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LOW-DOSE CHEMOTHERAPY COMBINED WITH NK CELL-BASED IMMUNOTHERAPY AS A TREATMENT FOR TRIPLE NEGATIVE BREAST CANCER

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A thesis submitted in partial fulfilment of the requirements of Nottingham Trent University for the degree of Doctor of Philosophy

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Copyright Statement

Signature ..... Date ....02-03-2020....

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Abbreviations

ADAM	A Disintegrin and Metalloproteinase
ADCC	Antibody-dependent cellular cytotoxicity
ALDH	Aldehyde dehydrogenase
AML	Acute Myeloid Leukaemia
BiKEs	BiSpecific Killer cell Engagers
CD	Cluster of Differentiation
CSCs	Cancer stem cells
CICs	Cancer initiating cells
CTLA-4	Cytotoxic T-lymphocyte–associated antigen 4
CTLA	Cytotoxic T lymphocyte-associated protein
CXCL	C-X-C motif Chemokine
DC	Dendritic cell
DMSO	Dimethyl Sulphoxide
DNAM	DNAX-accessory molecule
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
EMT	Epithelial to mesenchymal transition
ER	Oestrogen receptor
FACS	Fluorescent activated cell sorting
FcR	Fc Receptor
FCS	Fetal calf serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
FL	Fluorescence
FS	Forward Scatter
GITR	Glucocorticoid-induced TNFR related protein

HER2	Human epidermal growth factor 2
HLA	Human Leukocyte Antigen
ICAM	Intercellular Adhesion Molecule
IDO	Indoleamine Pyrrole 2,3-dioxygenase
IFN	Interferon
IFN-γ	Interferon Gamma
Ig	Immunoglobulin
IGF	Insulin Growth Factor
IL	Interleukin
IL	Interleukin
KIR	Killer-cell Immunoglobulin-like Receptor
LAMP	Lysosome asscociated Membrane Protein
LDMC	Low-dose metronomic chemotherapy
LFA	Lymphocyte function-associated antigen
L-Glut	L-Glutamine
mAb	Monoclonal antibody
MDSCs	Myeloid Derived Suppressor cells
MFI	Median Fluorescence Intensity
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
NK	Natural killer (cell)
PARP	Poly ADP-ribose polymerase
PBMC(s)	Peripheral blood mononuclear cell(s)
PI3K	Phosphoinositide 3-kinase
PR	Progesterone receptor
PRR	Pattern Recognition Receptor
PTEN	Phosphatase and tensin homolog
STAT	Signal transducer and activator of transcription
TGF	Transforming growth factor

TLR Toll-like receptor

TNF Tumour necrosis factor

TRAIL TNF-related apoptosis-inducing ligand

Treg Regulatory T (cell)

ULBP UL16-binding protein

VEGF Vascular endothelial growth factor

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Abstract

Despite considerable progress and the emerging use of immunotherapies, chemotherapy remains the only routine clinical option for treating triple negative breast cancer (TNBC). However, this is associated with severe toxicity, is not effective in all patients and chemo-resistance can develop. NK cells are emerging as powerful tools for cancer immunotherapy and we hypothesised that NK cell-based therapies could be improved by combining them with other approaches that modify various components of the tumour and its environment and renders tumours more sensitive to NK cell cytotoxicity.

In addition to being less toxic and having a lower impact on a patient's quality of life, evidence suggests that low-dose metronomic chemotherapy modulates adaptive and innate anti-tumour immune responses. Accordingly, the aim of this study was to enhance anti-tumour activity of adoptively transferred NK-cells using low-dose metronomic chemotherapy.

Herein, the effect of treatment with low-dose doxorubicin on the phenotype of human breast cancer cell lines corresponding to different molecular subtypes of disease [MCF-7 (Luminal A); SK-BR-3 (Her2+); MDA-MB-468 (Triple Negative); MDA-MB-231/RFP/LUC (Triple Negative)] and their sensitivity to NK cell killing was investigated, as was the capacity of combining low-dose doxorubicin and adoptive NK cell transfer to control the growth and metastasis of human MDA-MB-231 xenografts in immunedeficient mice.

We demonstrate that low-dose doxorubicin treatment halts the proliferation of breast cancer cells and triggers expression of natural killer (NK) cell ligands, enhances sensitivity to NK cell-mediated cytotoxicity *in vitro* and modulates important cancer pathways associated with invasion and metastasis. *In vivo,* the combination of low-dose doxorubicin with adoptive transfer of IL-2 activated human NK cells, reduced the growth of MDA-MB-231 cell-derived tumours in a pre-clinical murine xenograft model.

These findings confirmed the capacity of low-dose chemotherapy to sensitise tumours to NK cell cytotoxicity and indicated the therapeutic potential of combining low-dose chemotherapy and NK cell therapy for the treatment of patients with TNBC, for whom current therapies are largely ineffective.

Chapter 1: Introduction

1.1 Overview of the Immune system

The immune system is a collection of organs, cells and proteins that work in coordination within an organism to protect its host from threats. These can be external in the form of pathogens, such as viruses and bacteria, or internal, such as toxins and tumour cells. At the centre of this protection is the immune cells' ability to recognize and discriminate between non-self/altered-self and self/healthy molecules and to mount an adequate immune response. In an immunocompetent setting, an effector response will develop based on the type of the threat and will lead to its neutralization and elimination. The immune system is broadly divided into the innate and adaptive immune system, although these arms have overlapping functions and are closely related.

1.1.1 The innate and adaptive immune systems

The innate immune system represents the first line of defence, and is composed of physical and chemical barriers, circulating blood proteins and a panoply of cells. The cells of the innate immune system include phagocytic leukocytes (neutrophils, macrophages), dendritic cells (DCs), natural killer (NK) cells and other innate lymphoid cells. The activation of the innate response is based on the recognition of pathogen-associated molecular patterns (PAMPs), which are structures shared by various pathogens; or soluble factors, heat shock proteins and abnormal membranes that stressed cells typically release, called damage associated molecular patterns (DAMPs) by cognate pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and C type lectin receptors on the surface of innate immune cells (Boller, Felix 2009; Mogensen 2009). The innate response is able to quickly eliminate the majority of pathogens however, some pathogens can evade innate effectors. Protection against these involves the development of specific, more potent responses mediated by the adaptive immune system. Adaptive immune responses take longer to develop, are antigen-specific and are characterized by the establishment of long-term memory. They are divided into two types: humoral response, mediated by B lymphocytes that produce antibodies, and cell-mediated response, mediated by T lymphocytes which includes $CD4^+$ T helper (TH) cells and $CD8^+$ cytotoxic T lymphocytes and a panoply of other effector and regulatory cell populations and sub-populations.

1.1.2 Tumour immunology - Tumours and immunity

Evidence from animal models and clinical studies in cancer patients demonstrates the ability of the immune system to recognise and eradicate tumours. Although the idea that immune cells have the ability to control cancer growth was introduced more than 100 years ago by Paul Ehrlich (Ehrlich 1909), the actual concept of cancer immunosurveillance was conceived and validated later (Kaplan 1971; Stutman 1975; Thomas 1982; Burnet 1957). First, through results demonstrating the role of IFN-γ in promoting tumour recognition and control in mice implanted with IFN-γ-insensitive tumour cells *versus* mice implanted with wild-type IFN-γ-sensitive tumour cells (Dighe et al. 1994) and subsequently by various studies demonstrating the role of pre-existing immunity and various components of the immune system in protecting mice against different types of malignancies. The main findings from these studies which led to the establishment and development of the modern tumour immune-surveillance concept are reviewed by Vesely et al. (Vesely et al. 2011). The interaction between the immune system and tumours is a complex, dynamic process. Understanding the mechanisms governing this process, often referred to as the "cancer immune cycle" is essential for the development of immune modulatory therapies. The generation of a successful specific anti-tumour immune response depends on the dynamics of tumour growth, as tumour cells can escape both the innate and adaptive responses by a process called immunoediting.

1.1.2.1 Immunoediting

The three sequential phases in the cancer immunoediting theory consists of the elimination, equilibrium and escape phases. Elimination occurs when tumour cells are successfully eliminated by innate and adaptive immune cells, usually prior to the tumour becoming clinically apparent. However, the immune response might not achieve a complete elimination and might create a selection towards non-immunogenic cancer cells, which can survive leading to the equilibrium phase. The equilibrium phase is thought to be the longest phase of the process. Finally, resistant tumour cell variants with cumulative mutations may arise and allow them to escape immunosurveillance. Subsequently, in the escape phase, tumour cells form established primary tumours which can go on to metastasize (Dunn et al. 2004; Kim et al. 2007).

1.1.2.2 Immune escape mechanisms

Immune evasion is one the key hallmarks of cancer (Prosperi, Goss 2010; Hanahan, Weinberg 2011). Tumours have the ability to evade immune recognition and destruction via different mechanisms such as loss of antigenicity; this could be by downregulation of MHC class I expression, or antigenic

modulation where tumours acquire mutations in genes involved in antigen presentation and processing (Schreiber et al. 2011). Other mechanisms include, induction of tolerance; where tumour cells decrease their immunogenicity by upregulating the expression of immunoinhibitory molecules (such as PD-L1) (Blank et al., 2005), producing immunosuppressive molecules such as IL-10, Transforming Growth Factor beta (TGF beta) or Vascular Endothelial Growth Factor (VEGF) and through altering the balance between effector and regulatory cells, such as myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) which will contribute to the generation of immune tolerance and an immunosuppressive microenvironment (Pawelec et al., 2000 ; Itakura et al., 2011; Yang et al.,2010).

1.1.3 Anti-tumour immunotherapy

Cancer therapeutic modalities belong to five main categories; surgery, radiation, chemotherapy, targeted therapy, and immunotherapy. Immunotherapy represents a treatment modality based on harnessing the power of the patient's own immune system. The first immunotherapy concepts in the context of cancer treatment date back to more than 137 years ago, when it was first noticed by Busch (Busch,1868) and Fehleisen (Fehleisen,1882) that accidental infections by erysipelas induced tumour regression in cancer patients. This was followed by Coley's studies on several types of malignancies using bacterial toxins (Coley 1910; Wiemann, Starnes 1994). Exciting progress has been made in the field of immunotherapy since its conception (Oiseth, Aziz 2017; Eno 2017). This was highlighted by the US Food and Drug Administration (FDA) approval of different therapeutic agents, the emergence of new therapeutic concepts, and the award of 2018 Nobel Prize in Physiology and Medicine from the Nobel Committee to Tasuku Honjo and James Allison "for their discovery of cancer medicine by inhibition of negative immune regulation" (www.nobelprize.org/prizes/medicine/2018).

Although the actual concept of immunotherapy date back to the last century (Old 1996), it is only recently that it has gained a wide interest mainly due to the advances in the fields of immunology research and immune-oncology, but also the lack of success that other therapeutic modalities are encountering for some cancer types. In fact, most often for solid tumours, the primary therapeutic indication is surgery for operable tumours, which can be curative for local disease. However, advanced and metastasized cancers have a poor prognosis and such tumours continue to account for ~90% of cancer-related deaths(Chaffer, Weinberg 2011; Seyfried, Huysentruyt 2013).

Diverse strategies have been investigated to convert the anti-tumour properties of different elements of the immune system into therapeutic approaches. They broadly fall into five groups: vaccines, antibody-based therapies, immune targeted agents, cytokine-mediated therapies and adoptive cell

therapies (Zhang, Chen 2018; Riley et al. 2019; Couzin-Frankel 2013). Adoptive cell transfer therapy (ACT) is based on supplying autologous or allogeneic functional immune effector cells in high numbers to attack the tumour. The main approaches involve autologous tumour infiltrating lymphocytes (TILs) or autologous T cells genetically engineered with T cell receptors (TCRs) having a defined specificity or chimeric antigen receptors (CARs). However natural killer (NK) cells are also a powerful tool in this context.

1.2 Natural killer cells

Natural killer (NK) cells are the predominant innate lymphocyte subsets that contribute to the first line of defence against tumours and viruses. They comprise 5–15% of circulating lymphocytes and are characterized by a large, granular morphology. Derived from $CD34^{+}$ progenitor cells in the bone marrow, commitment to the NK cell lineages is associated with loss of CD34 and gain of the cytotoxicity receptors NKp44 and NKp46 (Freud, Caligiuri 2006). NK cells are found in different lymphoid and non-lymphoid tissues including the liver, lungs, gut and uterus. They are also one of the first immune cells to arrive at target inflamed organs (Carrega, Ferlazzo 2012; Shi et al. 2011). Phenotypically, human NK cells are defined by the absence of the T cell receptor CD3 and the expression of the neural cell adhesion molecule CD56 (Cooper et al. 2001). The relative expression of CD56 and the Fc gamma receptor 3 (CD16, FcγRIIIA) which enables antibody dependent cytotoxicity (ADCC) allows two main subpopulations of NK cells to be distinguished; $CD56^{dim}CD16^{bright}$ representing ≥90% of total NK cells, referred to as the cytotoxic subset, and cytokine producing $CD56^{bright}CD16^{dim}$ cells, although many other populations exist and the discrimination between cytotoxic effector functions and cytokine regulatory functions is not binary (Cooper et al. 2009; Caligiuri 2008). NK cells were originally defined by their natural cytotoxicity functions, but it was later shown that they were also able to mediate cytokine production and secretion (Vivier et al. 2008).

As they have a central role in the research programme described in this thesis, the main aspects of their effector functions and use in immunotherapy approaches will be discussed in this section.

1.2.1 Mechanisms of action of NK cells

1.2.1.1 Activating and inhibitory receptors

NK cell activation relies on a fine balance of signals originating from activating and inhibitory receptors expressed at their surface. Also, the co-engagement of various activating receptors is required for NK cell lysis of target tumour cells, as target lysis does not occur by default when inhibitory receptor/ligand interaction is absent (Bryceson et al. 2009).

Inhibitory receptors

Traditional NK cell inhibitory receptors belong either to the Ig superfamily - these are the killer immunoglobulin-like receptors (KIRs) that bind to human leukocyte antigen (HLA- A, -B or-C), or to the C-type lectin receptor NKG2A which forms a complex with CD94 (CD94/NKG2A) - these bind to the human leucocyte antigen HLA-E. Although they vary in their structure, upon engagement, these receptors share a common signalling pathway through the phosphorylation of their shared motif, the

immune tyrosine-based inhibitory motifs (ITIM). This leads to the recruitment of tyrosine phosphatases, commonly, the Src homology 2 domain-containing phosphatases (SHP1 or 2) that prevent Ca^{2+} influx and induce an inhibition of various NK cell effector functions (Vivier et al. 2008; Lanier 2005; Tomasello, et al. 2000). These receptors are central to the regulation of self-tolerance and activation of NK cells. Indeed, in order to avoid killing of self-cells, NK cells express at least one inhibitory receptor specific for self-MHC molecules (Tomasello, et al. 2000).

Other inhibitory receptors include Ig-like transcripts (ILTs) or leukocyte Ig-like receptors (LIRs). They are homologous to KIR receptors, as they contain the typical ITIMs motifs. However, they interact mainly with non-classical HLA-G molecules, and with a better specificity (Vitale et al., 1999). Additionally, NK cells express a killer cell lectin-like receptor G1 (KLRG1) that also contains an ITIM and regulates NK cell effector functions (Ito et al. 2006).

Activating receptors

NK cells acquire the expression of natural cytotoxicity receptors (NCRs) early during their maturation. These receptors belong to the Ig superfamily and are germline encoded transmembrane receptors. NCRs can mediate direct non-MHC-restricted killing of target cells and the secretion of cytokines and include NKp30, NKp44, NKp46, NKp65 and NKp80. Although NKp30 and NKp46 are constitutively expressed on NK cells in the peripheral blood, NKp44 was shown to be expressed on activated NK cells only (Lanier 2005). NCRs can bind to viral ligands, heat-shock associated proteins or tumour antigens and associate with immunoreceptor tyrosine-based activation motif (ITAM)-containing subunits. Their surface density was found to correlate with the magnitude of NK cell cytotoxicity against target cells (Baychelier et al. 2013; Pende et al. 1999; Mandelboim et al. 2001).

The second type of NK cell activating receptors belong to the lectin-like family. The NK group 2 member D and F (NKG2D and F). The NKG2D receptor associates with DNAX activation protein DAP-10 whereas NKG2F binds to DAP12. Of note, NKG2F does not translocate to the cell surface (Kim et al., 2004). When associated with the DAP10 adaptor protein, NKG2D can recruit the phosphoinositide 3-kinase (PI3K) and signal through its binding motif (Billadeau et al. 2003; Wu et al. 1999). The cellular ligands of NKG2D include MHC class I chain-related protein A MICA and MICB and the UL16-binding proteins 1-6 (UBLP1-6) (Mistry, O'Callaghan 2007; Biassoni n.d.). Additional receptors are important in the activation of NK cell cytotoxicity include the DNAX accessory molecule-1 (DNAM-1), CD2 and 2B4 (Bryceson et al. 2009).

NKG2D and DNAM-1 ligands are frequently expressed on stressed cells and various studies have demonstrated the key role played by these receptors in immune surveillance of tumours (Morisaki et al. 2012a; Fionda et al. 2015a). NKG2D has also been shown to contribute to shape tumour immunogenicity, as evidenced by a tumour cell's ability to prevent NKG2D-mediated recognition through NKG2D ligand shedding (Pegram et al. 2011). A comprehensive overview of various NK cell receptors is displayed in Figure 1.1.

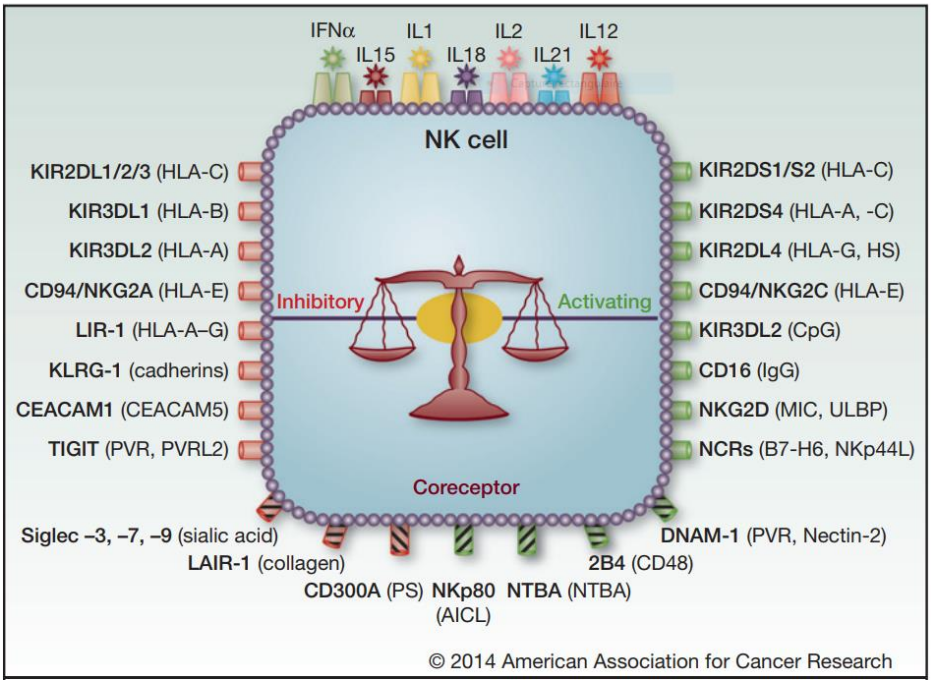

Figure. 1.1. NK cell surface receptors and their ligands. Representation of a human NK cell. Shown on top are cytokine receptors. Receptors' s ligands are shown within parentheses. Other known receptors not shown include chemotactic and adhesion receptors (CD2 and β1 and β2 integrins) as well as some co-receptors (CD96, CS1, and TLR). (Reprinted from Clinical Cancer Research,2014,20/13,3390-3400, Leung, Infusions of Allogeneic Natural Killer Cells as Cancer Therapy, with permission from AACR) (Leung 2014).

1.2.1.2 NK cell cytotoxicity

Unlike B and T cells, NK cells do not require prior sensitization and have the innate ability to eliminate cancer cells in a non-major histocompatibility complex (MHC) and non-tumour antigen restricted manner (Chiossone et al. 2018a; Vivier et al. 2011a; Raulet 1996; Moretta et al. 1992). NK cell recognition of target cells and cytotoxicity are mainly regulated by the interplay of multiple inhibitory and activating receptors that bind cognate ligands on target cells. The cytotoxic response can be divided into three main processes; target cell recognition, target cell contact and formation of immunological synapse, and induction of target cell death (Mace et al. 2014). Upon target cell recognition, NK cells can

respond via two types of actions (Figure 1.2) - the perforin-granzyme serine protease killing and caspase-mediated apoptosis. NK cells form an immune synapse at the point of contact with the tumour cell (Krzewski, Strominger 2008) and, with sufficient activation signals, the NK cell cytoskeleton rearranges leading to the polarization and release of their cytotoxic granules (degranulation) via exocytosis. The two main components of these cytotoxic vesicles are perforin and granzymes (Smyth et al. 1999). Once released at the immune synapse, perforin forms pores in the membrane of target cells through binding to the lipid bilayer. This allow granzymes, mainly the serine protease granzyme B to enter the tumour cell and induce apoptosis or programmed cell death (Orange 2008). Alternatively, endocytosis has been proposed as a different delivery pathway of granzymes into target cells (Bird et al. 2005; Thiery et al. 2011). Second, NK cells can induce caspase-mediated apoptosis via cell surface death receptor ligation mediated by Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL) (Lavrik et al. 2005). Moreover, NK cells can also exert their functions through antibody-dependent cellular cytotoxicity (ADCC) which involves ligation of the CD16 receptor by antibodies bound to the surface of tumour cells.

In addition to direct cytotoxic effects, NK cells can exert indirect anti-tumour effects by stimulating other immune cells through their release of pro-inflammatory cytokines, most notably interferon gamma (IFNγ) and tumour necrosis factor-alpha (TNFα).

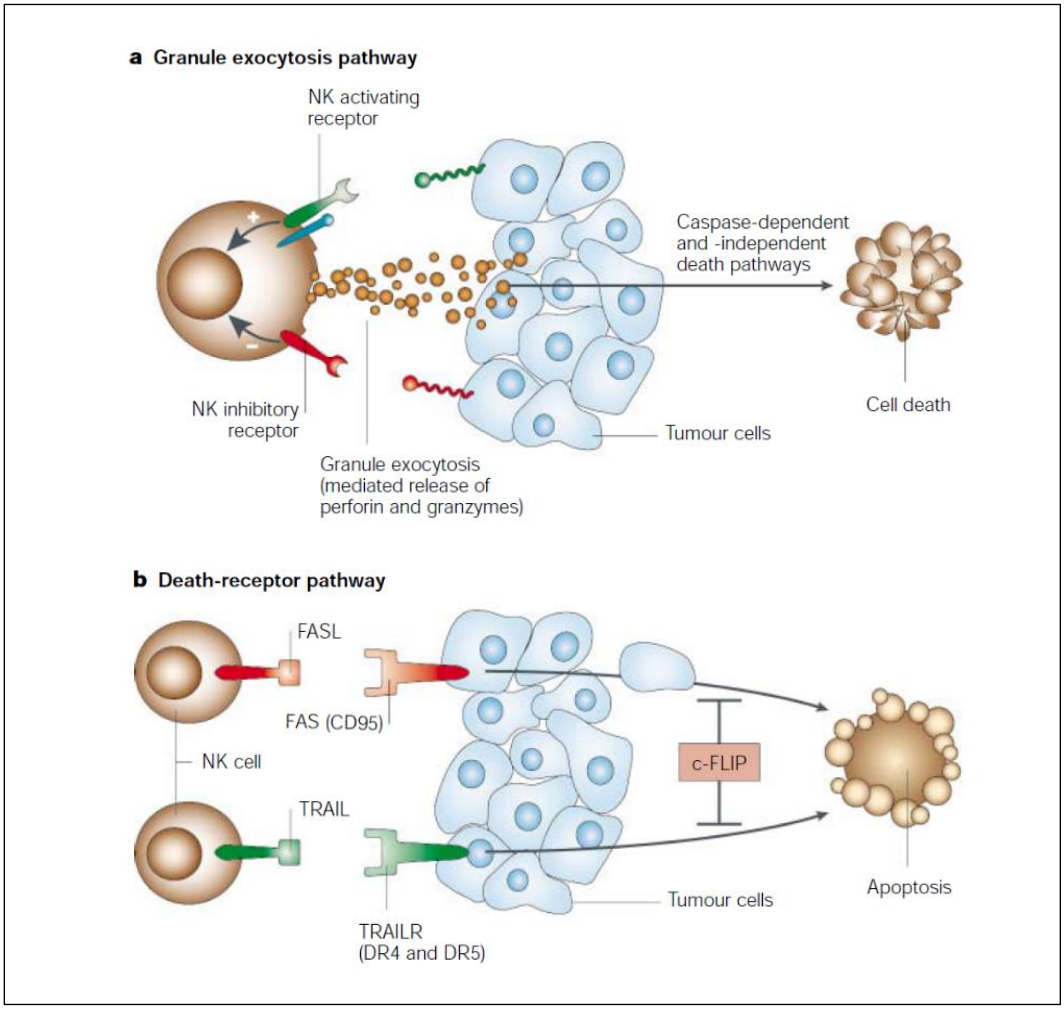

Figure 1.2 – NK cell cytotoxicity. (a) Perforin/granzyme mediated apoptosis (b) Caspase-mediated apoptosis via death inducing ligands. c-FLIP: Cellular FLICE-like inhibitory protein. Reprinted by permission from Springer Nature: on behalf of Cancer Research UK: [Nature Reviews Cancer] [New aspects of natural-killer-cell surveillance and therapy of cancer, Mark J. Smyth et al, [COPYRIGHT] (2002). (Smyth et al. 2002).

1.2.1.3 Cytokine secretion

In addition to their cytolytic activity, NK cells serve a number of regulatory functions via their release of various pro-inflammatory cytokines such as IFN-γ, TNF-α and of chemokines such as CCL3 and CCL4 that contribute to the development and regulation of adaptive immune responses. IFN-γ is known to be the signature cytokine of NK cells, it has a wide range of roles in immune responses, including anti-proliferative and anti-angiogenic effects, and it can also induce sensitivity to apoptosis (Chin et al., 1996; Beatty & Paterson, 2001; Chawla-Sarkar et al., 2003). In addition to contributing to the priming and presentation of antigens to antigen presenting cells (APCs) through upregulating MHC class I molecules on the surface of target cells (Seliger et al., 2008), IFN- γ can directly activate macrophages which leads to the production of chemokines responsible for recruiting other effector cells to the

inflammation site (Hu et al. 2008). TNFα is also produced by NK cells which initiates a cytokine cascade during an inflammatory response and can directly induce target cell death (Waters et al., 2013). Although cytotoxicity and cytokine secretion were regarded as two distinct functions of NK cells in the past, the combination of IFN-γ and TNF-α has been shown to synergistically enhance NK cell cytotoxicity through upregulation of ICAM-1 expression in target cells (Wang et al. 2012). Interestingly, when present simultaneously, TNF- α and IFN-γ have been shown to induce tumour senescence (Braumüller et al. 2013a).

1.2.1.4 Memory function of NK cells

NK cells are commonly considered as being incapable of mounting memory responses. This is mainly due to their use of germline-encoded receptors which are thought to lack antigen-specificity, a characteristic believed to be essential for the formation of immunological memory (Kurtz 2005). However, emerging evidence has supported the concept of a memory function for NK cells, including features of persistence, reactivation, and possibly the ability to respond to specific antigen stimulation (Vivier et al. 2011b; Peng, Tian 2017). Additionally, recent studies indicate that cytokine stimulation (a combination of IL-12, IL-15, and IL-18) can induce memory-like NK cells that display higher functional competence and longevity *in vitro and in vivo* (Cooper et al. 2009; Romee et al. 2012; Capuano et al. 2019a; Romee et al. 2016).

1.2.2 NK cells in cancer

The importance and pivotal role of NK cells in the immune surveillance of cancer is supported by several lines of *in vitro* and *in vivo* evidence. A large number of studies have demonstrated the capacity of NK cells to kill different types of tumour cell lines as well as primary tumour cells from patient biopsies *in vitro.* In most cases, this was found to be primarily mediated by engagement of NKG2D or DNAM-1 (Castriconi et al. 2004; Malmberg et al. 2017a). Antibody-mediated depletion experiments of NK cells in mouse models of cancer have demonstrated the contribution of NK cells in controlling the growth and metastatic spread of spontaneous, transplanted or carcinogen-induced tumours (Gorelik et al. 1982; Smyth et al. 2000; Kärre et al. 1986; Cerwenka et al. 2001; Malmberg et al. 2017a). On the other hand, in the context of human disease, analysis of NK cell infiltration in tumour samples demonstrated the correlation of high intra-tumoural NK cell levels with a good prognosis of patients with multiple cancer types. Of note, some of these studies used CD57 as NK cell marker, which is not ideal as the CD57 epitope is expressed on about 70% of $CD56^{dim}$ NK cells, but also on T cell subsets (Coca et al. 1997; Ishigami et al. 2000; Villegas et al. 2002; Hsia et al. 2005; Zhu et al. 2009).

A recent interesting finding, was the ability of NK cells to recognise and lyse cancer stem cell (CSC)-like populations, as defined on the basis of their expression of CSC-associated proteins CD44, CD24, CD326 (EpCAM) or the aldehyde dehydrogenase (ALDH) enzyme, also a cancer stem cell marker, in human cancer cell lines and in patient-derived tumour samples (Ames et al. 2015; Murphy Urayama et al. 2019; Luna et al. 2017; Pietra et al. 2009; Castriconi et al. 2009; Toledo-Guzmán et al. 2019). Furthermore, Bottcher et al have defined a new role for NK cells, demonstrating that they arrive early to the tumour microenvironment and recruit type 1 ($CD103^+$) dendritic cells (DCs) to the tumours, and that this cooperation results in the recruitment of $CD8^+$ T cells and induction of immune responses (Böttcher et al. 2018; Fessenden et al. 2018).

1.2.2.1 Tumour escape from NK cell surveillance

As discussed earlier, the ability of tumours to grow and spread is mainly due to the immune escape mechanisms they developed, including evasion from NK cell surveillance. Tumour cells establish a suppressive tumour microenvironment (TME) by modulating the expression of different factors that affect immune signalling and immune infiltration. Indeed, tumours are characterised as 'hot', 'altered' or 'cold' according to the presence and infiltration of immune cells (Galon, Bruni 2019).

