 mice[145]. It has been reported that a Ly49A-binding peptide that blocks receptor binding of H2-$D^{d/k}$ can enhance cytotoxicity of NK cells against H2-D^{d} expressing cancer cells[146]. On the other hand, a recent report has shown that NK cells in H2-M3 deficient mice produce a lower amount of IFN-γ upon activation and are less efficient in suppressing lung metastasis

of B16 melanoma cells, suggesting that the binding of host H2-M3 to Ly49A is required for development of functional 'licensed' NK cells[145]. LY49C and Ly49I are also inhibitory receptors and bind to H2-D^b and H2-K^b in C57Bl/6 mice[119,147]. It has been shown that blocking Ly49C/I leads to an increase in NK cell mediated tumour-cell rejection in a mouse model of lymphoma without causing autoimmunity, indicating that these receptors play a major role in tumour-cell tolerance[148]. Interestingly, a recent study has shown that the growth of LLC cells in syngeneic mice is significantly promoted by depletion of Ly49C/I expressing NK cells as well as genetic deletion of MHC-I, suggesting that Ly49C and/or Ly49I are also required for NK cell licensing[149].

NKG2A is another inhibitory receptor that binds classical MHC-I molecules such as HLA-E in human[150] and H2-Qa1 in mice[151]. It is reported that H2-Qa1 expression on activated $CD4^+$T cells protect them from NK mediated cytotoxicity[152] and upregulation of H2-Qa1 on B cells prevents NK cell mediated lysis of anti-viral T cells in virus infected mice[153]. These studies suggest that H2-Qa1 controls NK cell activation.

As previously mentioned, the human KIR family includes several inhibitory receptors. The inhibitory KIRs have long cytoplasmic domains (hence they have 'L' within their name) including ITIMs that transmit the inhibitory signals. These receptors specifically bind to either HLA-A, HLA-B, HLA-C, or HLA-G (**Table 1.3.1.**) whereas ligands for KIR3DL3 and KIR3DL5 have not yet been identified[118,154]. It has been shown that patients receiving allogeneic stem cell transplants have improved elimination of tumour cells compared to those receiving immunologically matched transplants due to what is known as the graft versus donor effect. This is because donor derived NK cells are no longer inhibited by patient derived KIR ligands due to immunogenetic mismatch[155].

In addition to the above-mentioned inhibitory receptors binding to MHC-I molecules, receptors for immune checkpoint ligands are also expressed by NK cells and mediate the inhibition of NK cell functions. For example, NK cells can express programmed cell death receptor 1 (PD-1) and cytotoxic T lymphocyte associated antigen 4 (CTLA-4) which binds to CD80/86. PD-1 is the receptor for the checkpoint molecules PD-L1 and PD-L2 that is expressed on activated cytotoxic lymphocytes in order to limit the excessive immune response and avoid collateral damage during inflammation[156]. It has been shown that PD-1 contains ITIMs within its intracellular domain which inhibits proliferation and cytokine production of $CD8^+$ and $CD4^+$ T cells[157]. Therefore, PD-1 signaling is known as a major suppressor for T cell mediated immune reactions. However, several studies have suggested that PD-1 also regulates NK cell functions. For example, primary multiple myeloma (MM) cells from patients express PD-L1, and preventing PD-1/PD-L1 interaction by anti-PD-1 blocking antibody increased NK cell cytotoxicity towards cancer cells but not normal cells[158]. In a mouse model of esophageal squamous cell carcinoma (ESCC), treatment with an anti-PD-1 blocking antibody suppressed tumour growth in mice and was abrogated by depletion of NK cells[159]. Although the involvement of PD-1 in NK cell function in breast cancer is currently unknown, it has been shown that high PD-L1 expression in primary breast cancers correlates with higher tumour grade and higher proliferation rate of the tumours. Interestingly, PD-L1 expression also associates with high infiltration of $CD68^+$ cells[160] that represent myeloid cells including macrophages[161].

Another checkpoint receptor CTLA-4 is a homolog of T cell co-stimulating receptor CD28 but it binds to CD80 and CD86 with much higher affinity than CD28. Binding of the ligands to CTLA-4 does not transmit activation signal like CD28, and instead leads to anergy of T cells[162]. It has been shown that CTLA-4 decreases IFN-γ production in murine NK cells, suggesting that this receptor also transmits NK cell suppressive signals[163]. Interestingly, high CTLA-4 expression in breast cancer

associates with poor-prognosis[164]. However, effects of CTLA-4 on human NK cells, especially their cytotoxicity against cancer cells, have not yet been identified.

1.3.2.6. NK Cell Suppression via Cytokines

It is well known that NK cell functions can also be inhibited by several cytokines. One of the most prominent cytokines responsible for NK cell suppression is transforming growth factor β (TGF-β). It has been demonstrated that expression of the NK activating receptor, NKG2D in cultured human NK cells is significantly reduced by incubation with plasma from patients with lung or colorectal cancers, and such a reduction is inhibited by anti-TGF-β blocking antibodies. Furthermore, incubation with recombinant human TGF-β reduces NKG2D expression in NK cells and thereby suppress NK cell mediated killing of human T lymphoblast or B cell lymphoma cell line[165,166]. In a mouse model of melanoma, blockade of active TGF-β suppressed tumour growth in an NK cell dependent manner[167]. In human breast cancer, high expression of TGF-β positively correlates with enhanced breast cancer progression, angiogenesis, and metastasis[168]. TGF-β has also been implicated to promote epithelial to mesenchymal transition (EMT) and tumour metastasis in mouse models of breast cancer[129, 130]. Although the contribution of TGF-β to the NK cell suppression in breast cancer is largely unknown, TGF-β reduces NK cell cytotoxicity against human breast cancer cells (MCF-7 and MDA-MB-231) *in vitro*[169]. It is therefore possible that TGF-β plays pivotal roles in NK cell suppression in the tumour microenvironment.

IL-10 is also known to maintain tolerance of NK cells within the liver to prevent inflammation caused by constant stimulation from pathogens from the gut[170]. In human breast cancer, high IL-10 expression is reported as a negative prognostic factor, whereas another study shows that IL-10 expression correlates with anti-tumour activity involving the augmentation of NK cell destruction of tumour cells[171].Therefore

the role of IL-10 in NK cell regulation of breast cancer is controversial. Interestingly, a recent study has shown that IL-4, IL-12, and IL-18 can significantly suppress NK cell cytotoxicity against leukemia cells in the presence of IL-15 *in vitro*[172]. However, the involvement of these cytokines in the NK cell suppression in breast cancer needs to be investigated.

1.4. Immunotherapy

1.4.1. T cell-based immunotherapies

Given the cytotoxic nature of $CD8^+$ T cells, there has been a range of therapeutic strategies established aimed at boosting their ability to attack tumour cells. Such T cell-based immunotherapies include therapeutic vaccination, immune checkpoint inhibitors, and the infusion of selected $CD8^+$ T cells.

As mentioned above (Chapter 1.3.1), $CD8^+$ T cells need to be activated and clonally expand through antigen presentation in order to eliminate target cells. Therapeutic vaccination aims to enhance this activation process by injecting tumour specific antigen with immune adjuvants. However, identification of targetable antigen that is selectively expressed by cancer cells and efficiently presented is still challenging, and thus therapeutic efficacy of this strategy is still very limited[173].

Checkpoint inhibitor therapy proposes to re-activate $CD8^+$ T cells which functions are suppressed through binding to immune checkpoint ligands expressed by tumour cells and pro-tumour immune cells (discussed in Chapter 1.3.1.2). To overcome this suppression, antibodies against these ligands (e.g., PD-L1) or their receptors (e.g., PD-1 and CTLA4) have been developed. These checkpoint inhibitors have dramatic therapeutic responses in the clinic in cancers such as metastatic melanoma, leading to the approval of checkpoint targeting drugs for a number of malignant tumours [174]. However, efficacy of this therapy is limited to certain types of tumour and therapeutic effects on metastatic breast cancer are not sufficient so far[175].

Another emerging T cell-based immunotherapy is transfer of $CD8^+$ T cells expressing chimeric antigen receptors (CAR) that consist of an intracellular signalling domain of the T cell receptor and extracellular single-chain variable fragments (scFv) that bind to a specific protein expressed on the surface of tumour cells. Since CAR expressing T cells can exert cytotoxicity against tumour cells expressing the target surface molecules without MHC-I restricted antigen presentation, they can target low antigenic cancer cells that are not detected by the intrinsic immune system[176]. Although this type of therapy was successful for B cell lymphoma[177], its applicability and efficacy in solid tumours including breast cancer has not been reported.

After the success of checkpoint inhibitors, immunotherapy has been considered as a promising therapy for malignant tumours that are refractory to current therapeutics. However, the above-mentioned T cell-based immunotherapies are not applicable to all types of cancers. As previously mentioned, $CD8^+$ T cells require antigen presentation via MHC-I and thus cannot target cancer cells that lack tumour antigens or down-regulate MHC-I expression such as metastatic breast cancer[178]. For these types of tumours, alternative strategies must be adopted to eliminate tumour cells.

1.4.2. NK cell-based immunotherapies

Due to the fact that NK cells are cytotoxic, not MHC restricted and can expand and exert their cytotoxic functions almost immediately, NK cells are attractive therapeutic tools to target cancer cells that express no or low levels of MHC-I or antigenic protein and thereby escape from surveillance of T cells[179]. Since malignant breast cancer cells frequently lose MHC-I expression, they are also targetable by NK cells.

Therefore, the infusion of *ex vivo* expanded and activated NK cells has been proposed as a novel immunotherapy.

Initially, NK cell-based immunotherapy aimed at stimulating NK cells within cancer patients. Namely, activating cytokines such as IL-2, IL-15, and IL-21 were administered to cancer patients in order to activate intrinsic NK cells. However, the responses were very limited and patients experienced life-threatening toxicities such as vascular leak syndrome[180,181]. As an alternative strategy to utilize NK cells as a therapeutic tool, NK cell infusion was then proposed in 2005. In this study, NK cells activated *ex vivo* were infused into leukemia patients who had already received lymphodepleting chemotherapy in order to make room for the NK cells. NK cells transferred into the patients expanded and persisted for up to a month, and some positive responses were observed. In this trial, patients also received IL-2 after the NK cell transfer in order to activate and maintain NK cells. However, another study had shown that IL-2 administration leads to an increase of immune suppressive T_{reg} cells as these cells express the high affinity receptor for IL-2[182]. For this reason, other cytokines such as IL-15 have been tested to enhance efficacy of infused NK cells. A recent study has shown that regimented doses of IL-15 are well tolerated and increases NK cell numbers in patients with advanced solid tumours such as renal cell carcinoma, non-small cell lung cancer and squamous cell carcinoma. Although no objective responses were observed, this study proposed that IL-15 administration was safe and supported NK cells in patients which can be combined with other NK-cell based immunotherapies[183].

Several different sources of NK cells are used or proposed for NK cell infusion therapy, i.e., autologous NK cells, allogenic NK cells (cells from the peripheral blood of another person), NK cell lines, and NK cells derived from induced pluripotent stem cell (iPSC) or embryonic stem cell (hESC). Although autologous or healthy donor-derived primary NK cells are most commonly used in the clinic so far, there are some

limitations in these types of NK cells. For example, healthy donor derived NK cells are not ideal to use for multiple rounds of treatment since NK cells account for a small proportion in blood therefore isolation requires multiple rounds of leukaphereses and there exists significant variation in NK cells from donor to donor[184]. In order to overcome this disadvantage, NK cell expansion methods have been improved by co-culturing NK cells with artificial antigen presenting cells that express membrane bound IL-21 (in contrast to membrane bound IL-15 which was also studied)[185]. Nevertheless, variation of NK cell status between donors makes it difficult to use donor-derived cells as a 'standard' product[184]. Moreover, autologous NK cells are suppressed by self-MHC-I expressed by the tumour or surrounding stromal cells. An alternative source of therapeutic NK cells are NK cell lines such as NK-92 that consistently exert cytotoxic capability against tumour cell targets[184] and enables a constant supply of clinical grade NK cells with minimum batch effects. In addition to this, NK cell lines are more susceptible to genetic manipulation compared to blood derived NK cells that usually display very poor transfection efficiency[184]. This feature allows for the generation of NK cells expressing chimeric antigen receptors (CARs) that can more efficiently kill cancer cells expressing target antigen such as CD19 in B cell lymphoma[186]. Therefore, NK-92 cells represent a valuable therapeutic tool in the field of immunotherapy. Another attractive source of NK cells are iPSC or hESC derived NK cells. A method to differentiate hESCs or iPSCs into functional NK cells had been reported where hESCs or iPSCs are cultured with stem cell factor (SCF), IL-3, IL-15, Fms-like tyrosine kinase 3 ligand (flt3L) and IL-7 for around 30 days with a feeder cell line[187,188]. hESC-NK cells have been shown to kill human tumour cells *in vitro* as well as *in vivo* and were significantly more effective than NK cells derived from umbilical cord blood[188]. These cells can also be genetically manipulated. For example, hESC-NK cells have been manipulated to express luciferase allowing trafficking and biodistribution studies to take place via *in vivo* bioluminescence imaging[187]. Similarly,

iPSC-NK cells have been shown to be effective in a number of different models of human ovarian cancer[187]. The biggest advantage to using these methods is that they represent an 'off-the-shelf' therapy, reducing problems associated with patient/ donor variability[187]. Similarly to NK cell lines, these cells are also relatively easy to genetically modify compared to peripheral blood NK cells. For example, one group modified iPSC-NKs to express a modified version of CD16 to improve ADCC toward HER2 expressing ovarian cancer cells[189]. The use of hESC/iPSC derived NK cells bears many advantages. As well as the aforementioned ease of manipulation (multiple enhancements are possible), these cells are defined and homogenous. They also do not need to be irradiated which means that they are able to expand *in vivo* [190]. From these studies, it is clear that NK cell immunotherapy represents a promising and exciting cancer targeting strategy.

Efficacy of clinical trials using NK cell infusion therapy have been evaluated. Although most trials are still on-going, infusion of autologous or allogenic NK cells in patients with solid tumours such as renal cell carcinoma, glioma[191] and breast cancer[177] have shown partial clinical responses without obvious side effects[192]. A phase 1 clinical trial using the NK-92 cell line has also been done and showed that these cells survive *in vivo* and are well tolerated without significant toxicities in patients with advanced cancer[156,168].

However, efficacy of NK cell infusion therapy in solid tumours including breast cancer is limited so far and is not sufficient to cure the patients. Although the mechanisms behind this limitation are currently unknown, inefficient delivery of infused NK cells into the tumour and the existence of immune suppressive tumour microenvironment have been suggested[178]. For example, T_{reg} cells in the tumour have been reported to suppress NK-cell mediated tumour rejection in mouse models of melanoma[193]. It is also reported that MDSCs from the peripheral blood of patients with

liver cancer can also suppress NK cell cytotoxicity *in vitro* in a cell-to-cell contact dependent manner [194]. Interestingly, TAMs from the primary mammary tumours in PyMT mice have also been shown to suppress NK cell activation status via the physical interaction with NK cells[195]. Although these data emphasize the involvement of the TME in the NK cell inefficiency, suppressive effects of macrophages on NK cell cytotoxicity, especially those within MBC, has not been well understood. In order to successfully apply the NK cell infusion therapy to MBC, a better understanding of NK cell suppression induced by metastasis-associated macrophages (MAMs) is needed. In this project we have investigated macrophage mediated NK cell suppression in mouse models of MBC and identified possible mechanisms that can lead to improving this therapy.

1.5. Hypothesis

As described above, metastatic breast cancer is a leading cause of cancer-related death in women and there is a desperate need to find new therapies to tackle this disease. An emerging therapy is immunotherapy. However, these immunotherapies are usually based on T cell activities and in many cases MBC cells cannot be targeted by T cells. An alternative immunotherapy for MBC can be NK cell infusion. The efficacy of NK cell therapy has been shown to be limited by the TME. The role of the suppressive TME at the metastatic site is largely unknown. Based on previous studies including our own, we hypothesize here that metastasis-associated macrophages play pivotal roles in NK cell suppression in metastatic tumours, and that targeting MAMs will improve NK cell cytotoxicity and thereby improve NK cell immunotherapy efficacy.

Chapter 2

Materials and Methods

2.1. Animals

All animal experiments were performed under UK Home Office project licenses issued under the Animals (Scientific Procedures) Act (1986) and the EU Directive 2010/63 in accordance with ARRIVE guidelines. Animals had unrestricted access to food and water, were housed in groups of up to 6 per cage and were subject to a 12-hour light/dark cycle.

C57BL/6NCrl mice were purchased from Charles River and were housed in our on-site animal facility for a 7 day 'settling period' before use.

rtTA:tetO-*Cre:Csf1r*$^{F/F}$ *(also called Csf1r*-cKO*)* mice were generated by crossing B6.Cg-*Csf1r*^{tm1Jwp}/J (*Csf1r*$^{F/F}$) mice with ROSA-rtTA and tetO-*Cre* mice (The Jackson Laboratory, Maine, USA). These mice were bred and maintained in-house. When treated with doxycycline (2µg/mL, Sigma, D9891) in 5% (v/v) sucrose water, the drug combines with transactivator rtTA which can in turn bind tetracycline-responsive promoter element TetO allowing transcription of Cre recombinase. Cre recombinase can cut the loxP sites which are located on either end of exon five of the *Csf1r* gene, leading to null allele of the *Csf1r.*

2.2. Culture of Cell Lines and Primary Cells

2.2.1. Tumour Cells

E0771 mouse mammary adenocarcinoma cells were originally derived from medullary cancer in C56BL/6 mice. E0771 were manipulated to express firefly luciferase plus a hygromycin resistance gene and highly metastatic derivatives were isolated from established metastases within the lung (called E0771-LG2:Fl≠4). These cells were also manipulated to express a nuclear red fluorescent protein (mKate2)[196] and were subsequently called E0771-LG2:Fl≠4-NLR. In order to isolate a clone of E0771-LG2:Fl≠4 cells that efficiently develop tumours in *Csf1rc*KO mice E0771-LG2:Fl≠4 cells were injected via tail vein into Csf1rcKO mice. Metastasized cells were isolated

from the lung and selected for using hygromycin (Thermofisher Scientific, 10687010) for 2 weeks in culture. The established cell lines (E0771-LG2:Fl#4 and E0771-LG2:Fl4-Csf1rcKO) were stocked in 1ml of 10% (v/v) dimethyl sulfoxide (DMSO) and 90%(v/v) fetal bovine serum (FBS) in liquid nitrogen until use. These stocks were carefully thawed and put into 9 mL of pre-warmed DMEM medium (Gibco, 41966) containing 10% FBS (Gibco, 10500) and 1% Penicillin/Streptomycin (Gibco, 15140122) (complete media) and spun down for 5 mins at 300g to get rid of remaining DMSO. Cells were resuspended in complete media and allowed to recover for a few days in the incubator at 37°C, 5% CO_2 and split every few days at 1:5-1:10 for maintenance. To subculture cells they were washed with 1x Phosphate buffered Saline (PBS), pH 7.4 and 0.25% w/v Trypsin-EDTA (Gibco, 25200056) was used to dissociate cells, followed by a wash in complete DMEM media. Cells were resuspended and counted for further procedures.