Tumour cells have the ability to secrete inhibitory cytokines such as transforming growth factor beta (TGF-β), IL-6 and IL-10, other immunomodulatory molecules such as prostaglandin E2 (PGE_2), indoleamine 2,3-dioxygenase (IDO), and recruit suppressive immune cells such as Treg cells and MDSCs. Tumour cells can also subvert NK cell anti-tumour activity by reducing the expression of NK cell activating receptor NKG2D and CD16, or shedding NKG2D ligands, namely MICA and B. Tumour cells can also express inhibitory receptor specific ligands such as glucocorticoid-induced TNFR-related protein (GITR) that can down modulate activating receptors NKG2D on NK cells. All of this translates into decreased numbers of NK cells and decreased tumour infiltration. A defective expression of activating receptors and overexpression of inhibitory receptors has been observed in various types of cancer (Guillerey et al. 2016a). Figure 1.3 summarises the main strategies that tumours employ to evade NK cell surveillance.

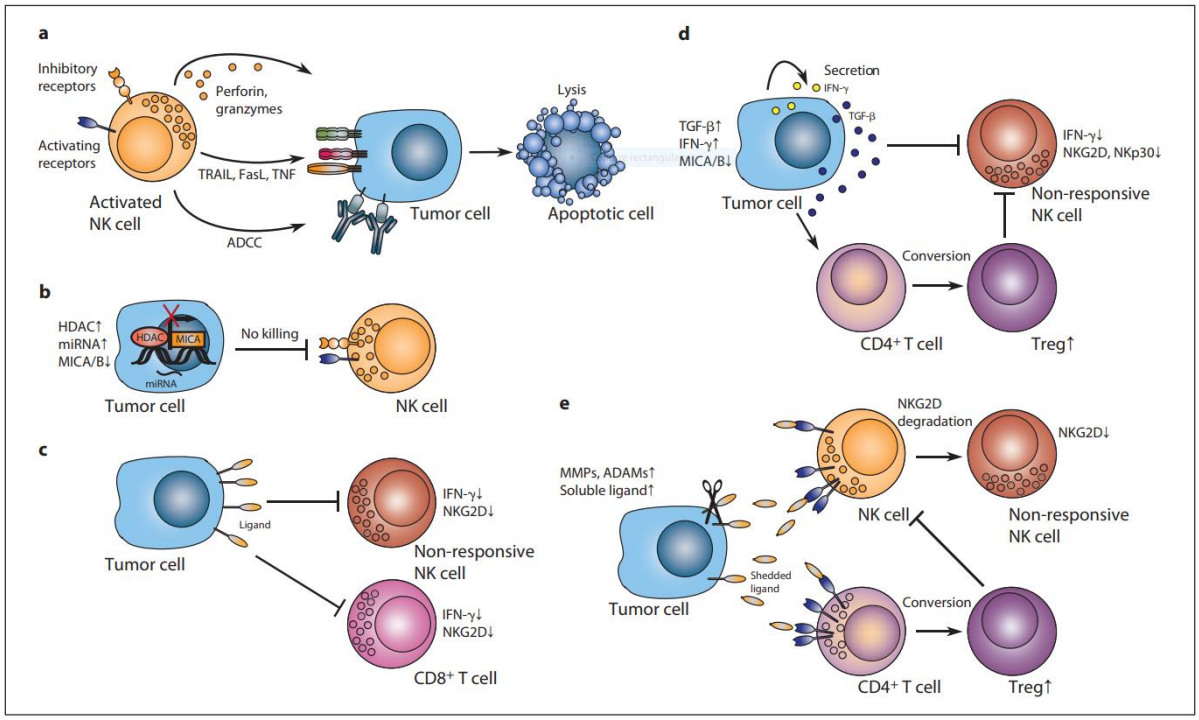

Figure 1.3. Mechanisms of tumour escape from NK cell surveillance. (a) NK cell recognition of target cells. (b) alterations in DNA modifying enzymes inhibit the expression of MICA/B (c) persistent expression of activating ligands resulting in hypo-responsiveness, decreased NKG2D expression and reduced IFN-γ production(d) Tumour-released cytokines such as TGF-β repress MICA/B expression, downregulate NKG2D and IFN-γ and promote the conversion of $CD4^+$ T cells into regulatory $CD4^+CD25^+FOXP3^+$ T cells (e) shedding of MICA ligands due to upregulation of the matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases ($ADAM_S$), leading to degradation of NKG2D and conversion of $CD4^+$ T cells into regulatory $CD4^+CD25^+FOXP3^+$ T cells. (Reprinted from (Groth et al. 2011). Copyright © 2011 Karger Publishers, Basel, Switzerland.

1.2.2.2 Approaches using NK cell for cancer immunotherapy and clinical outcomes

The important contribution of NK cells to anti-tumour immune responses has been illustrated by several pre-clinical and clinical studies which made them a very attractive tool for immunotherapy (Hu et al. 2019a). Different strategies have been investigated as NK cell-based treatments, these are aimed at either repairing, replacing or enhancing the effector functions of NK cells (Tonn et al. 2013a; Leung 2014; Hu et al. 2019b; Rezvani, Rouce 2015). Strategies aiming to enhance the activation, proliferation and cytotoxicity of patient's NK cells include the administration of immune stimulants to patients such as cytokines and antibodies. A variety of cytokines have been used in an attempt to improve NK cell function and activation including IL-2, IL-15, IL-12, IL-18, and IL-21 which have been described to regulate NK cell maturation, activation and survival (Zwirner, Domaica 2010a; Nayyar, Chu, Mitchell S Cairo 2019). In addition to the adoptive transfer of *ex-vivo* activated and expanded NK cells, recent pre-

clinical studies introduced the use of exosomes derived from NK cells as a promising method for inducing anti-tumour effects (Zhu, Kalimuthu, et al. 2017; Lugini et al. 2012; Hu et al. 2019a). The adoptive transfer of NK cells will be discussed further below, and the contribution of other NK cell-based approaches will be highlighted in this context.

1.2.2.2.1. NK cell adoptive transfer

For adoptive cell therapy, NK cells can be derived from different sources. Usually, they are generated from the peripheral blood of the patient or a donor (autologous or allogeneic). NK cells can also be derived from hematopoietic stem cells (HSCs) in umbilical cord blood, embryonic or induced pluripotent stem cells (iPSCs) (Knorr and Kaufman, 2010). Another source is NK cell lines, and these are gaining more attention as a potential "off-the shelf" therapy due to the ease of its application in terms of culture, cost-effectiveness and genetic modification (Rezvani, Rouce 2015; Cheng et al. 2013; Klingemann et al. 2016).

Autologous transfer

In autologous transfer, NK cells are generated from the patient, activated and expanded *ex vivo*, after which they are transferred back into the patient. In some instances, patients then receive cytokines, usually IL-2, for sustaining expansion and effector function of the infused cells. The transfer of autologous NK cells is a feasible approach, as it does not need prior immunesuppression, HLA-matching and avoids the risk of graft *versus* host disease (GvHD). However, despite an increase in circulating NK cell numbers in peripheral blood, limited therapeutic effects have been demonstrated. This was reported to be due to the inhibitory signals from self-HLA molecules (Parkhurst et al. 2011a; Rosenberg et al. 2008). Furthermore, the expansion efficiency and activation status of autologous NK cells are not optimal as they are obtained from heavily pre-treated patients. These challenges have motivated the evaluation of approaches involving allogeneic NK cells.

Allogeneic transfer

In allogeneic transfer, it is crucial to isolate NK cells at a high purity and deplete T and B cells in order to reduce the potential for GvHD and any risk of immune response to host erythrocytes and autoimmunity (Miller et al. 2005). The advantage of NK cells from healthy donors is that they are functional and not previously exposed to an immunosuppressive environment. In this context, HLA-matched or haploidentical (partially-matched) donors can be used. Haploidentical donors that do not express inhibitory KIRs able to recognize the patient's HLA molecules have more potential to generate

potent responses, as donor NK cells are not inhibited by a patient's tumour cells (W. Wang et al. 2017; Lupo, Matosevic 2019).

The first pieces of evidence demonstrating the *in vivo* efficacy of allogeneic NK cells following both donor hematopoietic stem cell transplantation (HSCT) and allogeneic infusions of isolated NK cells, was in patients with acute myeloid leukaemia (AML). An increased control of leukaemia was observed along with lower rates of relapse without NK cell-related GvHD in the allogeneic HSCT setting (Ruggeri et al. 2008). Despite these early signs of efficacy with HSCTs, studies carried out by Miller et al demonstrated better results in patients with advanced cancers (metastatic melanoma, metastatic renal cell carcinoma and acute AML) using haploidentical adoptive transfer of allogeneic IL-2 activated NK cells followed by 14 days IL-2 administration. Complete remission was observed in 30% of patients with AML having a poor prognosis (Miller et al. 2005).

Other studies have shown KIR ligand mismatching between patient and donor NK cells induces better anti-tumour effects. Rubnitz et al, infused KIR-HLA mismatched NK cells into paediatric patients with AML and demonstrated prolonged disease-free survival (DFS) and overall survival (OS) (Rubnitz et al. 2010). Infusion of haploidentical NK cells also induced remission in patients with non-Hodgkin's lymphoma (Bachanova et al. 2018). In a recent study by Cooley et al, infusion of haploidentical NK cells with recombinant human IL-15 (rhIL-15) achieved remission in 35% of patients with refractory AML (Cooley, He, Bachanova, Gregory M. Vercellotti, et al. 2019).

Induced pluripotent stem cell derived NK cells

With the aim of generating a more homogenous NK cell population, studies have used NK cells derived from induced pluripotent stem cells (iPSCs). Pre-clinical studies of these iPSC-NK cells have demonstrated effectiveness against leukaemia and ovarian cancer (Woll et al. 2005; Hermanson et al. 2016). Although these iPSCs show promise as an NK cell immunotherapy, the demonstration of efficacy and feasibility in clinical trials is still pending (Hu et al. 2019a).

NK cell lines

NK cell lines offer various benefits as a source of NK cells for adoptive transfer therapy. In addition to the ease of procurement, as they avoid the need for leukapheresis, they can be easily expanded enabling clinical scale manufacturing of homogenous cell populations (Tarn et al. 2003). They are also free of contaminating T and B cells, avoiding any potential GvHD effects associated with blood-derived

NK cells. Various ongoing clinical trials are demonstrating their safety and efficacy for use in adoptive transfer therapy. Among the established NK cell lines are NK-92, NK-YS, YT, YTS, NKG, NKL, KHYG-1, SNK-6, IMC-1, NKL and HANK-1. All of these cell lines have been derived from patients with leukemia/lymphoma, as a consequence of which cells have to be irradiated prior to infusion (Cheng et al. 2011). Although various studies are investigating the listed cell lines, only the NK-92 cell line has progressed to clinical trials and has received approval from the U.S. Food and Drug Administration (FDA) (Suck et al. 2016).

Originally developed by Klingemann's group and currently licensed by NantKwest (https://nantkwest.com), NK-92™ cells differ from peripheral blood-derived NK cells in a few points. They lack the expression of CD16 and so do not induce ADCC (Klingemann et al. 2016), along with some typical NK activating receptors such as NKp44 and NKp46. They are lack the expression of KIR inhibitory receptors, the only exception for this is the receptor KIR2DL (Suck et al. 2016), and this was shown to result in a more potent cytotoxic activity due to KIR mismatch following adoptive transfer (Maki et al. 2001). Multiple trials indicate that NK-92™ cells are safe and appear well-tolerated when used in adoptive therapy setting (Arai et al., 2008; Tonn et al., 2013; Tsartsalis et al., 2015; Boyiadzis et al., 2017; Williams et al., 2017; Tang et al., 2018; Chrobok et al., 2019; Törnroos, Hägerstrand And Lindqvist, 2019). Among the ongoing trials, many employ NK-92™ that are genetically engineered to induce more efficient anti-Tumour responses.

Genetic modification of NK cells

Genetic modification is a promising approach for creating more efficient NK cells. In this context, different strategies for enhancing different components of NK cell anti-tumour activity, including expression of autocrine cytokines which promote NK cell survival, over-expression of activating receptors, CAR arming for targeting tumour associated antigens and more recently expression of high affinity CD16 (haNK®) for improving ADCC activity have been investigated (Guillerey et al. 2016a; Klingemann 2014; Binyamin et al. 2008; Jochems et al. 2016). The major source of cells used for CAR-NK cell engineering are NK-92 cell lines, although CAR-NK cells based on primary NK cells have also been reported (Chu et al. 2014). More recently, Kaufman's group explored the use of iPSCs in this context and demonstrated that NK-CAR-iPSC-NK cells containing the transmembrane domain of NKG2D, the 2B4 co-stimulatory domain, and the CD3z signalling domain, were able to control tumour growth in an ovarian cancer xenograft mouse model (Y. Li et al. 2018). In addition to the previously mentioned advantages associated with the use of NK-92 cells, another benefit in this framework is the relative ease of their transfection, as they show a transfection efficiency of at least 50% with viral or non-viral methods, such as electroporation.(Ingegnere et al. 2019; Boissel et al. 2012; Klingemann et al. 2016).

Multiple pre-clinical studies have shown promising efficacy of CAR-NK cells targeting various tumour-associated antigens (TAAs) in haematological malignancies, but also in a few solid tumours (e.g. glioblastoma and breast cancer). TAAs tested include CD19, CD20, CD33, HER2, EGFR, EpCAM and CD138 (Romanski et al. 2016; Jiang et al. 2014; Mehta, Rezvani 2018; Zhang et al. 2017). Various CAR-NK cells have also progressed to clinical trials. For instance, transduction of a CD33-specific chimeric receptor in NK-92 cells has been shown to be safe in patients with AML (Tang et al. 2018), whereas clinical trials of CD19 CAR-NK cells have indicated potential efficacy for lymphoid malignancies (Romanski et al. 2016). In solid tumours, the clinical trial (NCT02839954) was the first to prove the safety and efficacy of CAR-NK cells. The trial included patients with lung, pancreatic, colon and ovarian cancer. These studies used dual-specific NK92 cells expressing CAR-Mucin1 and CAR-PD-1 with CD27 and 4-1BB (CD137) as signalling domains.

Overall, genetic modification provides more options for enhancing NK cell anti-tumour activity, albeit with various factors to consider for successful applications.

1.2.2.3 Methods for advancing NK cell therapies

Although it is clear that NK cell adoptive transfer is a promising tool for cancer treatment, some limiting factors and barriers need to be overcome for clinical practice. These include the expansion and activation of cells, their persistence once infused into the patient, their homing to tumour sites, and sustaining their activation and overcoming inhibition and suppression from the tumour microenvironment (Hu et al. 2019a). Various strategies have been developed in this sense, some of these will be discussed in this section.

1.2.2.3.1. NK cell expansion and activation

NK cell transfer therapy requires large number of functional cells. Except for NK cell lines, *ex vivo* expansion of NK cells is crucial for clinical application. Thus, several NK cell expansion protocols have been developed using either cytokines or feeder cells combined with cytokines. Although IL-2 and IL-15 were initially used, combining IL-12, IL-15, and IL-18 showed enhanced effector functions with increased IFN-γ secretion and cytotoxicity, but also an induction of memory properties (Romee et al. 2012). Feeder cell-based strategies applied in the clinic include irradiated peripheral blood mononuclear cells (PBMCs) and cell lines. Moreover, artificial antigen-presenting cells (aAPCs) have been developed as feeder cells for expanding NK cells *ex vivo*, such as gene-modified K562 feeder cells expressing membrane-bound IL-21 (K562-mbIL21, developed by Denman et al.,2012) which have been shown to induce intensive proliferation and a significant expansion of NK cells with a yield of 35,000-fold in 3 weeks (Denman et al. 2012; Somanchi, Lee 2016). Phase I clinical trials using these NK cells in

patients with leukaemia after transplantation of allogeneic hematopoietic stem cells have demonstrated their safety and efficacy in improving NK cell anti-tumour activity (Ciurea et al. 2017). The Lowdell group has proposed that NK cell cytotoxicity is dependent on a 'priming-triggering' signal (Kottaridis et al. 2015). For this, cell lysates from the leukaemia cell line CTV-1 (CNDO-109) were used to prime NK cells. A phase I/II clinical trial using activated haploidentical NK cells co-incubated with CNDO-109 prior to infusion in patients has indicated tolerability and a reduced incidence of disease relapse in patients with AML (Fehniger et al. 2018).

Another approach is to activate autologous NK cells *ex vivo* using a 14-mer peptide from the sequence of the heat shock protein 70 (Hsp70) in combination with low dose IL-2 (ENKASTIM-ev, multimmune GmbH, Munich)(Hsp70). This approach results in NK cell activation and the targeting of tumours expressing a membrane form of Hsp70 (Shevtsov et al. 2018). A Phase I study in patients with metastatic colorectal and non-small cell lung cancer demonstrated the safety of Hsp70-activated autologous NK cells (Krause et al. 2004) and a Phase II trial in patients with non small cell lung carcinoma is ongoing (Specht et al. 2015). Furthermore, combining this approach with checkpoint inhibition has induced long-term survival in a patient with non small cell lung carcinoma ((Kokowski et al. 2019). Furthermore, Romee et al. demonstrated that short-term cytokine stimulation (IL-12, IL-15, and IL-18) of NK cells generate populations with "memory-like" phenotypes that exhibit increased survival and effector functions, thereby proposing that this approach could enhance the efficacy of NK cells in cancer immunotherapy (Romee et al. 2012).

1.2.2.3.2. Reducing the inhibitory effect of the tumour microenvironment (TME) using regulatory checkpoint blockade

Advances in immunotherapy have included impressive clinical outcomes associated with the administration of immune cell checkpoint inhibitors in the form of monoclonal antibodies (mAbs) such as anti-CTLA-4 and anti-PD-1 (Korman et al. 2006). In addition to their conventional inhibitory receptors, NK cell functions are also regulated by non-MHC class I inhibitory receptors which represent targets for immune checkpoint blockade and have the potential to enhance the anti-tumour activity of NK cells (Kim, Kim 2018; André et al. 2018; Stojanovic, Cerwenka 2018). These emerging targets include NK cell and T cell-expressed checkpoints PD-1 (programmed death-1), TIGIT (T cell immunoreceptor with Ig and ITIM domains), LAG-3 (lymphocyte activation gene 3 protein) and TIM-3 (T cell immunoglobulin domain and mucin domain-3) and NK cell checkpoint receptors KIR, ILT and CD94/NKG2A (Kim, Kim 2018; Sanchez-Correa et al. 2019). The administration of immunomodulatory

mAbs to block NK cell inhibitory receptors may represent a potential strategy to augment NK cell mediated antiTumour responses.

1.2.2.3.3. Combinatorial therapies

Results from recent clinical studies indicate that optimal efficacy of anti-tumour therapy will likely be achieved through combinations of different therapeutic approaches. In regard to NK cell-based immunotherapy, various approaches have been evaluated including the use of cytokines to enhance the function and proliferation of NK cells (Cooley, He, Bachanova, Gregory M Vercellotti, et al. 2019; Nayyar, Chu, Mitchell S. Cairo 2019), the use of therapeutic antibodies, either tumour-targeted mAbs or Bi or Tri-specific Killer Engagers (BiKEs or TriKEs) to redirect NK cytotoxicity and mediate ADCC (Gleason et al. 2012; Kellner et al. 2011; Hu et al. 2019b) and also checkpoint blockade aimed at the restoration of NK cell functions (Benson et al. 2010; Stojanovic, Cerwenka 2018) . Further efforts to augment NK cell anti-tumour responses have included combination with other immunotherapy approaches, such as oncolytic viruses. In this regard, one group found that the adoptive transfer of NK cells combined with bortezomib and oncolytic HSV-1 significantly prolonged the survival of glioma-bearing mice (Yoo et al. 2016). Similarly, combining EGFR-CAR-NK-92 cells with oncolytic HSV-1 has been reported to enhance anti-tumour effects and survival of breast cancer-bearing mice (X. Chen et al. 2016). Some of the recent clinical trials with NK cell immunotherapy are summarized in Table 1.1. One of the methods that also has the potential to enhance NK cell function is low-dose chemotherapy, which will be reviewed in the next section.

Table 1.1 Recent clinical trials of cellular immunotherapy using NK cells (adapted from (Lupo, Matosevic 2019)

Trial	Source of NK cells	Setting/Disease	Therapy	Phase	status
NK cell adoptive transfer					
NCT01040026	Allogeneic haploidentical NK cells	Multiple myeloma	Ex vivo expanded NK cell infusions (30 days)	I/II	ANR
NCT02316964	Haploidentical PBMC-NK cells	Acute myeloid leukemia	Decitabine and aldesleukin + NK cells	I	ANR
NCT03068819	Cytokine-induced memory-like (CIML) NK cells	Pediatric acute myeloid leukemia (relapse after allogeneic HSCT)	Fludarabine, Ara-C, and G-CSF+CIML-NK cells	I	R
NCT01520558	Allogeneic PBMC-NK cells from first- or second-degree relative	Acute myeloid leukemia	Preparative chemotherapy prior to NK cell infusion	I/II	U
CAR-NK cells					
NCT03415100	NKG2D-CAR allogeneic NK cells (from parent or sibling donor)	Metastatic solid Tumours	CAR-pNK cell infusion	I	R
NCT02892695	CD19-CAR-NK-92 cells	$CD19^+$ leukemia and lymphoma	CAR-NK-92 cell infusion	I/II	R
NCT02742727	CD7-CAR- NK-92 cells	CD7 positive leukemia and lymphoma	CAR-NK92 cell immunotherapy	I/II	R
NCT02839954	MUC1-CAR-pNK cells	MUC1 positive advanced refractory or relapsed Solid Tumours	CAR-pNK cell immunotherapy	I/II	R
NCT03383978	NK-92/5.28.z (HER2.taNK) cells	Recurrent HER2-positive Glioblastoma	Intracranial NK cell injection	I	R
NK cells + therapeutic antibodies					
NCT02030561	expanded activated autologous NK cells	HER2+ breast and gastric cancer	NK cells + trastuzumab	I/II	U
NCT02843061	NS	Recurrent B-cell lymphoma	NK cells + rituximab	I/II	U
NCT02507154	expanded activated autologous NK cells	Refractory head and neck cancer	NK cells + cetuximab	I/II	R
NCT02857920	NS	Malignant solid tumours	NK cells + bevacizumab	I/II	U
NCT02843204	NS	Malignant solid tumours	NK cells + nivolumab	I/II	U
NCT02650648	Allogeneic NK cells	Recurrent non-small cell lung cancer	NK cells + cetuximab	I/II	R
NK cells + checkpoint blockade using mAbs against NK inhibitory receptors					
NCT03841110	FT500 (iPSC-derived NK cell product)	Advanced solid tumours	FT500+Nivolumab, Pembrolizumab, Atezolizumab	I	R
NCT03937895	allogeneic NK cells	Advanced biliary tract cancer	NK cells+ Pembrolizumab	I/II	ANR
NCT02843204	NS	Recurrent solid tumours	Nivolumab+ NK immunotherapy	I/II	U

ANR active, not recruiting, R recruiting, pNK primary NK cells, NS not specified, U unknown

1.3 Low-dose metronomic chemotherapy

1.3.1 The concept of low-dose metronomic chemotherapy

Conventional chemotherapy has been used for a long time in cancer treatment. Conventional chemotherapeutic drugs used in standard clinical protocols are typically administered at the maximum tolerated dose (MTD) which targets rapidly growing cells including tumour and normal cells (Hanahan et al. 2000). These high doses necessitate prolonged resting periods of generally 2–3 weeks between treatment cycles to allow for the recovery from the cytotoxic adverse effects on normal tissues. Consequently, these drug-free periods allow the development of therapeutic resistance, as chemo-resistant cancer cell populations regrow and elicit tumour neovascularization and metastasis (André et al. 2014; Kerbel, Kamen 2004). In fact, in addition to the high toxicity and adverse effects on the patients' quality of life, MTD chemotherapy often leads to disease relapse, despite initial tumour regression (Munoz et al. 2005; Hanahan et al. 2000).

The emergence of targeted therapies and recent advances in tumour biology changed the therapeutic goals for cancer treatment in clinical practice towards the delivery of prolonged responses rather than short-term tumour regression, which does not necessarily mean an increase in long-term survival for patients. One interesting alternative to MTD chemotherapy that has gained more attention recently is low-dose metronomic chemotherapy (LDMC). The term "metronomic chemotherapy", first used by Hanahan (Hanahan et al. 2000), currently refers to the frequent and regular administration of lower doses of chemotherapeutics. Indeed, in contrast to MTD therapy regimens, LDMC is characterized by the frequent administration (daily, weekly or several times per week) of chemotherapeutics at a lower, less toxic dose without long drug-free periods (André et al. 2014; Kerbel, Kamen 2004), the aim being to keep drug concentrations constant in plasma and maintain a continuous exposure of tumours to the chemotherapeutic agent. Initially, the anti-tumour mechanism of LDMC action was thought to be solely based on its anti-angiogenic activity (Kerbel, Kamen 2004). However, extensive studies have led to the identification of additional mechanisms, leading LDMC to be considered as a multi-targeted/multi-modal therapy. In addition to direct anti-proliferative effects on tumour cells and induction of tumour dormancy (Natale, Bocci 2018; Chen et al. 2017), other mechanisms include actions on stromal tissue, targeting of cancer stem cells and activation of the immune system/immunomodulation (Muthusamy 2016; Nars, Kaneno 2013; Chen et al. 2010a; Loven et al. 2013; Robert S Kerbel, Shaked 2017; Natale, Bocci 2018).

1.3.2 Immunomodulation by low-dose metronomic chemotherapy

In the past, chemotherapy was thought to be immunosuppressive, however various studies have demonstrated the positive effects on immune modulation of various chemotherapeutic agents, especially when administered at low-doses in a metronomic schedule (Sistigu et al. 2011; Damber et al. 2006a; Nars, Kaneno 2013; Chen et al. 2010a; Wu et al. 2016a). LDMC can activate the immune response against cancer by different means, including promoting the maturation and antigen presentation functions of DCs (Nars, Kaneno 2013; Tanaka et al. 2009), ablating immunosuppressive T-reg cells and MDSCs in the tumour bed (Sistigu et al. 2011; Ghiringhelli et al. 2007b) and, most importantly, enhancing the activation and function of $CD8^+$ T cells and NK cells (Ma, Waxman 2009; Wu, Waxman 2015; Wu, David J. Waxman 2014).

Various chemotherapy drugs currently used in standard chemotherapy regimens, such as cyclophosphamide, paclitaxel, doxorubicin, cisplatin, and temozolomide, have been studied/evaluated in the context of LDMC to modulate tumour immunity (Nars, Kaneno 2013; K Lien et al. 2013). Changing the dosage of these commonly used drugs was shown to affect APCs, such as DCs. In one study, vinblastine, which is highly immunosuppressive at high doses, was found to promote DC maturation when used at low concentrations (Tanaka et al. 2009). Another study also reported increased activity of $CD8^+$ T cells against melanoma cancer cells following LDM vinblastine (Cruz-Munoz et al. 2009). Low dose cyclophosphamide was also shown to convert $CD4^+$ T-helper type 2 to a type 1 phenotype and enhance T-cell memory responses via the secretion of IFNα (Chen, Emens 2013).

Several preclinical studies have demonstrated LDMC to inhibit and reduce immune suppressive populations of $CD4^+CD25^+$ T-reg cells and MDSCs and alleviate immune suppression and that this was typically accompanied by improved anti-tumour responses (Ghiringhelli et al. 2007a; Sistigu et al. 2011; Generali et al. 2009; Banissi et al. 2009; Nars, Kaneno 2013). In regard to innate immune responses, the treatment of brain tumour xenografts with LDM cyclophosphamide has been shown to induce significant regression of tumours and to be associated with recruitment and activation of NK cells, DCs and macrophages (Chen et al. 2014; Wu, David J Waxman 2014). Moreover, the stress induced by LDMC can also activate NK cells by inducing expression of NK cell activatory ligands, such as NKG2D (Khallouf, Märten, Serba, Teichgräber, Markus W. Büchler, et al. 2012; A. Soriani et al. 2009; Gasser et al. 2005a). Death receptors such as TRAIL can also be induced on the surface of tumour cells, thereby enhancing the susceptibility of tumour cells to immune cell attack (Fine et al. 2010; A. Soriani et al. 2009).

1.3.3 Targeting Cancer Stem Cells (CSCs)

Studies have reported the ability of LDMC to inhibit the tumour initiating/CSC subpopulation. For instance, Chan et al, demonstrated that LDMC inhibited tumour promoting chemokines affecting fibroblasts in the stroma and that are otherwise induced by MTD of doxorubicin, cyclophosphamide or paclitaxel, thereby improving therapeutic outcome (Chan et al. 2016). It has also previously suggested that inhibition of angiogenesis disrupts the CSC niche. Thus LDMC can also directly inhibit CSCs (Loven et al. 2013; Chan et al. 2019; Wu et al. 2016a).

1.3.4 Low dose metronomic chemotherapy in the clinic

Cyclophosphamide (CP) and methotrexate (MTX) used in metronomic regimens has shown clinical benefit in various studies in breast cancer (Colleoni et al. 2006; Orlando et al. 2006). In a study in patients with metastatic breast cancer, this combination yielded 5 and 7 months median PFS and OS, respectively, with 1 patient out of 48 experiencing a complete response (Hussein et al. 2017). A combination of metronomic CP, vinorelbine and capecitabine has been evaluated in patients with metastatic, hormone receptor positive breast cancer showed a significant activity with a median time to progression of 25.1 months when used as first-line therapy and 11.2 months as second-line treatment (Montagna et al. 2017). In a phase II trial, oral etoposide (60 mg/m_2 for 10 days, followed by 11 days rest) was used in patients with metastatic breast cancer. A clinical benefit rate of 21.3% was achieved with median PFS of 4.5 months (Yuan et al. 2015). In ovarian cancer, metronomic chemotherapy regimens have also shown encouraging results. One retrospective study using oral etoposide in platinum-resistant patients showed it to be effective and well-tolerated with a median PFS of 3.9 months and a median OS of 16.4 months (Kucukoner et al. 2012). Topoctecan has also shown promise as a single agent or in combination with the anti-angiogenic molecule Bevacizumab. Various other chemotherapeutics have been evaluated, either alone or in combination with other agents in various cancers. Table 1.2 represent a selection of clinical studies of LDMC.