2.2.2. NK Cell isolation and Culture

Spleens from C56BL/6 wild type (2-4 months) were isolated and pushed through a 70μM filter using the rubber end of a plunger from 5mL syringe into PBS and spun down at 300g for 5 minutes. Cells were resuspended in 1mL of MACS buffer (0.5% BSA, Sigma, A1470, 2mM EDTA, Sigma, E6758) and counted. $1x10^8$ cells in 1mL MACS solution were put into a 5ml round-bottom polystyrene tube. 50μL of antibody cocktail from the NK isolation kit (Stemcell Technologies, 19855) was added, mixed gently and incubated at room temperature (RT) for 10 minutes. 100μL of magnetic beads which bind antibodies from cocktail (same kit) was added and incubated for another 5 minutes at RT. The volume was made up to 2.5mLs using MACS buffer and the tube was inserted into MACS EasySep™ magnet for 5 minutes. The negatively selected cells in the supernatant were poured into a 15mL Falcon® tube

and put on ice. The tube in MACs magnet was removed, remaining cells were resuspended in 2.5mLs of MACS buffer and magnetic selection was repeated for a further 5 minutes before decanting into the 15mL tube with the previous supernatant. The 15mL tube (containing NK cells) was centrifuged at 300g for 5 minutes and supernatant discarded before resuspending with appropriate medium for the assay.

2.2.3. Bone Marrow-Derived Macrophages (BMMs)

Femurs and tibias from C57BL/6 wild type mice were isolated and flushed with cold PBS. Isolated bone marrow was centrifuged at 300g for 5 minutes, supernatant decanted and resuspended in 10mLs of alpha-MEM (Gibco, 22517) complete medium with 10% v/v FBS and 1% v/v Pen/Strep (as previously mentioned). 1000U/mL of CSF-1 (Chiron Corporation, 94608) (to make M-BMMs) or 25ng/mL GM-CSF (Peprotech, 31503) (to make GM-BMMs) was added to resuspended cells and was cultured for 24 hours in a tissue culture-treated 10cm dish (CORNING, 430167). Non-adherent cells were put into a 50mL Falcon® tube, 30mLs complete alpha-MEM was added with 1000U/mL CSF-1 or 25ng/mL GM-CSF and aliquoted to 4 petri dishes. After culture for a further 3 days, media was removed, fresh media added and adherent cells were scraped using a disposable scraper (Fisher-brand, 08-100-240). Cells were centrifuged at 300g for 5 minutes and resuspended for counting. $3x10^6$ BMMs/10cm petri dish were plated. These BMMs were cultured with either 1000U/mL CSF-1 in complete alpha-MEM for M-BMMs differentiation or 25ng/μL GM-CSF in complete alpha-MEM for GM-BMMs differentiation for a further 3-6 days until they became mature macrophages (Guc&Brownlie et al, accepted, Methods in Enzymology).

2.3. Animal models of metastatic breast cancer

$1x10^6$ E0771-LG2:FI≠4 or E0771-LG2:FI≠4-*Csf1rcKO* cells in 200µL cold PBS was injected into the tail vein of C57BL/6NCrl or *Csf1r*cKO mice. Mice were anaesthetized using isoflurane. The chest area of injected mice was shaved and depilated using a standard depilation cream followed by an intraperitoneal injection of luciferin (1.5mg/100µL in PBS, Gold Biotechnologies, LUCK). Animals were placed in PhotonIMAGER Optima machine (Biospace Lab) and bioluminescence imaging was carried out for 10-15 minutes to determine tumour load in the lungs. Mice were imaged at days 0, 1, 4, 7 10 and 14 to monitor tumour load and culled on day 10 or 14 for tissue (unless tumour load exceeded $1x10^5$ ph/s/cm^2/sr in which case animals were culled beforehand in compliance with animal protocols).

In some cases, macrophages were depleted as described in 2.1.3. Doxycycline was given to *Csf1r*cKO mice from Day 4 until the end of experiment to ensure that small foci were able to develop initially before macrophage depletion with doxycycline water being renewed every 2-3 days. The efficacy of macrophage depletion was quantified by flow cytometry (staining procedure, antibodies and gating strategy described in Section 2.5).

For adoptive transfer of NK cells, $2x10^5$ NK cells in 200µL were put into a round bottom 96-well plate (Thermofisher Scientific, 2202-02) with 1000U/mL IL-2 (Peprotech, 212-12) in MACS NK media (Miltenyi Biotec, 130112968) supplemented with 10% v/vFBS and MACS NK supplement as directed in manufacturer's instructions. Cells were cultured at 37°C, 5% v/v CO_2 for 48 hours. Cells were collected, washed, resuspended in PBS and injected via tail vein into mice ($1x10^6$/200µL).

2.4. Real-time *in vitro* Fluorescence Imaging

2.4.1. Plate Preparation and Culture of Cells

96-well glass bottom, black walled plates (NUNC, 165305) were coated with 30µL of Geltrex (Gibco, A1596-01) to allow cells to adhere. The plates were left for one hour at 37 degreesC prior to aspirating and adding cells/media in its place. E0771-LG2:Fl≠4-NLR containing a red nucleus were cultured beforehand in DMEM supplemented with 10% FBS and were not allowed to grow to more than 80% confluent by splitting every 2-3 days 1/10-1/20 dilution. NK cells were isolated and BMMs developed as described in 2.2.2 and 2.2.3, respectively. All cells were centrifuged at 300g for 5 mins and resuspended in alpha-MEM supplemented with 10% v/v FBS, 1% v/v penicillin/streptomycin (complete alpha-MEM). Complete alpha-MEM containing IL-2 (1000U/mL, Peprotech, 212-12) and fluorogenic caspase-3 substrate NucView 488 (2.5µg final, Biotum, 10402) was added to all wells. 1000 tumour cells, 1000 or 3000 BMMs, and 4000 NK cells were added to appropriate wells. All sample wells were in the middle 60 wells of the plate and made up to 100uL with additional alpha-MEM while empty wells were filled with 100µL PBS/well to maintain humidity to prevent evaporation. In some assays, blocking antibodies were used at concentrations listed in **Table 2.1.**

Table 2.1. Blocking antibodies used in cytotoxicity assays

Antibody	Company	Catalogue Number	Final Concentration ug/mL
IgG	BioLegend	401502	25
Ly49A	BioLegend	116803	25
Ly49C/I	BD	553273	25
H2-Db	BD	553600	25
H2-Kb	BioLegend	116502	25
TGF- β	BioLegend		2

2.4.2. Imaging Set-up

Plates were put into the Incucyte® live cell imaging system (Essen Bioscience) and Incucyte Zoom software (Essen Bioscience) was launched. Within the software, the position of the plate within the imaging system was selected. The software was instructed to take 4 non-overlapping images for every 3 hours using phase contrast, and fluorescence imaging (green: excitation 440nm, emission524nm. Red: 585nm excitation, 635nm emission). Plates were left to image for up 48-72 hours and data was stored automatically.

2.4.3. Image Processing and Analysis

When the time-course has finished, an image collection was prepared to train the software and make a 'processing definition' (2.4.4). Representative images, for example tumour only, BMMs only, NK only, as well as different combinations and timepoints were stored as an 'image collection'. A processing definition is a set of instructions given to the software in order to quantify each image. Using the image collection made previously, the software is trained to identify cells that are positive for the fluorescent marker versus background by manipulating different parameters set out in **Table 2.2**.

Analysis jobs are run on the collected data using the processing definition that has been set up for a particular set of experiments. Once finished, they contain quantified data from the images collected over the length of the experiment. It contains a wide range of information including the number of red cells, the number of green cells and the number of cells which have a red/green overlapping signal which can be exported into an excel sheet for further analysis by the user or configured into a graph by the Incucyte Zoom software.

Table 2.2. Processing definition for identification of dead tumour cells

Parameter	Explanation	Green (Apoptotic nuclei)	Red (E0771 nuclei)	Overlap (Red and Green)
Top Hat	Method of correcting uneven background fluorescence	On	On	
Radius (μM)	A slightly above average radius size is selected	12	12	
Threshold	The intensity of fluorescence that must be reached in order for an object to be 'real' and distinguish it from debris	0.5	0.2	
Edge Split	A scale at which you select the number that allows the software to delineate between one object, and many objects that are joined or close together. This looks for a reduction in intensity between two fluorescent objects and 'splits' them instead of counting them as one object	1	-7	
Area	Selection of minimum/ maximum area for objects	Minimum 100	Minimum 100	Minimum 120

2.5. Flow Cytometry

2.5.1. Lung Preparation and Dissociation

Mice were culled in CO_2 chamber. The femoral artery was cut and the chest was opened by cutting across the chest, just under the diaphragm and through the sternum up to the mouth. A 21G needle and syringe containing cold PBS was placed and injected into the right ventricle of the heart in order to flush the lungs of residual blood. The lungs were removed and placed in PBS on ice until ready to process.

Lungs were removed from PBS and blotted dry on tissue paper. The heart was removed, and lungs were cut into separate lobes before transferring into a petri dish on ice. Scissors were used to cut the lung up as until tissue pieces were less

than 1mm size. Using a single edge blade (Thermofisher Scientific, 119043ZS) lungs were minced into a consistency that resembles a viscous liquid.

Minced lungs were transferred to 15mL falcon tubes and 1.5mL/lung (tumour) or 1mL/lung (no tumour) of lung dissociation buffer (Miltenyi, 130095927) was added. Tubes were transferred to a thermomixer (Eppendorf ,thermomixer c) and kept at 37 degreesC and 600rpm. Cells were pelleted by centrifugation at 300g for 5 minutes and resuspended in 5mLs of 1X RBC lysis buffer (BioLegend, 420301) and left on ice for 5 minutes prior to adding 5mLs of flow cytometry buffer 2% v/v BSA PBS (Sigma, A1470 in PBS. The cell suspension was passed through a 40μM filter and centrifuged for 5 minutes at 300g and pelleted cells were resuspended in flow cytometry buffer prior to counting and staining (2.5.2)

2.5.2. Staining Protocol

Cells were resuspended at a concentration of $1x10^6$/100μL. 1μL of Fc Block (BD Pharmigen, 553142) was added per 100μL of cells/ buffer for 5-10 minutes on ice. Antibodies were added at appropriate concentrations (2.5.3.1, 2.5.4.2, 2.5.5.1) and left to incubate in the dark on ice for 30-60 minutes. 1mL of flow cytometry buffer was added to each tube and tubes were centrifuged at 300g for 5 minutes. Cells were resuspended in 300μL of flow cytometry buffer with/without 3μM DAPI (BioLegend, 422801).

2.5.3. MAM/RMAC Sorting

Lungs from tumour-bearing and non-tumour-bearing C57/Bl6 mice were isolated and processed and stained as described in 2.5.1 and 2.5.2 using antibodies listed in **Table 2.3**. Using the FACS Aria II (BD Biosciences) MAMS in the tumour bearing lung, and RMACs from non-tumour-bearing mice were isolated.

Table 2.3 Sorting antibodies

Antibody	Fluorophore	Company	Order No.
CD45	PerCPCy5.5	BioLegend	103130
Ly6C	APCCy7	BioLegend	128026
Ly6G	PE	BioLegend	127608
F4/80	AF647	BioRad	MCA497A647
CD11b	PECy7	BioLegend	101216
CD11c	BV650	BioLegend	117339

2.5.4. NK/BMM/MAM Flow Cytometry

In order to analyse MAMs and BMMs cells in suspension were counted and stained as described in 2.5.2 using antibodies listed in **Table 2.4.** In order to analyse NK cells, cells in suspension were counted and stained in the same way and using antibodies listed in Table 2.5 .Data was quantified using FlowJo software (FlowJo LLC, v10.5.3).

Table 2.4 Macrophage Characterisation Antibodies

Antibody	Fluorophore	Company	Order No.
CD45	Pacific Blue	BioLegend	103126
CD45	PerCPCy5.5	BioLegend	103130
F4/80	AF647	BioRad	MCA497A647
CD11b	BV605	BioLegend	101237
CD11c	BV650	BioLegend	117339
Ly6C	BV711	BioLegend	128037
Ly6G	BV510	BioLegend	127633
H2-Qa1	FITC	Novus	Nbp2-26649f
PDL1	PECy7	BioLegend	124313
PDL2	PE	BioLegend	107205
CD80	PE-Dazzle	BioLegend	400951
CD86	APCCy7	BioLegend	105029
H2-Kb	FITC	BioLegend	116505
H2-Db	PECy7	BioLegend	111515
H2-M3	AF594	BD Biosciences	551769
TGF- β	PE	BioLegend	141403

Table 2.5 NK characterization antibodies

Antibody	Fluorophore	Company	Order No.
CD45	PE-Dazzle	BioLegend	103145
CD3	Pacific Blue	BioLegend	100213
NK1.1	APCCy7	BioLegend	108724
F4/80	AF647	BioRad	MCA497A647
CD11b	BV605	BioLegend	101237
Ly6C	BV711	BioLegend	128037
Ly6G	BV510	BioLegend	127633
Ly49A	FITC	BioLegend	116805
Ly49C	PECy7	BioLegend	108209
NKG2A	PE	BioLegend	142803
CD27	PerCPCy5.5	BioLegend	123213
CD49b	AF700	eBiociences	56-5971-80
PD1	BV510	BioLegend	135241
CTLA4	BV605	BioLegend	106323
NKp46	PE	BioLegend	137603
NKG2D	FITC	BioLegend	115711
CD69	PECy7	BioLegend	104511
CD107a	PerCPCy5.5	BioLegend	121625
DNAM-1	APC	BioLegend	128809

2.6. Histological analysis

Lungs from Day 10 tumour bearing mice (Csf1rcKO, DOX+ and DOX-) were isolated and flushed as previously described (2.5.1.1) this time using 10% v/v Neutral buffered formalin (NBF) instead of PBS. Lungs were fixed in NBF for around 22 hours, followed by submersion in 70% v/v ethanol (EtOH). Slides were stained at CRUK Beatson Institutes histology facility by head of histology Colin Nixon for haematoxylin and eosin as well as NCR1 (RNAScope) and F4/80 (IHC).

2.6.1. *In Situ* Hybridisation

In situ-hybridisation detection for Mm-NCR1 (NK marker) and Mm-UBC (control) (Advanced Cell Diagnostics, Hayward, CA) mRNA was performed using RNAscope 2.5 LS (Brown) detection kit (Advanced Cell Diagnostics, Hayward, CA) on a Leica Bond Rx autostainer according to the manufacturer's instructions. Staining

was performed on 4um formalin fixed paraffin sections and placed in a 60 degreesC oven for 2 hours prior to staining.

2.6.2. Immunohistochemistry

IHC staining was performed on 4um formalin fixed paraffin embedded sections which had previously been ovened at 60ºC for 2 hours. 4um sections were stained for F4/80 (Abcam, UK) on the Leica Bond Rx autostainer. Sections were loaded onto the autostainer and underwent dewaxing and epitope retrieval on board using Enzyme 1 (Leica, UK) for 10 minutes at 37C. The sections were then stained using an Intense R kit (Leica UK) with F4/80 antibody used at a dilution of 1/200. After completion of staining sections were dehydrated through graded alcohols, taken through xylene and then mounted with a glass coverslip using DPX mountant for microscopy (CellPath, UK).

2.6.3. Image Processing and Analysis

Slides were stained at CRUK Beatson Institute's histology facility for haematoxylin and NCR1 using RNAScope as described and for haematoxylin and F4/80 using immunofluorescence. Stained slides were loaded into a Zeiss Axioscan.Z1 (Carl Zeiss, Oberkochen, Germany) and scanned using a 40x 0.95NA Plan-Apochromat objective lens in brightfield mode. Whole slide .czi images were imported into Definiens Tissue Studio 4.4.2 (Definiens AG, Munich, Germany) for processing and analysis. Tissue pieces on the slide images were segmented using automatic parameters and sub-tissue features (Lung Tissue, White space) were segmented using Tissue Studio's internal machine learning algorithms trained with parameters from 12 sub-regions of 2 images from each staining group (NCR-1 and F4/80). After automated detection of lung tissue and white/alveolar space, each image was put through manual quality control (QC) where artefacts and tumours were

manually drawn around to delineate tumour tissue from normal lung tissue. After QC, NCR-1 and F4-80 samples were split into separate analysis groups for nuclear detection and morphology filtration. Four NCR-1 and four F4/80 were used to train respective Tissue Studio internal machine learning algorithms for nuclear detection and morphologically filtered QC. Thresholds of positivity were then set for each stain type and data exported. This analysis was carried out by Dr Daniel Soong.

Chapter 3

Development and optimization of NK cell cytotoxicity assay

3.1. Aims

1. To isolate NK cells from the spleen of C57Bl/6 mice and determine their purity
2. To develop an *in vitro* assay that gives an accurate read-out of tumour cell apoptosis
3. To optimize the conditions for detecting NK cell-induced tumour cell apoptosis

3.2. Results

In order to determine NK cell cytotoxicity towards breast cancer cell lines we established an *in vitro* cytotoxicity assay. (**Figure 3.1** gives a general overview). Briefly, spleens isolated from C57Bl/6 mice were dissociated and NK cells were enriched through negative selection (Step 1). The isolated NK cells were cultured with E0771- LG2:Fl#4 mammary tumour cells expressing red fluorescent protein (E0771-LG2:Fl#4-NLR) in the presence of IL-2 that is required to maintain active NK cells. In this culture, fluorogenic caspase-3 substrate (NucView-488) was also added, which emits green fluorescence after cleavage by active caspase-3 and thereby labels apoptotic cells (Step 2). The cells were imaged by real-time fluorescence microscopy (IncuCyte Zoom) for 24-48 hours. The images were analyzed by IncuCyte software to quantify the number of red and green double positive nuclei that represent the apoptotic tumour cells within the assay (Step 3). In order to investigate the effects of macrophages on NK cell-induced cancer cell apoptosis, we optimized each step of the assay.