Table 1.2. Low dose metronomic chemotherapy in the clinic (adapted from Simsek et al.,2019) (Simsek et al. 2019a)

Author	Setting	Therapy/ regimen	N	SD	PR	CR	ORR	TTP	CB	PFS	OS
Studies evaluating Metronomic chemotherapy in Breast cancer											
Wong, 2010	Metastatic, pretreated or untreated	CP 50 mg qd po MTX 2.5 mg bid po two days q1w Prednisone 5 mg qd	41	7%	2%	15%	24%	10 w	24%		48 w
Orlando, 2006	Metastatic, pretreated, HER2 +	CP 50 mg qd po MTX 2.5 mg bid days 1, 4 q1w Trastuzumab 6 mg/kg q3w	22	46%	18%					6m	
Garcia-Saenz, 2008	Metastatic, pretreated, HER2 +/-	CP 50 mg qd MTX 1 mg/kg iv q14d Bevacizumab 10 mg/kg iv q14d Trastuzumab (in HER2 +)	22	32% (24 w)	32%				63.6%	7.5 m	13.6 m
Wang, 2012	Metastatic, pretreated	CP 65 mg /m_2 iv days 1-14 q3w Capecitabine 1000 mg/m_2 bid days 1-14 q3w	68				30.3%	5.2 m	53.0%		16.9 m
Taguchi, 2010	Metastatic, untreated recurrent	Capecitabine 825 mg/m_2 bid days 1-21 q 28d	33	24% (> 6 m)			18%			6.9 m	24.8 m
Masuda, 2014	HR-, ER-, preoperative	Paclitaxel 80 mg/m2 days 1. 8. 15, 4 cycles Cyclophosphamide 50 mg qd po 4 cycles Capecitabine 1200 mg/m_2 qd 4 cycles 5-FU 500 mg/m_2 q3w, 4 cycles Epirubicin 100 mg/m_2 q3w, 4 cycles CP 500 mg/m_2 q3w, 4 cycles	33		54.5%				31 (93.9%)		
Mutlu, 2015	Metastatic, pretreated	CP 50 mg qd po Etoposide 50 mg bid 2 days per week	-							7.03 m	32.5 m
Ambros, 2014	Metastatic, pretreated, HER2-	Capecitabine 1000 m_2 bid po days 1-14 q21d	86				24.3%	7 m	55.8%		24.0 m
Yuan, 2015	Metastatic, pretreated	Etoposide 60 mg/m_2 po days 1-10 q 21d	75	39%	9%					4.5 m	
Studies evaluating Metronomic chemotherapy in Ovarian cancer											
Garcia, 2008	Platinum refractory	Bev 10 mg/kg q14d CP 50 mg qd po	17		17 (24%)			7.2 m		6 m (56%	16.9 m
Sanchez-Munoz, 2010	Pretreated, recurrent	Bev 10 mg/kg q14d CP 50 mg qd po	38	3 (8.1%) (6 w)	12 (32.4%)	3 (8.1%)				4.5 m	10.7 m

Table 1.2. Continued.

Roque, 2015	Uterine/Ovarian-fallopian-peritoneal	Ixabepilone 16-20 mg/ m_2 days 1, 8, 15, q28d Vs Ixabepilone 16-20 mg/ m_2 days 1, 8, 15, q28d Bev 10 mg/kg q14d	8/3 16/33			41.7%		3.0 m/- 6.5 m/ -	9.6 m
Studies evaluating Metronomic chemotherapy in glioblastoma multiforme (GBM)									
Kesari, 2007	GBM and AG	Etoposide 35 mg/m_2 days 1-21 CP 2 mg/kg days 22-42, Talidomide Celecoxib	48	59%	11%			11 w (GBM) 14 w (AG)	41.5 w (GBM) 42 w (AG)
Kong, 2010	Pretreated GBM	TMZ 40 mg/ m2 m2 qd or 50 mg/ m2qd	38					32.5%	56.0% (6 m)
Zustovich, 201	Pretreated GBM	TMZ 40 mg/ m2qd Sorafenib 400 mq qd	43					26%	7.5 m
Studies evaluating Metronomic chemotherapy in Prostate cancer									
Author	**Setting**	**Therapy/ regimen**	**N**	**PSA**	**DS**	**PR**	**TTP**	**PFS**	**OS**
Nishimura, 2001	Hormone refractory	CP 100 mg qd UFT 400 mg qd Estramustine 560 mg qd	21	57%	7 m				
Fontana, 2009	Hormone refractory	CP 500 mg/m_2 bolus than 50 mg qd po Celecoxib 200 mg bid Dexamethasone 1 mg/day po	28	32%				3 m	21 m
Gebbia, 2011	Castration resistant,	CP 50 mg qd MTX 2.4 mg po two times a week LHRH analogue	58	25%	24%	28%			
Yashi, 2014	Castration resistant, metastatic	CP 50 mg qd po Dexamethasone 1 mg qd po	24	33.3 %				5.0 m	19.0 m
Petrioli, 2015	Castration resistant	Abiraterone 25 mg qd po Prednisone 5 mg qd po	26	69.2 %				6.4 m	14.3 m

SD Stable disease, PR Partial response, CR complete response, OS Overall survival, PFS progression free survival, TTP time to progression, EPS event free survival, ORR overall response rate, CB clinical benefit. DS duration of response.

1.3.5 Potential synergy between LDMC and immunotherapy

The immunomodulatory properties of LDMC make it a good combination candidate to immunotherapy, as it has the potential to enhance immunogenicity of tumour cells, the recruitment of immune cells and address immunosuppression issues by depleting immunosuppressive cells. Emerging evidence is supporting the idea that immunotherapy may be synergistic with LDMC. Indeed, LDMC has been shown to increase potency of immunotherapeutic modalities such as vaccines. Doxorubicin (5 mg/kg) was shown to enhance anti-tumour responses when given 7 days after vaccination in neu-transgenic FVB/n female mice. Responses were associated with polarization of macrophages to an anti-tumour M1 activation status (Machiels et al. 2001). In a phase II trial, low dose cyclophosphamide and celecoxib enhanced responses to a DC-based vaccine in patients with advanced melanoma, doubling the number of patients with stable disease and significantly increasing survival compared to trials with the vaccine alone (Chen et al. 2010b; Ellebaek et al. 2012). In the context of checkpoint molecule blockade, pre-clinical studies have demonstrated the impact of combining CTX with anti-CTLA4 in a model of breast cancer (Parra et al. 2017b). A large proportion of clinical trials have used LDM cyclophosphamide. In a prospective trial, LDM cyclophosphamide (50 mg/day, orally) combined with G-CSF, a sulfhydryl donor, a Cox2 inhibitor, and a preparation of autologous tumour antigens resulted in higher anti-tumour reponses and improved survival in cancer patients (advanced, unresectable pancreatic adenocarcinoma, non-small cell lung cancer, or prostate cancer) compared to standard chemotherapy alone (Lasalvia-Prisco et al. 2012). Many other clinical trials evaluating LDMC and immunotherapy combination are ongoing (https://clinicaltrials.gov), indicating that LDMC has potential to increase the contribution of immunotherapy responses, especially for solid tumours where targeting the tumour and overcoming immunosuppression is a major challenge.

1.4 Breast cancer

Breast cancer is a heterogeneous disease, and represents one of the most frequently diagnosed cancers and the second leading cause of cancer-related mortality among women worldwide (Siegel et al. 2016; Torre et al. 2015). The aetiology behind breast cancer is complex and involves environmental, lifestyle and genetic factors that collectively influence the risk of developing the disease (Ripperger et al. 2009; Rojas and Stuckey, 2016). Although the prognosis is generally favourable for breast cancer patients due to early detection and treatment, around 20%–30% of patients will still develop metastasis leading to a poor prognosis and low survival rate for cases with progressive disease (Kennecke et al. 2010; Eckhardt et al. 2012).

1.4.1 Breast cancer metastasis

Breast cancers can arise from epithelial cells of different parts of the breasts: ducts, lobules and connective tissues. Metastasis occur when cells leave the original tumour site and spread to the distant organs of the body through circulatory or lymph systems (Benson et al. 2009; Melzer et al. 2017).

It is believed that breast cancer-related deaths are predominantly due to metastatic progression, and despite advances in treatment modalities, it remains largely incurable (Weigelt et al. 2005; Lu et al. 2009; Torre et al. 2015). This is particularly challenging as tumour spread can both be an early event in breast cancer, where tumour cells dissemination can start 5-7 years prior diagnosis of the primary tumour (Hüsemann et al. 2008; Hosseini et al. 2016) and a late event, as metastasis can also develop decades after the time of diagnosis and primary treatment. In fact, the risk of distant recurrence from breast cancer was shown to be persistent for at least 20 years after diagnosis (Engel et al. 2003; Pan et al. 2017). Breast cancer cells disseminate to different sites and organs, including brain, lungs and liver, with the most common first site being the bones (Redig, Mcallister 2013; Schwartz, Erban 2017).

A range of factors can indicate progression, invasiveness and metastatic behaviour of different breast tumours, among these, a molecular classification is used to predict the prognosis and direct the treatment of breast cancer patients (Sorlie et al. 2001; Akrami et al. 2017). This classification is based on the expression of three different receptors, including estrogen receptor-alpha (ERα), progesterone receptor (PR), and overexpression of HER2. There are 3 distinct molecular subtypes, Luminal (ERα+/PgR+, HER2−/+), HER2 (ERα−/PgR−, HER2+), and triple-negative (ERα−/PgR−, HER2−) (Perou et al. 2000; Prat et al. 2010).

1.4.2 Triple negative breast cancer

Triple-negative breast cancer (TNBC) accounts for 15-20% of all breast cancers (Brown et al. 2008; Garrido-Castro et al. 2019). Compared with other breast cancer subtypes, TNBC affects a young patient population (<40 years) and is more prevalent in women of African and Hispanic descent (Carey et al. 2006; Dent et al. 2007). There is also an enrichment of TNBC phenotype in the subset of patients with BRACA1 germline mutations (80-85% of BRCA1 BCs are triple negative) (Foulkes et al. 2010). TNBC represent the most aggressive subtype of breast cancer due to its poor prognosis, high rates of local and systemic relapse and the lack of available targeted treatments (Rakha et al. 2009; Gonzalez-Angulo et al. 2011). Histologically, the majority of the TNBCs are high-grade invasive ductal carcinomas (grade III) of no special type, with distinctive features such as central necrosis, pushing borders, high mitotic rate and prominent tumour lymphocytic infiltrate (Dent et al. 2007).

Given that TNBC as a group is defined by the absence of the known markers, it is only plausible to imagine that there would be different subtypes within TNBC. Sub-classification of TNBC tumours can be based on molecular, prognostic signatures and lymphocytic infiltrate. In a seminal study, using gene expression microarray, Lehmann and colleagues classified TNBC into 7 subtypes (6 defined subtypes and one unstable group) based on gene ontologies and differential gene expression. Known as the Vanderbilt classification, the 6 subtypes have been shown to display distinct biology and responded differently to various therapies. They included two basal-like subtypes (BL1 and BL2), one immunomodulatory (IM), one mesenchymal (M), one mesenchymal stem-like (MSL), and one luminal androgen receptor (LAR) class (Lehmann et al. 2011a). Following further studies and observations suggesting that the IM phenotype was not an isolated cluster, Lehmann et al., later refined their classification to only include four subtypes (BL1, BL2, M, LAR) (Lehmann et al. 2016). Other molecular groups were identified based on different approaches. A group from Baylor University proposed additional clusters, incorporating immune signatures and labelled as basal-like immune-suppressed (BLIS) and basal-like immune-activated (BLIA) associated with poorest and best prognosis, respectively (Burstein et al. 2015). Other similar classifications include the PAM50 algorithm (Liu et al. 2016) and the French subtypes (Jézéquel et al. 2015). More recently, the 3 groups of the French subtypes were further characterized, C1 as a molecular apocrine cluster, with luminal, PIK3CA-mutated, and HER2E features. Although C2 and C3 are associated with basal-like hallmarks, they are substantially different in terms of immune responsiveness, with the C3 subtype being associated with a more anti-Tumourigenic immune response and C2 being more pro-Tumourigenic (Jézéquel et al. 2019).

The classification of TNBC into subtypes is not only useful to better understand the disease, dissect its heterogeneity and aid to direct therapeutic decisions, but also to identify new targets for treatments

(Ahn et al. 2016). However, the predictive role of this molecular classification in response to treatment remains elusive (Lehmann et al. 2016; Burstein et al. 2015).

1.4.2.1 Treatment options and challenges in TNBC

Since TNBC tumours are negative for ER/PR and HER2, TNBC patients cannot benefit from endocrine and anti-HER2 agents. In clinical practice, chemotherapy remains the only treatment option available. First-line systemic treatment include various chemotherapy regimens (anthracyclines, taxanes, and/or platinum compounds) and single-agent response rates range from 20 to 50% (Zeichner et al. 2016). Despite aggressive treatment with adjuvant and neoadjuvant chemotherapies, and the prolonged survival found to be associated with the pathological complete response (pCR) to neoadjuvant chemotherapy (Cortazar, et al. 2014), the mortality rate for patients with advanced stage TNBC who do not achieve pCR remains very high, as tumour relapse occurs usually within 2 years (Groheux, et al. 2015).

The recent classifications of TNBC and improved understanding of their heterogeneity has been changing the treatment landscape toward more targeted alternative approaches such as poly (ADP-ribose) polymerase (PARP) and tyrosine kinase receptor inhibitors, antibodies targeting Notch or PI3K/AKT/mTOR pathways (Bianchini et al. 2016; Vidula, Bardia 2017; Jhan, Andrechek 2017; Zhang et al. 2016). A selection of approaches in clinical trials is listed in Table 1.3. Despite these treatment modalities in clinical trials, no significant survival improvements have been proven. To date, there is still a significant shortage of viable treatment options for TNBC patients (Lee, Djamgoz 2018) .

Table 1.3. Selected active clinical trials using targeted therapy for TNBC

Trial	Setting	Therapy	Phase	Status
Studies evaluating PARP inhibitors				
NCT03167619	Platinum treated advanced TNBC	Olaparib in combination with durvalumab	II	R
NCT02484404	mTNBC	Olaparib +/- Cediranib + MEDI4736(anti-PD-L1 antibody)	I/II	R
NCT03330847	mTNBC	Olaparib+ DNA damage/repair targetting agents	II	R
Studies evaluating VEGF/VEGFR inhibitors (Tyrosine kinase inhibitors)				
NCT02624700	Reccurent/mTNBC	Sorafenib + Pemetrexed	II	ANR
NCT03394287	mTNBC	Apatinib (tyrosine kinase inhibitor targeting VEGFR2) + SHR-1210(anti-PD-1 antibody)	II	ANR
NCT03316586	mTNBC	Cabozantinib+Nivolumab	II	ANR
Studies evaluating EGFR inhibitors				
NCT01097642	TNBC	Cetuximab+ Ixabepilone	II	ANR
NCT03692689	mTNBC	SCT200 (recombinant anti-EGFR mAb)	II	R
Studies evaluating multi tyrosine kinase inhibitors				
NCT01639248	mTNBC	ENMD2076	II	R
NCT03797326	TNBC	lenvatinib (E7080/MK-7902)+Pembrolizumab (MK-3475)	II	R

ANR active, not recruiting, R recruiting

In addition to the vast inter-and intra-tumour heterogeneity, the main obstacles facing the development of effective treatment for TNBC are the lack of specific biomarkers and the aggressive nature of the disease, making it more difficult to develop treatment strategies. Moreover, conventional chemotherapy has been shown to be associated with an enrichment of CSCs (Jia et al. 2017; Zhou et al. 2018). Hence, a significant challenge is to eliminate CSC populations as they are the main drivers of disease relapse. Indeed, CSCs have the ability to initiate new tumours through their self-renewal and differentiation properties (Matsuda et al. 2014). The involvement of CSCs in invasion and metastasis and their resistance to treatment has been demonstrated and various studies are looking at their targeting and elimination such as through targeting Notch pathway. Therefore, to ease the burden of TNBC treatment, an optimal therapeutic approach focusing on overcoming these obstacles should be found.

One modality having potential in this area is metronomic chemotherapy (MC). Preclinical studies have demonstrated the potential of MC for TNBC treatment, as it has been shown to modulate angiogenesis, proliferation and drug resistance (Di Desidero et al. 2015). In the clinical setting, metronomic cyclophosphamide with paclitaxel after neoadjuvant epidoxorubicin-cisplatin-fluorouracil (ECF) has

been reported to decrease proliferation (41-91% decrease of Ki-67) in patients that had a complete pathological response (Cancello et al. 2015). For advanced, pre-treated TNBC, Viale et al. reported that the combination of metronomic CP with cisplatin yielded a 23.3% clinical benefit at 6 months after treatment (Viale et al. 2017). On the other hand, emerging evidence has highlighted the role of tumour-infiltrating lymphocytes in predicting the prognosis of TNBC (Loi et al. 2019; Criscitiello et al. 2016; Loi et al. 2013). These data, together with the recent encouraging results from the IMpassion130 trial on the implementation of immune checkpoint blockade for TNBC which demonstrated an improved OS with the addition of anti-PD-L1 to first line nab-paclitaxel in patients with PD-L1 positive TNBC (Schmid et al. 2018) provide a rational for the use of immunotherapy for treating TNBC, and various phase II and III clinical trials are ongoing in this area (Table 1.4).

Table 1.4. Ongoing phase II/III randomized immunotherapy trials in triple negative breast cancer (adapted from Marra et al.,2019) (Marra et al. 2019)

Trial	Setting	Therapy	Phase	Status
NCT03639948 (NeoPACT)	Neoadjuvant	Carboplatin + docetaxel + pembrolizumab	II	R
NCT03289819		Pembrolizumab + Nab-paclitaxel ➔ pembrolizumab + epirubicin and cyclophosphamide	II	R
NCT03356860 (B-IMMUNE)		Paclitaxel + epirubicin + cyclophosphamide ± durvalumab	II	R
NCT03036488 (KEYNOTE-522)	Neoadjuvant/Adjuvant	Carboplatin + paclitaxel + (anthracycline) + cyclophosphamide ± pembrolizumab➔ pembrolizumab	III	ANR
NCT03281954		Doxorubicin + cyclophosphamide + paclitaxel + carboplatin ± atezolizumab ➔ atezolizumab	III	R
NCT03197935 (IMpassion031)		Doxorubicin + cyclophosphamide + nab-paclitaxel ± atezolizumab ➔ atezolizumab	III	ANR
NCT02954874	Adjuvant only for patients with residual disease after neoadjuvant chemotherapy	Pembrolizumab vs. observation	III	R
NCT03756298		Capecitabine ± atezolizumab	II	R
NCT03498716 (IMpassion030)	Adjuvant	Paclitaxel ➔ dose-dense doxorubicin/epirubicin + cyclophosphamide ± atezolizumab	III	R
NCT02926196 (A-Brave)		Avelumab vs. observation	III	R
NCT02768701	Locally advanced or metastatic TNBC	Cyclophosphamide + pembrolizumab	II	ANR
NCT03121352		Carboplatin, nab-paclitaxel and pembrolizumab	II	R
NCT02819518 (KEYNOTE-355)		Abraxane or paclitaxel or carboplatin/gemcitabine ± pembrolizumab	III	ANR
NCT02555657 (KEYNOTE-119)		Capecitabine, eribulin, gemcitabine, or vinorelbine as TPC vs. pembrolizumab	III	ANR
NCT03644589		Cisplatin + pembrolizumab	II	NYR
NCT02755272		Carboplatin + gemcitabine ± pembrolizumab	II	R

Trial	Setting	Therapy	Phase	Status
NCT02447003 (KEYNOTE-086)		Pembrolizumab monotherapy	II	ANR
NCT03125902 (IMpassion131)		Paclitaxel ± atezolizumab	III	R
NCT03164993 (ALICE)		Pegylated liposomal doxorubicin + cyclophosphamide ± atezolizumab	II	R
NCT03206203		Carboplatin + gemcitabine	II	R
NCT03606967		Nab-paclitaxel + durvalumab ± neoantigen vaccine	II	NYR
NCT03616886 (SYNERGY)		Paclitaxel, carboplatin, durvalumab ± oleclumab	II	R
NCT03371017 (IMpassion132)		Carboplatin + gemcitabine or capecitabine ± atezolizumab	III	R
NCT03167619 (DORA)		Durvalumab + olaparib	II	R

ANR active, not recruiting, NYR not yet recruiting, TPC therapy per physician's choice, R recruiting.

1.5 Thesis aims:

The central objective of the research programme described in this thesis was to determine the therapeutic potential of combining low-dose chemotherapy (LDC) with NK cell-based immunotherapy and thereby provide novel insights for improving existing NK cell-based immunotherapy strategies. The experimental aims of this programme were:

1. To establish a LDC treatment protocol for human breast cancer cell lines and characterize the phenotype of treated cells *in vitro* (Chapter 3);
2. To characterize the influence of LDC on the expression of NK cell ligands on breast cancer cell lines and determine the sensitivity of untreated and treated cells to NK cell cytotoxicity *in vitro* (Chapter 4);
3. To interrogate the biology of treated cells and mechanisms involved in the response to the treatment using gene expression analysis (Chapter 5);
4. To evaluate the influence of LDC and adoptive NK cell transfer on the growth of implanted human TNBC cell-derived xenografts in immunedeficient mice *in vivo (*Chapter 6).

CHAPTER 2 : Materials and Methods

2.1 Blood donors

Ethical approval for taking blood samples from healthy volunteers was provided by Nottingham Trent University's Ethical Committee (Humans) under the application Number: 435- 25/01/2016, and informed consent obtained.

2.2 Mouse strains

The pre-clinical studies have been approved by the Home Office under the Animals (Scientific Procedures) Act under the Project Licence (PPL): PB26CF602, granted on the 28th of November 2016 and valid until the 28th of November 2021. Female nonobese diabetic (NOD)-scid gamma (NSG) mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) have a background of SCID mice and IL-2 receptor gamma chain deficiency. These mice are one of the most immunodeficient strains due to the lack of mature B, T, and NK cells; they also lack cytokine signalling. NSG mice were purchased from Charles River Laboratories. The other mice strain used in these studies is the NOD,B6.SCID Il2rg$^{-/-}$ Kit$^{W41/W41}$ (NBSGW) mice, which was developed by the intercrossing of the NSG mouse strain with mice homozygous for the Kit^{W41} allele C57BL/6J-Kit^{W-41J}/J (C57BL/6.Kit^{W41}). NBSGW mice support engraftment of hematopeoetic cells without requiring myeloablative irradiation due to the mutant Kit^{W41} and were purchased from Jackson Laboratories (USA). All mice were 6-8 weeks old at the start of the studies.

2.3 Cell lines

Cell line	Breast cancer subtype	Culture conditions		Supplier
MCF-7	Luminal A	EMEM, 10% v/v FCS, w/v 1% L-glutamine	37°C in 5% v/v CO2	ATCC
SKBR-3	Her2+	McCoy's 5, v/v 10% v/v FCS	37°C in 5% v/v CO2	ATCC
MDA-MB-468	Triple negative	Leibovitz's L-15, v/v 10% FCS, 1% w/v L-glutamine	37°C without CO2	ATCC
MDA-MB-231	Triple negative	Leibovitz's L-15, v/v 10% FCS, 1% w/v L-glutamine	37°C without CO2	ATCC
MDA-MB-231/RFP/LUC	Triple negative	Leibovitz's L-15, v/v 10% FCS, 1% w/v L-glutamine, 1% w/v NEAA	37°C without CO2	AMSBIO
K562 (erythroleukemia)		RPMI, 10% v/v FCS, 1% w/v glutamine	37°C in 5% v/v CO2	ATCC

Effector cell lines	Source-Characteristics	Culture conditions		Supplier
NK92	Natural Killer Cell line (IL-2 dependent) derived from peripheral blood mononuclear cells from a 50 year old Caucasian male with rapidly progressive non-Hodgkin's lymphoma	75% alpha-MEM + 12.5% v/v FCS + 12.5% v/v horse serum + 2mM L-glutamine + 5 ng/ml IL-2	37°C in 5% v/v CO2	DSMZ
NK92-MI	IL-2 independent Natural Killer Cell line derived from the NK-92 cell line by transfection.	75% alpha-MEM + 12.5% v/v FCS + 12.5% v/v horse serum + 2mM L-glutamine + 0.2 mM inositol+0.1 mM 2-mercaptoethanol+0.02 mM folic acid.	37°C in 5% v/v CO2	ATCC

2.4 Methods

2.4.1 Cell culture

2.4.1.1 Cell culture maintenance (thawing, subculture and freezing)

Cryovials of cells were withdrawn from liquid nitrogen and thawed quickly by transfer into Falcon tube with added medium. The cells were centrifuged (300g, 5min, RT). Following removal of the supernatant, the cells were resuspended in fresh, pre-warmed medium and transferred into cell culture flasks for culture (37°C, with or without 5% v/v CO_2). Cells were subcultured once they reached around 80-90% confluency.

Adherent cells were passaged using trypsin-EDTA treatment. After the culture medium was decanted the cell layer was gently washed with sterile PBS and pre-warmed trypsin-EDTA was added until it covered the cell layer, the cells were then incubated at 37°C until the all cells detached from the surface. Complete medium was added to neutralise the activity of trypsin-EDTA and the cell suspension was then transferred into Falcon tubes and was centrifuged (300g, 5min,RT). The supernatant was removed and the cell pellet resuspended in fresh medium and either counted or used for assays or split at appropriate ratios for culture (37°C, without or without 5% v/v CO_2).Cultures of suspension cells were maintained by addition of fresh medium with an appropriate split ratio.

Suspension of cells to be frozen down were centrifuged (300 or 400g, 5min, RT) and following the removal of the supernatant, the cell pellet was resuspended in an appropriate volume of freezing medium (90% v/v FCS+ 10% DMSO) and transferred into cryovials (1ml aliquots). The cryovials were stored in -80°C freezer and transferred into liquid nitrogen for long-term storage.

2.4.1.2 Counting of cells

Cells were checked for viability and counted using either a Haemocytometer or the NucleoCounter®. For Haemocytometry counting, the cell suspension was pre-diluted with an appropriate volume, and 10µl were added to 90µl of trypan blue solution (0.05 % w/v) mixed 1:1 with PBS. Cells were transferred into the counting chamber and live cells were counted. The concentration of cells was calculated using the following formula:

$$\text{Cell concentration} = \text{Counted cells/counted squares} \times \text{dilution} \times 10^4$$

For NucleoCounter®cell counting. The cell suspension was pre-diluted with an appropriate volume, from which 50µl of cells were mixed with 2.5µl of Solution 18 AO.DAPI which contains Acridine Orange and DAPI for living and dead cells staining, and transferred into the counting slide. The slide

was inserted into the NucleoCounter® and viability percentages and cell concentration were obtained from the software.

2.4.2 *In vitro* experiments

2.4.2.1 Doxorubicin treatment of cells

Cells were seeded into tissue culture plates and then allowed to adhere for 24h before being treated with a range of doxorubicin concentrations (15-500 nM) for 24h-72h or with 1 or 2µM for 2h. Concentrations that maintained the viability of cells >90%, as determined using Trypan Blue dye exclusion, were selected. Following treatment, cells were washed free of doxorubicin and provided with fresh complete medium. The cells were then cultured in drug-free medium for an additional 24h to 7 days and used for assays.

2.4.2.2 Flow cytometry cell surface and intracellular staining

The expression of senescence, DNA damage markers and NK cell ligands on tumour cells was assessed using flow cytometry (See Appendix 8.1.6 for antibodies details). For the analysis of cell surface marker expression, cells were harvested, counted and 0.5-1x10^6 cells for each condition were washed with 2ml PBS then incubated with a panel of Fluorochrome-conjugated monoclonal antibodies at recommended concentrations for 30min on ice in the dark, after which they were washed once with PBS by centrifugation (300g, 5min,4°C) and the supernatant was discarded. The cell pellet was resuspended in 200-300 µl Isoton™ (cytometer running buffer) and analysed on the flow cytometer.

For intracellular antigens, cells were fixed and permeabilised using an eBioscience™ Intracellular Fixation & Permeabilization Buffer Set, according to the manufacturer's protocol. After fixation, cells were incubated with FcR blocking reagent for 10 min, after which they were incubated with antibodies diluted in permeabilisation buffer for 30 min on ice. Cells were finally washed with buffer before data acquisition. Antibody panels included LIVE/DEAD™ Fixable Dead Cell Stain to exclude dead cells from the analysis. Data was acquired on the Gallios flow cytometer (Beckman Coulter) and Kaluza 1.3 version (Beckman Coulter) was used for all flow cytometry data analysis. Figure 2.1 represent the basic gating strategy used for flow cytometry analysis.

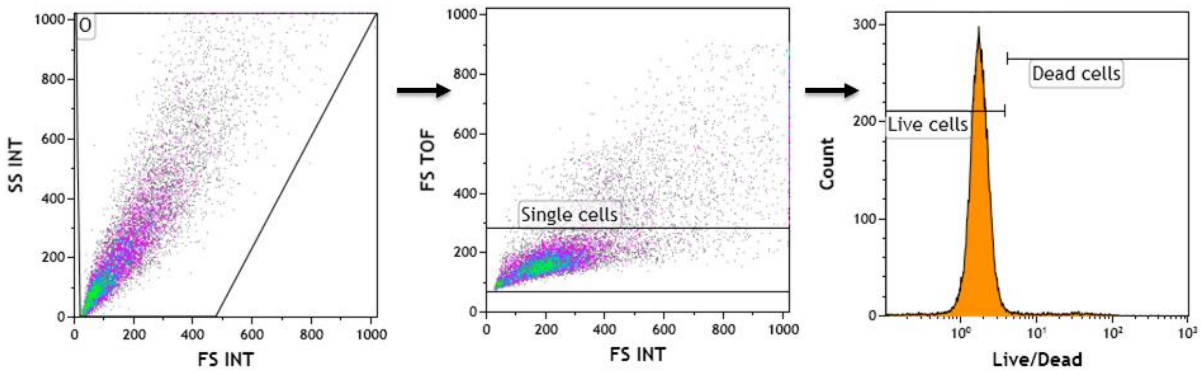

Figure 2.1 Basic gating strategy for flow cytometry analysis. Following the identification of cells on a forward scatter (FS INT) vs side scatter (SS INT) plot, Single cells are selected by exclusion of cell aggregates/doublets using cell height and areas. Viable tumour cells are then identified for analysis using LIVE/DEAD Cell Stain.