3.2.1. NK Cell Isolation and Purity Check (Step1)

To prepare effector cells for the cytotoxicity assay, NK cells were isolated from the spleen of C57Bl/6 mice and cultured *in vitro* (details of which are in Chapter 2). The purity of NK cells was determined by flow cytometry (**Figure 3.2**). **Figure 3.2A** shows the gating strategy to detect NK cells that are characterized as $CD45^{+}CD3^{-}NK1.1^{+}$.

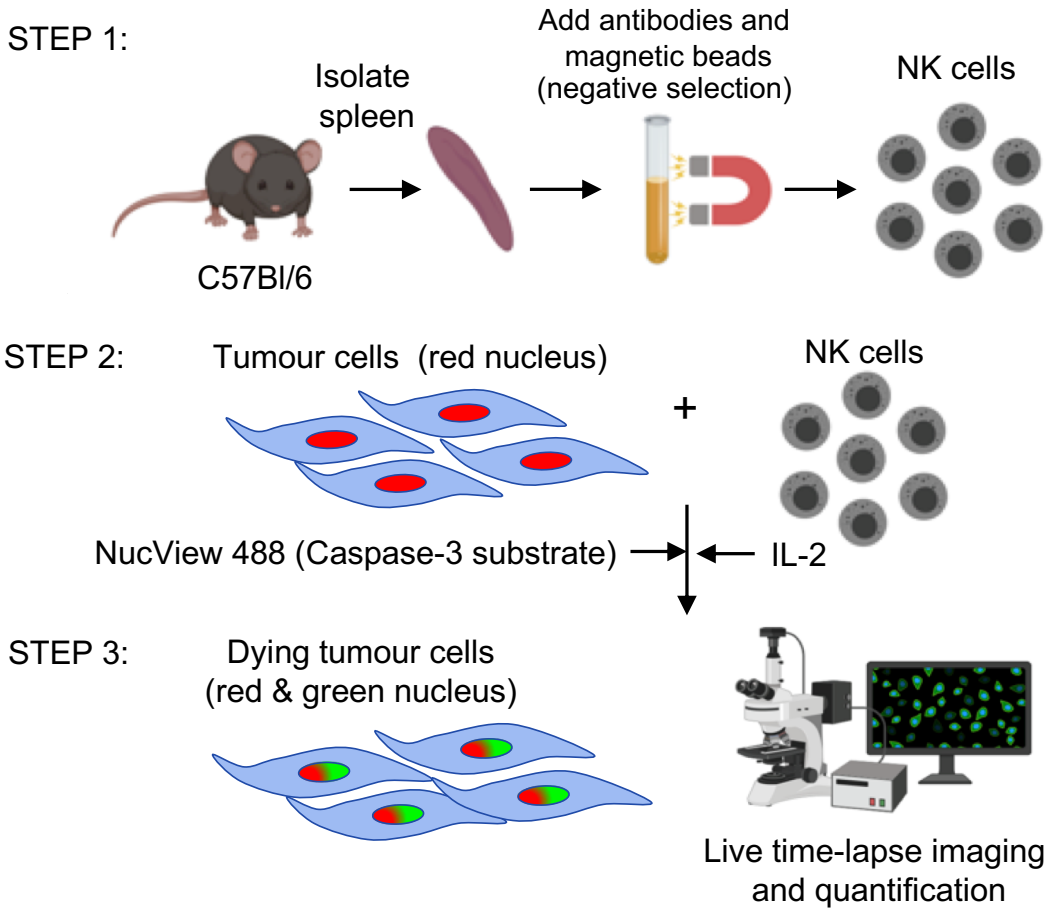

Figure 3.1. Outline of NK cell cytotoxicity assay. (STEP 1) The spleen from C57Bl/6 mice is smashed, and splenocytes are incubated with antibodies for non-NK cells and magnetic beads that bind to the antibodies. The non-NK cells are trapped in a magnetic field, whereas NK cells are eluted from the cell suspension. **(STEP2)** The isolated NK cells are cultured with tumour cells expressing red fluorescent protein (mKate) in the presence of IL-2 and an apoptosis marker, green fluorogenic caspase-3 substrate (NucView 488). **(STEP 3)** Cells are imaged over time by real-time fluorescence microscopy (IncuCyte) and apoptotic tumour cells expressing red and green fluorescence are enumerated.

Briefly, leukocytes were separated from cell debris and dead cells ($FSC^{high}SSC^{low}DAPI^{-}$), and single cells detected by FSC-A/FSC-H and SSC-A/SSC-H gate. These were separated into CD45 positive cells (total leukocytes). NK cells in the $CD45^{+}$ population were detected as $CD3^{-}NK1.1^{+}$. $CD3^{+}NK1.1^{-}$ and $CD3^{-}NK1.1^{-}$ populations were efficiently reduced by magnetic separation (after enrichment) compared to total splenocytes (before enrichment). Before NK cell isolation, NK cells

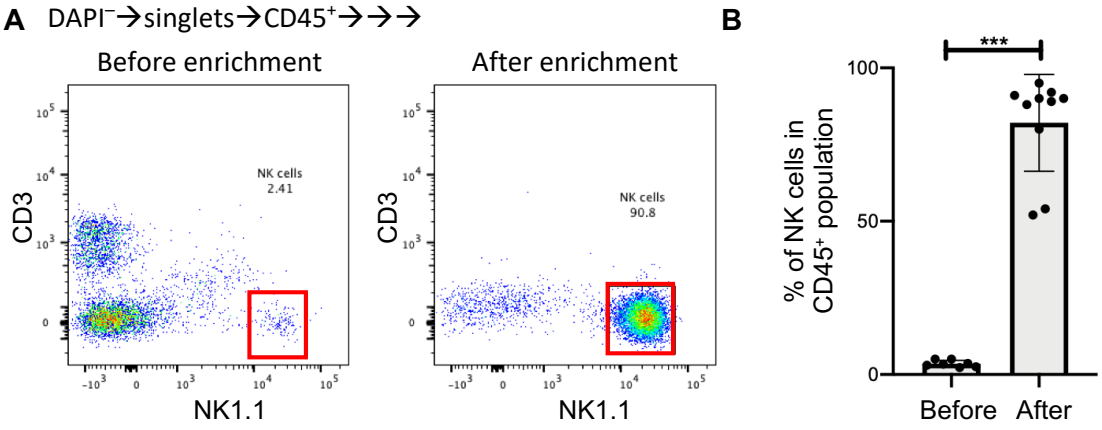

Figure 3.2 Efficacy of NK cell enrichment. (A) Representative dot plots showing NK cells ($DAPI^{-}CD45^{+}CD3^{-}NK1.1^{+}$) in the cell suspension before (left) and after (right) NK cell negative selection. **(B)** Percentage of NK cells within the $CD45^{+}$ population before and after NK cell enrichment (n=7-10, mean ± SD, ***p<0.001, Mann-Whitney test).

represented 3.5±1.1% of the total $CD45^{+}$ population. In contrast, the NK cell population after magnetic bead negative selection accounted for 82±15.8% of the total $CD45^{+}$ population (**Figure 3.2B**). Therefore, NK cells were successfully enriched from the spleen by this method, which can be used for future *in vitro* assays.

3.2.2.Establishment of an *in vitro* assay to Detect Tumour Cell Apoptosis(Step2)

In order to detect tumour cell apoptosis we established an *in vitro* assay using microscopy where apoptotic cells were detected by green fluorescence in their nuclei from a fluorogenic caspase-3 substrate (NucView488). To identify the target cancer cells, E0771-LG2:Fl#4 cells were manipulated to express red fluorescent protein (mKate) in their nuclei (E0771-LG2:Fl#4-NLR). We cultured E0771-LG2:Fl#4-NLR cells with or without staurosporine, a commonly used apoptosis inducer and imaged via fluorescence microscopy (IncuCyte). **Figure 3.3A** shows representative images of tumour cells cultured for 24 hours on their own (top row) or with staurosporine (bottom row) taken from the red and green channels as well as a phase contrast

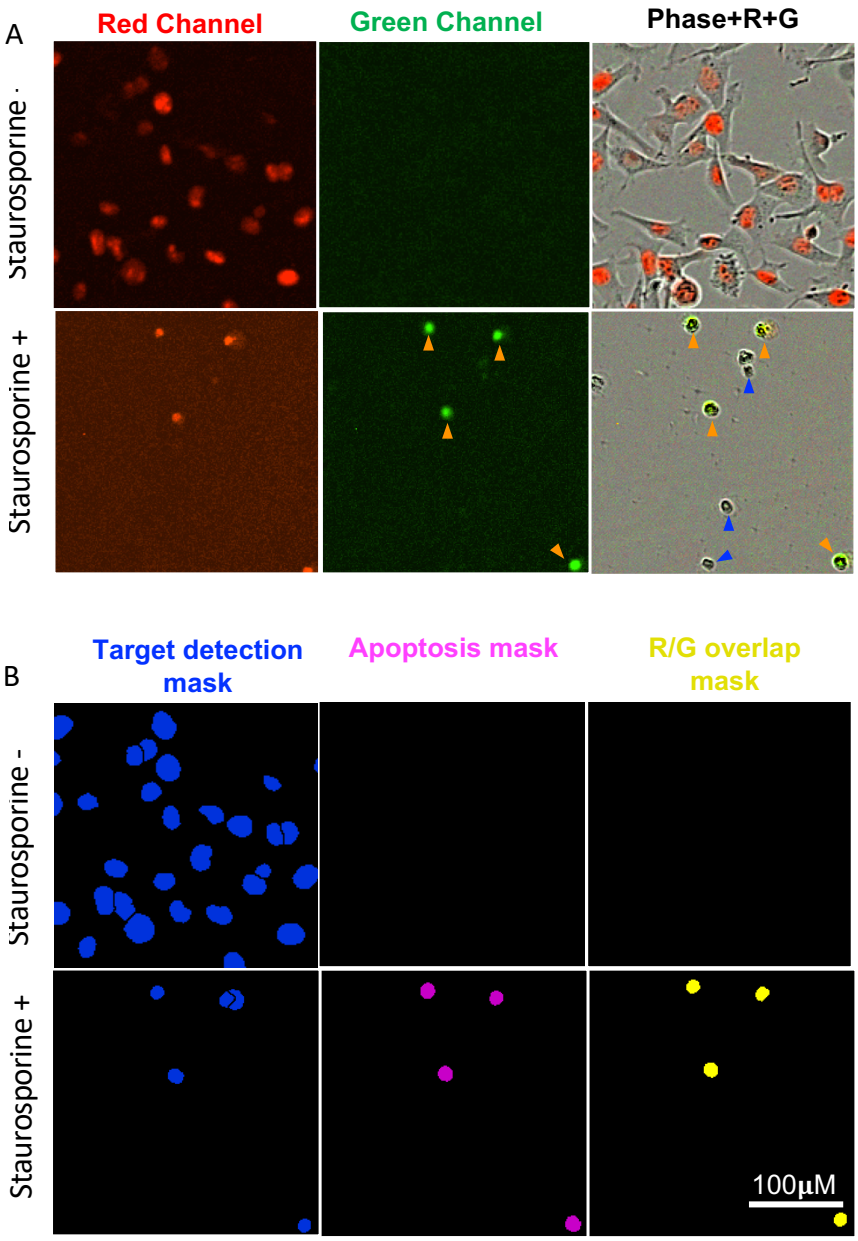

Figure 3.3 Establishment of *in vitro* assay to validate tumour cell apoptosis. (A) Representative images of E0771-LG2:Fl#4-NLR cells cultured without (top) or with 5uM staurosporine (bottom) for 24 hours. Panels show images in the red channel, green channel, and those merged with phase contrast image. Orange arrowheads show apoptotic tumour cells, blue arrowheads show cells that are completely dead and no longer going through active apoptosis. **(B)** Images shown in A applied with 'Masks' to detect apoptotic tumour cells. First, an analysis procedure to detect target cells shown in the red channel (left) was set up (target detection mask: blue). Second, an analysis procedure to detect apoptotic nuclei shown in the green channel (middle) was set up (apoptosis mask: pink). Last, an analysis procedure to detect apoptotic target cells expressing both red and green fluorescence (right) was set up (R/G overlap mask: yellow).

image merged with red and green channels (left to right). Orange arrows show a tumour cell undergoing apoptosis, which is positive for the green apoptotic nucleus marker (NucView-488) as well as the red tumour nucleus marker (Nuclight-Red). In order to quantify the number of apoptotic target cells, we set up several masks using the image analysis software, IncuCyte Zoom (**Figure 3.3B**). In the red channel image, we set up a mask to detect fluorescent nuclei within target cells (blue target detection mask) with the average radius of 12μM and area of at least 100μM. In the green channel image, we set up a mask to distinguish apoptotic target nuclei (pink apoptosis mask). Apoptotic target cells were detected via a Red/Green overlap mask where red fluorescent signal and green fluorescent signal were combined (Yellow R/G overlap mask). As shown in **Figure 3.3**, cancer cells treated with staurosporine are round in shape with red and green signals in their nuclei, and such apoptotic cells were picked up by the masks. On the other hand, dead cells that showed rounded cell shape but lost red or green signals (blue arrows) were not included in the Red/Green overlap mask. These data indicate that our analysis procedure enables selective detection of target cancer cells that are actively going through apoptosis.

3.2.3. Optimisation of NK Cell Cytotoxicity Assay(Step3)

To test the applicability of the above-mentioned assay system to detect NK cell-induced tumour cell apoptosis, we cultured E0771-LG2:Fl#4-NLR cells with NK cells at different Tumour:NK (Tu:NK) ratios, and imaged the cells after 24 hours of co-culture. We found that dying NK cells fluoresced green (white arrows) and it was necessary to remove this from the analysis. Therefore the apoptosis mask was modified to include a size restriction to ensure that only dying tumour cells (orange arrows) were included in the analysis (**Figure 3.4A**). Total (red nuclei) and apoptotic (red/green overlapping nuclei) target cell numbers were enumerated and the

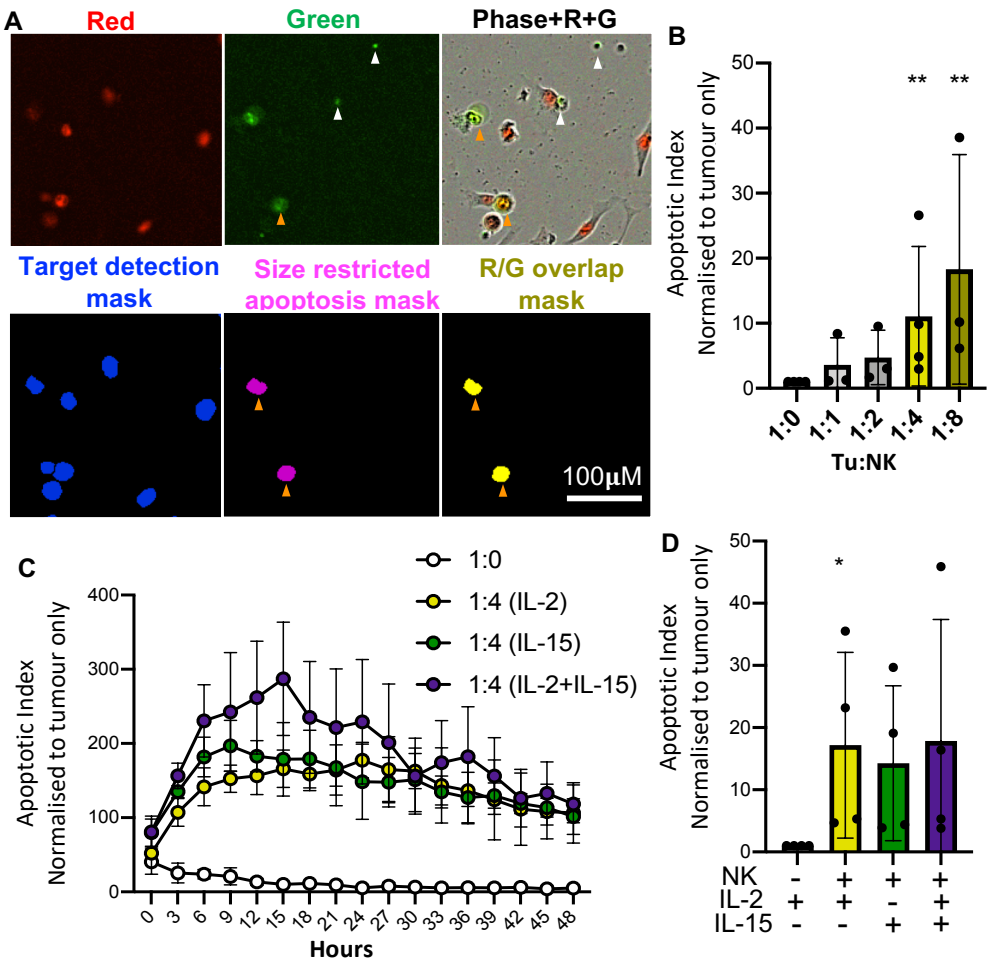

Figure 3.4 Optimisation of *in vitro* assay to detect NK cell-induced tumour cell apoptosis. (A) Representative images of E0771-LG2:Fl#4-NLR cells cultured with NK cells. Panels in top row show images in the red channel, green channel, and those merged with phase contrast image. Panels in bottom row show 'Masks' applied to images in A as described in Fig. 3.3. The analysis procedure to detect apoptotic nuclei (middle) was modified to distinguish target tumour cells and NK cells ('size restricted' apoptosis mask: pink). Orange arrowheads show apoptotic tumour cells, white arrowheads show apoptotic NK cells. **(B)** Apoptotic index of tumour cells at 24 hours after co-culture of E0771-LG2:Fl#4-NLR cells without or with NK cells at different Tu:NK ratios. The apoptotic index was calculated by dividing the number of Red/Green double positive cells/well by the number of total red cells/well and multiplying by 1000. **(C)** A graph showing representative changes in apoptotic index over time. E0771-LG2:Fl#4-NLR tumour cells were cultured on their own (1:0, white) or with NK cells at 1:4 of Tu:NK ratio in the presence of IL-2 (yellow), IL-15 (green), or their combination (purple). Data show mean±SD from technical triplicates. **(D)** Mean apoptotic index of tumour cells at 24 hours after co-culture in the same condition as that in C. (n=3-4 biological replicates, mean ± SD, *p<0.05, **p<0.01, Kruskall-Wallis with Dunn's post-

apoptotic index calculated where the number of apoptotic target cells were divided by the total number of target cells within the well. As shown in **Figure 3.4B**, we found a negligible level of spontaneous apoptosis of target cells in the absence of NK cells (Tu:NK=1:0). In contrast, the apoptotic index was significantly increased by co-culture with NK cells at the 1:4 and 1:8 Tu:NK ratios, suggesting that there has to be at least a 1:4 Tu:NK ratio to evaluate NK cell cytotoxicity within this assay. In this assay we added IL-2 in culture to maintain and activate NK cells. As well as IL-2, several studies have suggested that IL-15 also maintains and activates NK cells (Chapter 1.4.2). We thus co-cultured E0771-LG2:Fl#4-NLR cells with NK cells in the presence of IL-15 and/or IL-2 and imaged the cells over a 48-hour period. We found that co-culture with NK cells increased apoptotic index by 16 hours which plateaued by 24 hours in all conditions (**Figure 3.4B**). However, there was no significant difference in cytotoxicity between NK cells cultured with IL-2 and those cultured with IL-15 or the IL-2/IL-15 combination (**Figure 3.4C and D**).