1.2.2.3.4. Assessment of SA-B-gal expression

On day 6-7 post-treatment, culture medium was removed and replaced by pre-warmed fresh culture medium. As SA-β-gal is detected at ~pH 6, the internal pH of lysosomes was first neutralized using a lysosomal inhibitory drug, Bafilomycin-A1 at 100nM for 1 h at 37°C, 5% v/v CO2. Cells were then incubated for 1 h with 30 µM 5-Dodecanoylaminofluorescein Di-β-D-Galactopyranoside (C12FDG) a β-galactosidase substrate which becomes fluorescent after cleavage by the enzyme. Following incubation, cells were washed twice with phosphate buffered saline (PBS), harvested by trypsinization and centrifuged at 300g for 5 min at 4°C. Cells ($1x10^5$) were resuspended in 250µl Isoton™. SA-β-gal positive cells were detected and quantified on the flow cytometer (Gallios-Beckman Coulter). Kaluza 1.3 version (Beckman Coulter) was used for all flow cytometry data analysis.

1.2.2.3.5. Cell cycle analysis

Cells were harvested and washed in PBS, after which they were fixed in 1 mL ice cold 70% v/v ethanol for 30 min on ice. Ethanol was added dropwise to the cell pellet whilst vortexing to avoid clumping and ensuring an even fixation. Fixed cells were then treated with 50 µL of 100 µg/ml Ribonuclease A to ensure that only DNA was stained, after which cells were incubated with propidium iodide (200 µl, 50 µg/ml, Invitrogen) for 10 min at room temperature. Samples were then analysed by flow cytometry (Gallios-Beckman Coulter).Kaluza 1.3 version (Beckman Coulter) was used for all flow cytometry data analysis.

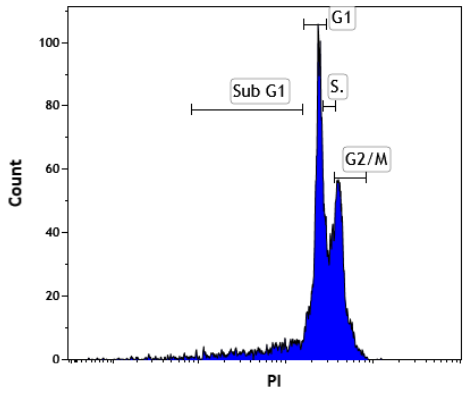

Figure 2.2 Gating strategy for cell cycle analysis. Staining with propidium iodide allows for the identification of the percentage of cells in G1, S, G2/M phases of the cell cycle.

2.4.2.3 Isolation of human PBMCs

Peripheral blood was collected from human healthy volunteers into 100 U/ml of heparin by a trained phelebomist and diluted with PBS at a 1:1 ratio. Peripheral blood mononuclear cells (PBMCs) were isolated by layering diluted blood over Ficoll-Paque density gradient medium and centrifuging tubes at 800xg for 20 min at room temperature with the brake off. PBMCs were harvested from the interface layer into 50ml Falcon tubes using Pasteur pipettes. Cells were then washed twice with PBS and centrifuged at 400xg for 10 min at room temperature. The resulting cell pellet was resuspended in RPMI culture medium cells and counted using the NucleoCounter®.

2.4.2.4 Isolation of human NK cells

Human NK cells were isolated from PBMCs by immunomagnetic negative selection using EasySep™ Human NK-Cell Isolation Kit. For this, PBMCs were transferred into a 5 ml polystyrene round-bottom tube at a concentration of $5x10^7$ cells/mL in 0.25-2 mL, 50 µL of isolation cocktail was added per ml of sample, mixed and incubated for 5 min, RapidSpheres beads were vortexed and added to the sample at 50 µl/ml after mixing, the sample volume was made up to 2.5 ml with recommended medium (PBS with 2% v/v FBS and 1 mM EDTA) and mixed by gentle pipetting. The sample tube was then placed into the magnet for 3 min, after which isolated cells were collected by pouring the enriched cell suspension into a new tube. Cells were counted using the NucleoCounter® and checked for purity using flow cytometry (Gallios-Beckman Coulter), on the basis of CD3 and CD56 expression (Figure 2.3).

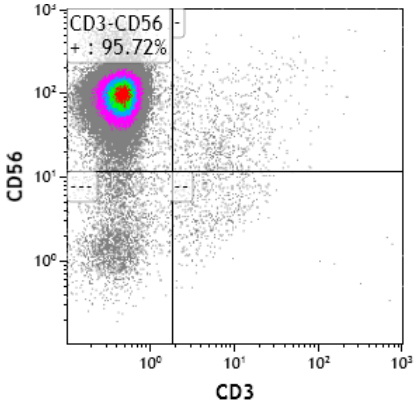

Figure 2.3. Purity of $CD3^{-}CD56^{+}$ NK cells after isolation from PBMCs

2.4.2.5 *In-vitro* cytokine stimulation of NK cells

Following isolation, NK cells were either used in their resting state or after stimulation for 24h/48h with IL-2 (100 U/ml or 200 U/ml).

2.4.2.6 Cytotoxicity assays

NK cell cytotoxicity was determined using a flow cytometry-based technique, the principle of which is that target cells are fluorescently labelled and the viability of these is determined by flow cytometry following the inclusion of a viability stain.

1.2.2.3.6. CellVue™ labelling of target cells

Target cells were washed with serum-free medium and labelled with CellVue™ NIR780 cell labelling kit (633-779nm). These are lipophilic dyes that can be used to label the cell membrane for the purpose of identifying and tracking labeled cells. For staining, the cell pellet was resuspended in 200 µl of Diluent C and pipetted gently to ensure a single cell suspension. The dye solution (2 µl of the 1 mM dye stock to 200 µl of Diluent C) was rapidly added to the sample and mixed well by pipetting to ensure uniform labelling. Cells were incubated for 3 min and the staining was stopped by the addition of an equal volume of foetal calf serum (FCS) for 1 min. Cells were then centrifuged at 300x g for 5 min and the supernatant discarded. Cells were then washed twice with complete medium, counted and transferred to sterile conical poly-propylene tubes.

1.2.2.3.7. Cytotoxicity assay

CellVue™ dye-labelled target cells, at a minimum of $1x10^5$ cells in 100 µl medium were added to $5x10^5$ primary NK cells or NK-92® cells in 100 µl (effector: target [E:T] cell ratio of 5:1, other E:T of 1:1 and 10:1 were also tested), and incubated for 3 h at 37°C and 5% v/v CO2. As controls, untreated and treated cells alone without NK cells were used to measure spontaneous cell death. After the incubation

period, the cells were resuspended in a solution of LIVE/DEAD® Fixable Dead Cell Stain for 20 min and then centrifuged for 5 min at 300xg, resuspended in Isoton™ Sheath Fluid and analysed by flow cytometry (Figure 2.4). At least three independent experiments were performed unless otherwise stated. NK cell cytotoxic activity was calculated using the following equation:

$$\%\ \textbf{Cytotoxicity} = (\%\ \textbf{sample cell death} - \%\ \textbf{spontaneous cells dead})\ \textbf{x100}/$$
$$\textbf{100} - \%\ \textbf{spontaneous cell death}$$

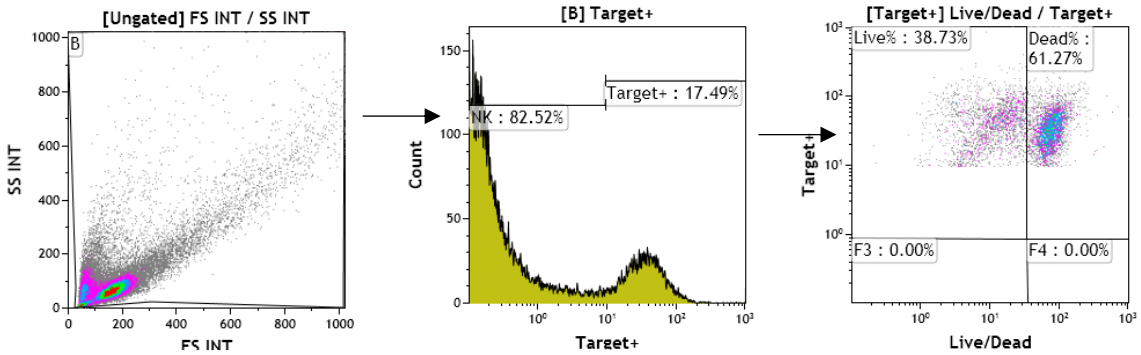

Figure 2.4. Gating strategy of the cytotoxicity assay. Percentage of cell death was analysed by gating on the cellvue+ target cells (target +) first and then the number of positive and negative LIVE/DEAD™ cells.

2.4.2.7 Flow cytometric assessment of intracellular cytokines and CD107a degranulation assay

To determine the expression levels of IFN-γ, perforin, granzyme B and CD107a secretion/expression. NK-92® MI cells were incubated alone, with K562 target cells (as a positive control target) or with treated / untreated MDA-MB-231 cells at a 1:1 ratio for 4 h at 37°C. After 1 h, Brefeldin A and monensin were added to allow the accumulation of CD107a protein in the Golgi complex, as well as CD107a antibody. Cells were washed, stained with CD56 antibody and LIVE/DEAD™ stain, followed by fixation and permeabilisation using PerFix-nc assay kit and intracellular staining for the expression of IFNγ, perforin and granzyme B. Cells were washed and analysed on the flow cytometer (Gallios-Beckman Coulter).Data analysis was carried out using Kaluza 1.3 version software (Beckman Coulter).

2.4.2.8 NanoString nCounter™ amplification-free gene expression profiling

1.2.2.3.8. Fluorescence activated cell sorting (FACS)

For sorting treated cells by size using the forward and side scatter scale (FS^{high} and FS^{low}), at least $2x10^6$ cells were re-suspended in cell-sorting sorting medium (PBS + 1% v/v FCS + HEPES (25mM) + EDTA (2mM)), then sorted into FS^{high} and FS^{low} populations using MoFlo™ cell sorter, onto a plate containing collection medium (normal cell medium + 20% v/v FCS). Sorted cells were washed with PBS twice and cell pellets were frozen at -80°C until used for RNA extraction.

1.2.2.3.9. RNA extraction and Nanostring gene expression profiling

The transcriptomes of untreated and low-dose doxorubicin-treated breast cancer cells were determined using NanoString nCounter™ FLEX platform. The nCounter™ analysis system detects the expression of up to 800 genes in a single reaction with high sensitivity across a wide range of expression levels and allows for direct multiplexed measurements of gene expression without the need for amplification by PCR. For the analysis, total RNA was extracted from cell pellets ($5x10^5$ cells) using the RNAqueous™-micro total RNA-isolation kit, following the manufacturer's recommended protocol. RNA was assessed for quantity and purity using a NanoDrop spectrophotometer. An OD260/280 ratio between 1.8-2.2 was considered for further processing.

The PanCancer Pathways Panel, which includes 770 genes from 13 cancer-associated canonical pathways was used. Samples were analysed in 3 biological replicates. For each sample 150ng of RNA in 5µl (diluted in nuclease-free water) was hybridized to the probes at 65°C for 17 h in 8 µl of a Master Mix solution (containing 3 µl of reporter CodeSet and 5 µl of hybridization buffer) and 2 µl of capture ProbeSet. Hybridised samples were then transferred to an automated fluidic handling system (nCounter™ prep-station), in which excess of probe was removed using a magnetic beads-based purification process. Biotinylated capture probe/target sample complexes were bound, immobilized and aligned on a streptavidin-coated cartridge. The cartridge was then transferred for quantification of the abundance of specific target molecules using digital image processing within the nCounter™ Digital Analyser instrument (555 fields of view (fov) collected for each sample). The reporter probe counts, were tabulated for analysis using the nSolver™ package and Advanced Analysis module. The analysis software automatically performs quality controls, normalization, data analysis and creates reports with the options of performing advanced analyses including pathway applications. Principle component analysis (PCA) was used for assessing sample grouping. Genes with a false discovery rate below 0.05 were considered as being significantly differentially expressed. Further Gene ontology enrichment analysis was carried out using MetaCore software.

2.4.3 *In vivo* assays

2.4.3.1 Preparation of cells for tumour injections into mice

Prior to implantation into animals, tumour cells were checked for mycoplasma contamination and for luciferase signal. MDA-MB-231/RFP/LUC cells were harvested while in the exponential phase (no more than 70% confluency), washed once with sterile PBS, checked for good viability (≥95) and counted. Cells were then resuspended in appropriate volumes of sterile PBS and extracellular matrix (ECM) (1:1 v/v). $5x10^6$ MDA-MB-231/RFP/LUC cells in a final injection volume of 100µl (PBS+ECM) for each animal were implanted subcutaneously into the 4th inguinal mammary fat pad.

2.4.3.2 Animal treatment

Implanted cells were allowed to grow until tumours became palpable, at which time animals were imaged and randomised into treatment groups based on tumour size (Bioluminescent signal, total Flux [p/s]), in order to ensure that there was no statistically significant differences in tumour mass within the different groups of mice at the start of treatment.

Animals received 2 cycles of treatment consisting of; 3 doses of doxorubicin (0.5 mg/kg) per week, administered through intraperitoneal injection (IP), followed by 1 intravenous injection (IV) of adoptively-transferred donor-derived NK cells or NK-92® MI cells ($10x10^6$ cells). Control mice received PBS alone. Doxorubicin only group were only treated with doxorubicin, and NK alone group only received NK cell infusions (Figure 2.5). Tumour growth was monitored twice a week using calliper measurements and at regular intervals using bioluminescence-based in vivo imaging. The development of metastasis was also assessed using ex vivo whole body imaging after culling. Tumour implantations, monitoring of animals, NK cell infusions as well as most of Doxorubicin injections were undertaken by the Manager, Deputy Manager and technicians in the Biological Support Facilities (BSF) unit.

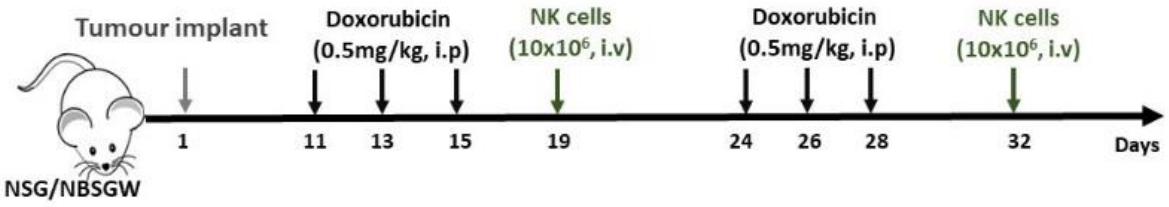

Figure 2.5. Treatment schedule for MDA-MB-231/LUC/RFP cell-based xenograft model.

2.4.3.3 Preparation, cytokine activation and VivoTrack680 labelling of NK cells

Primary NK cells were isolated from PBMCs from healthy volunteers blood and were activated by culturing overnight with IL-2 (PeproTech, 100U/ml, 37°C, 5% v/v CO2) prior to adoptive transfer. In order to monitor NK cells migration to the tumour using in vivo imaging, primary NK cells and NK-92® MI cells were fluorescently labelled with the VivoTrack 680 NIR fluorescent imaging agent prior to adoptive transfer. For this, cells were first washed with sterile PBS and resuspended (up to 250 x 106 cells/mL) in 2ml PBS. 2ml of VivoTrack680 cell labelling solution (0.2mg of dye in 1g of PEG) was added to the cells and mixed by gentle vortexing. Cells were then incubated for 15 min in the dark, at room temperature. 20ml PBS with 1% FCS was used to wash the cells 3 times. Cells were then resuspended in PBS alone for counting before resuspension in appropriate volumes for injection, $10x10^6$ cells in 100ul PBS were injected for each animal.

2.4.3.4 *In vivo* imaging

Mice were imaged using the IVIS® imaging system. D-luciferin at a concentration of 30 mg/ml was injected i.p. at 150 mg/kg (typically 120 µl) 10min before the acquisition of the bioluminescent signal intensity (BLI). General anaesthesia was induced with 5% isoflurane in a chamber and then continued with 2.5% isoflurane via nose anaesthetic cones during the acquisition. Luminescent images were acquired using the IVIS Living Image Software. For analysis, regions of interest (ROI) were drawn around the signal to quantify the emitted photons (Figure 2.6). Luminescence was expressed as Total flux (Photons/secs) in all respective experiments. Fluorescence images for VivoTrack were taken in the same imaging sessions as bioluminescent images.

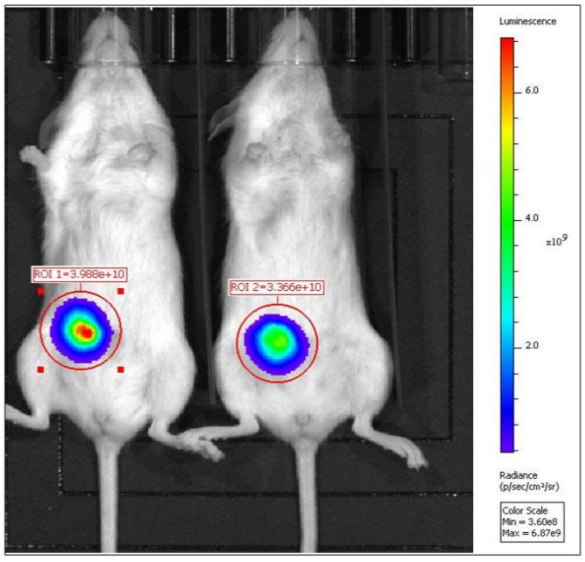

Figure 2.6. *In vivo* imaging analysis

2.4.3.5 Tumour processing and preparation of single cell suspensions

Tumours were collected from treated and untreated mice at the time of culling, cut into pieces using forceps and scalpels and were dissociated in digestion medium containing collagenase 4 (10u/µl) and DNase-I (50ug/ml) in a shaking incubator (37°C,180 rpm) for 45min. Digested tissues were minced through a 70 µm strainer, washed with PBS (400g, 10 min, 4°C) and resuspended in complete medium prior to counting and use for assays.

2.4.3.6 Analysis of tumour samples

The phenotype of isolated tumour cells including proliferation potential (KI67), NK cell ligand MICA/B expression, as well as their sensitivity to NK cell killing was determined using flow cytometry as described previously. Infiltration of tumours by NK cells was also determined using flow cytometry and *in vivo* fluorescence imaging.

The proportion of cancer initiating cells (CIC) using CD44 and EPCAM, was determined using flow cytometry surface staining as described above. Data was acquired using Gallios flow cytometer (Beckman Coulter) and analysed using Kaluza 1.3 version (Beckman Coulter).The gating strategy for CIC population is presented in Figure 2.7.

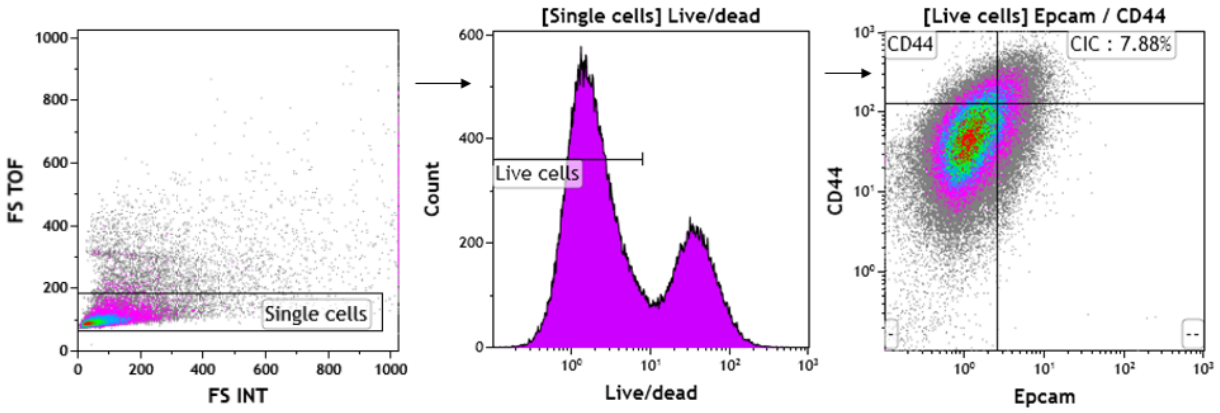

Figure 2.7. Gating strategy for the identification of Cancer initiating cells population.

2.4.4 Statistical analysis

Except for gene expression data, for which statistical analysis was carried out using the Nanostring nsolver software. p-values were calculated using either Student's t-test with two-tailed distribution or Two-way/one-way ANOVA as stated, using GraphPad Prism. Values of $p < 0.05$ were considered to be statistically significant.

Chapter 3: Effect of treatment with low-dose doxorubicin on the phenotype of human breast cancer cells.

3.1 Introduction

Cancer cells are characterized by their immortality and their aberrant cell growth. This uncontrolled proliferation is due to dysfunctions in a range of checkpoint regulators that control progression through the different phases of the cell cycle (Zhou, Elledge 2000). Given that the majority of normal cells are in a resting state, traditional chemotherapies were developed with the aim of interfering with the cell cycle machinery to selectively prevent proliferation and eradicate rapidly dividing cancer cells.

Doxorubicin, also known as Adriamycin is a natural anthracycline antibiotic produced by *Streptomyces peucetius var. caesius* (Arcamone et al. 1969). Since it was first extracted in the 1970 's it became routinely used in the treatment of several cancer types including ovarian, lung, multiple myeloma and it is one of the most commonly used chemotherapeutics for breast cancer (Tacar et al. 2013a; Weiss 1992). Doxorubicin is a DNA-damaging agent as it acts by intercalating DNA along with inhibition of topoisomerase II and the formation of free radicals (Gewirtz 1999; Fornari et al. 1994). Doxorubicin treatment therefore leads to a range of cytotoxic and anti-proliferative effects (Buchholz et al. 2002). Although delivering therapeutic success in some cases, doxorubicin at the maximal tolerated dose (MTD) levels causes toxicity to various major organs, most importantly cardiotoxicity and bone marrow depression (Carvalho et al. 2009; Thorn et al. 2011). Much work has therefore been focused on devising strategies that eliminate these deleterious side-effects on healthy tissue while maintaining their effects on tumour cells.

As discussed in the general introduction, an alternative approach to the delivery of chemotherapeutics at their MTDs is the use of metronomic chemotherapy which involves the administration of low doses of the drug. *In vitro* evidence indicates that some chemotherapeutic agents given at a low dose still elicit anti-proliferative effects on cancer cells and can induce a state of "therapy-induced senescence" in which they remain viable, but do not proliferate (Collado, Serrano 2010).

3.2 Experimental Aims

A key hypothesis of this study is that the induction of a senescence phenotype in breast cancer cells using low-dose chemotherapy (doxorubicin) changes the phenotype of these cells to render them more sensitive to the cytotoxic effects of NK cells. The aim of the experiments described in this chapter was

to develop and optimise a protocol for low-dose doxorubicin treatment of human breast cancer cell lines derived from different subtypes of the disease, namely (MCF7 (Luminal A, ER+), SKBR3 (Her2+), MDA-MB-468 and MDA-MB-231 (triple negative, TNBC)) and characterize the response of the different cell lines to the treatment. The influence of treatment on the induction of a senescence-like phenotype was studied using a panel of senescence and DNA damage response markers, alongside the analysis of cell cycle progression and proliferation.

3.3 Results

3.3.1 Optimisation of drug concentrations and treatment duration

The aim of the initial set of experiments was to optimize the concentrations of doxorubicin and the duration of treatment required to generate a senescence phenotype based on SA-β-gal expression, one of the main markers of cellular senescence (Dimri et al. 1995; Lee et al. 2006). Although SA-β-gal is a lysosomal enzyme that catalyzes the hydrolysis of X-gal at an optimal pH 4 in young/immortal cells, it is activated at pH 6 in senescent cells. It is a commonly used biomarker of senescent cells in culture as well as *in vivo* (Dimri et al. 1995). In order to assess SA-ß-Gal activity following doxorubicin treatment, cells were incubated with C12FDG, a substrate of SA-ß-Gal which becomes fluorescent when it is cleaved by the enzyme. As the initial aim was to induce the expression of SA-ß-Gal without affecting cell viability, doxorubicin concentrations and treatment duration were first optimized for the different cell lines, based on post-treatment cell viability. Cells were treated with a range of doxorubicin concentrations (15-500 nM) for 24h-72h or with 1 or 2μM for 2h. Following 24h of treatment, concentrations that retained cell viability > 90% were selected for the 48h and 72h treatment time points. As cell viability decreased following 72h with most of the doxorubicin concentrations assayed, the 48h treatment time period was selected. After which, SA-β-gal expression was assessed at different time points post-treatment (24h, 48h, 72h, 120h (5d) and 168 (7d)), Results showed that SA-ß-Gal activity remained low directly following treatment, but increased rapidly following several days, with the highest SA-β-gal positivity being observed at 7 days (168h) post-treatment, in line with previous protocols for senescence induction (Noren Hooten, Evans 2017) This time point was used for all subsequent experiments. Optimal doxorubicin concentrations and treatment duration, based on retention of cell viability and high SA-β-gal expression, were found to be 50 nM and 25 nM for 48 h for MCF7 and SKBR3 respectively, and 1 μM for 2h for MDA-MB-468 and MDA-MB-231 cells (Figure 3.1).

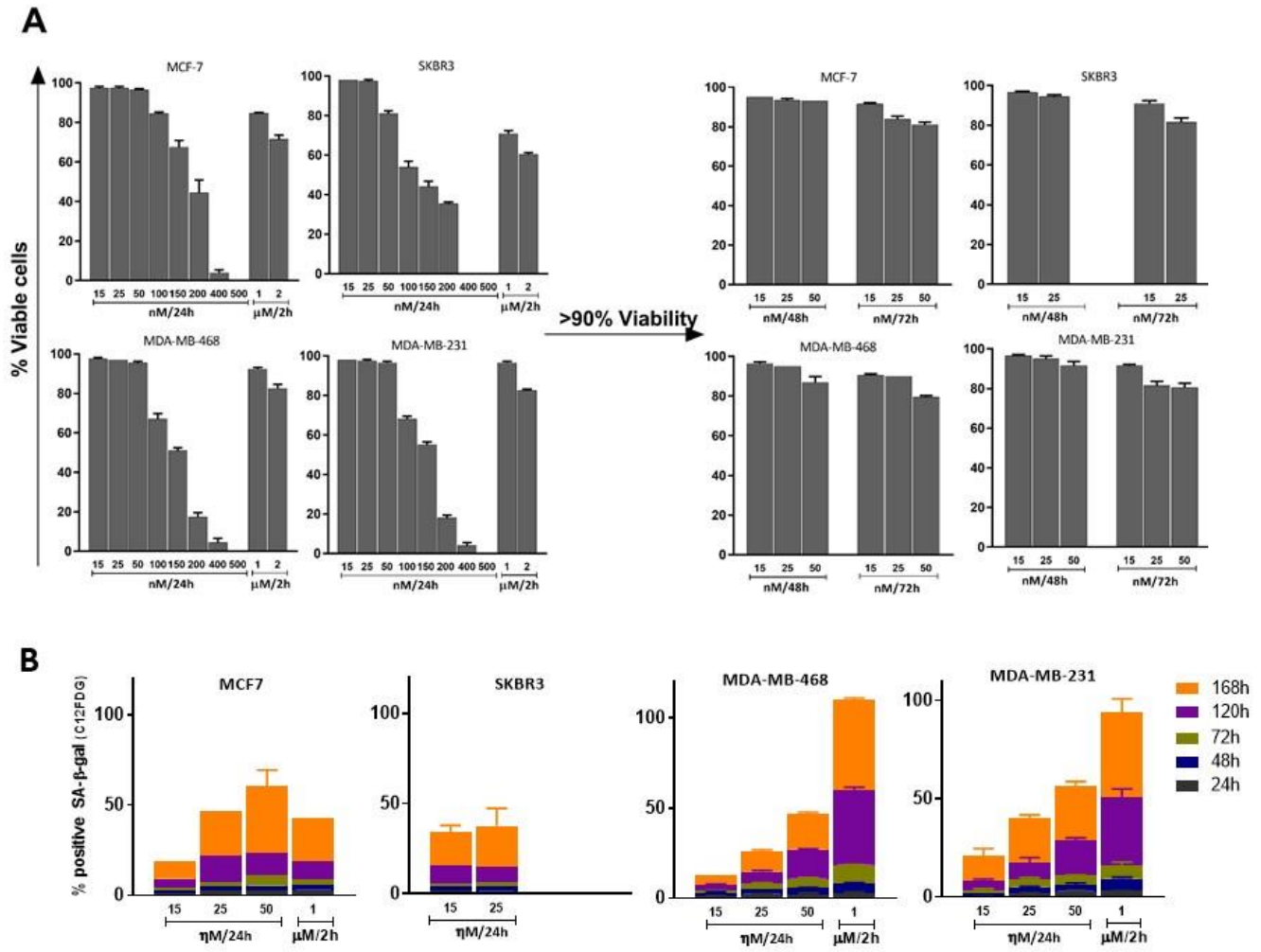

Figure 3.1. Doxorubicin treatment optimization. A. Cells were treated using different concentrations of doxorubicin and their viability was assessed using Trypan blue staining. Following 24h of treatment, concentrations resulting in more than 90% cell viability were selected for 48h and 72h treatment time points, since the cell viability was decreasing following 72h of doxorubicin treatment, the 48h treatment time point has been selected. **B.** SA-β-gal expression was assessed at different time points post-treatment (24h, 48h, 72h, 120h (5d) and 168 (7d)) using the galactosidase substrate C12FDG. Data are presented as mean +SD, from two independent experiments.

3.3.2 Influence of low-dose doxorubicin treatment on cell morphology and SA-β-gal expression

Treatment with low doses of doxorubicin induced a large and flattened morphology (Figure 3.2), a feature which is known to be typical of a senescent phenotype (Cristofalo, Pignolo 1993). Treatment also increased the activity of senescence-associated β-galactosidase (SA-β-Gal) in these cells, the level of which varied between the different cell lines. The highest differential expression of SA-β-Gal was observed in the MDA cell lines corresponding to the triple negative subtype of breast cancer (Figure 3.3).