3.3. Discussion

In order to validate the ability of NK cells to kill E0771 metastatic breast cancer cells, we optimized a protocol for NK isolation. It has been reported that enrichment of NK cells by negative selection enables a high purity enrichment in a shorter time compared to other methods to isolate NK cells (e.g., positive selection kits) that show higher levels of contamination, especially of $F4/80^+$ cells[197]. Another advantage to using negative selection is that the NK cells are essentially 'untouched', i.e., NK cells do not have any antibodies attached that may alter the phenotype or function of isolated cells. To this end, we tested negative selection to enrich NK cells from total splenocytes for our assay. By this method we could isolate NK cells that accounted for 82±15.8% of total cells whereas NK cells were just 3.5±1.1% of total splenocytes before enrichment. Since contaminating cells were negative for CD3 these cells

should not be T cells and might be neutrophils and/or B cells given their abundance in the spleen. It has been reported that B cells can directly kill tumour cells via a Fas/FasL mechanism[198]. However, our previous data indicates that activation of Fas signaling by NK cells does not induce apoptosis in E0771 cells[64], suggesting a minor contribution of B cells to cancer cell apoptosis *in vitro*. Furthermore, most granulocytes cannot survive *in vitro* for more than 24 hours. It is also reported that *in vitro* culture with IL-2 can increase the purity, viability and activation of NK cells[197]. These data suggest that a small level of contamination may not affect the evaluation of cancer cell apoptosis induced by enriched NK cells.

In order to detect NK cell-mediated tumour cell apoptosis, several methods have been established. For example, chromium release from target cells has been commonly used to determine NK cell cytotoxicity. However, this method has a low level of sensitivity and a high level of variation, and uses radioactive isotopes raising disposal concerns[196]. Flow cytometry has also been commonly used to detect apoptotic target cells since this method is advantageous to detect other parameters such as target cell death (e.g., propidium iodide) and effector cell activation status (e.g., CD69 expression) in addition to the apoptosis in target cells[199]. However, this method requires higher numbers of cells and the detachment of adherent target cells that can bias the results. Compared to these standard methods, our *in vitro* assay system is more advantageous. For example, our assay requires a very low cell number for adequate apoptosis detection (i.e., 1,000 target tumour cells: 4,000 effector NK cells/well). Our assay using the IncuCyte also enables to detect real-time apoptosis in the adherent cells with high sensitivity, which is essential to determine the time point when apoptotic cell number reaches its maximum and that is suitable to evaluate the effects of suppressor cells on the NK cell-induced cancer cell apoptosis.

Using this novel assay system, we could detect tumour cell apoptosis induced by NK cells cultured with IL-2 and/or IL-15 that have been shown to promote NK cell activation and proliferation (Chapter 1.4.2). Although it has been reported that IL-15 can stimulate NK cells at 50-100 times less concentrations of IL-2[200], we did not find a significant difference between apoptosis induction by NK cells cultured with IL-2, IL-15 or IL-2 in combination with IL-15. It might be possible that the concentration of IL-15 was too high in our assay since NK cells are known to be overstimulated and exhausted with continuous cytokine treatment[201]. However, it is unlikely that NK cells in our conditions became exhausted, as cytotoxicity of NK cells treated by IL-2 combined with IL-15 was comparable with that of NK cells stimulated with IL-2 alone. We also compared a time point when NK cell cytotoxicity reaches its maximum and found that NK cells cultured with IL-2 or IL-15 both increase cancer cell apoptosis by 12 hours after co-culture and plateau after 24 hours. Although we could not find clear differences between IL-2 and IL-15 in tumour killing ability of NK cells, tumor cell apoptosis at 24 hours was statistically significant only in the presence of IL-2 stimulated NK cells, suggesting this condition is more stable. We thus decided to culture NK cells with IL-2 and detect cancer cell apoptosis after 24 hours post-co-culture in the current project.

In terms of the target to effector (Tu:NK) ratio, we decided to use 1:4 for the rest of the experiments as this ratio gave a significant increase in apoptosis which was not further enhanced by the addition of more NK cells. Importantly, this ratio is lower than that used in previous studies that determine tumour cell apoptosis by chromium release assay or flow cytometry (1:5 to 1:100)[195,202]. Therefore, our *in vitro* assay seems to be more sensitive compared to standard methods although it is also possible that our target cells (E0771-LG:Fl#4 mouse mammary tumour cells) are more susceptible to NK cells.

It is also important to note that different NK cell preparations yielded large variations in terms of tumour cell killing ability. This did not correlate with the age of the mice, time taken from isolation of spleen to the enrichment of NK cells, different IL-2 stocks/ storage conditions or the addition of double IL-2 concentration (data not shown). We are not sure why this is the case and this causes problems in terms of data analysis further down the line as even with normalization results were still very varied. However, it is even more important to note that despite this variation, the same trends were always seen here and further on in the thesis.

Chapter 4

Effects of macrophages on NK cell cytotoxicity towards tumour cells

4.1. Aims

1. Determine whether MAMs isolated from metastatic breast tumours in the lung suppress NK cell cytotoxicity *in vitro*
2. Develop and characterize bone marrow derived macrophages as a model of MAMs
3. Investigate the effects of bone marrow-derived macrophages on NK cell cytotoxicity

4.2. Results

4.2.1. MAM Suppression of NK Cell Cytotoxicity *in vitro*

Using the *in vitro* NK cell cytotoxicity assay, we investigated the effects of MAMs from metastatic tumours on NK cell cytotoxicity. C57Bl/6 mice were injected via tail vein with $1x10^6$ of E0771-LG2:Fl#4 cancer cells (**Figure 4.1A**), which develop tumours in the lung by 14 days as indicated by bioluminescence from luciferase expressing cancer cells (Chapter 2.3.1). On day 14 after tumour injection, lungs with metastatic tumours were dissociated, and metastasis-associated macrophages (MAMs; $F480^+CD11b^+Ly6C^-$) and alveolar resident macrophages (RMACs; $F4/80^+CD11b^-$ $Ly6C^-CD11c^+$) were isolated by FACS (**Figure 4.1B**). The sorted cells were then co-cultured with E0771-LG2:Fl#4-NLR tumour cells and NK cells at a ratio of 1:3:4 (Tumour:Macrophage:NK) (**Figure 4.2A**), and the number of apoptotic tumour cells were determined. We found a tendency that MAMs reduced NK cell cytotoxicity towards tumour cells at 24 hours (**Figure 4.2B**), whereas we did not see this tendency when RMACs were added to the co-culture. These experiments must be repeated and statistics carried out to confirm these results.

Figure 4.1

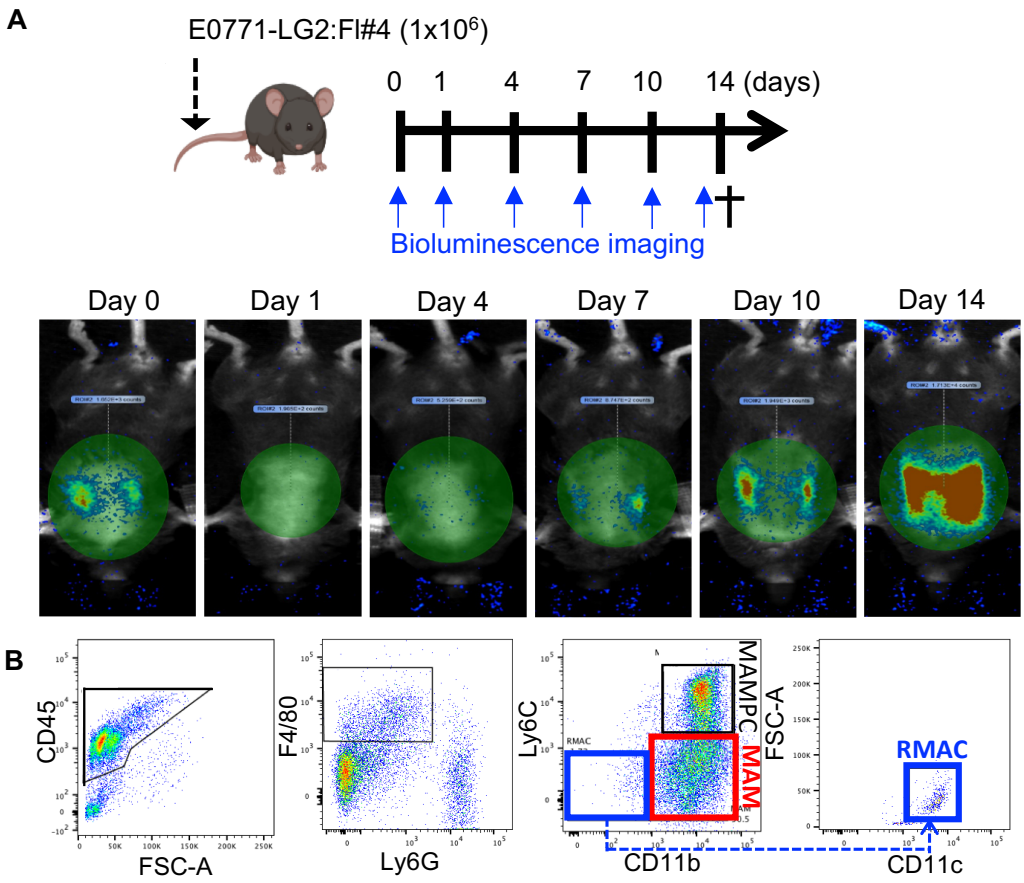

Figure 4.2

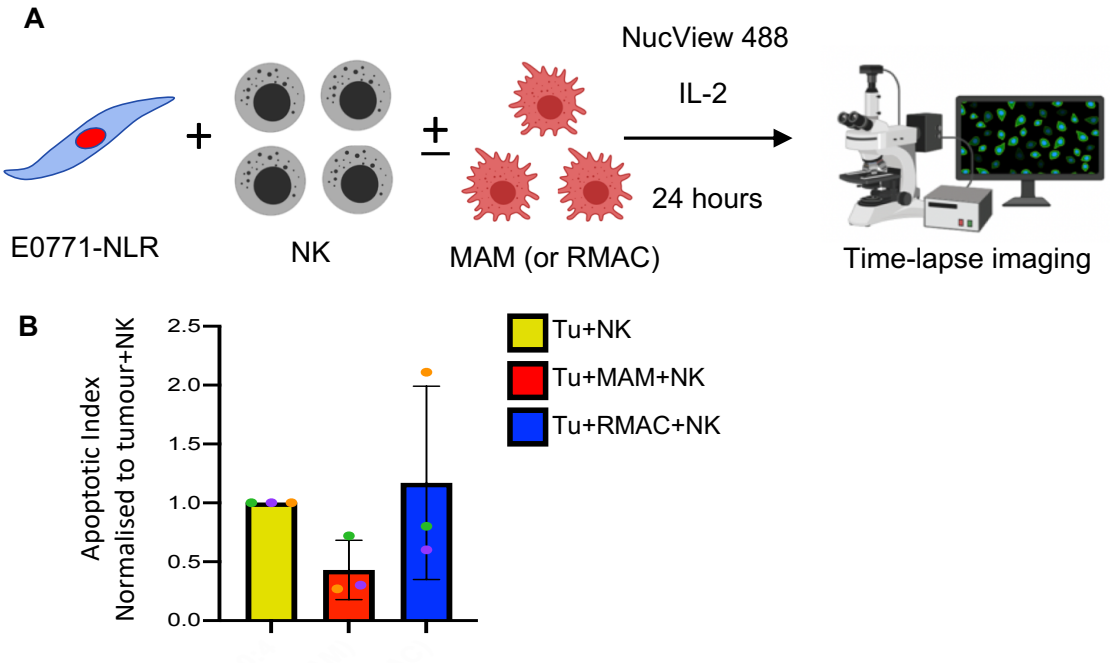

Figure 4.1 Isolation of macrophages from the metastatic lung in an animal model of breast cancer metastasis. (A) Representative images showing tumour loads in the lung at days 0, 1, 4, 7, 10 and 14 after tumour injection. A schematic overview of the metastasis model is also shown on top. **(B)** Representative dot plots showing a gating strategy to detect metastasis-associated macrophages (MAM: F4/80^{+}CD11b^{high}Ly6C^{low}), MAM progenitors (MAMPC: F4/80^{+}CD11b^{high}Ly6C^{high}), and alveolar resident macrophages (RMACs: F4/80^{+}CD11b^{low}Ly6C^{low}CD11c^{high}) within the tumour bearing lung.

Figure 4.2. Effects of macrophages from the metastatic lung on NK cell cytotoxicity *in vitro.* (A) A scheme showing outline of the assay. E0771-LG2:Fl#4 tumour cells and NK cells from the normal spleen were cultured with MAMs or RMACs sorted from the metastatic lung along with NucView-488 and IL-2. Tumour cell apoptosis was detected by the time-lapse fluorescence microscopy. **(B)** Apoptotic index of tumour cells cultured with NK cells without (yellow) or with MAMs (red) or RMACs (blue) (n=3 from biological replicates, mean ± SD).

4.2.2. Development of Macrophage Models

In order to investigate the mechanisms by which macrophages suppress NK cell cytotoxicity *in vitro*, we utilized bone marrow-derived macrophage (BMM) models, instead of MAMs from the metastatic lungs, given their unlimited availability. It has been reported that BMMs cultured with M-CSF bear some resemblance to TAMs[203] and are more anti-inflammatory/pro-tumour due to a higher production of anti-inflammatory cytokines such as IL-10 and a lack of production of more pro-inflammatory cytokines such as TNFα and IL-23 whereas those cultured with GM-CSF are more pro-inflammatory/anti-tumour with the opposite cytokine production profile[204]. Based on these reports, it was hypothesized that these two distinct macrophage populations might possess different effects on NK cell functions, and thereby be good models to identify NK-suppressive mechanisms.

Thus, M-BMMs and GM-BMMs were prepared by a well-established method (**Figure 4.3A**)[203,205,206], and were characterised. As shown in **Figure 4.3B**, the majority of cells cultured with the cytokines expressed F4/80 (95±4.2% and 73±7.7% in

Figure 4.3

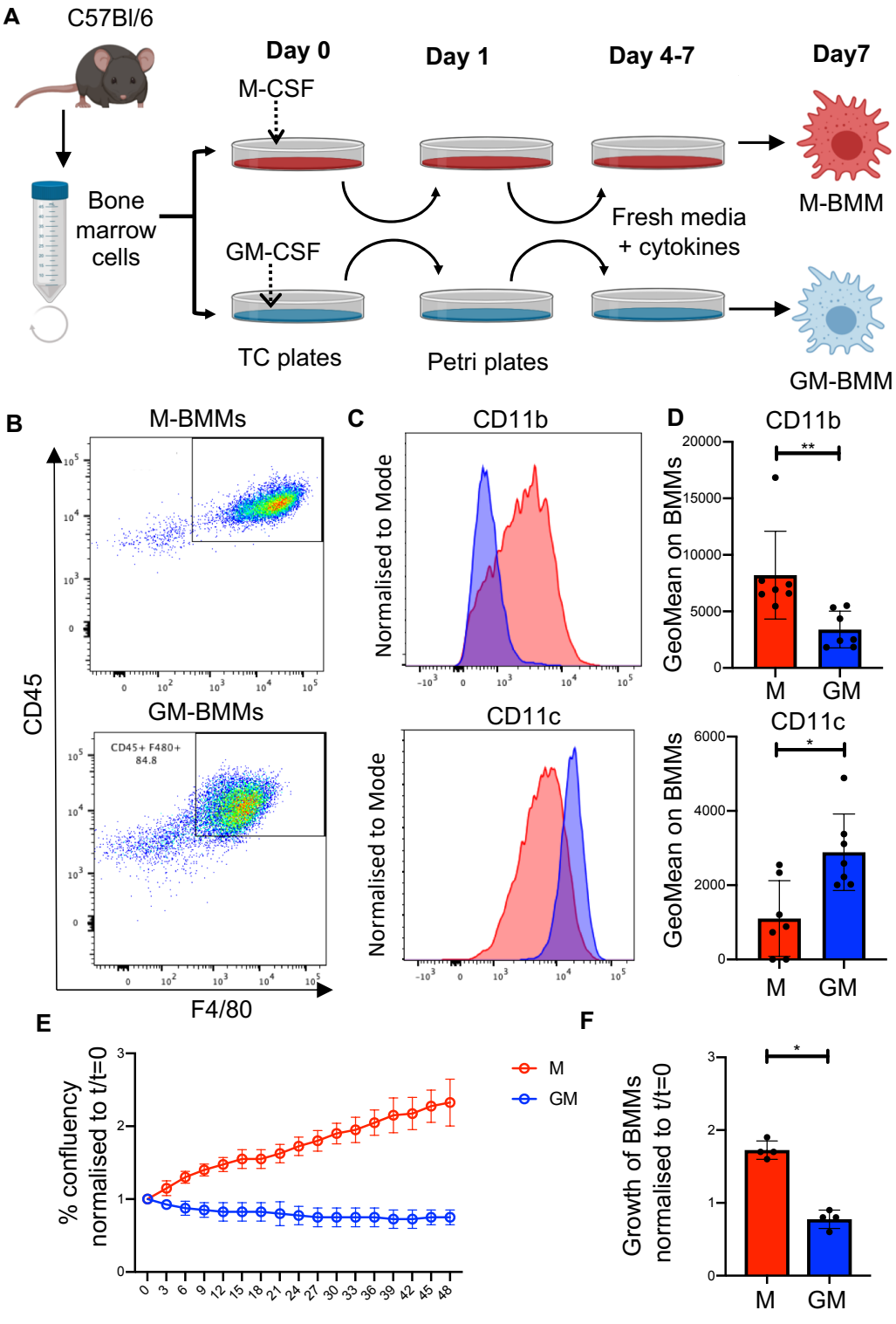
A
C57Bl/6
Day 0
Day 1
Day 4-7
Day7
M-CSF
Bone marrow cells
Fresh media + cytokines
M-BMM
GM-CSF
TC plates
Petri plates
GM-BMM
B
M-BMMs
GM-BMMs
CD45+ F480+ 84.8
CD45
F4/80
C
CD11b
CD11c
Normalised to Mode
D
CD11b
CD11c
GeoMean on BMMs
M
GM
E
% confluency normalised to t/t=0
F
Growth of BMMs normalised to t/t=0