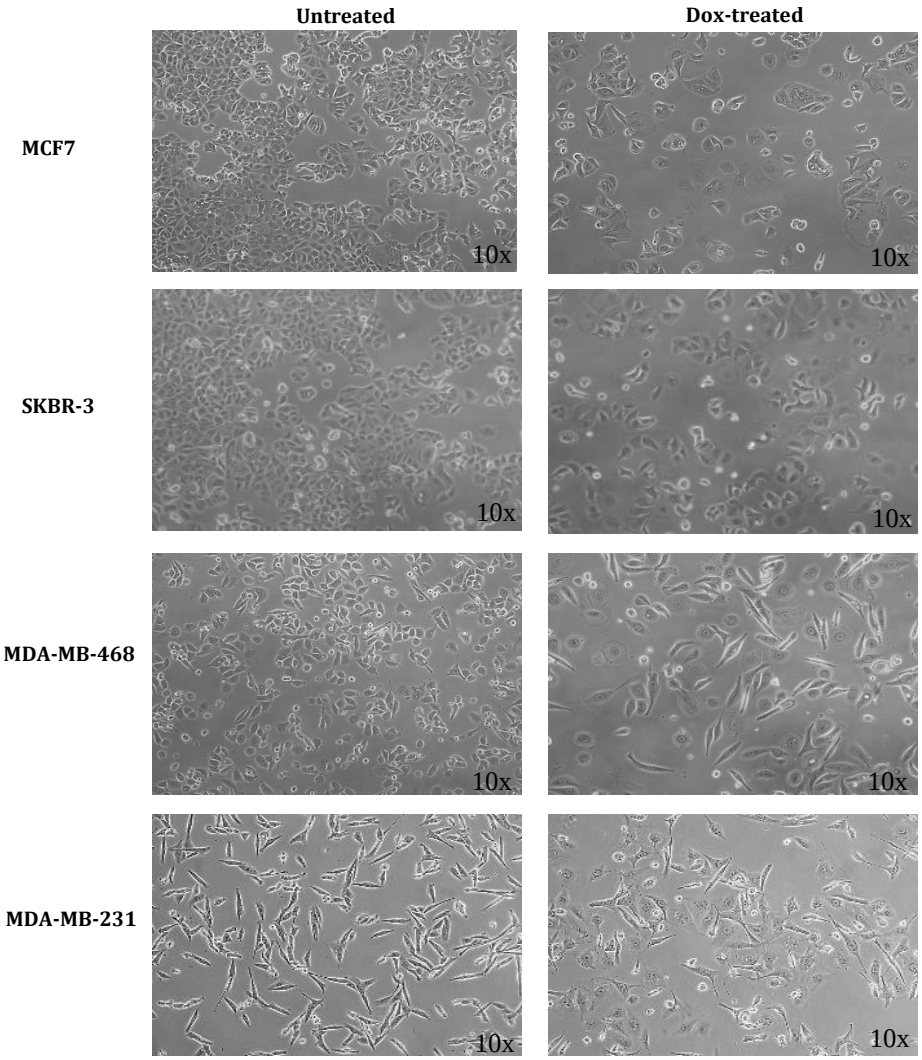

Figure 3.2. Morphological changes of Doxorubicin-treated cells. Breast cancer cells were treated with low-doses of doxorubicin: 25 nM and 50 nM for 48h for SKBR3 and MCF7, respectively and 1 µM for 2h for MDA-MB-468 and MDA-MB-231 cells. Photomicrographs of untreated (left panel) and doxorubicin-treated (right panel) cells were taken on day 7 post-treatment. Images are representative of at least three independent experiments

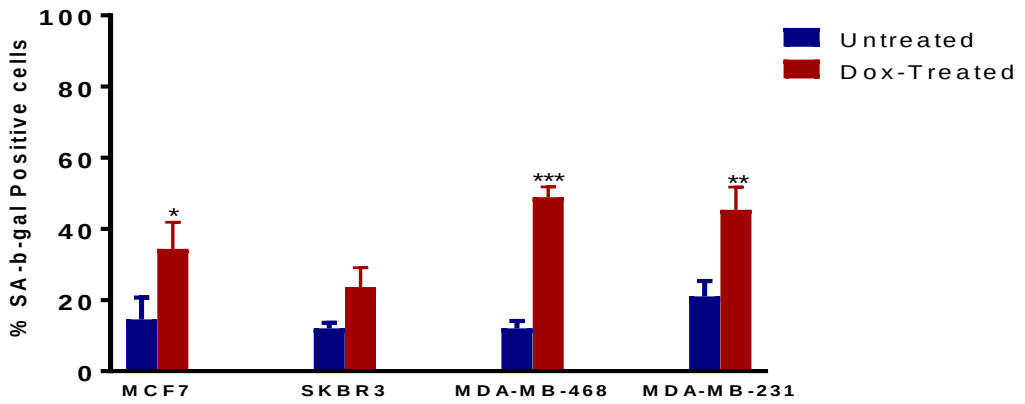

Figure 3.3. Influence of doxorubicin treatment on the expression of SA-β-gal. Untreated and treated cells were assessed for their expression of SA-β-gal by flow cytometry using the galactosidase substrate C12FDG. Data are presented as means+SD. of three independent experiments. Statistical analysis and significance determined by a two-way Anova (* $P \leq 0.0332$, ** $P \leq 0.0021$, *** $P \leq 0.0002$).

3.3.3 Influence of low-dose doxorubicin treatment on the expression of senescence-related and DNA damage response markers

3.3.3.1 p53, p21 and p16 expression

As the tumour suppressor pathways p53/p21 and/or p16INK4a/pRB are known to be involved in senescence (Chang et al. 1999), the influence of low-dose doxorubicin treatment on the intensity of p53 and CDK-inhibitors p21 and p16 expression was determined by flow cytometry (Figure 3.4). A significant increase in the expression of p16 was only observed for the MDA cell lines, and an increase in p21 expression for MDA-MB-231 cells. A slight increase in the expression of p53 on MDA-MB-468 and MDA-MB-231 was also observed.

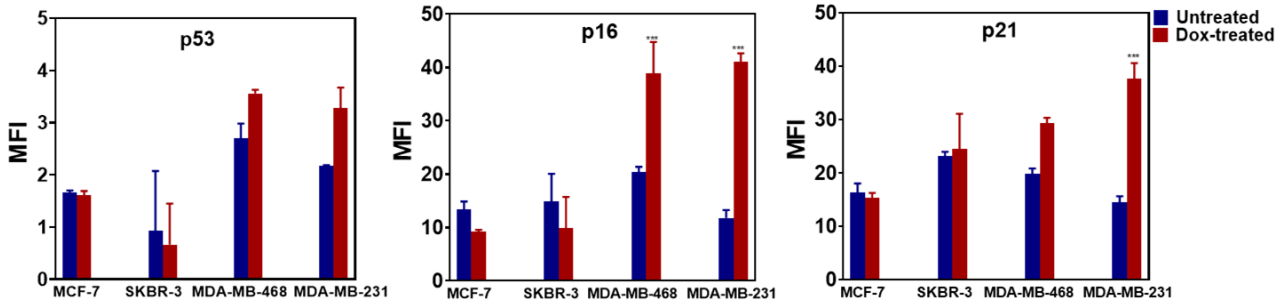

Figure 3.4. Influence of doxorubicin treatment on the expression of common senescence markers. The intensity of p53, p16 and p21 expression by untreated and treated cells (7 days after treatment) was determined using intracellular flow cytometry staining. The Median Fluorescence Intensity (MFI) value was used to compare levels of expression. Data are presented as means+SD. of three independent experiments. Statistical analysis and significance determined a Two-way Anova (*** $P \leq 0.001$).

3.3.3.2 Influence of low-dose doxorubicin treatment on Notch-3 and γ-H2AX expression

Notch-3 is a member of Notch family transmembrane receptors which has been shown to have tumour suppressor functions and to be a mediator of the senescence response, in that elevated levels of Notch-3 expression in cells during senescence have been reported (Cui et al. 2013). Low dose-doxorubicin treatment had no effect on the proportion of cells expressing Notch-3, apart from MDA-MB-231 cells in which it was upregulated following treatment (Figure 3.5).

H2AX is a histone variant of the H2A protein family and the generation of double-stranded breaks (DSBs) as a result of DNA-damaging agents is followed by the phosphorylation of H2AX at Ser139 and the generation of γ-H2AX (Redon et al. 2002). We have detected levels of γ-H2AX by flow cytometry and found that low-dose doxorubicin treatment significantly increases in the proportion of MDA-MB-468 and MDA-MB-231 cells expressing γ-H2AX (Figure 3.5).

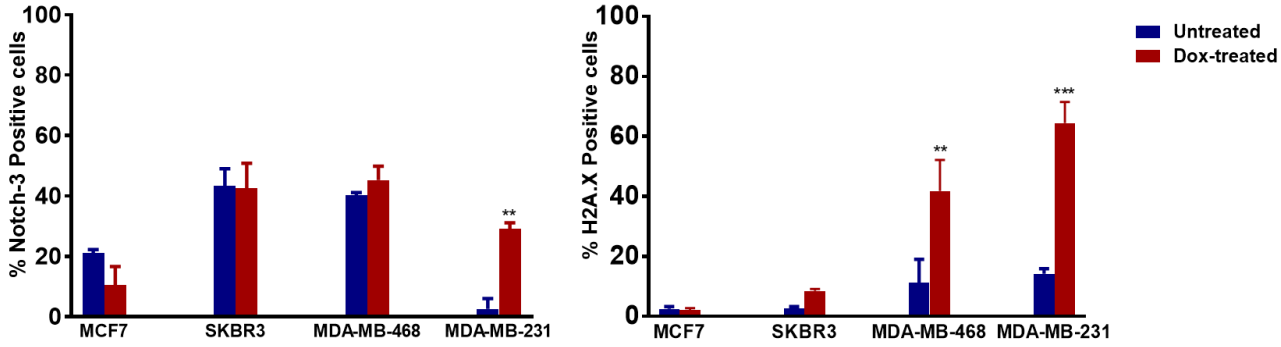

Figure 3.5. Influence of doxorubicin treatment on NOTCH-3 and γ-H2AX expression. Cells were analyzed for the expression of NOTCH-3 and γ-H2AX by flow cytometry. Data are presented as mean+S.D from three independent experiments. A two-way ANOVA was performed for statistical comparison (** $P \leq 0.01$, *** $P \leq 0.001$).

3.3.4 Influence of low-dose doxorubicin treatment on breast cancer cell proliferation

The influence of low-dose doxorubicin treatment on progression of cells through the cell cycle was determined by profiling the DNA content of permeabilized cells stained with the DNA binding dye propidium iodide using flow cytometry. Analysing the fluorescent intensity profiles enables different phases of the cell cycle to be profiled. Low-dose doxorubicin treatment led to the accumulation of cells in the G2/M phase, compared to untreated cells (Figure 3.6).

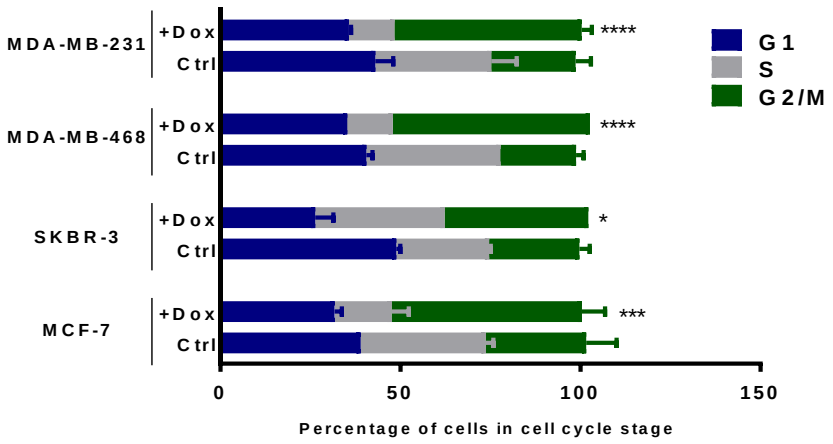

Figure 3.6. Influence of doxorubicin treatment on cell cycle progression. Untreated (Ctrl) and doxorubicin-treated (+Dox) were fixed, stained with propidium iodide (PI) and the proportion (%) of cells in each phase of the cell cycle determined by flow cytometry. Data are presented as means+SD from two to three independent experiments. Statistical comparison between the proportions of untreated and treated cells in G2 was performed using a two-way ANOVA (* $P \leq 0.0332$, **** $P \leq 0.0001$).

Ki-67 is a cell proliferation antigen, that is highly expressed in proliferating mammalian cells and is downregulated in resting cells, as a consequence of which it is widely used in grading tumours (Dowsett et al. 2011). As shown in Figure 3.7, low dose-doxorubicin treatment reduced the proportion of Ki-67$^+$ cells by ~50% for all four BC cell lines.

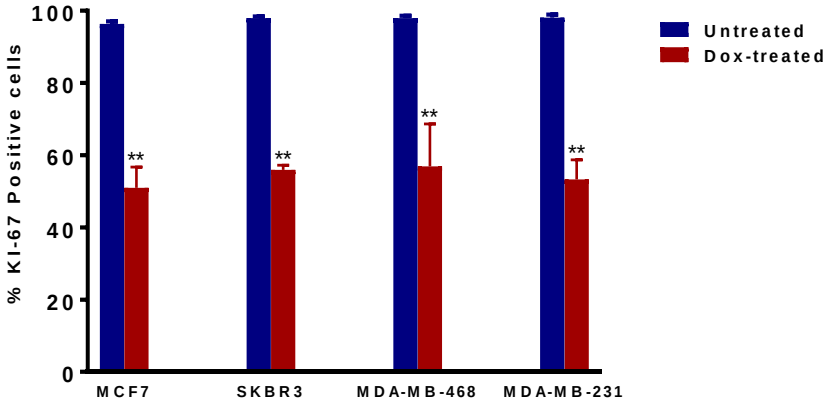

Figure 3.7. Influence of doxorubicin treatment on Ki67 expression. The proliferation of cells was assessed by determining the percentage of Ki67$^+$ cells by flow cytometry. Data are presented as means+SD from three independent experiments. A two-way ANOVA was performed for statistical comparison (** $P \leq 0.01$).

3.3.5 Low-dose doxorubicin treatment does not lead to development of resistance to higher doses of doxorubicin

In order to test if the sub-lethal doses of doxorubicin selected would generate resistance to subsequent treatments with toxic doses of doxorubicin (an important factor for clinical deployment of this approach), MDA-MB-231 cells were treated with the low-doses of doxorubicin, as per the described protocol, 7 days after which they were treated with concentrations of Doxorubicin that were shown to induce cell death in the preliminary titration experiments (500nM) – these concentrations would be equivalent to the maximum tolerated doses *in vivo*. As shown in Figure 3.8, cells that were pre-treated with low doses of doxorubicin remained sensitive to subsequent treatment with high dose doxorubicin.

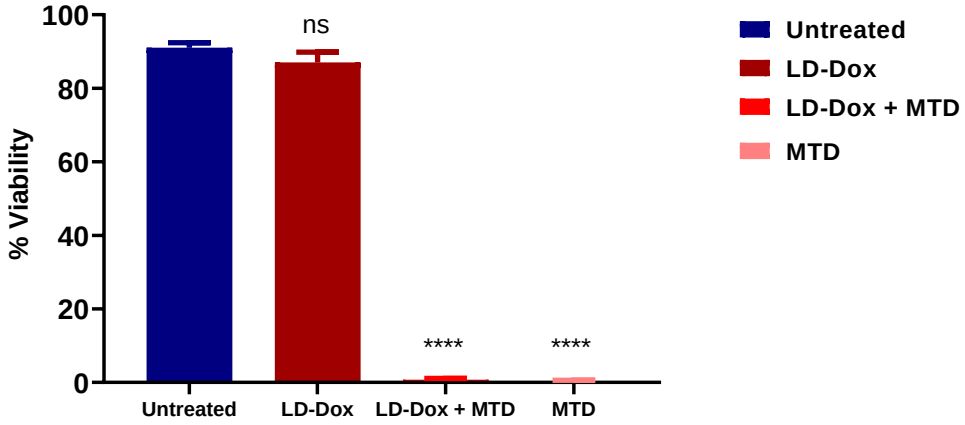

Figure 3.8. Low-dose doxorubicin treatment does not lead to the development of resistance to higher doses of doxorubicin *in vitro*. The viability of MDA-MB-231 cells pre-treated low-dose doxorubicin to subsequent treatment with a toxic, high dose (MTD) doxorubicin (500nM) was compared to untreated, low-dose and MTD treated cells. Data are presented as means+SD from 2 independent experiments. A One-way ANOVA was performed for statistical comparison (ns, not significant, **** $P \leq 0.0001$).

Summary

In this Chapter, we describe a protocol that was developed for low-dose doxorubicin treatment of breast cancer cell lines and characterized its effect on the phenotype of the cells. Low-doses of doxorubicin were previously shown to induce a state of ”therapy-induced senescence” in which cells stop proliferating (Collado, Serrano 2010). This led us to optimize low-dose doxorubicin treatment on the basis of SA-β-gal expression, as it represents the most commonly used marker for the identification of senescent cells. Optimal drug concentrations and treatment durations were established based on achieving the greatest proportion of SA-β-gal positive cells while maintaining high levels of cell viability. A high SA-β-gal activity was demonstrated following treatment of all the cell lines tested.

The first observed effect of the treatment was the increased cell size with a flattened morphology. Cells were also found to accumulate in G2 phase of the cell cycle following treatment, which correlated with the decrease in the expression of the proliferation marker Ki67. A significant increase in the expression of the cell cycle inhibitors p16 was observed in treated MDA-MB-468, MDA-MB-231 cells and p21 in treated MDA-MB-231. Similarly, Notch-3 levels were only elevated in treated MDA-MB-231 cells. Studies have previously shown that Notch-3 upregulation is required for p21 expression (Cui et al. 2013), and this is reflected in these data, where we found elevated levels of p21 only in MDA-MB-231 cells in which Notch-3 was also up-regulated. Another marker of a DNA-damage response and cellular senescence is the DNA double-strand break γ-H2AX foci, which we found to be significantly up-regulated in both MDA-MB-468 and MDA-MB-231 cell lines following treatment. Overall, these findings suggest that cells acquire a senescent-like phenotype following treatment with low-dose doxorubicin and that the mechanisms involved may be cell line-dependent.

Chapter 4: Influence of low-dose doxorubicin treatment on the sensitivity of breast cancer cells to NK cell-mediated cytotoxicity

4.1 Introduction

Natural killer (NK) cell activation and cytotoxicity are primarily regulated by the integration and balance of signals from inhibitory and activating NK cell surface receptors that are triggered following interactions with their respective ligands that are expressed on stressed/tumour cells. The combination of ligands on the surface of tumour cells and the degree of intensity of their expression direct NK cell cytotoxic responses and determine the susceptibility of target cells to NK cell killing (Bryceson et al. 2009). The HLA Class I human leukemic cell line K562 is the most commonly used NK cell-sensitive cell line which provides the combination of ligands which allow for engagement of NK cell activating receptors. This engagement results in the triggering of downstream signaling pathways that leads to the secretion of lytic granules and a selection of cytokines such as IFN-γ which not only act as a co-stimulatory signal for other immune cells, but also enhance NK cytotoxicity by upregulating specific receptors on tumour cells (Cooper et al. 2009).

The potentially weakened killing ability of NK cells to kill cancer is partly due to the capacity of tumour cells to escape NK cell recognition by altering the balance between NK cell activating and inhibitory receptors and proteolytic shedding of ligands; mainly MICA and MICB proteins which are recognized by the NK cell activating receptor NKG2D (Salih et al. 2002; Raulet et al. 2013).

Extensive studies have explored different strategies to improve NK cell function and the capacity of NK cells to control tumour growth in an immunotherapeutic setting by manipulating the expression of these ligands (Morisaki et al. 2012b; Lupo, Matosevic 2019; Hu et al. 2019b). One potential approach to enhance the effect of immunotherapies would be to harness the ability of chemotheraputic agents to activate responses to cellular stress and render cells more sensitive to NK cell-mediated cytotoxicity. Indeed, responses to cellular stress, such as activation of the DNA damage response and the senescence programme have been shown to upregulate ligands for activating receptors in tumour cells and consequently increase their elimination by NK cells (Soriani et al. 2014a; Sagiv et al. 2016).

Studies reported in Chapter 3 demonstrated that low-dose doxorubicin treatment generates a senescence-like phenotype, induces a DNA-damage response in breast cancer cells and markedly decreases their proliferation. In this Chapter, the effect of low-dose doxorubicin on the expression of NK cell ligands by breast cancer cells and the sensitivity of treated breast cancer cells to NK cell killing is described.

4.2 Experimental Aims

The aim of the experiments described in this Chapter was to determine the effect of low-dose doxorubicin treatment on the expression of NK cell ligands by breast cancer cells and the sensitivity of treated cells to killing by NK cells derived from healthy donors and the NK-92™ MI cell line. Preliminary experiments compared the cytotoxic potential of resting NK cells and NK cells stimulated with IL-2, IL-15 or IL-2 and IL-15. In addition to cytotoxicity, effector cell function was determined based on expression of the degranulation marker CD107a, perforin, granzyme B and IFN-gamma.

4.3 Results

4.3.1 Influence of low-dose doxorubicin treatment on the expression of NK cell ligands by breast cancer cells

As part of our investigations into the susceptibility of treated cells towards NK cell mediated lysis we first looked at the effect of low-dose doxorubicin on the expression of a panel of the most important NK cell ligands that bind and trigger NK cell receptors NKG2D, 2B4, DNAM-1, KLRG1, KIRs (see Table 4.1). For these experiments, cells were treated with low dose doxorubicin as described previously, after which the expression of NK ligands was determined by flow cytometry and compared to that by untreated cells.

The expression of ligands for NKG2D and DNAM-1 activating receptors (MICA/B, CD155) were of particular interest, as they play a crucial role in enhancing NK cell-mediated recognition of cancer cells (Fionda et al. 2015a; Morisaki et al. 2012a). Across the four cell lines, treated cells expressed higher levels of ligands for activatory NK cell receptors compared to their untreated counterparts in terms of percentage of positive cells (Figure 4.1). The most upregulated ligands being MICA/B, ULBP1-3 and CD155. The expression of CD112 was significantly higher in treated MCF-7 cells, but not in others. A modest upregulation in CD48 expression was only observed on treated MDA-MB-231 and MDA-MB-468 cells. In terms of intensity of expression (MFI) on the positive cell population, a significant increase was only observed for CD112 in MCF-7 and MDA-MB-468 cells, MICA/B for MDA-MB-468 and MDA-MB-231 and CD155 for MDA-MB-231 cells, presented in Figure 4.2 is the Influence of doxorubicin treatment on the MFI of NK cell ligand expression, only the ligands that showed significant or close to significant differences are shown. Treatment induced a more ‘activatory’ phenotype on the MDA-MB-231 and MDA-MB-468 cells, in that ligands for the NKG2D receptor and the CD155 ligand for DNAM-1 receptor were consistently upregulated.

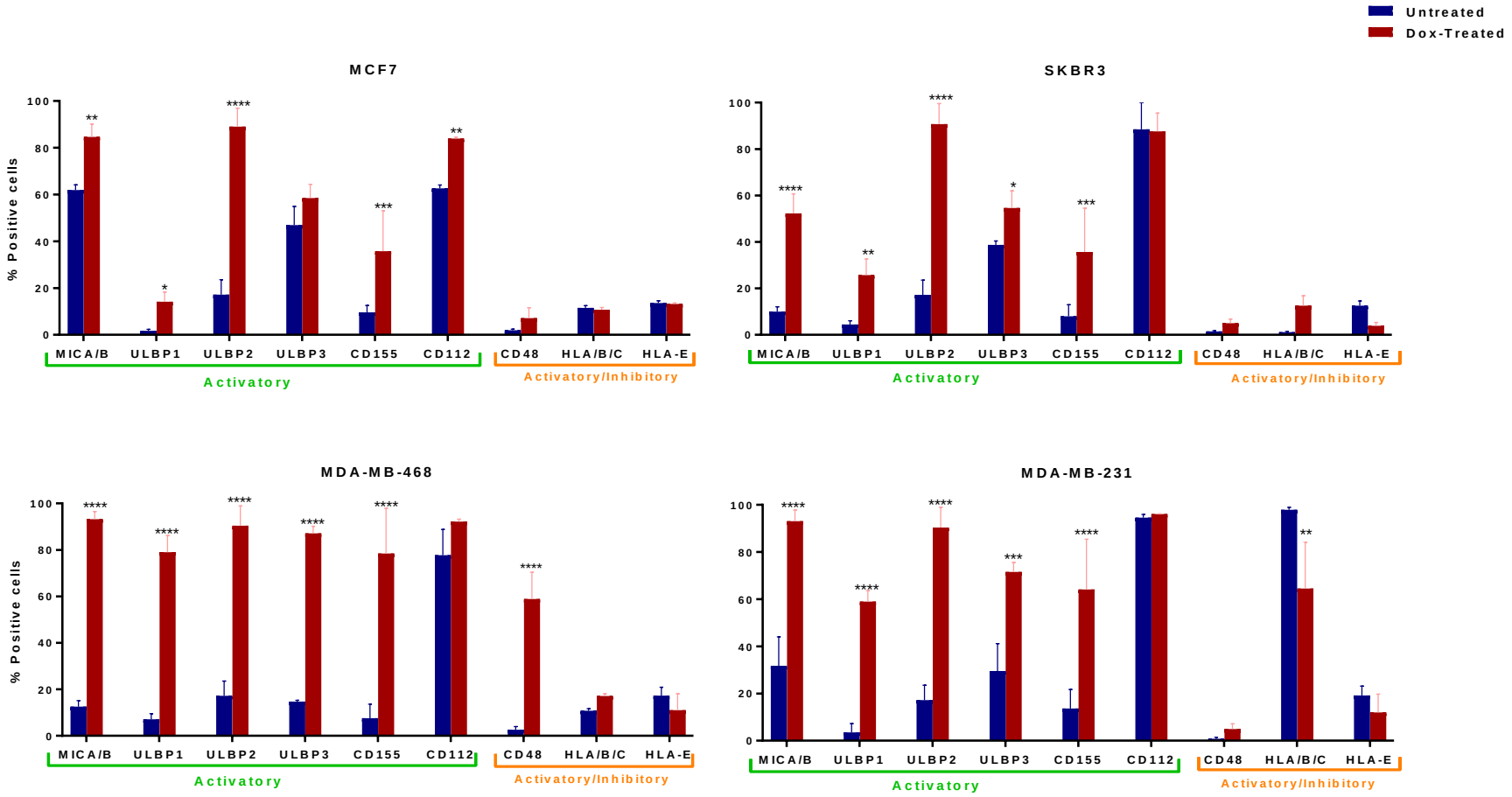

Figure 4.1. Influence of doxorubicin treatment on the expression of NK cell ligands. Cell were treated with previously described concentrations of doxorubicin and were then tested for cell surface expression of the ligands by flow cytometry. Data (percentage of positive cells) are presented as mean+SD. from three independent experiments. Statistical comparison was performed using a two-way ANOVA (** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

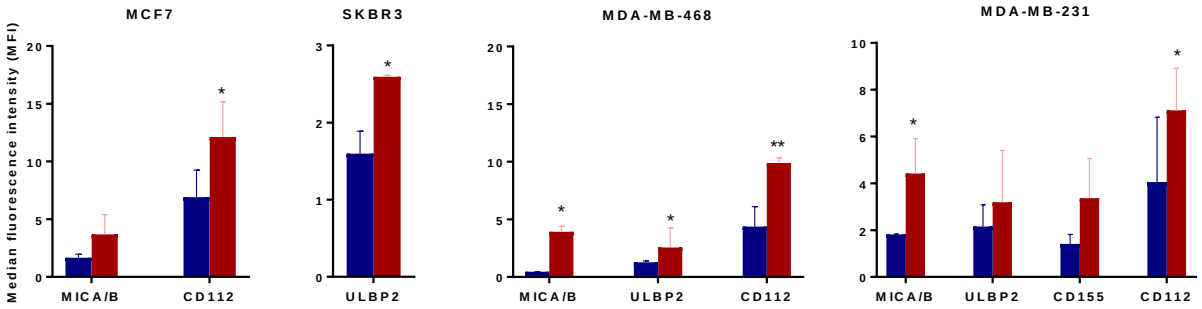

Figure 4.2. Influence of doxorubicin treatment on the intensity of NK cell ligand expression. Cell were treated with previously described concentrations of doxorubicin and the cell surface expression of ligands determined by flow cytometry. Data are presented as mean (±S.D) median fluorescence intensity of cells expressing ligands from three independent experiments. Statistical comparison was performed using a two-way ANOVA (*P ≤ 0.1, ** P ≤ 0.01).

4.3.2 Influence of low-dose doxorubicin treatment on the sensitivity of breast cancer cells to NK cell-mediated cytotoxicity

Having demonstrated that low-dose doxorubicin treatment induces an activatory phenotype, we determined if this observed effect induces sensitivity to NK cell cytotoxicity. For this, NK cell cytotoxicity was determined by flow cytometry using two sources of NK cells; primary NK cells isolated from healthy donors and the NK-92™ cell line (parental and NK-92™ MI cells). The NK cell-sensitive, human erythroleukemic cell line K562 was used as positive target cell control for NK cell activation and lysis, as it lacks expression of HLA class I (an inhibitory ligand for NK cells) and provides the necessary ligands for NK cell engagement and lysis.

4.3.2.1 Influence of low-dose doxorubicin on the sensitivity of breast cancer cells to killing by primary NK cells

Although allogeneic NK cells have been used in several pre-clinical and clinical studies and their safety and efficacy has been demonstrated against different hematological malignancies and solid tumours, we initially examined the effect of low-dose doxorubicin treatment on the sensitivity of breast cancer cells to killing by freshly-isolated, resting donor-derived NK cells. For this, NK cells were isolated from PBMCs prepared from the blood of healthy volunteers using immunomagnetic (negative) selection and their purity (>90% $CD3^-/CD56^+$) confirmed by flow cytometry, immediately after which their capacity to kill treated and untreated cells was determined in a 3-hour cytotoxicity assay. As shown in Figure 4.2, treatment of cells with low-dose doxorubicin increased the sensitivity of MCF7, MDA-MB-468 and MDA-MB-231 cells to killing by freshly isolated, resting NK cells. In contrast, low-dose doxorubicin treatment had no effect on the sensitivity of SKBR3 cells to killing by freshly isolated, resting NK cells.