Figure 4.3. Preparation of bone marrow-derived macrophages (BMMs) with different phenotypes. (A) A scheme showing a protocol to prepare BMMs. Bone marrow cells are flushed from the femur and tibia of C57Bl/6 mice and are collected by centrifugation. The bone marrow is resuspended with either M-CSF or GM-CSF and cultured for 24 hours in a tissue culture (TC) plate. After 24 hours the floating cells are transferred to petri plates and cultured for additional 3 days. On day 4 adherent cells were given fresh media including cytokines. On day 7 adherent cells are used as mature macrophages called M-BMMs (M-CSF treated BMMs) and GM-BMMs (GM-CSF treated BMMs). **(B)** Representative dot plots showing expression of CD45 and F4/80 on M-BMMs (top) and GM-BMMs (bottom). Note: both BMMs are characterized as $CD45^{+}F4/80^{+}$. **(C)** Representative histograms showing expression of CD11b (top) and CD11c (bottom) on M-BMMs (red) or GM-BMMs (blue) within the $CD45^{+}F4/80^{+}$ gate **(D)** Geometric mean fluorescence intensities of CD11b (top) and CD11c (bottom) on M-BMMs (red) and GM-BMMs (blue) (n=7 biological replicates, mean ± SD, *p<0.05, **p<0.01, Mann-Whitney test). **(E)** Changes in confluency of M-BMMs (red) and GM-BMMs (blue) over 24 hours (n=4 biological replicates, mean±SD). Data show fold change relative to time 0 (t=0). **(F)** Growth rate of M-BMMs and GM-BMMs at 48 hours after the culture (n=4 biological replicates, mean±SD, *p<0.05 Mann-Whitney).

M-BMMs and GM-BMMs respectively), indicating that the bone marrow cells become mature macrophage-like cells during this time period. We also found that M-BMMs expressed significantly higher levels of CD11b compared to GM-BMMs, which in turn showed significantly higher levels of CD11c than M-BMMs (**Figure 4.3C and D**). We also validated the growth rate of M-BMMs and GM-BMMs over 48 hours and found that M-BMMs proliferated faster than GM-BMMs (**Figure 4.3E**). The confluency of GM-BMMs at 24 hours is around half of that of M-BMMs at the same time point (**Figure 4.3F**). These results indicate that this method enables preparation of two distinct macrophage subtypes from the bone marrow (potential problems associated with the difference in proliferation rate is addressed in the next section).

4.2.3. Effects of M-BMMs and GM-BMMs on NK Cytotoxicity Toward Tumour cells

We then investigated whether the two distinct macrophage populations had different capabilities to suppress NK cell cytotoxicity. To this end, we co-cultured tumour cells, each macrophage population, and NK cells at the ratio of 1: 3: 4 (Tumour: BMM: NK). As shown in **Figure 4.4A**, the apoptotic index was significantly reduced in the presence of M-BMMs compared to that without BMMs (0.5±0.2 relative to no-BMM control). In contrast, the apoptotic index of cancer cells cultured with GM-BMMs remained high and no statistical significance was found compared to control (0.9±0.42 relative to no-BMM control), suggesting that the suppression of NK cells is specific for a distinct macrophage population (i.e., M-BMM). However, it is possible that this

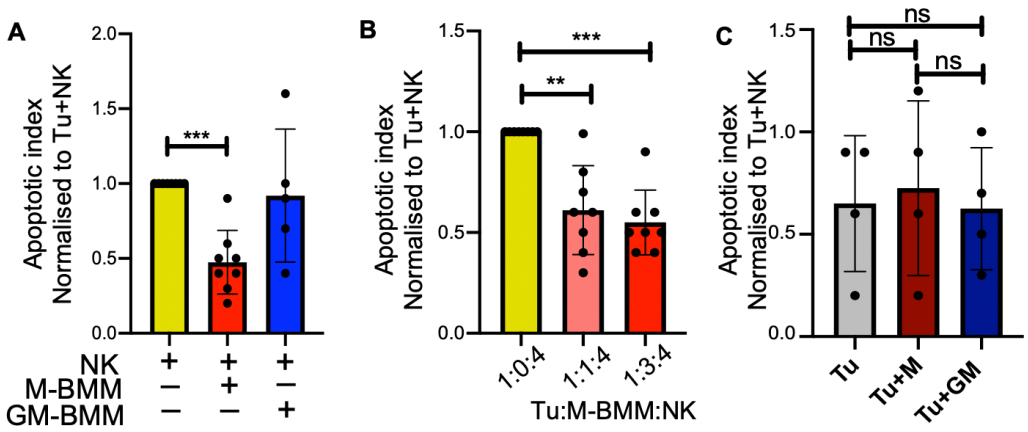

Figure 4.4. Effects of M-BMMs and GM-BMMs on NK cell cytotoxicity *in vitro.* **(A)** Apoptotic index of tumour cells cultured with NK cells in the absence (yellow) or presence of M-BMMs (red) or GM-BMMs (blue) at a tumour:BMM:NK ratio of 1:3:4. Data are shown as fold-change relative to Tu+NK only. (n=5-8 biological replicates, mean±SD, ***p<0.001, Kruskall-Wallis with Dunn's post-test). **(B)** Apoptotic index of tumour cells cultured with NK cells without (yellow) or with different ratio of M-BMMs (tumour:BMM:NK = 1:1:4 (pale red) and 1:3:4 (bright red)) Data are shown as fold-change relative to Tu+NK only. (n=8 biological replicates, mean±SD, **p<0.01, ***p<0.001, Kruskall-Wallis with Dunn's post-test). (C) Apoptotic index of tumour cells cultured on their own (grey), with M-BMMs (dark red) or with GM-BMMs (dark blue) (n=4 biological replicates, mean±SD, ns=not significant, Kruskall-Wallis with Dunn's post-test).

difference might be due to a difference in growth rate between M- and GM-BMMs (**Figure 4.3F**). We thus investigated whether a lower number of M-BMMs could also suppress NK cell cytotoxicity, and found that M-BMMs were still able to suppress NK cell cytotoxicity towards tumour cells even at 1:1:4 of Tumour:M-BMM:NK ratio (**Figure 4.4B**), which was comparable with 1:3:4 ratio. Therefore, it is unlikely that the difference in the NK suppressive effect was due to the increase in growth of M-BMMs compared to GM-BMMs. These results indicate that M-BMMs but not GM-BMMs suppress NK cell cytotoxicity towards the E0771 breast cancer cell line, however experiments involving GM-BMMs must be repeated to confirm this conclusion as their effects on NK cells were variable compared to M-BMMs. To confirm that macrophages did not cause apoptosis of tumour cells we co-cultured tumour cells with M- or GM-BMMs. There was no significant increase in apoptosis compared to the tumour only control.

4.3. Discussion

Studies in patients with Hodgkin's lymphoma and diffuse large B cell lymphoma showed that TAM-like monocytes from patient blood reduced NK cell activation *in vitro*[207]. In human colorectal cancer, TAMs together with cancer-associated fibroblasts can suppress NK cell cytotoxicity towards colorectal adenocarcinoma cells *in vitro*[208]. It is also reported that macrophages in primary tumours can suppress the maturation, activation and cytotoxic capabilities of NK cells in mouse models of primary breast cancer[209]. These studies suggest pivotal roles of macrophages in NK cell suppression in the primary tumours. However, the effects of macrophages in metastatic tumours have not been identified. Here, we have shown that MAMs from metastatic mammary tumours can suppress NK cell-induced tumour cell apoptosis *in vitro*. To our knowledge, this is the first study showing that MAMs in metastatic breast cancer impair NK cell cytotoxic function. Interestingly, we have reported that MAMs isolated

from pulmonary metastases in the same mouse model can also suppress cytotoxicity of $CD8^+$ T cells *in vitro*[31], suggesting that MAMs are immune suppressive cells in the metastatic tumour microenvironment. On the other hand, our previous data shows that RMACs can also suppress cytotoxicity of T cells to some extent although their suppressive effects were lower than MAMs[31]. Interestingly, a study using 4T1 mouse mammary tumour cells has shown that alveolar macrophages can suppress anti-tumour T cell responses in the lung of tumour-injected mice and thereby promote tumour cell accumulation in this organ[210]. However, in another mouse model of pulmonary metastasis using Met-1 mouse mammary tumour cells, depletion of RMACs does not affect metastasis formation[61]. Furthermore, RMACs locate in the luminal surface of the alveolar spaces rather than the interstitium[211] where metastatic lung tumours are established, and the number of RMACs in the metastatic lung is lower than that of MAMs in mouse models using E0771-LG2:Fl#4 tumour cells[31]. This may suggest that the contribution of RMACs to the NK cell suppression is minor in our model however staining of MAMs and RMACs within tumours will give more indication about the exact location of these cells within tumours. In the experimental pulmonary metastasis models using E0771-LG2:Fl#4 tumour cells, MAMs are recruited to and accumulate in the metastatic site and make contact with metastasized cancer cells, which promotes the metastatic tumour growth[30,63]. It is therefore possible that the accumulation of MAMs in the tumour creates an immune suppressive tumour microenvironment at the metastatic site and thereby helps the outgrowth of metastasized cancer cells.

It has been reported that BMMs cultured with M-CSF (also known as CSF1) are a 'pro-tumour' type of macrophage. For example, M-BMMs can enhance tumour cell migration through an endothelial monolayer in an *in vitro* trans-endothelial migration assay[63]. Furthermore, our recent data demonstrated that M-BMMs can secrete hepatocyte growth factor (HGF) which contributed to the growth and survival

of metastatic mammary tumour cells[64]. In addition to their phenotype ($F4/80^{+}CD11b^{high}CD11c^{low}$), these pro-metastatic functions of M-BMMs represent MAMs in metastatic tumours. In this chapter, we demonstrated that M-BMMs as well as MAMs suppress NK cell cytotoxicity, which suggest that M-BMMs resemble MAMs not only in their pro-tumour ability but also in their immune suppressive functions. We also investigated whether human iPSC-derived and THP-1 derived macrophages could suppress cytotoxicity of the human NK-92 cell line towards human breast cancer cell lines. We found here that these macrophages also suppress NK cell cytotoxicity, therefore this is not a murine-only phenomenon (data not shown).

Interestingly, GM-BMMs did not significantly change the NK cell induced tumour cell apoptosis. Although GM-CSF has been used for differentiation of bone marrow cells into dendritic cells (DCs)[212], recent studies demonstrate that bone marrow cells cultured with GM-CSF have both macrophage and DC properties[203,206]. A recent transcriptomic study also showed that bone marrow cells cultured with GM-CSF were more closely related to macrophages than DCs[206]. Furthermore, several reports have demonstrated that a more 'pro-inflammatory' type of macrophage can be developed using the growth factor GM-CSF (also known as CSF2). These reports showed that GM-derived populations expressed more genes involved in inflammatory responses and also expressed higher levels of pro-inflammatory cytokines versus anti-inflammatory cytokines compared to M-BMMs with similar findings in humans too[204]. This is in line with our findings as these BMMs did not suppress NK celsl within our assay. As well as this, M-BMMs have also been described to be similar to TAMs compared to GM-BMMs which the authors suggest are more tumouricidal based on gene-expression prolifing[203]. This is in keeping with our results which show they suppress NK cells and are therefore more 'pro-tumour' as described.

Collectively, our data suggest that suppression of NK cell function is a specific characteristic of a distinct pro-tumour macrophage population. Our results also

indicate that M-BMMs resemble an immune suppressive phenotype of MAMs and can be utilized to investigate their NK cell suppression mechanisms.

Chapter 5

Mechanisms behind macrophage mediated NK cell suppression: Effects on NK cell inhibitory ligands expressed by macrophages

5.1. Aims

1. To investigate whether M-BMM mediated NK cell suppression depends on cell contact or secreted factors.
2. To investigate expression of NK cell inhibitory ligands on M-BMM and GM-BMMs as well as effects of cancer cells on their expression
3. To determine whether MAMs in the metastatic lung express NK cell inhibitory ligands
4. To investigate the involvement of key NK regulatory ligands/receptors in the macrophage-mediated NK cell suppression
5. To investigate the involvement of membrane bound TGF-β in macrophage-mediated NK cell suppression

5.2. Results

5.2.1. Effects of M-BMMs on NK Cell Cytotoxicity under Non-Contact Conditions

To determine whether macrophage-mediated NK cell suppression is caused by secreted factors or cell-to-cell contact, we performed the NK cell cytotoxicity assay using transwell chambers (**Figure 5.1A**). Briefly, M-BMMs were cultured within the upper chamber of a transwell with a permeable membrane, which separate macrophages from E0771-LG2:Fl#4-NLR and NK cells that were co-cultured in the bottom chamber (non-contact condition). As a control, M-BMMs were cultured together with tumour and NK cells within the bottom chamber (contact condition). Consistent with our previous results, the apoptotic index was reduced to around 50% in the contact condition compared to the non-BMM (tumour and NK cell only) control. In contrast, M-BMMs cultured in the non-contact condition did not reduce the NK cell-induced tumour cell apoptosis (around 90% compared to the control), which was significantly higher than that in the contact condition (**Figure 5.1B**). These results

indicate that macrophages require cell-to-cell contact in order to exert their full suppressive effects on NK cells.

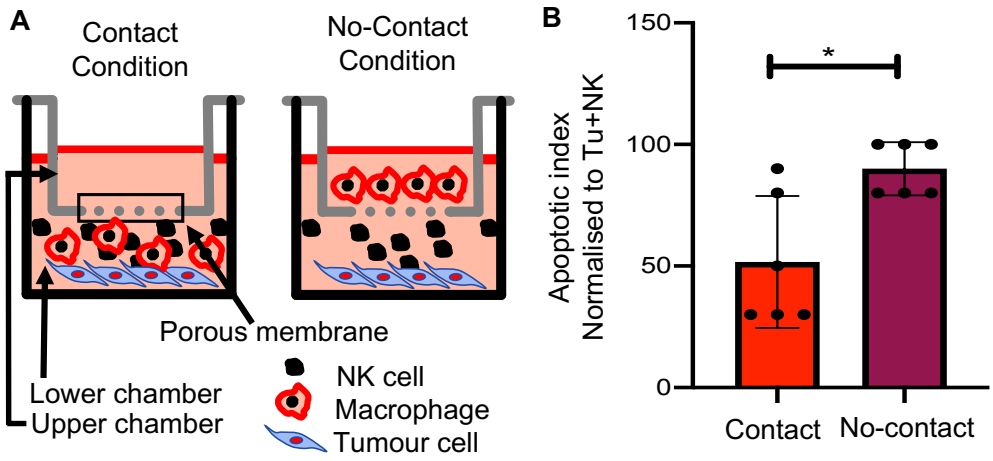

Figure 5.1. M-BMM mediated NK cell suppression under cell-to-cell contact or non-contact conditions. (A) Schematic showing the overview of the experiment. In the no-contact condition, tumour cells and NK cells cultured in the lower chamber are separated from M-BMMs by a porous membrane (right), whereas all three cell types are cultured together in the lower chamber in the contact condition (left) **(B)** Apoptotic index of tumour cells cultured in the conditions shown in A (n=6 biological replicates, mean±SD, *p<0.05 Mann-Whitney test).

5.2.2. Expression of NK inhibitory ligands on BMMs and tumour cells

It has been reported that NK cell functions are inhibited by several ligands on suppressor cells that bind to their cognate receptors on NK cells. For example, inhibitory ligands such as H2-Kb and H2-Db bind to Ly49C/I on NK cells and transmit inhibitory signals to NK cells. Likewise, H2-Qa1 and H2-M3 negatively regulate NK cells upon binding to NKG2A and Ly49A on NK cells respectively (Chapter 1.3). It is also reported that binding of PD-L1 and PD-L2 to their receptor PD-1 and binding of CD80 and CD86 to CTLA-4 can lead to the reduction of NK cell activity (Chapter 1.3). Based on the results from **Figure 5.1B**, we hypothesised that M-BMMs (NK suppressive) expressed a higher level of such inhibitory ligands on their surface

compared to GM-BMMs (NK non-suppressive). To investigate this hypothesis, we cultured M-BMMs and GM-BMMs in the presence or absence of tumour cells for 48 hours and determined levels of NK inhibitory ligands on the macrophages as well as tumour cells using flow cytometry. BMMs (red gate) were distinguished from cancer cells (grey gate) based on their high expression of CD45 and F4/80 (**Figure 5.2A**). We found that expression of H2-Kb, H2-Db and CD86 was significantly higher in M-BMMs compared to GM-BMMs (**Figure 5.2B**), suggesting the involvement of these molecules in NK cell suppression induced by M-BMMs. Interestingly, levels of H2-Qa1 and PD-L1 in M-BMMs were increased by the co-culture with tumour cells, whereas we did not find significant differences compared to GM-BMMs. Levels of PD-L2 and CD80 were significantly higher in GM-BMMs rather than M-BMMs in monocultures and were not changed by the co-culture with tumour cells. We could not investigate the expression of H2-M3 in BMMs since a reliable antibody for this molecule is not available. We also investigated whether BMMs enhance the expression of inhibitory ligands in tumour cells, as it can be another mechanism behind macrophage-mediated NK cell suppression (**Figure 5.2C**). Interestingly, expression of H2-Kb and PD-L1 in E0771 cells was significantly increased by co-culture with M-BMMs but not GM-BMMs. Levels of CD80 on tumour cells was also significantly increased by co-culture with M-BMMs but it was also increased by GM-BMMs. We did not find significant increases in the expression of H2-Db, H2-Qa1, PD-L2, or CD86 by co-culture with M-BMMs. Given these data, it is possible that M-BMMs increase expression of some NK inhibitory ligands on target cancer cells and thereby protect them from NK cells. However, levels of the tested NK cell inhibitory ligands in cancer cells were much lower than those in M-BMMs. Therefore, regulation of inhibitory ligands on tumour cells might play a minor role in NK cell suppression at least in our model.

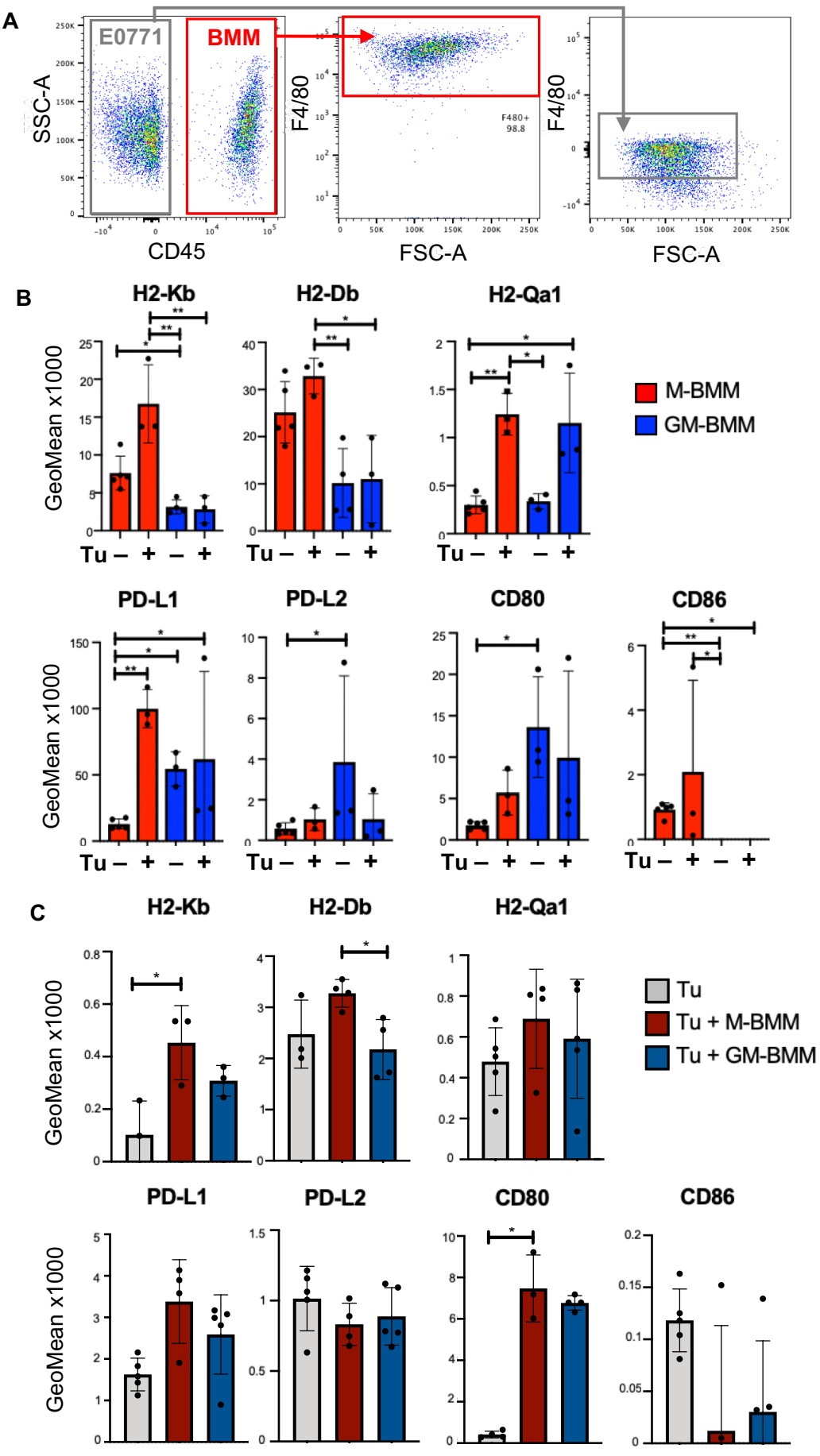
A
E0771
BMM
SSC-A
CD45
F4/80
FSC-A
F480+
98.8
B
H2-Kb
H2-Db
H2-Qa1
GeoMean x1000
Tu – + – +
M-BMM
GM-BMM
PD-L1
PD-L2
CD80
CD86
C
Tu
Tu + M-BMM
Tu + GM-BMM

Figure 5.2. Expression of NK cell inhibitory ligands on BMMs and E0771-LG2:Fl#4 cells. (A) Representative dot plots showing gating strategy to separate BMMs from tumour cells. E0771-LG2:Fl#4 tumour cells are characterized as $CD45^-$ $F4/80^-$, and BMMs are characterized as $CD45^+$ $F4/80^+$ (red gate). **(B)** Geometric mean fluorescence intensities of NK cell inhibitory ligands on M-BMMs (red) and GM-BMMs (blue) cultured with (+) or without (–) tumour cells. **(C)** Levels of NK cell inhibitory ligands on tumour cells cultured on their own (Tu: grey) or co-cultured with M-BMMs (dark red) or GM-BMMs (dark blue) (n=3-5 biological replicates, mean±SD, *p<0.05, **p<0.01, Kruskall-Wallis with Dunn's post-test).

5.2.3. Expression of NK inhibitory ligands on MAMs in the metastatic lung

We further investigated expression of the NK cell inhibitory ligands in MAMs and compared their levels to major immune cell populations in the metastatic tumours, i.e., RMACs and neutrophils (**Figure 5.3**). We found that MAMs expressed high levels of H2-K^b, H2-D^b H2-Qa1, PD-L1, PD-L2, and CD86 at significantly higher levels compared to neutrophils. Interestingly, we also found that these molecules were highly expressed in RMACs as well even though these cells did not have clear suppressive effects on NK cells **(Figure 4.2).** Since M-BMMs and MAMs commonly express high levels of H2-K^b, H2-D^b and CD86, binding of these ligands to their cognate receptors on NK cells could be a potential mechanism behind NK cell suppression by macrophages.

5.2.4. Effects of Blocking Antibodies Against H2-K^b, H-2D^b, and Their Receptors in M-BMM Mediated NK Cell Suppression

Given the high levels of H2-K^b and H2-Db in M-BMMs and MAMs, we investigated the effects of blocking antibodies against these NK cell inhibitory ligands and their receptor (Ly49C) on the M-BMM mediated NK cell suppression in our *in vitro* assay (**Figure 5.4A**). Because we could not rule out the expression of H2-M3 in M-BMMs, we also blocked its receptor Ly49A. Consistent with our previous data, NK cell-

induced tumour cell apoptosis was reduced by M-BMMs (approximately 50% suppression) and it was not altered by isotype control IgG (**Figure 5.4B**). Interestingly, blocking antibodies against Ly49A and H2-Db showed a tendency to rescue NK cell cytotoxicity from approximately 50% suppression (IgG control) to approximately 10-20% suppression, however such differences were not statistically significant (**Figure 5.4C**). Antibodies against either Ly49C/I or H2-Kb did not show any significant differences compared to IgG control. These results suggest the existence of another mechanism behind M-BMM mediated NK cell suppression rather than through the actions of H2-Db and H2-Kb.

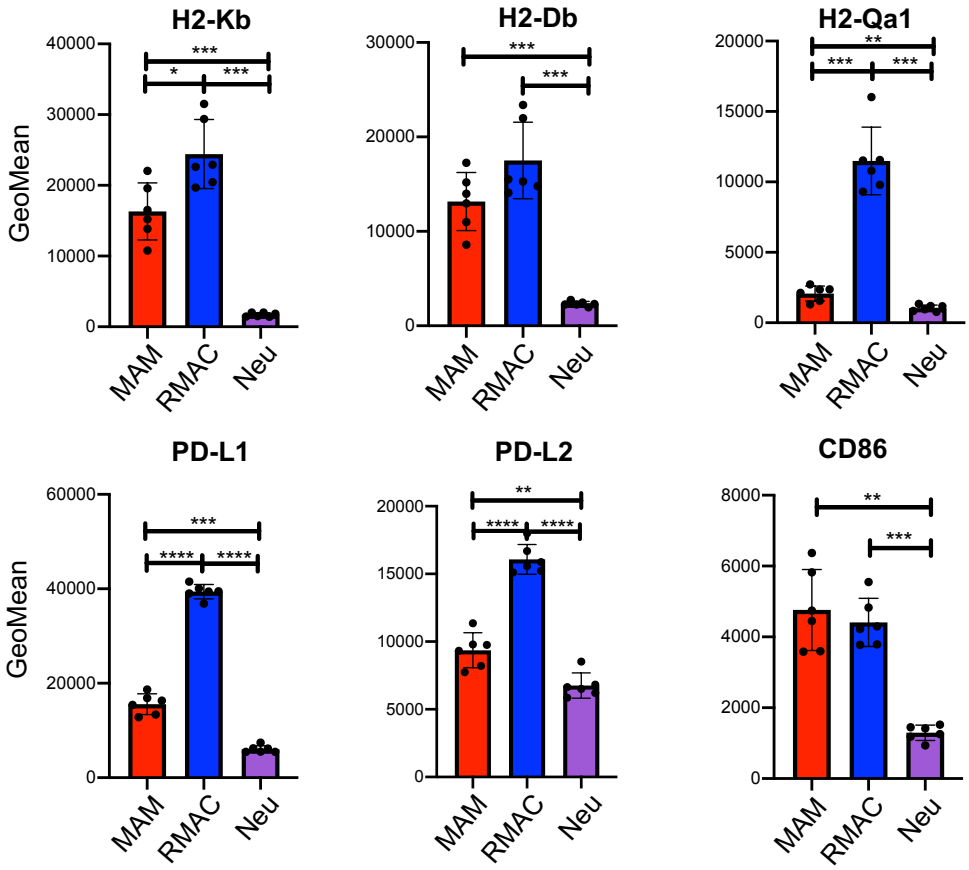

Figure 5.3. Expression of NK cell inhibitory ligands on MAMs within the tumour bearing lungs. Geometric mean fluorescence intensities of NK cell inhibitory ligands on MAMs (red) and neutrophils (purple) from the tumour bearing lungs and RMACs (blue) from non-tumour bearing lungs (n=6, mean±SD, *p<0.05, **p<0.01, ***p<0.001, Kruskall-Wallis with Dunn's post-test).

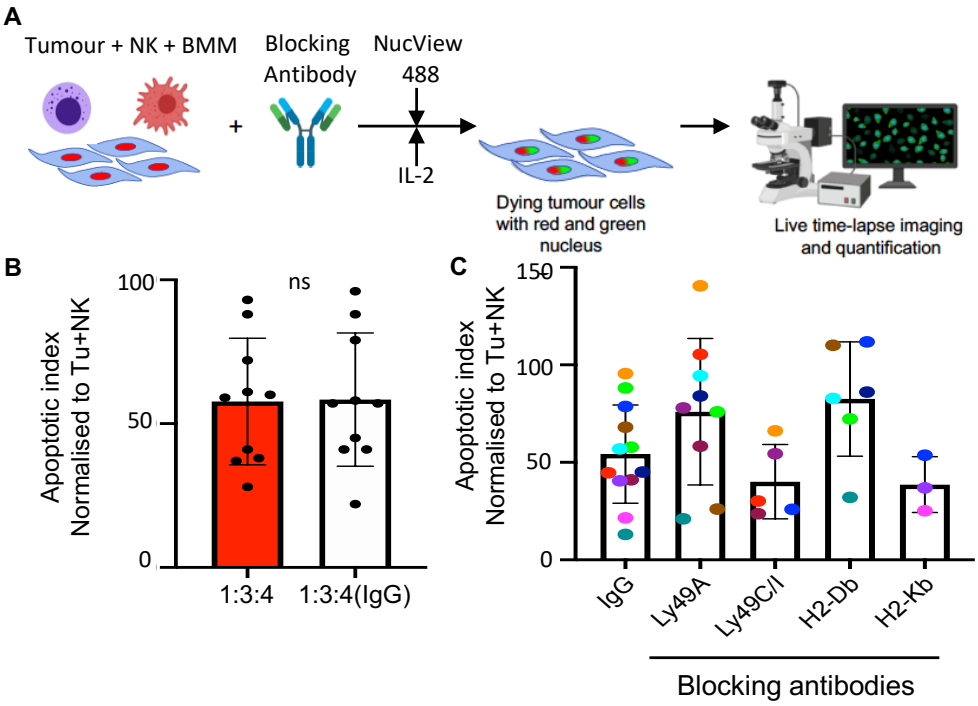

Figure 5.4. Involvement of NK cell inhibitory ligands in the M-BMM mediated NK cell suppression. (A) Schematic overview of experiment. Tumour cells, NK cells and M-BMMs were co-cultured with blocking antibodies against NK inhibitory ligands or receptors or control IgG in the presence of NucView 488 and IL-2. Apoptotic tumour cells were detected by microscopy, and apoptotic index (normalized to Tu+NK only) was determined. **(B)** Apoptotic index of tumour cells cultured with NK cells and M-BMM with (white) or without (red) isotype IgG. (n=10 biological replicates, mean±SD, no significance, Kruskall-Wallis with Dunn's post-test). **(C)** Apoptotic index of tumour cells cultured with NK cells and M-BMMs with blocking antibodies against Ly49A, Ly49C/I, H2-Kb, H2-Db or isotype IgG control. Biological replicates have different coloured dots within bars (n=3-12 biological replicates, mean±SD, no significance, Kruskall-Wallis with Dunn's post-test).

5.2.5. Expression of membrane bound TGF-β in M-BMMs and its contribution to their NK suppressive function.

A recent study has shown that macrophages cultured with IL-4 and IL-13 suppress NK cell activation *in vitro,* and that such suppression is reduced in the non-contact condition or treatment with anti-TGF-β blocking antibody[195]. Although TGF-β is a secreted factor, it can also be expressed on the cell surface in a membrane-bound

form which has been shown to suppress NK cell cytotoxicity via cell-to-cell contact[213].

We thus determined the expression of membrane-bound TGF-β in M-BMMs (NK suppressive) and GM-BMMs (non-suppressive) by flow cytometry. As shown in **Figure5.5A**, cell surface expression of TGF-β protein was significantly higher in M-BMMs compared to GM-BMMs, raising the possibility of its involvement in M-BMM mediated NK cell suppression. To address this possibility blocking antibodies against TGF-β were added to our *in vitro* assay. Consistent with previous data, M-BMMs reduced the number of apoptotic cancer cells induced by NK cells in the presence of control IgG (**Figure 5.5B**). In contrast, treatment with anti-TGF-β blocking antibodies tended to rescue NK cell cytotoxicity in the presence of M-BMMs. Although the average difference was not statistically significant, the apoptotic index in the presence

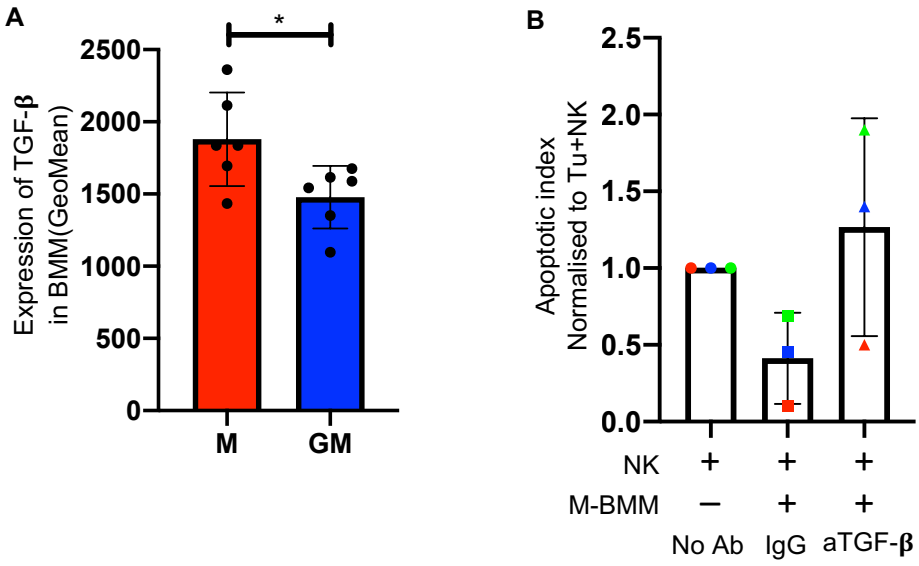

Figure 5.5 Expression of membrane bound TGF-β on M-BMMs and its contribution to the M-BMM mediated NK cell suppression. (A) Geometric mean fluorescence intensity of membrane bound TGF-β on M-BMMs and GM-BMMs (n=6, mean±SD, *p<0.05, Mann-Whitney). **(B)** Apoptotic index of tumour cells co-cultured with NK cells and M-BMMs in the presence of anti-TGF-β blocking antibodies or isotype IgG control. Data are normalized to Tu+NK only. Biological replicates have different coloured dots within bars (n=3 biological replicates, mean±SD).

of anti-TGF-β blocking antibodies was always higher than IgG control (see coloured dots representing independent experiments). These experiments, however, are preliminary and need to be repeated to confirm the results or not with sufficient replicates.