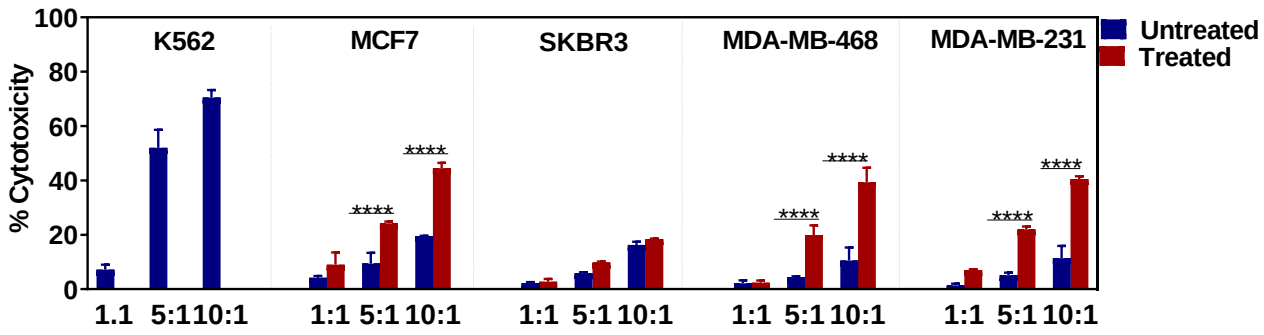

Figure 4.3 Influence of doxorubicin treatment on the sensitivity of breast cancer cells to killing by freshly isolated, resting NK cells. NK cell cytotoxic activity towards doxorubicin-treated (Red) and untreated cells (blue) was evaluated at 3 different effector to target (E:T) ratios, 1:1 5:1 10:1, using a flow cytometry-based killing assay. Data are presented as mean+SD from 3 independent experiments. A two-way ANOVA was used for statistical comparisons (**** $P \leq 0.0001$).

In vitro stimulation of primary NK cells (IL-2+IL-15 stimulation)

The phenotype and function of NK cells are also influenced by the presence of cytokines in the microenvironment and one of the approaches/possibilities to improve NK cell cytotoxicity is via cytokine stimulation. Preliminary experiments therefore determined the effect of stimulating NK cells with IL-2, IL-15 of IL-2 and IL-15 on their capacity to kill target cells. IL-2 and IL-15 both stimulate NK proliferation and activation are commonly used to activate NK cells, with IL-15 also promoting NK cell development and survival (Zwirner, Domaica 2010b). For this experiment, NK cells were pre-activated by incubating with IL-2 (100 U/ml), IL-15 (10 ng/ml) or a combination of the two cytokines for 24 h prior to incubation with target cells. As shown in Figure 4.4, although IL-2 and IL-15 stimulation both enhanced NK cell cytotoxicity, the effect was greater following IL-2 stimulation. Stimulation with both IL-2 and IL-15 had no greater effect than IL-2 alone. However, it should be noted that these data are preliminary and were derived from a single experiment. Given that IL-2 is already approved for clinical use, we focused on examining the influence of low-dose chemotherapy on the sensitivity of breast cancer cells to IL-2 activated NK cells.

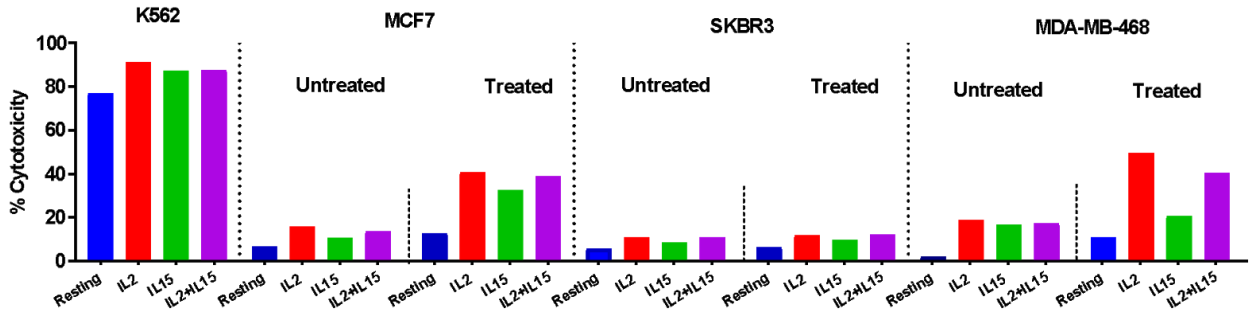

Figure 4.4 Influence of doxorubicin treatment on the sensitivity of breast cancer cells to cytotoxicity by cytokine-activated NK cells. NK cells were pre-activated using either IL-2 (100 U/ml), IL-15 (10 ng/ml) or a combination of both for 24h before being used in a cytotoxicity assay against untreated and doxorubicin-treated breast cancer cells (E:T=5:1). Data are derived from a single experiment.

Since IL-2 pre-activation enhanced the cytotoxic potential of NK cells, we optimized the concentration of IL-2 and the exposure time. For this, the influence of two concentrations of IL-2 (100 U/ml and 200 U/ml) and stimulation periods (24h, 48h) on the capacity of NK cells to kill MCF7 and MDA-MB-468 cells were compared. IL-2 stimulation greatly enhanced the ability of NK cells to kill doxorubicin-treated cells. However, no significant differences were observed when comparing the effects of the two concentrations and time points (Figure 4.5).

We also compared NK cytotoxicity against breast cancer cells following IL-2 stimulation at a range of effector to target ratios (1:1, 5:1, 10:1) using untreated MDA-MB-231 cells and MDA-MB-231 cells treated with low-dose doxorubicin (Figure 4.6). As with the previous studies, IL-2 stimulation increased the cytotoxicity of NK cells toward treated cells. Notably, at a 5:1 E:T ratio, IL-2 stimulated NK cells displayed a better killing efficiency than resting NK cells at an E:T ratio of 10:1. Overall, as summarized in Figure 4.7, IL-2 stimulation greatly enhanced NK cell killing of breast cancer cells treated with low-dose doxorubicin.

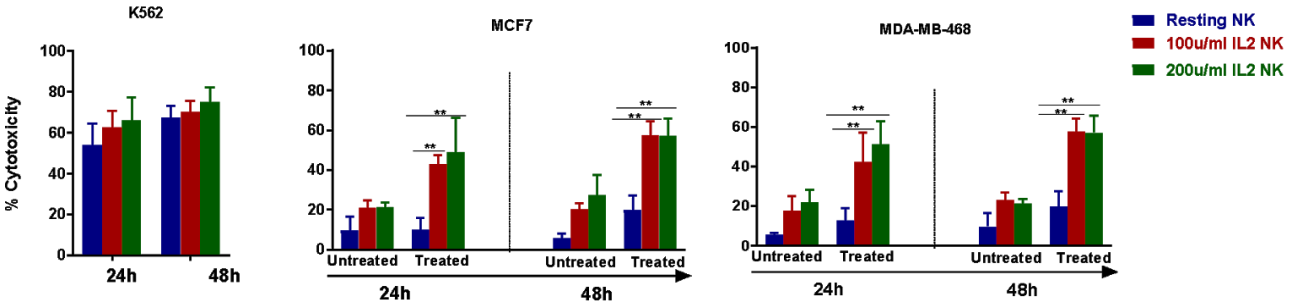

Figure 4.5 Influence of doxorubicin treatment on the sensitivity of breast cancer cells to killing by cytokine-activated NK cells: Optimisation of IL-2 stimulation protocol (E:T=5:1). NK cells were stimulated with IL-2 (100 U/ml, 200 U/ml) for 24h or 48h before being co-incubated with their target cells (untreated and doxorubicin-treated MCF7, MDA-MB-468 cells, and K562 cells as positive control) (E:T= 5:1). Statistical significance is

represented for comparisons between IL-2 concentrations and resting NK cells. Data are means+SD from 3 independent experiments. A two-way ANOVA was used for statistical comparisons (* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001).

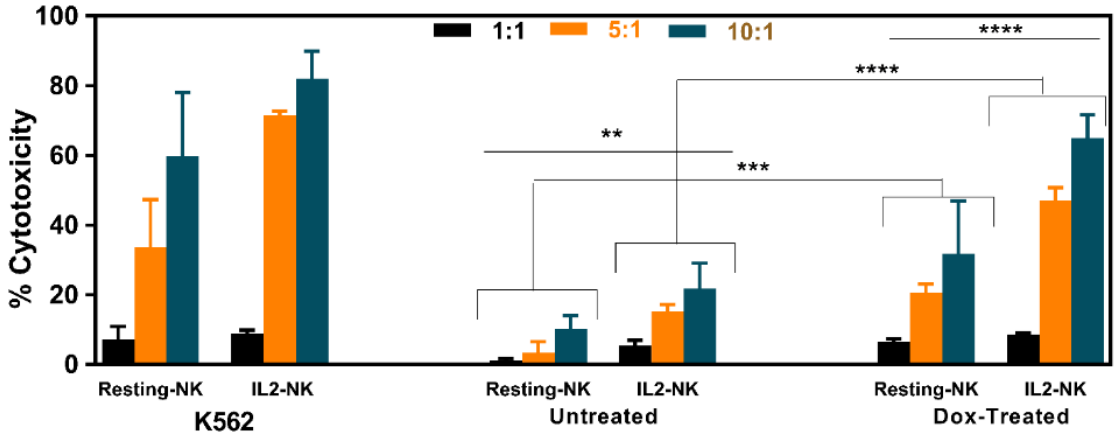

Figure 4.6 Influence of doxorubicin treatment on the sensitivity of MDA-MB-231 cells to killing by cytokine activated NK cells: (E:T = 1:1, 5:1, 10:1). The cytotoxicity of resting and IL-2 stimulated NK cells (200 U/ml for 48h) against MDA-MB-231 cells was assessed at E:T ratios 1:1, 5:1, 10:1. Data are presented as mean+SD from 3 independent experiments. A two-way ANOVA was used for statistical comparisons (* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001,**** P ≤ 0.0001).

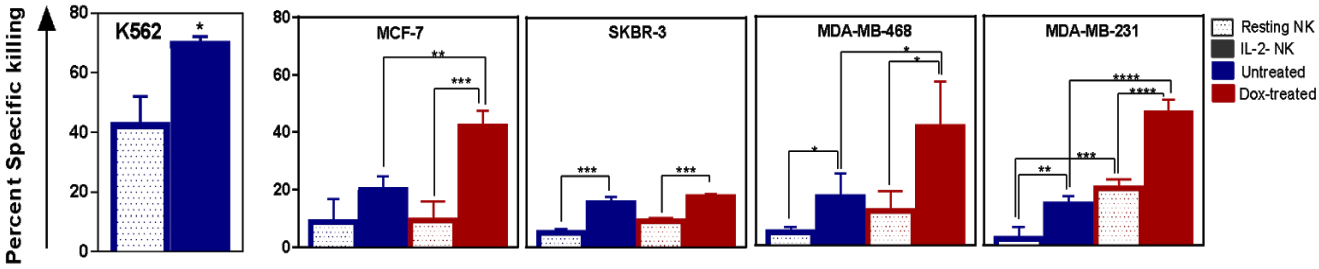

Figure 4.7 Sensitivity of breast cancer cells to killing by resting *versus* IL-2 stimulated donor-derived NK cells. Summary of the cytotoxicity of resting and IL-2 activated NK cells against untreated and low-dose doxorubicin treated cells. Data are presented as mean+SD from 3 independent experiments. A two-way ANOVA was used for statistical comparisons (* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001,**** P ≤ 0.0001).

4.3.2.2 Influence of low-dose doxorubicin on the sensitivity of breast cancer cells to killing by the NK-92™ cell line

The established human cytotoxic NK-92™ NK cell line is emerging as a potential alternative to primary NK cells in immunotherapeutic strategies and is being further developed for clinical trials (Klingemann et al. 2016; Tonn et al. 2013c). We therefore determined the influence of low-dose chemotherapy treatment on the sensitivity of breast cancer cells to killing by NK-92™ cells (either the parental cell line

or the NK-92™ MI cell line, as indicated) using the same approaches described above for primary, donor-derived NK cells.

Cytotoxicity

As indicated in Figure 4.8, low-dose chemotherapy treatment increased the sensitivity of cells to killing by NK-92™ cells (mean increase ~22% across the three cell lines MCF-7, MDA-MB-468 and MDA-MB-231). These findings are in broad concordance with the data obtained using primary, donor-derived NK cells. Low-dose doxorubicin had no effect on the sensitivity on the HER-2 positive SKBR3 cells to NK-92™ cells (mean difference of 5.5%).

We also determined whether the treatment of cells isolated from MDA-MB-231/Luc tumours harvested from immunodeficient NSG mice with low-dose doxorubicin also increased their sensitivity to killing by the NK-92™ MI cell line. For this, tumours generated by implanting MDA-MB-231/LUC cells into NSG mice were harvested 4 weeks following tumour implantation, dissociated into single cell suspension and the cells derived from each animal were either left untreated or were treated with low-dose doxorubicin and then used as targets in a cytotoxicity assay. As shown in Figure 4.9, treatment enhanced the sensitivity of cells to killing by NK-92™ MI cells.

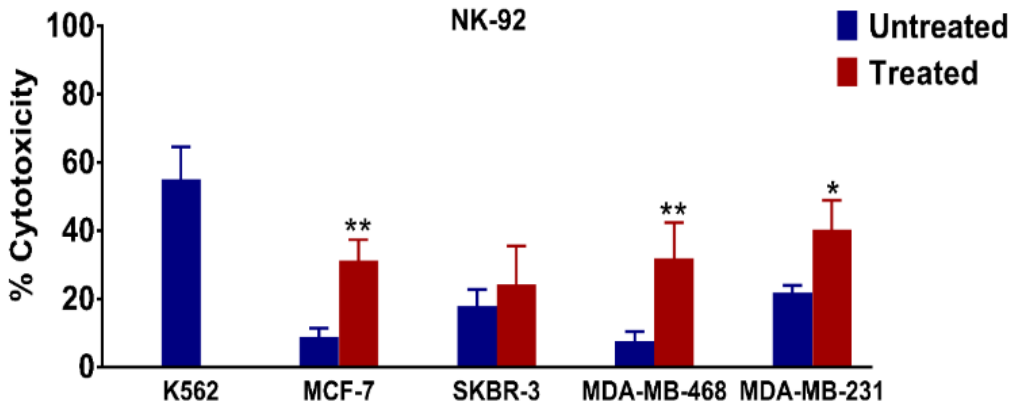

Figure 4.8 Influence of doxorubicin treatment on the sensitivity of breast cancer cells to killing by NK-92™ MI cells. The sensitivity of untreated and doxorubicin-treated cells to NK-92™ MI cell-mediated cytotoxicity was determined at an E:T ratio of 5:1. Data are presented as mean+SD from three independent experiments. A two-way ANOVA was used for statistical comparisons (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

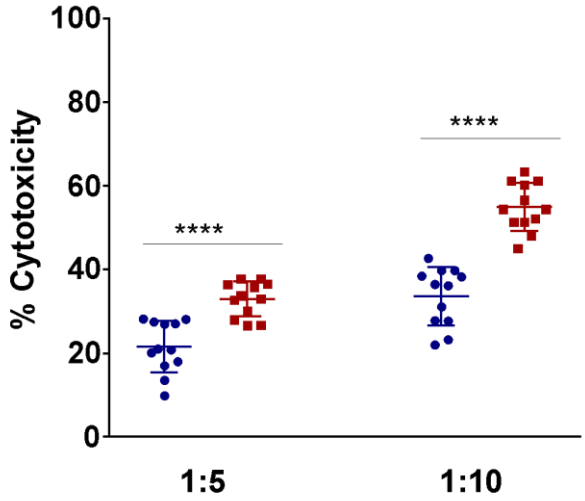

Figure 4.9 Influence of doxorubicin treatment on the sensitivity of MDA-MB-231/LUC breast cancer cells isolated from MDA-MB-231/LUC cell-derived tumours in NSG mice to killing by NK-92™ MI cells. The sensitivity of untreated (blue) and doxorubicin-treated (red) MDA-MB-231/luc cells derived from MDA-MB-231/luc xenografts to NK-92™ MI cell-mediated cytotoxicity was determined at an E:T ratio of 5:1 and 10:1. Data are presented as mean±S.D, n=12. One-way ANOVA (**** $P \leq 0.0001$).

Degranulation and cytokine function

NK cells control and kill target cells by secreting granzyme-containing granules and a range of pro-inflammatory cytokines. We therefore compared the expression of the degranulation marker CD107a, granzyme B, perforin and IFN-γ in NK-92™ MI cells following exposure to untreated MDA-MB-231 cells, MDA-MB-231 cells treated with low-dose doxorubicin and K562 cells (as control). As indicated in Figure 4.10, exposure of NK-92™ MI cells to MDA-MB-231 cells treated with low-dose doxorubicin triggers higher levels of IFN-γ, perforin, granzyme B and CD107a (indicative of degranulation) expression than that triggered by untreated cells. Of note, exposure to low-dose doxorubicin treated cells triggered higher levels of IFN-γ, CD107 and perforin expression that exposure to K562 cells.

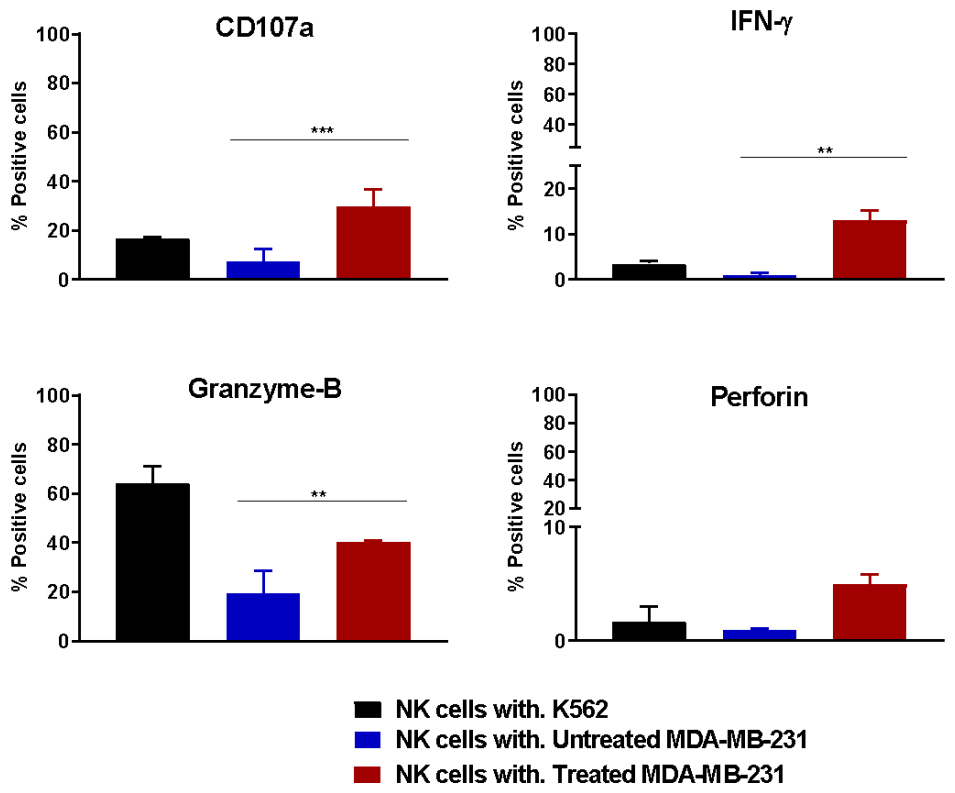

Figure 4.10 Effect of exposing NK-92™ MI cells to untreated MDA-MB-231 cells, MDA-MB-231 cells treated with low-dose doxorubicin and K562 cells on degranulation and cytokine expression. NK-92™ MI cells were co-incubated with untreated/treated MDA-MB-231 cells or K562 cells (as a positive control) at an E:T ratio of 1:2 for 5 hours, after which the expression of IFN-γ, perforin, granzyme B and CD107a (indicative of degranulation) was determined by intracellular flow cytometry (mean+SE,* P≤ 0.03, ** P ≤ 0.002, *** P ≤ 0.0002).

4.3.3 Low-dose doxorubicin treatment does not affect the viability or phenotype of NK-92™ MI cells

One potential concern related to the administration of low-dose doxorubicin followed by NK cell transfer in the clinical setting would be the effect that doxorubicin could have on the viability and function of NK cells. To assess this, NK-92® MI cells were treated with the same doses of doxorubicin as those used for treating the TNBC cells (1uM) and its effect on viability determined using trypan blue dye exclusion and on the expression of the NK cell activation receptors NKG2D and NKp30 using flow cytometry. As shown in Figure 4.10, treatment had no effect on the viability of NK-92® MI cells, nor did it have any effect on their expression of NKG2D and NKp30.

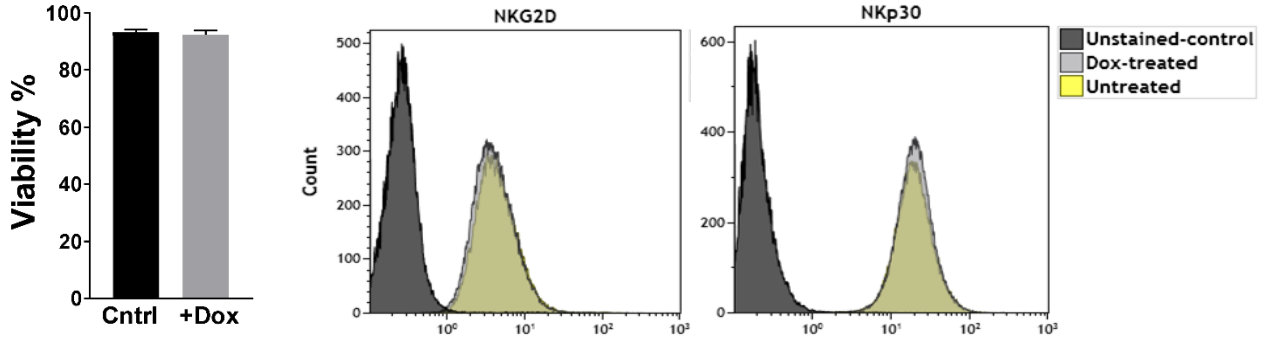

Figure 4.11 Low-dose doxorubicin treatment has no impact on the viability of NK92™ MI cells or on their expression of the activation receptors NKG2D and NKp30. NK-92™ MI cells were treated with 1uM doxorubicin for 24h, after which their viability was determined using trypan blue exclusion and their expression of NKG2D and NKp30 receptors using flow cytometry. Data are representative of 3 experiments.

4.3.4 Sensitivity of SA-β-gal positive cells

In order to determine if the sensitivity of doxorubicin-treated cells aligns with SA-β-gal positivity, and to assess its potential use as a marker for NK cell sensitivity, we determined the killing of SA-β-gal positive and negative populations by donor-derived NK cells and the NK-92™ MI cell line. For these experiments, cells treated with low-dose doxorubicin were stained for SA-β-gal prior to exposure to NK cells and the percentages of killed SA-β-gal positive and negative cells determined at the end of the standard cytotoxicity assay. Both primary NK cells and NK-92™ MI cells preferentially killed SA-β-gal$^+$ MCF7 cells and MDA-MB-468 cells, whereas there was no preferential killing of SA-β-gal$^+$ SKBR3 and MDA-MB-231 cells (Figure 4.11).

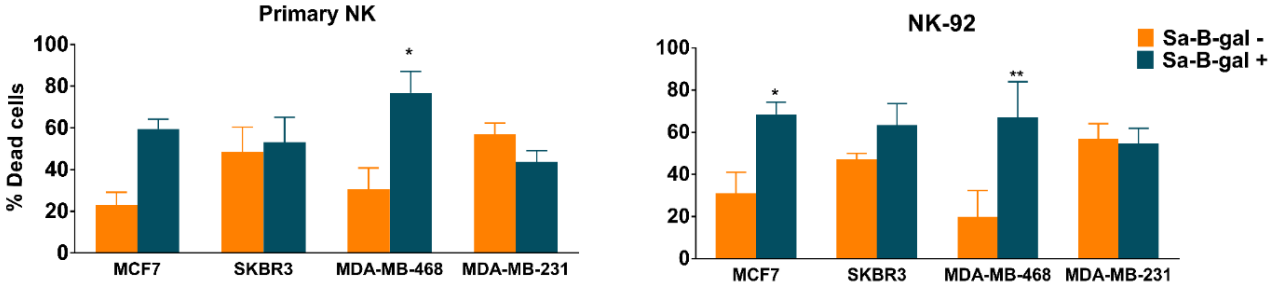

Figure 4.12 Sensitivity of SA-β-gal positive cells to NK cell-mediated cytotoxicity. Breast cancer cells treated with low-dose doxorubicin were stained for the expression of SA-β-gal using C12FDG prior to their inclusion as targets for primary, donor-derived NK cells or NK92™ MI cells in a flow cytometry-based cytotoxicity assay. The percentages of dead cells in the SA-β-gal positive and negative populations was determined by flow cytometry. Data are presented as mean+SD from 3 independent experiments. A two-way ANOVA was used for statistical comparisons (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$).

4.4 Summary

The aim of these experiments was to determine the effect of low-dose doxorubicin treatment on the sensitization of breast tumour cells to killing by primary, donor-derived NK cells and the NK-92™ MI cell line. We first observed that low-dose doxorubicin treatment increased the expression of ligands for major NK cell activatory receptors (NKG2D, DNAM-1). It was expected that this upregulation would act as a "kill me" signal to NK cells and thereby increase their sensitivity to NK cell-mediated killing. We therefore compared the killing of untreated and treated breast cancer cells by primary, donor-derived NK cells and confirmed that treated cells were indeed more sensitive to NK cytotoxicity, but with the exception of SKBR3 cells. We also demonstrated that IL-2 stimulation greatly enhanced NK cell-mediated cytotoxicity of treated cells. Low-dose doxorubicin treatment also rendered cells more sensitive to killing by the NK92™ MI cell line.

Further experiments focused mainly on MDA-MB-231 cells, as it represented one of the most responsive cell lines to NK cell killing following low-dose doxorubicin treatment. This cell line was also selected for tumour model studies (presented is chapter 6) as MDA-MB-231 cell-derived breast cancer xenograft models are one of the most commonly used and easily generated models for studying TNBC (Iorns et al. 2012) . MDA-MB-231 cells were shown to be highly aggressive and prone to metastasis (Simmons et al. 2015; Minn et al. 2005). We have also shown that treatment of cells derived from MDA-MB-231 xenografts harvested from NSG mice with low-dose doxorubicin increased their sensitivity to killing by NK92™ MI cells, and observed that the exposure of NK92™ MI cells to MDA-MB-231/LUC treated with low-dose doxorubicin increased their expression of CD107a (a degranulation marker), granzyme B, perforin and IFN-gamma, as determined with flow cytometry.

Furthermore, we addressed one potential concern regarding the effects that doxorubicin might have on NK cells, in terms of viability and activation receptor expression by demonstrating that exposure of NK92™ MI cells to doxorubicin had no effect on their viability or expression of NKG2D and NKp30 receptors.

In an attempt to identify markers of response and sensitivity to NK cells, we asked if the SA-β-gal positive cells induced by low-dose doxorubicin were more sensitive to NK killing. Although this was the case for MCF-7 and MDA-MB-468 cells, it was not for SKBR3 and MDA-MB-231 cells, for reasons that are currently unclear.

Taken together, these findings support the proposition that low-dose doxorubicin treatment represents a potential way to increase the sensitivity of breast cancer to NK cell-based immunotherapies.

Chapter 5: Gene expression profiling of low-dose doxorubicin treated breast cancer cells using the NanoString nCounter™ platform.

5.1 Introduction

Tumour development and progression is commonly accompanied and/or dictated by epigenetic changes (DNA alterations and histone modifications) which affect gene expression in tumour cells and cause perturbations in their associated signalling cascade and pathways (Vogelstein and Kinzler, 2004). Pathway deregulation may contribute to the initiation and progression of cancer by silencing tumour suppressor genes and activating oncogenic genes (Edelman *et al.*, 2008; Vogelstein *et al.*, 2013). Thus, identifying pathways that are deregulated in tumours and/or deregulated in response to treatment is crucial for advancing the understanding of tumour biology and progression, and may lead to the identification of potential treatment targets and/or insights into mechanisms of therapeutic resistance (Edelman *et al.*, 2008; Yauch and Settleman, 2012).

Many studies have demonstrated that several signalling pathways play important roles in the development, progression and metastasis of breast cancer. These include estrogen receptor signalling, EGFR (epidermal growth factor receptor) signalling, PI3K/AKT/mTOR, MAPK (mitogen-activated protein kinases), nuclear factor-κB (NF-κB), Notch and canonical Wnt pathways (Foley *et al.*, 2010; Haagenson and Wu, 2010; Prosperi and Goss, 2010; Rosen, Ashurst and Chap, 2010; Guo, Liu and Gonzalez-Perez, 2011; Vitale Gobbi *et al.*, 2015; Papa and Pandolfi, 2019; Saha *et al.*, 2019).

Considering the effect of low-dose doxorubicin on the phenotype of breast cancer cells and their susceptibility to NK cell killing, as described in previous chapters, it would be expected that treatment has an impact on the breast cancer cell transcriptome (gene expression) and that the interrogation of this will provide insight into the mechanism of action and reveal predictive markers of susceptibility to NK cell cytotoxicity and thereby as actionable targets for therapy.

High-throughput gene expression analysis can identify gene transcription which is markedly changed from a normal state and can generate insights into the mechanisms and pathways underlying these changes. The Nanostring nCounter™ amplification-free gene expression profiling platform (NanoString Technologies Inc., Seattle, USA) enables the direct quantification of target RNA transcripts using unique digital colour-coded barcodes that hybridize to specific targets and can be detected using single-molecule imaging. This technology gives direct multiplex measurement of mRNA expression levels

without the need for amplification and been shown to be highly precise, sensitive and reproducible (Geiss *et al.*, 2008).

5.2 Experimental Aims

The aim of the experiments described in this chapter was to investigate the effect of low-dose doxorubicin treatment on the gene expression patterns in breast cancer cells and thereby determine its influence on relevant cancer-related pathways using the NanoString PanCancer pathways panel. This panel encompasses 770 genes representing major cancer pathways. The overall gene expression signatures for the different pathways were compared and further analysed in enrichment studies. The most significantly differentiated genes, as well as their respective pathways, were defined.

5.3 Results

In order to gain a better understanding of the biology and the effect of low-dose doxorubicin treatment on breast cancer cells, we have performed a NanoString nSolver™ gene expression analysis using the PanCancer Pathways gene code set (Geiss et al., 2008). This panel simultaneously analyses 770 genes representing the activity of 13 key cancer pathways (606 critical genes from 13 canonical cancer pathways, 124 cancer driver genes, and 40 reference genes). The pathways covered by this panel are provided in Table 5.1, with the number of genes included in the panel in relation to the total number of known genes identified by KEGG (Kyoto Encyclopedia of Genes and Genomes) for each pathway. It should be noted that some genes belong to multiple pathways.