5.3. Discussion

NK cell killing can be suppressed in a number of ways. In order to find a mechanism behind the suppression induced by macrophages (MAMs), first we narrowed down whether suppression was dependant on cell-to-cell contact, or a secreted factor. For example, a recent publication showed that thyroid cancer cells secrete prostaglandin E2 which suppressed NK cells by decreasing their expression of NKp44 and NKp30 as well as reducing their expression of TRAIL[214]. Alternatively, it has also been shown that physical contact via membrane-bound TGF-β on myeloid derived suppressor cells can also cause suppression of NK cells in *in vitro* and *in vivo* models of liver cancer. This caused a reduction in cytotoxicity and expression of NKG2D as well as IFN-γ production[215]. We therefore carried out a transwell assay where M-BMMs could physically touch tumour and NK cells, or were isolated from the other cells in culture by a permeable membrane. We found that NK cells were only suppressed during the contact condition and therefore this appears to be the dominant mechanism (secreted factors may be necessary but not sufficient).

Based on these results, we hypothesized that M-BMMs express high levels of cell surface proteins that transmit inhibitory signals into NK cells through the direct contact. In human breast cancer, it has been reported that levels of NKG2A (a NK inhibitory receptor) is increased during progression of cancer whereas expression of NKG2D and DNAM-1 (NK activation receptors) are decreased, which correlates with the decrease in cytotoxicity[115]. Interestingly, TGF-β in the tumour microenvironment has been shown to reduce the expression of NKG2D in NK cells in human lung and

colon cancers[165]. Given these findings, it is possible that macrophages expressing membrane-bound TGF-β can inhibit the activation of NK cells via skewing expression of NK cell regulatory receptors. However, MAMs in our model may not utilize this mechanism since the levels of NK cell activating and inhibitory receptors on NK cells were not changed following macrophage depletion.

We demonstrated that NK-suppressive M-BMMs as well as MAMs express high levels of H2-Kb, H2-Db, and CD86. These ligands can suppress NK cell functions through their receptors Ly49C, Ly49I, and CTLA-4 (Chapter 1.3). However, NK cells cultured in our condition did not express detectable levels of CTLA-4 (data not shown) making this latter protein an unlikely candidate. Furthermore, treatment with blocking antibodies against H2-Kb, H2-Db, or their receptor Ly49C/I did not significantly rescue the macrophage-mediated NK suppression. These results suggest that the expression of MHC-I molecules or checkpoint ligands is not the main mechanism of macrophage mediated NK cell inhibition.

It has been reported that TGF-β is an inhibitor of NK cell function in the tumour microenvironment (Chapter 1.3). Although TGF-β acts as a soluble immune suppressor, as previously mentioned, some studies indicate that membrane-bound TGF-β is also functional in regulatory T cells and required for their contact-dependent immune suppression. Data reported in this chapter demonstrates that NK suppressive M-BMMs express higher levels of membrane-bound TGF-β than NK non-suppressive GM-BMMs, and blockade of TGF-β prevented macrophage-mediated suppression of NK cell cytotoxicity. Interestingly, a recent study has shown that mouse BMMs cultured with M-CSF in combination with IL-4 and IL-13 suppress NK cell activation via contact and TGF-β dependent manner[195]. Collectively, these findings suggest that the expression of membrane-bound TGF-β is one of the major mechanisms behind the contact-dependent NK cell suppression by a certain population of macrophages such as M-BMMs and MAMs. As mentioned above, however, *in vitro* assays using

anti-TGF-β blocking antibodies must be repeated to confirm this conclusion. In a mouse model of breast cancer metastasis, MAMs are differentiated from a subpopulation of "myeloid-derived suppressor cells" (MDSCs) that accumulates in the metastatic lung[31]. Interestingly, it has been reported that MDSCs from the spleen of liver cancer bearing mice suppress cytotoxicity and IFN-γ secretion of NK cells through membrane-bound TGF-β *in vitro*[215]. In order to improve efficacy of NK cell infusion therapy, it will be important to determine expression of membrane-bound TGF-β in MAMs and their progenitors in metastatic tumours and investigate whether TGF-β inhibitors can restore NK cell activities *in vivo*.

On the other hand, our data does not exclude the involvement of other mechanisms in the macrophage-mediated NK suppression depending on cell-to-cell contact. It has been reported that M-BMMs as well as MAMs make direct contact with metastatic mammary tumour cells[30,61]. Furthermore, a recent study suggests that direct contact between breast cancer cells and stromal cells can induce pro-metastatic cytokines in cancer cells[216].

Given these findings, it might be possible that cell-to-cell contact between cancer cells and macrophages induce NK suppressive molecules in tumour cells or prevent physical contact of NK cells to target cancer cells. These possibilities are now under investigation in our lab.

Chapter 6

Mechanisms behind macrophage mediated NK cell suppression: Effects of macrophages on NK cell regulatory receptor expression, maturation and localization *in vivo*

6.1. Aims

1. To investigate the effects of MAMs on the expression of inhibitory or activating receptors on NK cells *in vivo*
2. To determine the effects of MAMs on the activation and maturation of NK cells *in vivo*
3. Determine whether the depletion of macrophages alters the localisation of NK cells within metastatic pulmonary tumours

6.2. Results

6.2.1. Effect of MAM Depletion on NK Cell Regulatory Receptor Expression, Activation and Maturation Status.

As another possible mechanism by which macrophages suppress NK cell cytotoxicity, we hypothesized that MAMs might change NK cell phenotypes *in vivo*, i.e., decrease or increase the expression of activation or inhibitory 'receptors' on NK cells and/or prevent functional differentiation of NK cells. To investigate this hypothesis, we utilized mice with a *Csf1r* conditional allele in which the *Csf1r* gene is ablated by the administration of doxycycline (Dox) that induces the expression of cre-recombinase (cKO)[217]. To perform these experiments E0771-LG2:Fl#4 cells were injected into the tail vein of *Csf1r*-cKO mice, and mice were treated with Dox from day 4 to deplete MAMs in the lung. After a further 10 days, we collected the lung with metastatic tumours and performed flow cytometry for infiltrating immune cells (**Figure 6.1A**). Consistent with our previous report[31], the number of MAMs within the metastatic lungs was reduced by 75% on average (**Figure 6.1B**). We then analysed expression of NK inhibitory receptors by flow cytometry. As shown in **Figure 6.1C** NK cells in the metastatic lung expressed detectable levels of inhibitory receptors such as Ly49A, Ly49C/I, NKG2A, and PD-1 whereas CTLA4 was not detectable (data not shown). However, there were no significant differences in their expression between NK cells

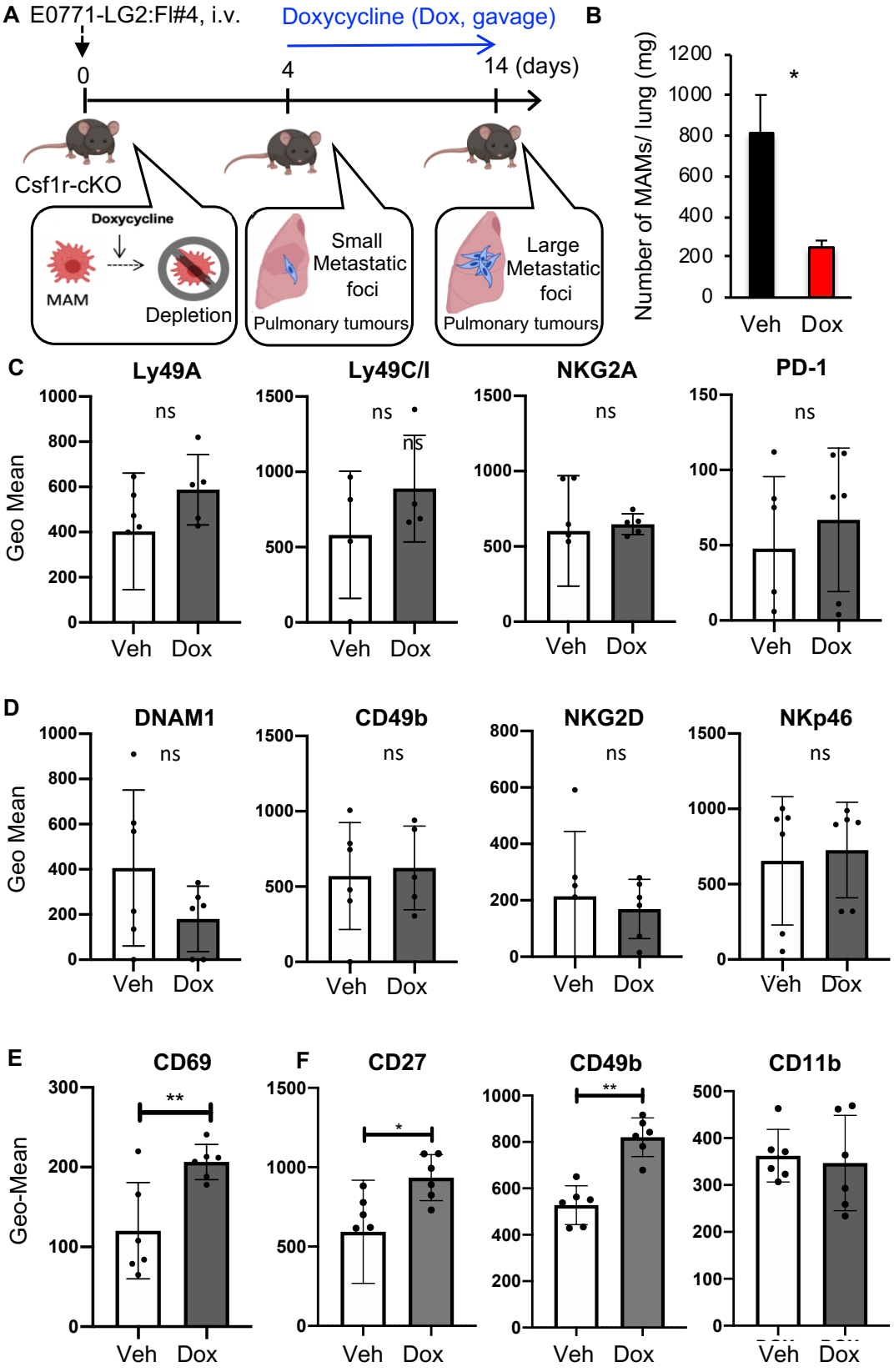
A
E0771-LG2:Fl#4, i.v.
Doxycycline (Dox, gavage)
0
4
14 (days)
Csf1r-cKO
Doxycycline
MAM
Depletion
Small Metastatic foci
Pulmonary tumours
Large Metastatic foci
Pulmonary tumours
B
Number of MAMs/ lung (mg)
Veh
Dox
C
Ly49A
Ly49C/I
NKG2A
PD-1
Geo Mean
ns
D
DNAM1
CD49b
NKG2D
NKp46
E
CD69
F
CD27
CD49b
CD11b
Geo-Mean

Figure 6.1. Effects of MAM depletion on NK cell regulatory receptor expression, activation and maturation. **(A)** Schematic overview of experiment. E0771-LG2:Fl#4 tumour cells were intravenously injected into *Csf1r*-cKO mice in which MAMs are depleted by doxycycline (Dox) treatment. From day 4 after tumour injection, water including Dox was given to the animals, and on day 10 or day 14 the lung with metastatic tumours were dissected for flow cytometry. **(B)** Number of MAMs in the lung of mice treated with vehicle (Veh) or doxycycline (Dox) **(C)** Expression of NK cell inhibitory receptors and **(D)** activating receptors on NK cells from the lung of vehicle and DOX treated tumour bearing mice at day 14. **(E)** Expression of CD69 or **(F)** maturation markers on NK cells from vehicle or and DOX treated tumour bearing mice at Day 10. (n=6 biological replicates, Mann-Whitney, ns= no significance, **p<0.001). The same NK gating strategy as Figure 3.2 was used.

with doxycycline (MAM depleted) and vehicle (MAM intact). Furthermore, levels of activating receptors such as DNAM-1, CD49b, NKG2D, and NKp46 on NK cells were not changed by the MAM depletion (Day 14) (**Figure 6.1D**). We also analysed the lung with tumours at an earlier time-point (day 10) but did not find significant differences in levels of these receptors (data not shown).

In order to investigate the effects of MAM depletion on the NK cell activation status, we determined the expression of CD69, a commonly used marker of activated NK cells[218] in mice at day 14 after tumour injection (10 days after Dox treatment). We found that levels of CD69 on NK cells were significantly higher in Dox-treated mice (**Figure 6.1E**), suggesting that depletion of MAMs promotes NK cell activation in the lung with metastatic tumours. Using this model, we investigated effects of MAM depletion on the expression of major activating and inhibitory receptors on NK cells.

We then investigated whether MAMs had an impact on NK cell maturation within tumour bearing lungs. To this end, we determined the levels of CD27 and CD49b in NK cells since high expression of these markers are known to represent mature NK cells[219]. Interestingly, we found that expression of CD27 and CD49b were significantly higher in NK cells from the metastatic lung of mice in which MAMs were depleted (Day 10) (**Figure 6.1F**). These results suggest that effects on NK cell

regulatory receptors may not be the major mechanism of NK cell suppression by MAMs, whereas alteration of NK cell differentiation status could be another mechanism behind MAM-mediated NK cell suppression *in vivo*.

6.2.2. Effects of MAM Depletion on the Number of NK Cells Within Tumour Areas

A recent study using a mouse model of mammary tumour development has reported that tumour-associated macrophages can reduce motility and infiltration of $CD8^+$ T cells into the primary tumours, and that depletion of macrophages enhances therapeutic efficacy of checkpoint inhibitors[220]. Given these findings in primary tumours, another potential mechanism was investigated where MAMs might prevent NK cell infiltration into the tumour area and thereby prevent NK cell mediated tumour cell elimination *in vivo*. To this end, we utilized the *Csf1r*-cKO model as previously described. Briefly, we injected $1x10^6$ of E0771-LG2:Fl#4-Csf1rcKO cells into *Csf1r*-cKO mice via the tail vein, and the mice were treated with Dox from 4 days after tumour injection, a time point when injected cancer cells establish micro-metastasis in the lung. 10 days post-tumour injection, tumour-bearing lungs were isolated and the expression of NKp46 (a marker for NK cells) and F4/80 (a macrophage marker) was analysed by RNAScope. Using image analysis software (Definiens), tissue sections were classified into 'tumour' area and 'normal lung' area based on the differences in their nuclear density (**Figure 6.2A**). In each area macrophages and NK cells were detected based on the distinct expression of F4/80 and NKp46 (**Figure 6.2B and C**).

Consistent with our previous data, the number of $F4/80^+$ macrophages in the tumour area but not lung parenchyma was reduced in *Csf1r*-cKO mice treated with Dox compared to those in mice treated with vehicle (**Figure 6.2D**). Importantly, the number of NK cells within the tumour area and lung parenchyma was significantly higher in the Dox-treated mice compared to untreated animals (**Figure 6.2E),**

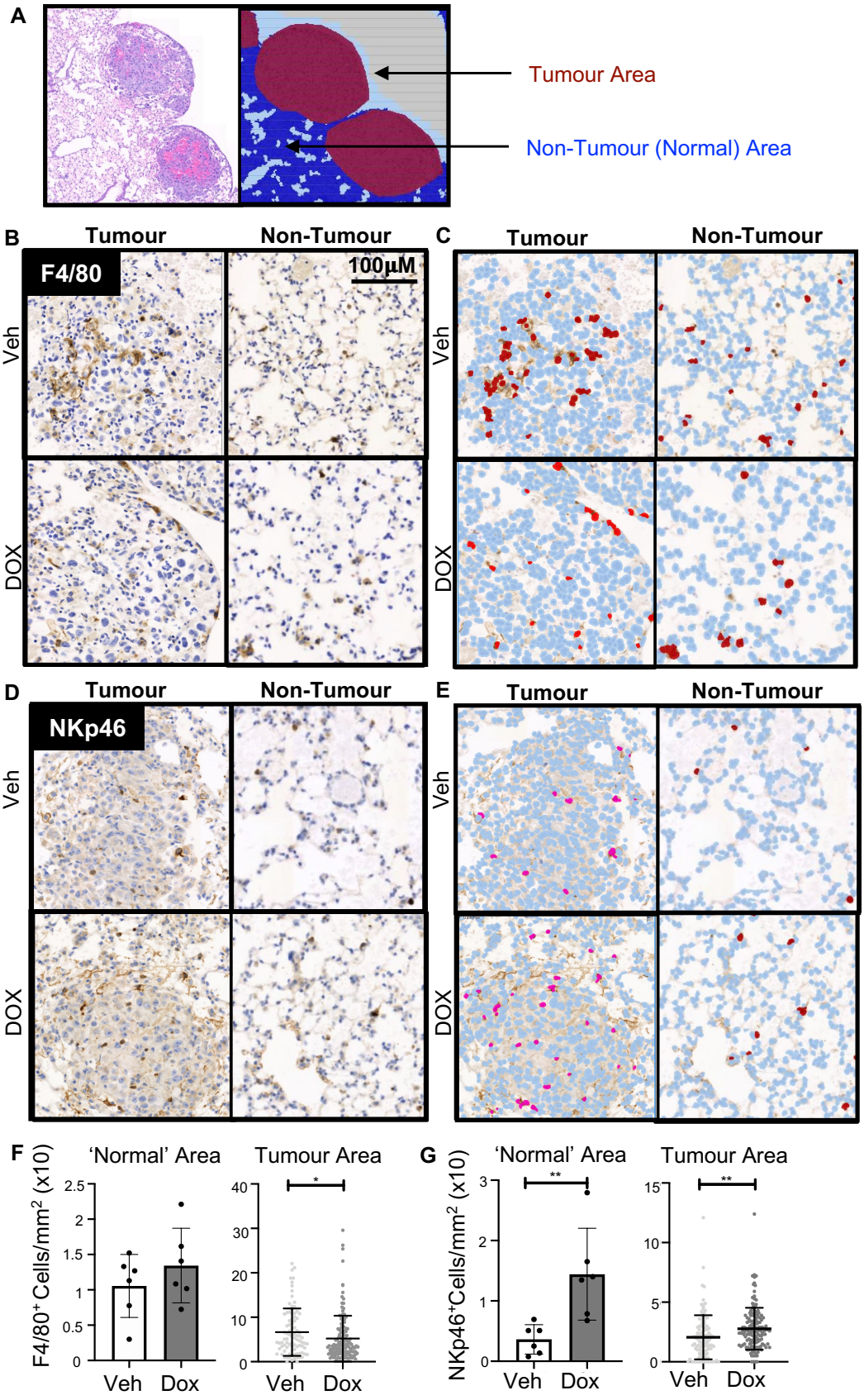
A
Tumour Area
Non-Tumour (Normal) Area
B
Tumour
Non-Tumour
F4/80
100µM
Veh
DOX
C
Tumour
Non-Tumour
Veh
DOX
D
Tumour
Non-Tumour
NKp46
Veh
DOX
E
Tumour
Non-Tumour
Veh
DOX
F
'Normal' Area
Tumour Area
F4/80+Cells/mm2 (x10)
Veh
Dox
*
G
'Normal' Area
Tumour Area
NKp46+Cells/mm2 (x10)
**
**

Figure 6.2. Macrophage depletion in Csf1rcKO animals allows more NK cells to enter the tumour bearing lung. (A) Left: H&E image of tumour bearing lung. Right: Overlay of tissue classification into 'normal' (blue) and 'tumour' (red) areas. **(B)** Representative raw images of F4/80 IHC staining between vehicle (top) and DOX (bottom) treated animals. Images on the left show tumour areas. Images on the right show adjacent non-tumour areas. **(C)** Quantification of F4/80 from (B). Nuclear detection (light blue) and F4/80 detection (red). **(D)** Representative raw images of NKp46 RNAScope staining between vehicle (top) and DOX (bottom) treated animals. Images on the left show tumour areas. Images on the right show adjacent non-tumour areas. **(E)** Quantification of NKp46 from (D). Nuclear detection (light blue) and NKp46 detection (pink). **(F)** Number of $F4/80^+$ cells/mm^2 (x10) of lung in 'normal' and 'tumour' areas (n=6 biological replicates, mean±SD, *p<0.05, Mann-Whitney). **(G)** Number of $NKp46^+$ cells/mm^2 (x10) of lung in 'normal' and 'tumour' areas (n=6 biological replicates, mean±SD, **p<0.001, Mann-Whitney).

suggesting that MAMs can prevent the accumulation of NK cells in the metastatic lung.

6.3. Discussion

In this chapter we found that expression of CD27 and CD49b were significantly increased in NK cells from tumour bearing lungs where MAMs had been depleted. It is known that mouse NK progenitor cells ($CD11b^-CD27^-CD49b^-$) give rise to immature NK cells ($CD11b^-CD27^+CD49b^-$) that further differentiate into transitional ($CD11b^+CD27^+CD49b^+$) and fully mature ($CD11b^+CD27^-CD49b^+$) NK cells. Since high expression of CD27 and CD49b is a characteristic of the initial stage of mature NK cells[219], our data suggest that MAMs prevent differentiation of NK cells recruited to the metastatic tumours. Although NK cells were initially thought to develop in the bone marrow and exit as mature cells, a recent study has suggested that peripheral NK-cell populations originate from site-specific immature NK cells more than from BM-derived mature NK cells[221]. Although maturation status or origin of NK cells in human cancer is unclear yet, a recent study using a mouse model of breast cancer has shown

that NK cells in the primary tumours express lower levels of CD27 and CD49b compared to NK cells in the spleen[111], suggesting the recruitment of immature NK cell to the tumour microenvironment. Interestingly, a recent report has shown that CD27 expression is reduced in NK cells that are co-cultured with macrophages from the primary mammary tumours in mice[195]. Furthermore, it has been reported that $CD27^{high}$ NK cells demonstrate higher cytotoxic capacity against target cancer cells compared to $CD27^{low}$ NK cell[222]. Taken together, these results suggest that prevention of NK cell differentiation within the metastatic site may be another mechanism behind MAM mediated NK cell suppression *in vivo* in addition to the direct suppression via membrane-bound TGF-β. To our knowledge, this is the first report suggesting that NK cells in the metastatic site are less mature and that might be caused by macrophages. It will be important to investigate maturation status of NK cells and its association with numbers of distinct populations of macrophages within human metastatic tumours.

A recent study has reported that the number of tumour-infiltrating NK cells is very low in most cases of breast cancer[223], suggesting that the tumour microenvironment in breast cancer excludes NK cells. In this project, we have demonstrated that the number of NK cells infiltrating into the metastatic tumours is increased following MAM depletion. In mouse models of breast cancer, macrophages in the tumour stroma reduce $CD8^+$ T cell motility via direct contact with them and thereby suppress the T cell infiltration into the tumour nodules[220]. However, MAMs and NK cells are not restricted to the border between tumour foci and the lung parenchyma in our experimental metastasis model Therefore, it is unlikely that depletion of MAMs enhances NK cell infiltration by releasing NK cells from macrophage-mediated retention in the lung parenchyma. It is instead possible that the absence of MAMs can enhance migration of NK cells throughout the metastatic lung since MAM depletion increases the number of NK cells in normal (non-tumour) area in addition to the tumour area. Although it is unclear how NK cells reach the

metastatic tumours, pre-activated NK cells transferred into the circulation evenly distribute in the lung initially and then accumulate in the metastatic tumours in lung metastases established by B16 melanoma cells as well as several other different types of cancer cells[224]. It is thus possible that depletion of MAMs might promote the recruitment of NK cells into the metastatic lung through blood vessels outside the tumour followed by their redistribution into the metastatic foci. It has been reported that the recruitment of NK cells into the tumour is regulated by inflammatory chemokines which bind CXCR1, CXCR2 and CX_3CR1 on NK cells[225]. Further investigation is required to identify whether the accumulation of MAMs negatively regulates levels of these chemokines (or other regulators of NK cell recruitment) in the metastatic tumour microenvironment.

Chapter 7

Effects of macrophage depletion on the efficacy of infused NK cells in a mouse model of metastatic breast cancer

7.1. Aims

Investigate whether the depletion of macrophages improves the efficacy of NK cell infusion therapy

7.2. Results

In the previous chapters, we have suggested that MAMs can prevent NK cells from exerting their full cytotoxicity and that macrophages reduce NK cell infiltration into the lung. We therefore hypothesised that depletion of macrophages prior to NK cell infusion therapy would significantly improve the efficacy of this type of treatment. To this end, we utilized the *Csf1r*-cKO mice (**Figure 7A**). In order to prepare cohorts of animals with the same age and to restrict genetic alterations to blood cells, we transferred bone marrow cells from *Csf1r*-cKO mice into lethally irradiated recipient C57Bl/6 mice (4-5-week-old, female). 6 weeks after bone marrow transplantation (BMT), the animals were injected with E0771-LG2:Fl#4 tumour cells via the tail vein. After 4 days, cohorts of animals were treated with Dox to deplete MAMs. On Day 7, the Csf1r-cKO:BMT mice treated with or without Dox were intravenously injected with $5x10^5$ of NK cells that were isolated from the spleen of normal C57Bl/6 mice and pre-cultured with IL-2 for 24 hours. Tumour loads in the lung were determined by bioluminescence imaging on day 4 and day 10 after tumour injection. As shown in **Figure7B**, metastatic tumour loads in the lung were not reduced by the single treatment with Dox (MAM depletion) or NK cell infusion. Importantly, however, NK cell infusion in combination with MAM depletion significantly reduced the metastatic tumour loads in the lung.

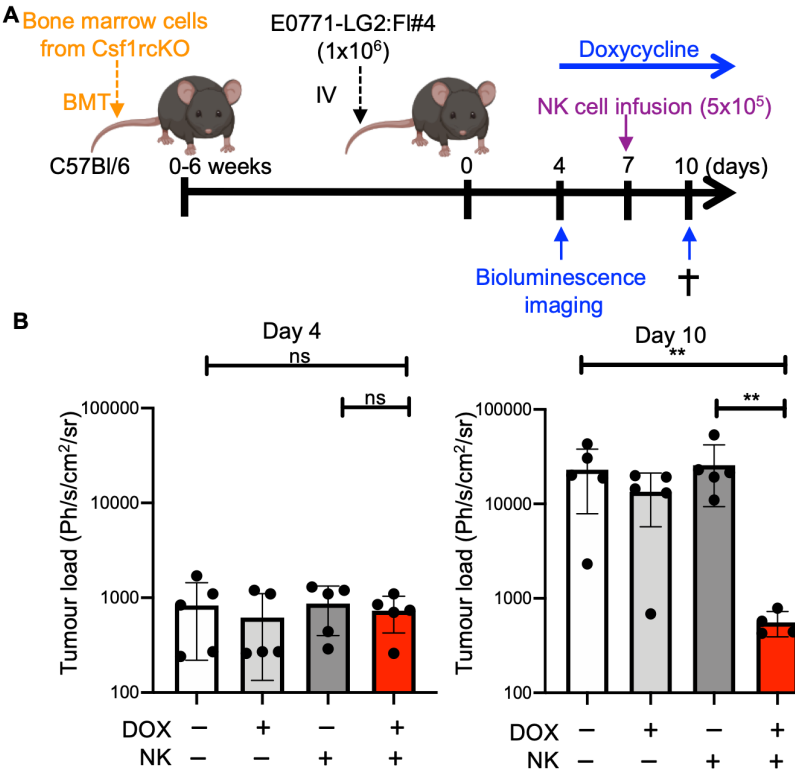

Figure 7. NK cell infusion efficacy can be improved by depletion of macrophages. (A) Schematic overview of the treatments. Bone marrow cells from *Csf1r*-cKO mice were transplanted (BMT) into irradiated recipient C57Bl/6 mice. After 6 weeks, the BMT mice were intravenously injected with E0771-LG2:Fl#4 cells and allocated into four cohorts. Cohorts of mice were treated with doxycycline (Dox) from day 4 after tumour injection and/or intravenously injected with *ex vivo* activated NK cells on day 7. Metastatic tumour loads in the lung of mice were determined on days 4 and day 10 by bioluminescence imaging. **(B)** Metastatic tumour loads on days 4 (left) and 10 (right) in the lung of mice from following groups. Group 1: Vehicle, No NK infusion (white). Group 2: DOX, no NK infusion (light grey). Group 3: Vehicle, NK infusion (dark grey). Group 4: DOX, NK infusion (Red) (n=5, mean±SD, **p<0.01, Kruskall-Wallis, Dunn's post-test).

7.3. Discussion

It has been shown that a high number of circulating NK cells in metastatic triple-negative breast cancer patients correlates with low number of circulating tumour cells as well as lower risk of lung and visceral metastases[226], suggesting that NK cells can recognize and eliminate tumour cells that enter the circulation which in turn controls the development of lung metastasis. Therefore, NK cells have been considered as promising therapeutic tools to eliminate malignant cancer cells. In acute myeloid leukaemia, adoptive transfer of NK cells along with IL-2 after lymphodepleting chemotherapy resulted in a complete remission in 5 out of 19 patients with a poor prognosis[227]. However, the efficacy of NK cell infusion immunotherapy is limited in solid tumours including metastatic breast cancer as previously mentioned. Consistent with these clinical data, we found that a single treatment with NK cell infusion is not sufficient for suppression of metastatic tumour growth in our mouse model.

A recent study has shown that NK cells transferred into mice with mammary tumours have significantly reduced expression of activation marker (NKp46) when they infiltrate into the tumour whereas transferred NK cells that arrived at the spleen maintained high expression of NKp46[111]. These results suggest that mammary tumour cells can establish a tumour microenvironment that suppresses NK cell function and thereby limit therapeutic efficacy of NK cell infusion.

There is increasing evidence that leukocytes in the tumour microenvironment such as regulatory T (T_{reg}) cells, myeloid-derived suppressor cells (MDSCs) as and tumour-associated macrophages (TAMs) can restrict anti-tumour immune response by NK and $CD8^+$ T cells as previously discussed. In a lung metastasis model using CT26 mouse colon cancer cells, elimination of T_{reg} cells by denileukin diftitox (Ontak) treatment can enhance therapeutic effects of intravenously injected NK cells[228]. This study indicates that it is feasible to augment efficacy of NK cell infusion therapy by depletion of immune suppressor cells from the tumour microenvironment. However,

in our experimental metastasis model using E0771-LG2:Fl#4 mouse mammary tumour cells, T_{reg} cells in the metastatic lung are very few compared to MAMs (0.1±0.03 and 8.5±0.5% in $CD45^+$ cells, Kitamura et al. unpublished data). Furthermore, we have demonstrated that MAMs suppress cytotoxicity of NK cells in this project. Importantly, we have shown here that metastatic tumour growth is significantly suppressed by adoptive transfer of NK cells in combination with genetic depletion of MAMs via deletion of *Csf1r* gene under DOX treatment. Therefore, our data has shown we believe for the first time, that targeting MAMs could be a promising strategy to improve NK cell infusion therapy for metastatic tumours.

On the other hand, our data showed that MAM depletion on its own did not suppress metastatic tumour expansion even though it enhances activation of NK cells in the metastatic lung. Although our previous studies have shown that MAM depletion by clodronate or loss of *Csf1* or *Csf2* gene [61,63,30] can suppress metastasis formation, in these models macrophages are not present, or are depleted before tumour cells were given. In contrast, we depleted MAMs after Day4 when tumour cells have already formed small metastatic foci within the lung[31] which may be too late to suppress metastatic tumour expansion. It is possible that strategies targeting the tumour microenvironment do not directly induce tumour cell death, and thus additional treatment such as adoptive transfer of 'external' NK cells is required. It is also possible that another type of immune suppressor cell compensates for the NK cell suppressive effects of MAMs, and thus re-activation of 'intrinsic' NK cells by MAM depletion alone is not enough to prevent metastasis formation. As described above, the metastatic lung in our model contains a significant amount of MAMPCs that can differentiate into MAMs. Furthermore, we have shown that a short (10 days) treatment with DOX can eliminate MAMs but not MDSCs[31] in the metastatic lung established in *Csf1r*-cKO mice[10]. Since several studies have shown that MDSCs can suppress NK cell

functions, depletion of both MDSC and MAMs might more effectively boost intrinsic NK cell functions or augment infused NK cell efficacy.

Chapter 8

Conclusions and Future Directions

In this project, we have suggested that macrophages in the metastatic tumour microenvironment (i.e., MAMs) suppress NK cell cytotoxicity *in vitro,* and that depletion of MAMs by targeting the CSF-1 receptor can improve therapeutic efficacy of infused NK cells in a mouse model of breast cancer metastasis. Using an *in vitro* macrophage model mimicking MAMs, we also identified that the NK suppressive macrophages require cell-to-cell-contact and membrane-bound TGF-β to suppress NK cell cytotoxicity. Furthermore, we have shown that MAMs can restrict the infiltration and differentiation of NK cells in the metastatic lung. Although some experiments must be repeated to confirm our conclusion, our findings highlighted MAMs as one of the key components of the NK cell suppressive tumour microenvironment at the metastatic site. In addition to repeating the incomplete or preliminary experiments described above, the presence of TGF-β on MAMs within the metastatic lung tumours should be confirmed. This should be combined with blocking TGF-β in our mouse models of pulmonary metastatic breast cancer along with NK cell infusion which should also be investigated to support our updated hypothesis that targeting TGF-β in MAMs can improve NK cell infusion efficacy.

We have performed an experiment to investigate whether MAM depletion affects the recruitment of infused NK cells in our mouse model. However, results are yet to be analysed.

Since CSF1 signalling is essential for the differentiation and survival of certain populations of macrophages including tumour-associated macrophages[229], several CSF1 receptor antagonists (e.g., BLZ945, PLX3397 and PLX7486) have been developed to deplete disease-promoting macrophages and tested in phase I or phase II studies in patients with malignant cancers. These drugs were well tolerated, and partial anti-tumour effects were observed in some cases[66]. Although the clinical benefits of CSF1 receptor antagonists in immunotherapy has not yet been reported, a mouse study involving pancreatic tumours has shown that treatment with PLX397

in combination with immune checkpoint inhibitors (blockade of PD-1 and CTLA-4 by antibodies) markedly reduced the size of established tumours[230]. This study emphasizes that macrophage targeting is a promising treatment option to augment immunotherapy efficacy. Currently, studies using a mouse model of breast cancer metastasis are ongoing in the Kitamura lab in order to identify the effects of CSF1 receptor antagonists on the efficacy of infused NK cells in suppressing metastatic tumour expansion.

Although macrophage depletion by CSF1 receptor inhibitors is an efficient strategy for MAM targeting, it is not specific for MAMs and may also affect other macrophage populations that are involved in a wide range of physiological and pathological processes (Chapter 1). It is thus possible that prolonged treatments with CSF-1 receptor antagonists can cause toxic side effects in patients[231]. An alternative and more specific approach to target MAMs to improve immunotherapy is the blockade of MAM-derived suppressive factors. Our data suggest that TGF-β is an attractive candidate. There are several ongoing clinical trials using TGF-β inhibitors in a range of advanced solid tumours (NCT03834662, NCT03192345, NCT03470350). Although outputs of these trials have not been posted yet, a recent trial using anti-TGF-β antibody (Fresolimumab) in patients with malignant melanoma has shown that this treatment does not cause dose-limiting toxicities[232]. Further pre-clinical studies investigating the effects of TGF-β inhibitors on cytotoxicity of infused NK cells *in vivo* may lead to the improvement of current NK cell-based immunotherapies. On the other hand, it is possible that other molecules contribute to MAM-mediated NK cell suppression in addition to TGF-β.

Interestingly, our data have also suggested that MAMs can prevent the recruitment of NK cells in the lung with metastatic tumours. Since efficient delivery of infused NK cells is another important aspect to improve NK cell infusion therapy, it is necessary to investigate the effects of MAMs on the recruitment of infused NK cells.

Since NK cell recruitment can be regulated by chemokines, it is possible that MAM depletion might change chemokine profiles in the metastatic TME to favour NK cell recruitment. Further studies investigating potential mechanisms by which MAMs prevent NK cell infiltration would also lead to the improvement of NK cell infusion therapy. On the other hand, several groups have been trying to improve NK cell homing to the tumours by manipulating them to express chemokine receptors specific for chemokines within the TME. A combination of this strategy with MAM depletion may further improve NK cell delivery to the tumour.

This project has highlighted that macrophages within metastatic tumours prevent NK cells from exhibiting their full cytotoxic capabilities and has provided important information about the mechanisms that may be responsible for this reduction in cytotoxicity, for example, suppression via macrophage-derived membrane bound TGF-β. Further studies based on this project would accelerate the establishment of effective NK cell-based immunotherapy for metastatic breast cancer as well as other types of tumours.

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