Three cell lines were selected for the study, the two triple negative cell lines, MDA-MB-231 and MDA-MB-468 as well as the Luminal A cell line MCF7, in order to compare the transcriptional impact of treatment on cells derived from different subtypes of breast cancer.

As described previously, one common feature of low-dose doxorubicin treated cells was an increase in cell size. Thus, in addition to comparing the gene expression profiles of untreated and doxorubicin-treated cells, treated cell populations were sorted into 'low' and 'high' forward light scatter (FS^{low}, FS^{high}) populations using a Beckman Coulter MoFlo™ cell sorter (Figure 5.1). Three replicate samples for each population and experimental condition were analysed.

Table 5.1. Pathways of the PanCancer Pathways Panel

Pathway	#genes	Pathway	#genes
Chromatin modification	22/46	RAS	142/226
Hedgehog	28/51	Pi3K	201/345
Wnt	78/139	STAT	86/156
Notch	24/48	MAPK	157/258
Apoptosis	66/86	TGF-β	51/80
Cell cycle	73/124	DNA Damage Control	50/106
Transcriptional Regulation	101/179		

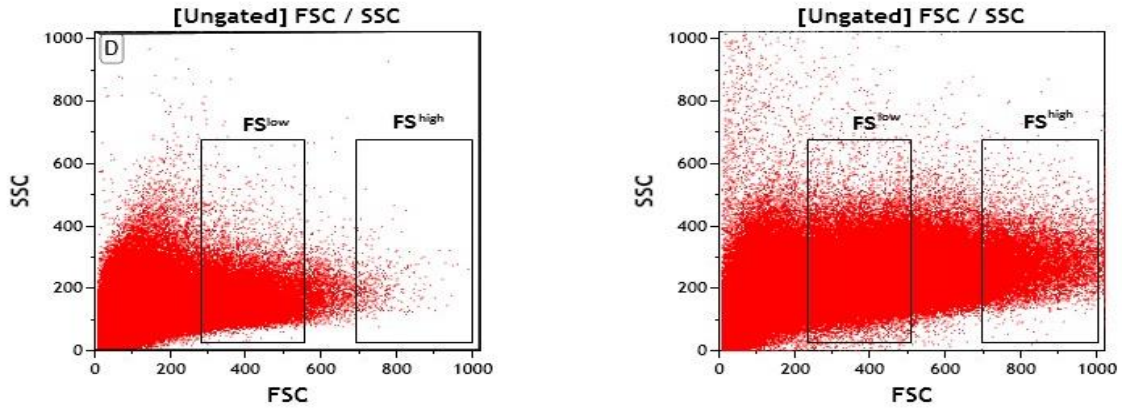

Figure 5.1. Representative Scatter Plot illustrating the regions used to define and sort FS^{low} and FS^{high} populations in MDA-MB-231 cells. The same sorting strategy was used for MDA-MB-468 and MCF-7 cells. Left: Untreated cells used to set the gates according to the baseline. Right: Treated cells with doxorubicin (1 μM for 2h, cells used at day 7 post treatment).

5.3.1 Influence of low-dose doxorubicin treatment on pathways gene signatures and scores

First, we compared the overall gene expression signatures for control, treated and FS ^{high} and FS^{low} categories. As shown in Figure 5.2, low-dose doxorubicin treatment resulted in a differential gene expression for the three cell lines tested in most pathways. However, cells in the FS^{high} gate did not exhibit the most differentially expressed gene profile in terms of pathway signatures in every case. For MDA-MB-468 cells, for example, FS^{low} cells appear to have a more distinct gene expression profile than FS^{high} populations, as most pathways appears to be upregulated, when compared to baseline untreated cells. Cell size might therefore not be the best indicator of a response to the treatment. For this reason, further analysis only compared the transcriptome covered by the PanCancer Pathways gene code set of the entire low-dose doxorubicin treated cells with that of their untreated counterparts at baseline. Figure 5.3 only include untreated versus treated samples to show better the differential pathway gene signatures.

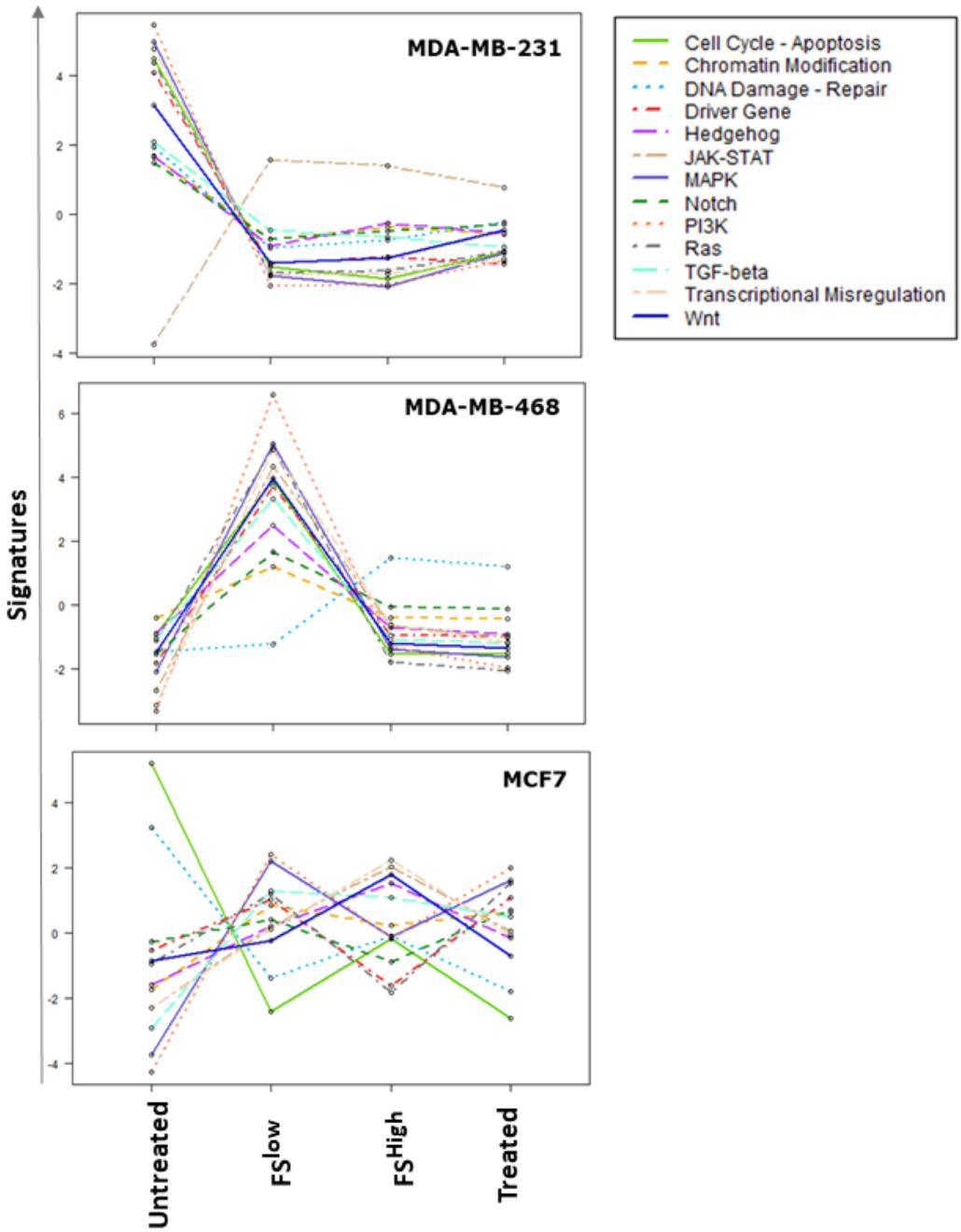

Figure 5.2. Comparison of overall gene expression signatures. Trend Plots of pathways signatures of the 3 cell lines tested provide an overview of the influence of treatment on pathway deregulation.

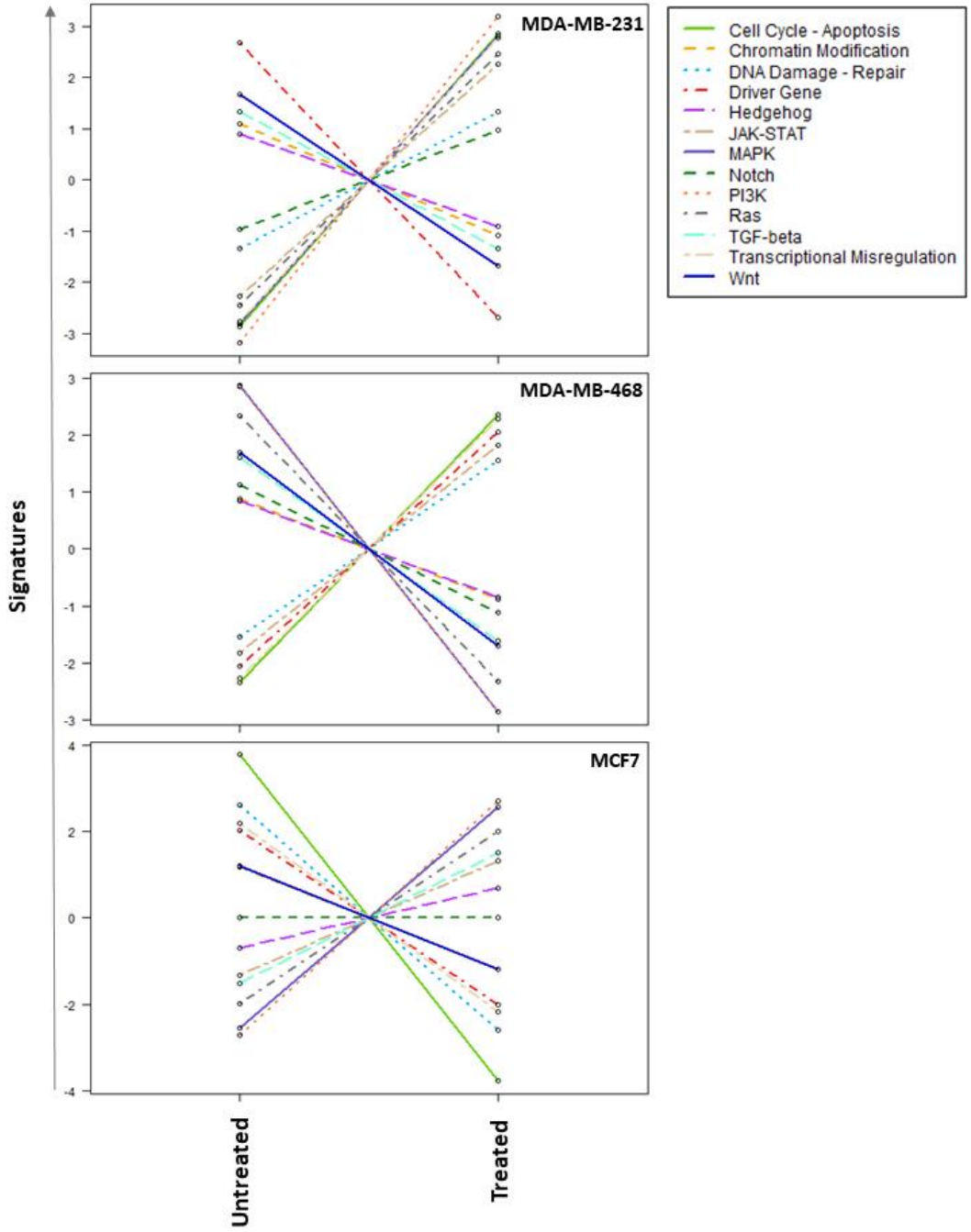

Figure 5.3. Comparison of overall gene expression signatures between untreated and treated samples. Trend Plots of pathways signatures of the 3 cell lines tested provide an overview of the influence of treatment on pathway deregulation.

In order to gain an initial insight into the impact of low-dose doxorubicin at the pathway, rather than individual gene level, pathway score analysis was performed first. This approach condenses the data from the different genes in a pathway into a single score, allowing changes of pathway scores across experimental samples to be visualized. Presented in Figure 5.4-A is a heatmap of pathway scores for MDA-MB-231 cells presenting the pathways that were most influenced by low-dose doxorubicin treatment. This can be divided into two sets, a set of pathways that had a lower score in treated cells (TGF-beta, Wnt, Chromatin modification and Hedghog pathways and driver genes) and a set that has a higher score in treated cells (DNA-Damage-Repair, Notch, JAK-STAT, Ras, PI3K, Transcriptional Misregulation, Cell Cycle-Apoptosis and MAPK).

Gene ontology enrichment analysis using MetaCore provides a more detailed overview of the signalling pathways influenced by low-dose doxorubicin. MetaCore is software that offers different tools, such as Pathway and Process Network Enrichment Analysis, to reveal which pathways are affected by differences in gene expression. The input genes from the NanoString nSolver™ analysis were filtered using $p<0.05$ as the cut-off for differential expression. Enrichment analysis was mainly in line with pathway score data. As depicted in Figure 5.4-B, which shows the biological processes and pathways influenced by low-dose doxorubicin treatment in MDA-MB-231 cells. Pathway analysis indicated that the differential gene expression induced by low-dose doxorubicin treatment significantly correlated with multiple signal pathways, the top 10 of which are presented for both Networks and Pathway Maps. The top three networks that were most significantly influenced were the Cell Cycle G1-S growth factor regulation, Notch signalling, and Cell Cycle G1-S Interleukin Regulation. For Pathway maps, the top three enriched pathways also included Cell Cycle Regulation and EGFR Signalling Pathway.

For treated MDA-MB-468 cells, fewer pathways were of a higher score than treated MDA-MB-231 cells, these were Cell Cycle-Apoptosis, DNA-Damage-Repair, JAK-STAT, Transcriptional Misregulation pathways and driver genes. The remainder of the pathways were of a lower score in treated cells (Figure 5.5-A). Like MDA-MB-231, the 3 top networks in MDA-MB-468 cells which were most significantly influenced by low-dose doxorubicin treatment were also the Cell Cycle G1-S Growth Factor Regulation, Notch Signalling, and Cell Cycle G1-S Interleukin Regulation. Pathway maps analysis indicated that differentially expressed genes were primarily enriched in EGFR, Canonical Leptin Pathways and Androgen Receptor (Figure 5.5-B).

Interestingly, opposite results were observed for MCF7 cells. Most of the previous pathways that were of a lower score in TNBC cells were higher in MCF7 treated cells and vice-versa. Scores for Cell Cycle-Apoptosis, Chromatin Modification, Wnt, DNA Damage-Repair, Transcriptional Misregulation pathways and driver genes were of a higher score in untreated controls (Figure 5.6.A). In terms of Network

Process Enrichment Analysis, among the top 10 most significant processes, 6 out of 10 were associated with cell cycle and 2 of 10 were associated with the DNA damage response. The two others, NOTCH and EMT (epithelial to mesenchymal transition), were common to the three cell lines (Figure 5.6-B).

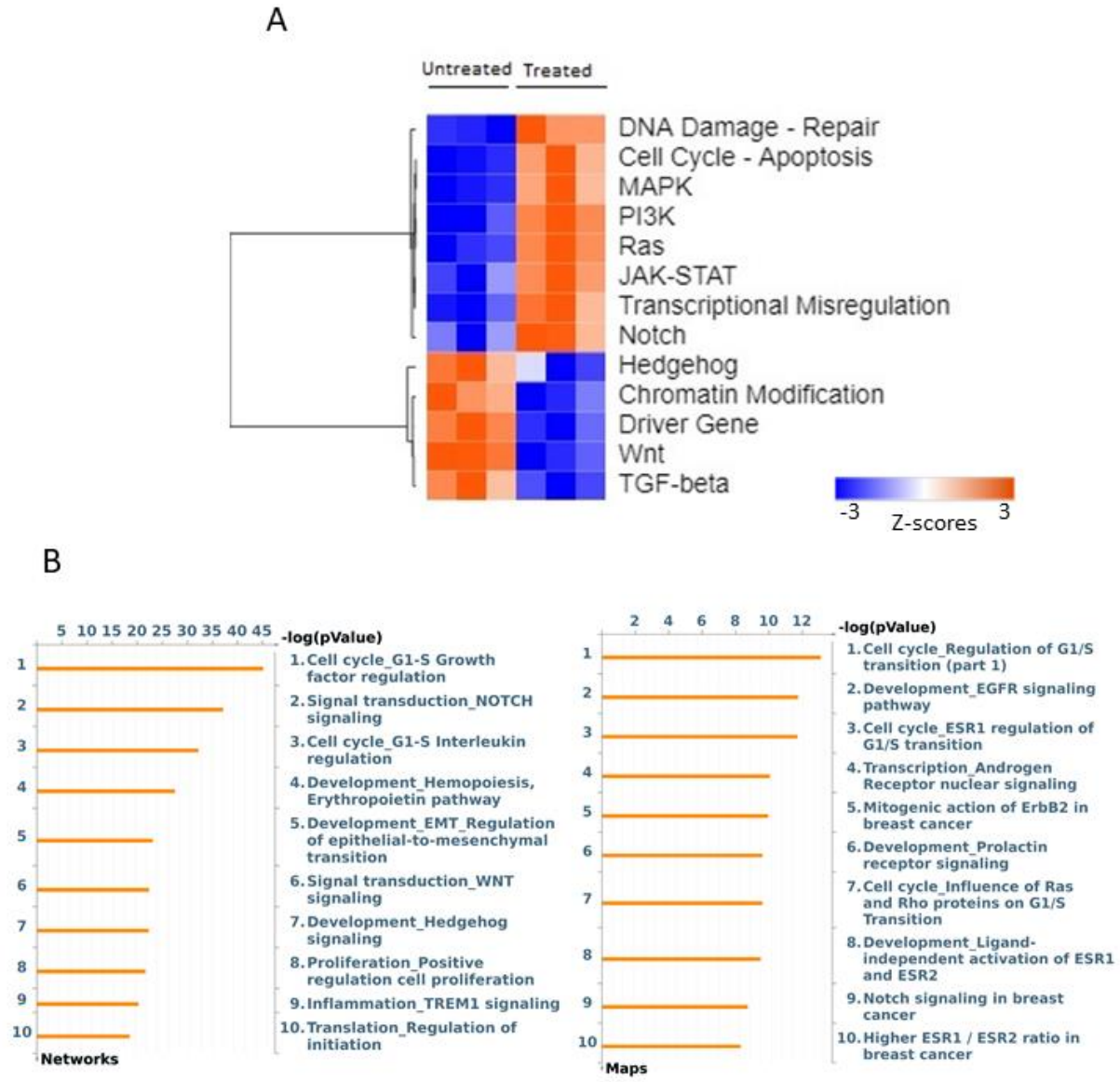

Figure 5.4. Influence of low-dose doxorubicin treatment on pathways gene signatures in MDA-MB-231 cells. **A.** Heatmap of Pathway scores of MDA-MB-231 cells treated with low-dose doxorubicin *versus* untreated controls. Overview of pathway scores changes. Orange indicates high scores; blue indicates low scores. Scores are displayed on the same scale via a Z-transformation. The Morpheus online tool (Broad Institute, USA) was used to visualise pathway score data. **B.** Enrichment analysis of for Network processes and Pathway Maps. MetaCore pathway analysis of gene expression differences between treated and untreated cells. Orange bars represent the significance of difference by –log (p value).

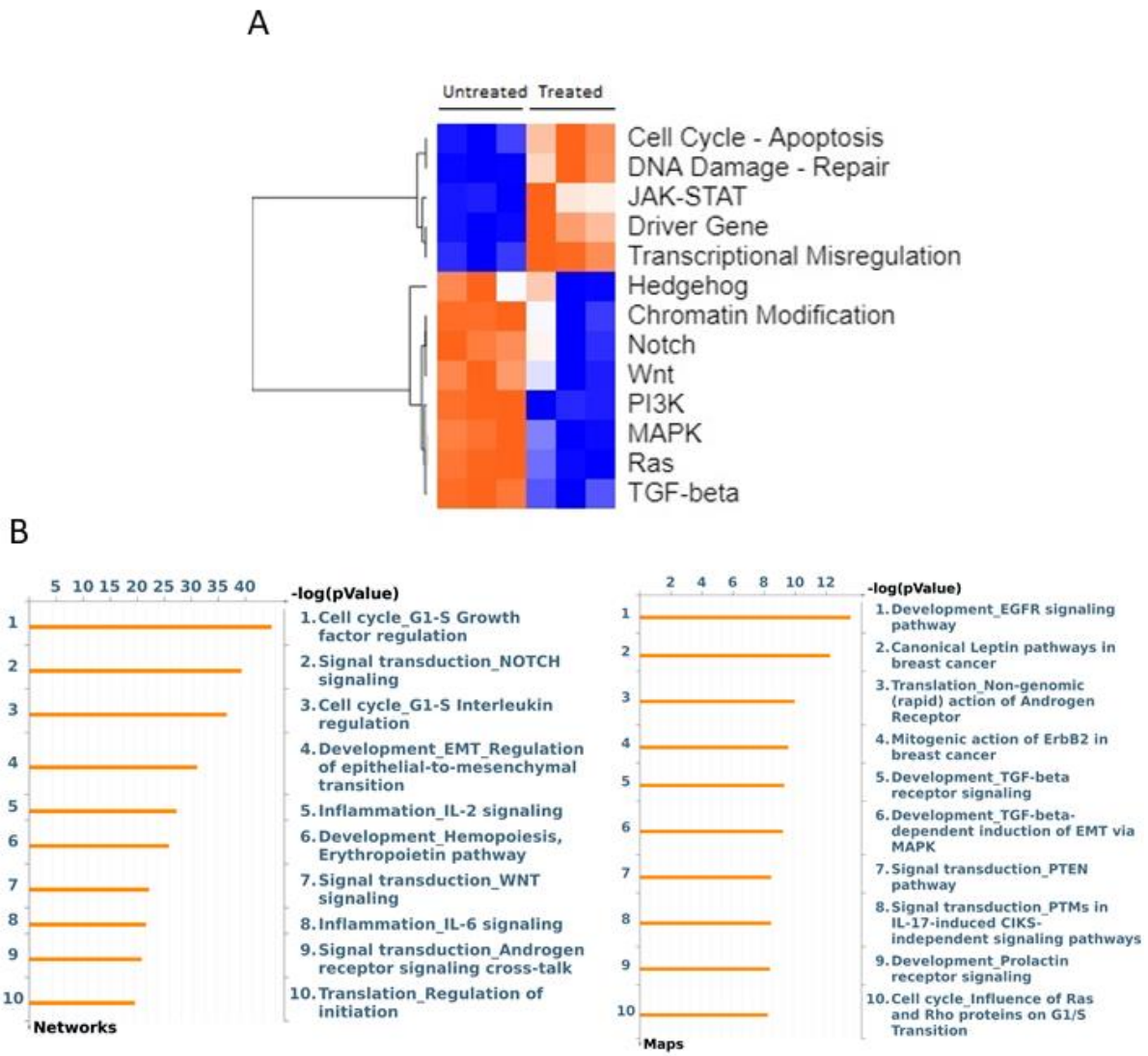

Figure 5.5. Influence of low-dose doxorubicin treatment on pathways gene signatures in MDA-MB-468 cells. **A.** Heatmap of Pathway scores of MDA-MB-468 cells treated with low-dose doxorubicin *versus* untreated controls. Overview of pathway scores changes. Orange indicates high scores; blue indicates low scores. Scores are displayed on the same scale via a Z-transformation. The Morpheus online tool (Broad Institute, USA) was used to visualise pathway score data. **B.** Enrichment analysis of for Network processes and Pathway Maps. MetaCore pathway analysis of gene expression differences between treated and untreated cells. Orange bars represent the significance of difference by –log (p value).

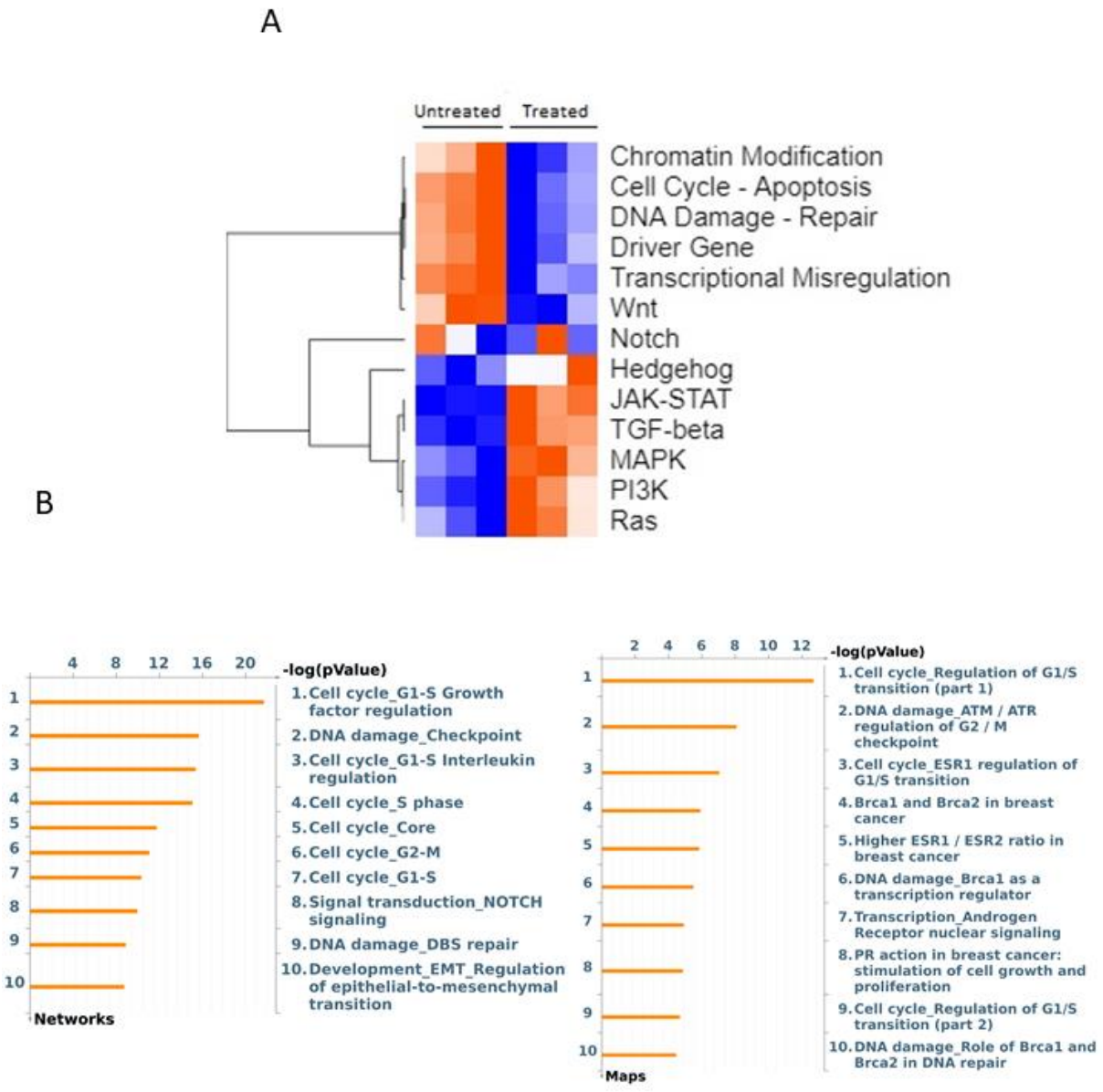

Figure 5.6. Influence of low-dose doxorubicin treatment on pathways gene signatures of MCF7 cells. **A.** Heatmap of Pathway scores of MCF7 treated with low-dose doxorubicin *versus* untreated controls. Overview of pathway scores changes. Orange indicates high scores; blue indicates low scores. Scores are displayed on the same scale via a Z-transformation. The Morpheus online tool (Broad Institute, USA) was used to visualise pathway score data. **B.** Enrichment analysis of for Network processes and Pathway Maps. MetaCore pathway analysis of gene expression differences between treated and untreated cells. Orange bars represent the significance of difference by –log (p value).

5.3.2 Influence of low-dose doxorubicin treatment on differential gene expression

Differential expression analysis was carried out to identify the changes in the expression levels of the genes belonging to the previously reported pathways. For MDA-MB-231 cells, amongst the 770 genes tested, there was evidence of differential expression at $p<0.05$ in treated cells relative to normal untreated cells for 257 genes. A volcano plot was generated (Figure 5.7-A) in which gene expression in treated cells was compared to that in untreated cells assigned as reference. The Benjamini-Yekutieli False Discovery Rate (FDR) approach was employed to generate an adjusted p-value. The top 10 most significantly differentiated genes with an adjusted p-value of <0.16 are presented in Figure 5.7-B. From these, the highly differentially expressed genes with a fold change value of >2 or <-2 were mainly downregulated (HIST1H3H, NPM1, E2F5, PRKCA and HMGA1), with the exception of PTPRR which was upregulated in treated cells. Interestingly, these most significantly downregulated genes are mainly associated with invasiveness and metastasis of tumours. NPM1 (nucleophosmin 1) which encodes for one of the most abundant proteins of the nucleoli and plays a role in different central cellular functions such as DNA replication, transcription and repair (Lindström 2011) is overexpressed in many solid tumours including liver, colorectal and cervical cancer, and its expression has been reported to correlate with cancer development and metastasis (Zhu et al. 2015). Furthermore, siRNA-reduction of NPM1 expression has been shown to inhibit the migration and invasiveness of colon cancer cell lines (Liu et al. 2012). In a study by Zeng et al, expression level of NMP1 was significantly higher in TNBC tissues versus other breast cancer subtypes. And the knockdown of NPM1 impaired the proliferation of TNBC cells via activation of the CDH1/Skp2/p27kip1 pathway (Zeng et al. 2019). Moreover, it was shown that NMP1 expression conferred resistance to TNBC cells to platinum chemotherapeutics cytotoxicity (Malfatti et al. 2019). E2F5 is an important transcription factor which has been shown to be overexpressed in tumours of patients with breast cancer compared to normal tissues and was associated with tumour development and metastasis (Liu et al. 2018). High E2F5 expression levels have been reported to be significantly associated with a TNBC phenotype and poor clinical outcome (Umemura et al. 2009). Xu et al, demonstrated that targeting of E2F5 with miR-154 inhibited breast cancer cells growth and invasion (Xu et al. 2016). Also shown to be involved in breast cancer invasion is PRKCA. PRKCA, encodes for a Protein kinase C alpha (PKCα), which is a serine-threonine protein kinase also implicated in cancer metastasis. PKCα expression was shown to be elevated in TNBC and to be responsible for chemotherapy resistance and metastasis (Tonetti et al. 2012; Tam et al. 2013). In a study by Pham et al., PKCα was shown to enhance migration of breast cancer cells through PKCα/FOXC2-dependent repression of p120-catenin (Pham et al. 2017).

Similarly, HMGA1, the high mobility group A1 gene is also involved in cell proliferation and motility of breast cancer cells and has been shown to drive metastatic and aggressiveness of triple negative breast tumours (Shah et al. 2013; Huang et al. 2015). Zanin et al, showed that through interaction with FOXM1, HMGA1 promoted breast cancer angiogenesis by increasing VEGFA transcription levels (Zanin et al. 2019). The most downregulated gene in treated MDA-MB-231 cells was HIST1H3H (histone cluster 1 H3 family member H), a member of the histone family genes. Various studies demonstrated the prognostic value of these genes as higher expression of histone gene sets was associated with poor survival and distant metastasis-free survival of breast cancer patients (Xie et al. 2019).

In contrast, PTPRR was upregulated in treated cells. PTPRR encodes protein tyrosine phosphatase receptor type R (PTPRs) which represent a subgroup of protein tyrosine phosphatases (PTPs). They play a central role in regulating signal transduction of physiological processes such as cell proliferation, apoptosis, migration, and invasion (Du, Grandis 2014; Guan et al. 1990). They are reported to be involved in several cancer types (Julien et al. 2011). Interestingly, downregulation of PTPRR is associated with tumour progression across multiple cancer types (Menigatti et al. 2009). In ovarian cancer, PTPRR was found to act as a tumour suppressor by inactivating the Wnt/β-catenin pathway (Y. Wang et al. 2019).

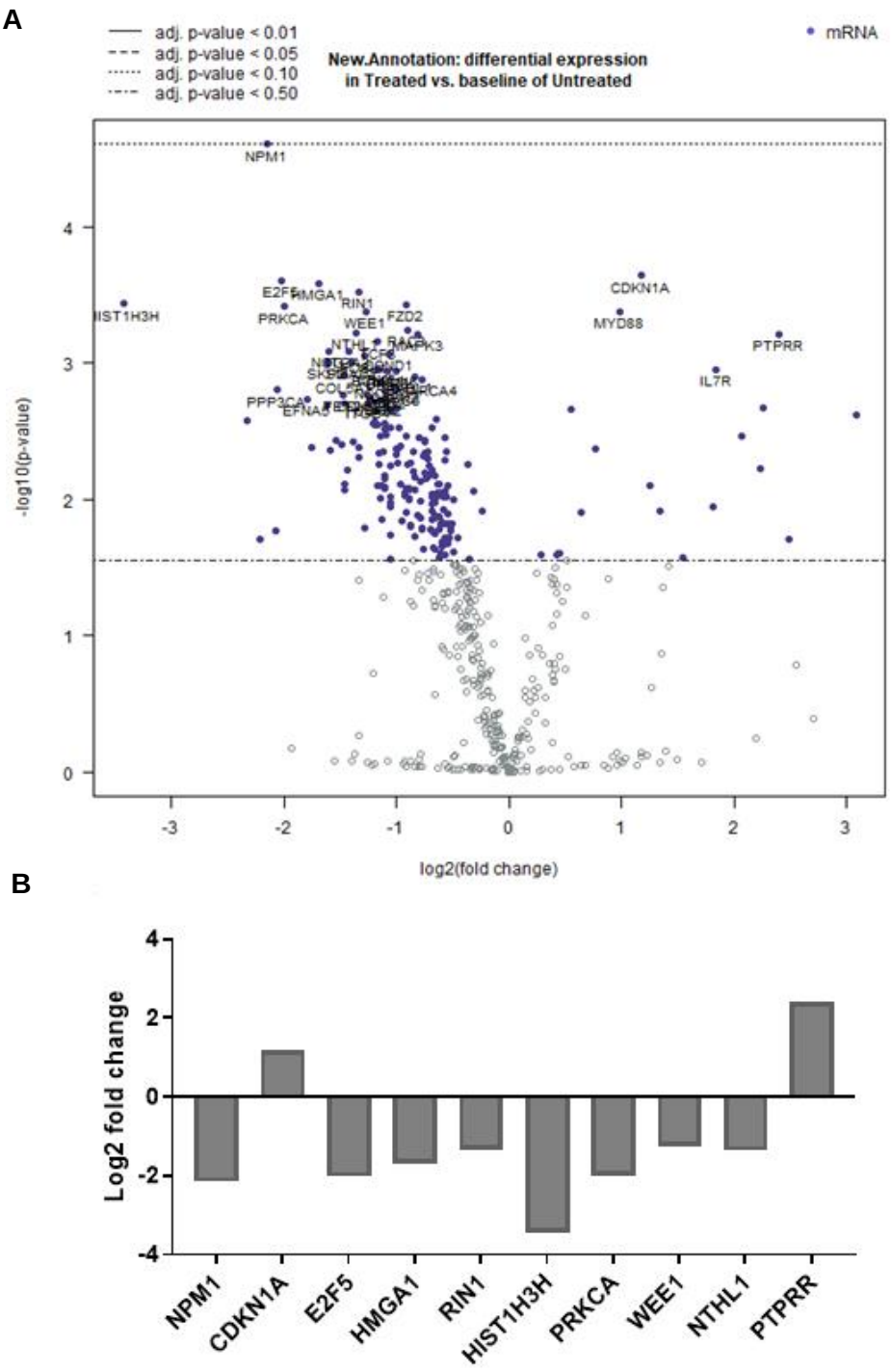

Figure 5.7. Differentially expressed genes in low-dose doxorubicin treated MDA-MB-231 cells. A. Volcano plot displaying -log10 (p-value) and log2 fold change for each gene. Highly statistically significant genes fall at the top of the plot above the horizontal lines, and highly differentially expressed genes fall to either side. Horizontal lines indicate various False Discovery Rate (FDR) thresholds. The 40 most statistically significant genes are labelled in the plot. **B.** Graph of the top 10 most differentially expressed genes with their fold change (adjusted p-value≤0.16, log2 fold change of >1 or <-1).

For MDA-MB-468 cells, amongst the 770 genes tested, there was evidence of differential expression at $p<0.05$, in low-dose doxorubicin treated cells relative to normal untreated cells for 228 genes. The top 10 most significantly differentiated genes with the Benjamini-Yekutieli adjusted p-value of ≤0.16 and a log2 fold change of >1 or <-1 are presented in figure 5.8-B. These 10 top differentially expressed genes were all downregulated in treated samples. Most of these genes belong to the Cell Cycle-Apoptosis and DNA Damage pathways**.** The top highly downregulated gene was TGFB3 (transforming growth factor, beta 3) (Log2 fold change -2.91), TGFβ signaling is at the center of tissue homeostasis maintenance and many physiological processes, which makes it frequently exploited by cancer cells to promote survival and tumour progression (Massagué 2008). TGFB3 encodes for TGFβ3, one of the three isoforms of TGFβ. In cancer, TGFβ3 was shown to have a role in the EMT (epithelial to mesenchymal transition) process (Kaartinen et al. 1995; Massagué 2012; Zu et al. 2012; Hao et al. 2019). In a study by Chen et al, TGFB3 expression was found to be greatly upregulated in premalignant tumour cells, as TGFB3 mRNA levels were increased by 84 % in breast tumours compared with adjacent normal tissues (Chen et al. 2015). TGFβ3 was also found to be expressed at higher levels in invasive breast cancer compared to normal tissues, highlighting its role in the promotion of breast cancer invasion and metastasis (Hachim et al. 2018). Interestingly, treatment of triple negative breast tumours with MTD paclitaxel has been reported to increase TGFB3 gene expression (Bhola et al. 2013) highlighting the low-dose chemotherapy as a potential alternative free of these adverse effects. Here as well, many of the downregulated genes are associated with proliferation and invasiveness across different cancers. CREB3L4, member of the CREB/ATF transcription factor family, regulate various cellular processes including cell proliferation, differentiation and apoptosis (Velpula et al. 2012) and has been reported to be expressed at high levels in invasive breast cancers and to be associated with reduced RFS and disease-specific survival (DSP) (Jing et al. 2019). PBX1 (Pre-B-cell leukemia homeobox 1), a member of three amino acid loop extension (TALE) family of homeodomain proteins, is involved in various developmental processes and at the center of ERα signaling (Delval et al. 2011). It was also shown to be highly expressed in breast cancer (J. Wang et al. 2017; Magnani et al. 2011) and has been reported to be a driver of metastasis in ER positive breast cancers (Magnani et al. 2015). IL20RA (Interleukin 20 receptor, alpha subunit), encodes for a subunit for the IL-20 receptor composed of IL20RA/IL20RB dimer, it's also a receptor for IL-19 and IL-24 (Rutz et al. 2014). IL-20 is a proinflammatory cytokine and its expression was associated with advanced tumour stage, metastasis and poor survival in breast cancer patients (Hsu et al. 2012). A few histone deacetylase genes were also found downregulated in treated cells, such as the gene encoding histone deacetylase 6 (HDAC6). HDAC6 is a cytoplasmic enzyme deacetylase involved in various biologic processes such as cell survival

and migration (T. Li et al. 2018). In MDA-MB-231 breast cancer cells, HDAC6 was shown to play a critical role in the invasive apparatus of tumour cells (Rey et al. 2011).

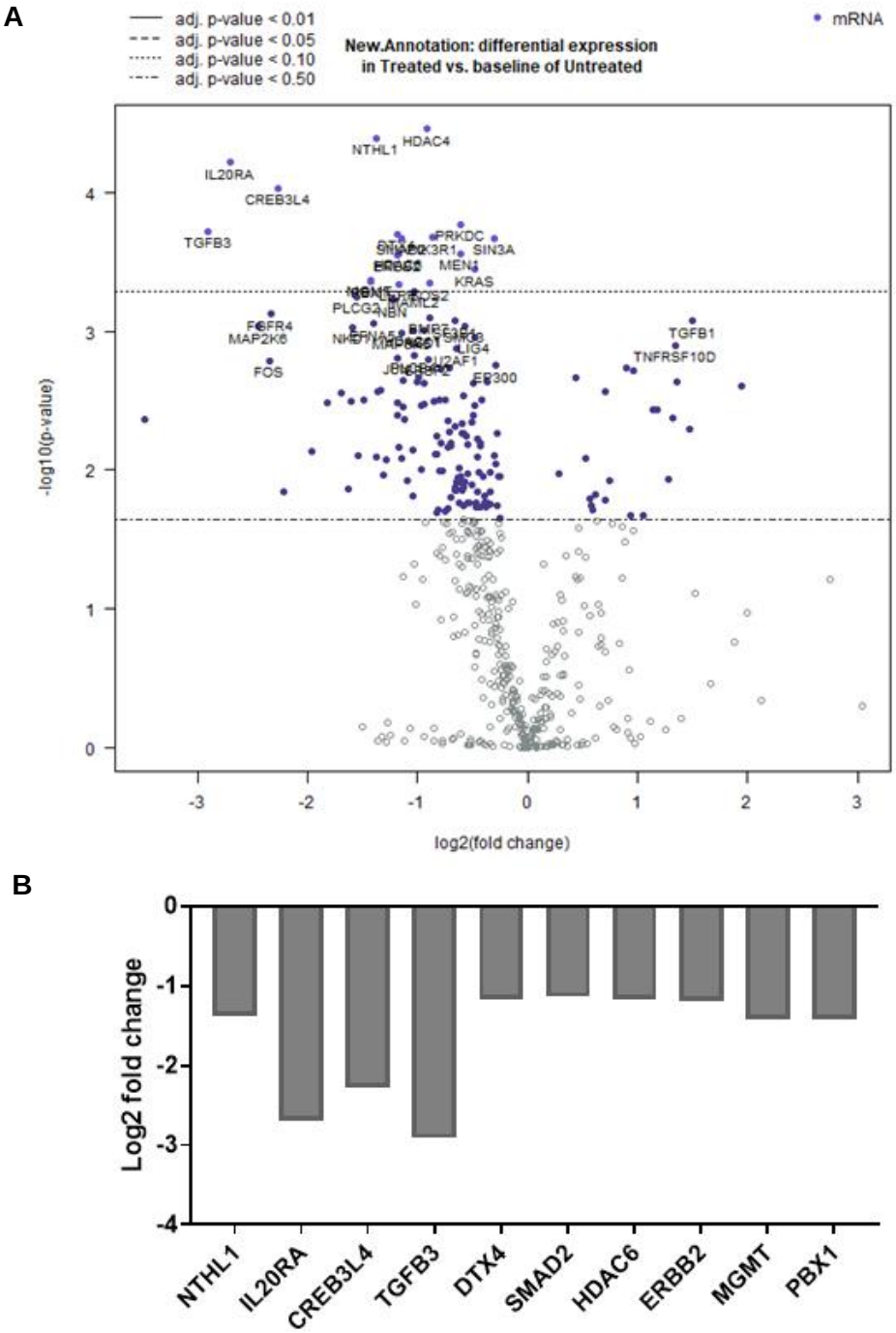

Figure 5.8. Differentially expressed genes in low-dose doxorubicin treated MDA-MB-468 cells. A. Volcano plot displaying each gene's -log10(p-value) and log2 fold change. Highly statistically significant genes fall at the top of the plot above the horizontal lines, and highly differentially expressed genes fall to either side. Horizontal lines indicate various False Discovery Rate (FDR) thresholds. The 40 most statistically significant genes are labelled in the plot. **B.** Graph of the top 10 most differentially expressed genes with their fold change (adjusted p-value≤0.16, log2 fold change of >1 or <-1).

With respect to MCF7 cells, amongst the 770 genes tested, there was evidence of differential expression at $p<0.05$ in low-dose doxorubicin treated cells relative to normal untreated cells for 118 genes. For this analysis, there were only three highly significantly expressed genes (TNFRS10C, EGFR, CDKN1A) based on the stringent corrected p-value≤0.16 and so we increased the FDR threshold to a p-value ≤0.3. Presented in Figure 5.9-B are the top 10 most differentially expressed genes having a log2 fold change of >1 or <-1. Contrary to the TNBC cell lines, these were all upregulated in treated cells compared to untreated controls. However, similar to the other cell lines, most of these genes belong to the cell cycle-apoptosis pathways. The genes that were significantly upregulated and having the highest fold change were CDKN1A and TNFRS10C (Log2FC= 3.43, 3.24 respectively). As with the other cell lines, CDKN1A, the cyclin-dependent kinase inhibitor 1 which encodes the cell cycle regulator p21 was upregulated in response to DNA damaged caused by doxorubicin, although no upregulation of p21 protein levels for MCF-7 cells were observed by flow cytometry (Chapter 3). TNFRSF10C (tumour necrosis factor receptor superfamily, member 10c), one of many TRAIL (TNF-related apoptosis inducing ligand-like) decoy receptors, which encodes for TRAIL receptor 3, is a p53-regulated DNA damage-inducible gene and functions as an antagonistic receptor, protecting cells from TRAIL-induced apoptosis (Ruiz de Almodóvar et al. 2004). A significantly reduced expression of TNFRSF10C is associated with multiple tumour types (Macartney-Coxson et al. 2008; Tanenbaum et al. 2016). Also involved in apoptosis are GADD45A and RASGRP1. GADD45A encodes for the Growth arrest and DNA-damage-inducible protein GADD45 alpha, a member of the GADD45 family of genes which are described as stress sensors that modulate cellular responses to various stress stimuli (Cretu et al. 2009; Li et al. 2017). GADD45A is a downstream target gene of p53 and BRCA1 (breast cancer susceptibility gene 1), playing a central role in genomic stability and tumorigenesis in various cancers (Cretu et al. 2009; Reis et al. 2015; Cui et al. 2017; Wingert, Rieger 2016). It has been reported to act as a tumour suppressor in Ras-driven breast tumorigenesis via increasing JNK-mediated apoptosis and p38-mediated senescence (Tront et al. 2010). Also, within the Ras pathway, RASGRP1 encoding for the RAS guanyl-releasing protein 1, is involved in proliferation, differentiation and apoptosis of tumours (Chang, Karin 2001). Interestingly, a higher expression of RASGRP1 has been associated with better overall survival in TNBC in various studies (Wang et al. 2018; J. J. Wang et al. 2019; Gupta et al. 2019). SHC2 (Src homology 2 domain) encode for the adaptor protein Shc 2 which is implicated in mediating several intracellular signalling cascades; involved in cell proliferation, survival and migration (Ravichandran 2001; Ahmed, Prigent 2017).TGFBR2 encoding for TGFβ2, one of TGFβ isoforms, was also upregulated. mRNA and protein levels of TGFBR2 were previously shown to be significantly decreased in breast cancer tissues compared with adjacent non-tumorous breast tissues, this downregulation in tumour

tissue was correlated with larger tumour size, lymph node metastasis and poor prognosis in breast cancer (Wei et al. 2015).

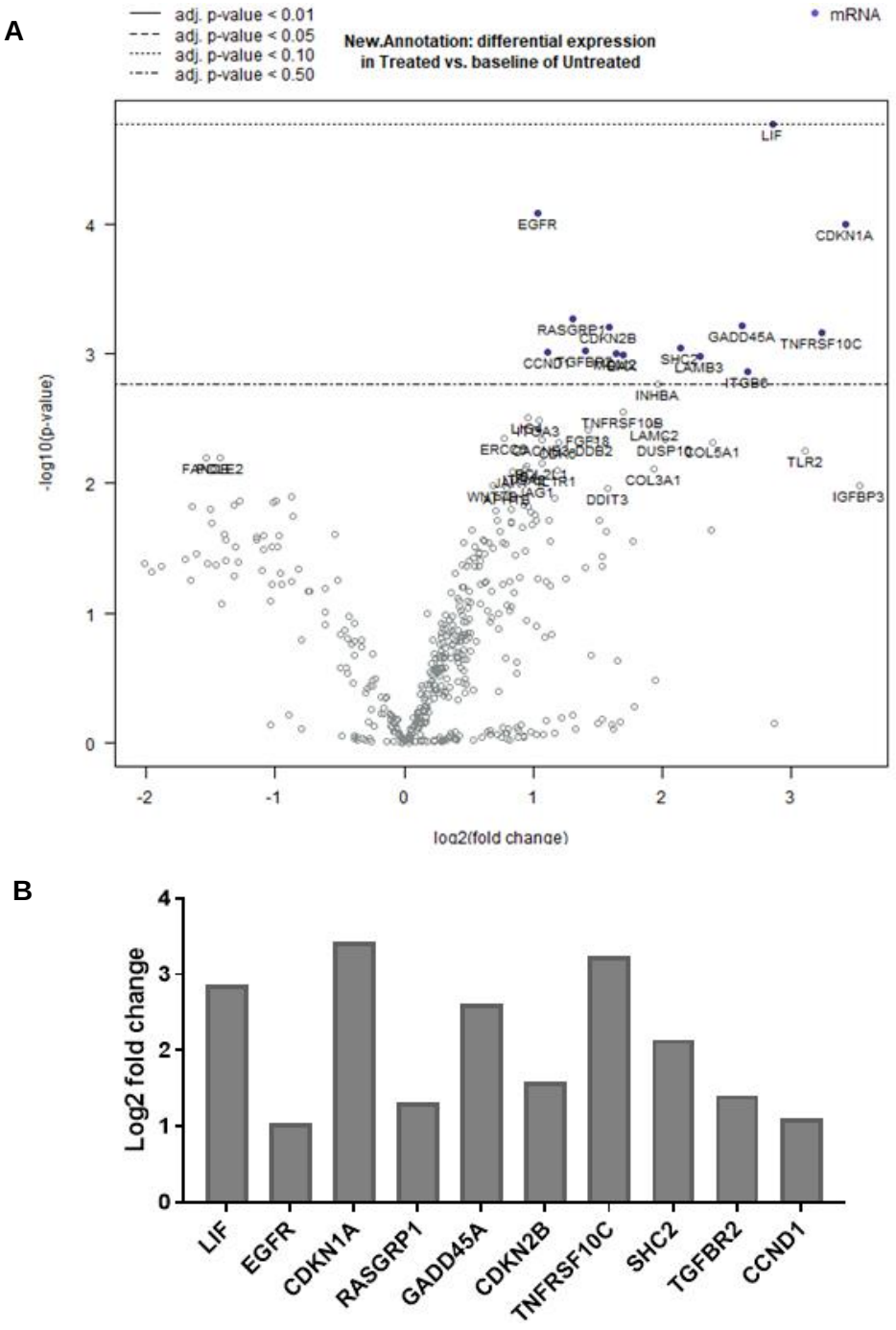

Figure 5.9. Differentially expressed genes in low-dose doxorubicin treated MCF7 cells. A. Volcano plot displaying each gene's -log10(p-value) and log2 fold change. Highly statistically significant genes fall at the top of the plot above the horizontal lines, and highly differentially expressed genes fall to either side. Horizontal lines indicate various False Discovery Rate (FDR) thresholds. The 40 most statistically significant genes are labelled in the plot. **B.** Graph of the top 10 most differentially expressed genes with their fold change (adjusted p-value≤0.3, log2 fold change of >1 or <-1).

5.4 Summary

Pathway and gene expression profile analysis provides essential information for identifying subtle changes in the biology of cancers and distinguishing cancer subtypes (Sorlie et al., 2001; Gatza et al., 2010). With the purpose of understanding the impact of low-dose doxorubicin treatment on cancer-related gene changes and identifying which cell properties and processes are modified by this treatment, we used NanoString nSolver™ amplification-free gene expression profiling using the Cancer Pathways code set which includes critical genes from 13 key cancer pathways. In this analysis, mRNA expression profiles of breast cancer cell lines treated with low-dose doxorubicin treatment were analysed and compared to control untreated cells, we identified the genes and their associated pathways that were deregulated for each of the three cell lines tested MDA-MB-231, MDA-MB-468 and MCF-7.

Pathway analysis identified the top consistently upregulated pathways in the two TNBC cell lines; DNA Damage-Repair, Cell Cycle-Apoptosis, Transcriptional Misregulation and JAKK-STAT, with PI3K, Notch, MAPK and Ras signaling pathways being upregulated in low-dose doxorubicin treated MDA-MB-231 and MCF7 cells. In terms of downregulated pathways, Chromatin Modification and Wnt were both downregulated in all cell lines following treatment, Hedgehog and TGFβ pathways were downregulated in both TNBC cells. Although upregulated in TNBC cell lines, DNA Damage-Repair and Cell Cycle-Apoptosis were downregulated in MCF7 cells.

In order to supplement the NanoString nSolver™ Analysis and further evaluate the functional pathways of deregulated genes, we analysed Pathway Maps, and Networks using MetaCore software. The three top networks with the most significant changes were common to both MDA-MB-231 and MDA-MB-468 cells and were associated to Cell Cycle and Notch signalling. Among these, the cell cycle pathway wasalso on the top three deregulated networks of MCF7 cells, in addition to DNA damage checkpoint. Other processes that were significantly altered in the three cell lines were Notch and EMT signalling. Pathway analysis using the breast neoplasms folder (MetaCore) was also concordant with NanoString nSolver™ analysis results. The three top pathways displaying significant alterations in MDA-MB-231 were associated with Cell Cycle Regulation and the EGFR Signalling Pathway. The EGFR Signalling Pathway was also the top deregulated pathway in MDA-MB-468 cells, followed by the Canonical Leptin Pathway and Androgen Receptor. For MCF7 cells, genes for Cell Cycle G1/S Transition and DNA Damage ATM/ATR Regulation of G2/M Checkpoint Signalling Pathways were highly enriched following low-dose doxorubicin treatment.

Differential expression analysis identified the highly altered gene levels in treated cells compared to untreated controls. A notable observation was that many of these alterations belong to signalling pathways that govern cell proliferation and cell differentiation. Interestingly, many of the downregulated genes were associated with cancer proliferation and invasiveness, and were shown to affect tumour metastasis. Overall, MCF7 cells showed a much weaker response to low-dose doxorubicin treatment in terms of significantly differentially expressed genes; as only 3 genes were found to be significantly altered following Benjamini-Yekutieli correction for multiple comparison - these genes were involved in cell cycle regulation and NDA-damage response. This weaker response could be due to the fact that MCF7 cells were treated with a lower concentration of doxorubicin than MDA-MB-231 and MDA-MB-468 cells. The concentration was based on the levels of SA-B-gal expression as previously explained. This is particularly interesting as it sheds light on the importance of drug dosing, as different doses of doxorubicin differently influence gene regulation. This might explain the differences found between the TNBC and MCF7 cells.

Considering results from previous chapters, showing the effect of low-dose doxorubicin on breast cancer cells. It was expected that doxorubicin treatment would have an impact on the transcriptome of the cells and induce changes in gene expression that would highlight the decrease in proliferation potential and the enhanced susceptibility to NK cell killing. Overall, Pathway and gene expression profile analysis gave insight into the altered pathways and molecular changes induced by low-dose doxorubicin treatment. Treatment with low-dose doxorubicin downregulated many genes involved in tumour progression and invasiveness. These findings are particularly interesting, as they provide insight into the mechanisms of action of low-dose doxorubicin and reveal potential predictive markers of sensitivity to NK cell cytotoxicity and potential targets/candidates for combination treatment. This effect on downregulating invasion and metastasis-related genes is further interrogated in tumour growth studies in the next chapter.

Chapter 6: Effect of the combination of low-dose doxorubicin and adoptive NK cell transfer on the growth of MDA-MB-231 cell-derived tumours in a murine xenograft model

6.1 Introduction

The efficacy of Immunotherapy for cancer treatment has been proven by different studies, especially the blockade of immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA4), programmed cell death protein 1 (PD1), and programmed cell death 1 ligand (PD-L1) (Chen, Han 2015; Topalian et al. 2015; Hoos 2016). However these studies also showed that only a subset of patients can benefit from this treatment in a sustainable manner, with some patients failing to respond completely to such treatment (Braun et al. 2016). Furthermore, the effectiveness of checkpoint inhibitor-based monotherapies is dependent on the nature of the cancer entity and its inherent immunogenicity (i.e. 'hot' versus 'cold'). Broadening the spectrum of patients that can benefit from immunotherapy treatment and achieving higher rates of tumour control will require the use of combined approaches. In TNBC, only a minority of patients benefit from these treatment options. This is mainly due to failure in overcoming the well-evolved mechanisms of immune escape in these advanced tumours, such as decreased expression of MHC class I which result in reduced immunogenicity and recognition (Borcherding et al. 2018) . Until the recent Impassion130 phase-3 trial which tested nab-paclitaxel combined to atezolizumab (Schmid et al. 2018), very few studies addressed immunotherapeutic approaches for TNBC treatment. Also, despite the encouraging results yielded in the Impassion130 trial, the median PFS was only improved in a subset of patients (PD-L1 positive for tumour –infiltrating immune cells) (Schmid et al. 2018). Therefore, further studies focusing on enhancing immunotherapy response are urgently needed (Chrétien et al. 2019; Sylvia Adams et al. 2019).

Identifying and exploiting potential synergies between treatment modalities is critical to increase the anti-tumour efficacy of existing cancer treatment approaches and reduce resistance and disease relapse. A potentially effective option is to combine immunotherapy with low-dose metronomic chemotherapy. As reviewed in the introduction chapter, some chemotherapeutic agents given in a

metronomic schedule have been shown to positively modulate anti-tumour immune responses by rendering tumour cells more immunogenic and more sensitive to NK cell-mediated cytotoxicity (Pol et al. 2015; Heinhuis et al. 2019; Ghiringhelli et al. 2007b). Given that immunotherapies that are based on triggering/restoring NK cell function have been shown to generate robust immune responses against a number of tumour entities (Souza-Fonseca-Guimaraes, 2016; Fang and Xiao, 2017; Hu *et al.*, 2019; Lupo and Matosevic, 2019), combining low-dose metronomic chemotherapy and NK cell-based immunotherapy is likely to improve therapeutic outcomes (Cifaldi et al. 2017).

The basis of such an approach is to sensitize tumours to NK cell-mediated cytotoxicity by administering low doses of the chemotherapeutic prior to the infusion of autologous or allogeneic NK cells, thereby enhancing tumour control. Until now, few studies have reported on the clinical efficacy of this approach. Pre-clinical studies have reported synergistic activity between low-dose chemotherapy and checkpoint inhibition. For example, Parra et al, demonstrated the efficacy of combining anti-CTLA4 and metronomic chemotherapy in a breast cancer model (Parra et al. 2017a).

Murine preclinical models are a crucial step in the development of cancer therapeutics. Different mouse models can be used for modelling cancer therapies, from cancer cell line and patient-derived xenografts to sophisticated humanized mice, each with its own advantages and disadvantages (Perrin 2014). The goal with animal models is to mimic human tumour biology, interactions of the tumour with the microenvironment and response to therapeutic concepts (Landis et al. 2013). Xenograft models are one of the main models used in cancer research and are based on the implantation of human cells or tissues into immunodeficient mice. The development of immunodeficient mouse strains bearing a mutation in the IL2 receptor common gamma chain gene (*IL2rγnull*) (Ohbo et al. 1996) combined with the *scid, Rag1null,* or *Rag2null* mutations, generate a mouse which is completely deficient in adaptive immunity and severely deficient in innate immunity and is highly receptive to engraftment of human cells, tissues, and primary tumours (Shultz et al. 2014).

In the previous chapter, the capacity of low dose doxorubicin to sensitize human breast cancer cells to NK cell killing was demonstrated, in this chapter, the experiments to examine the capacity of combining low-dose chemotherapy and adoptive NK cell transfer to control the growth of MDA-MB-231 cell-derived tumours in a murine xenograft model are described.

6.2 Experimental Aims

The aim of the experiments described in this chapter was to evaluate the capacity of low-dose doxorubicin (LD-Dox) combined with adoptive NK cell transfer to control the growth of MDA-MB-231

cell-derived tumours in a murine xenograft model. The effect of treatment on tumour growth and metastasis were assessed against controls receiving no treatment. Furthermore, to examine the *in vivo* immunological consequences of treatment, its influence on the expression of ligand for the main activatory NK cell receptor NKG2D (MICA/B) and proliferation marker Ki67, as well as the proportion of cancer initiating cells (CICs) and sensitivity of isolated tumour cells to *ex-vivo* killing by NK cells were assessed and compared between tumours that received either LD-Dox alone, NK cells alone or the combination treatment. The effect of treatment on NK cell infiltration into tumours was also studied using *in vivo* fluorescence imaging.

6.3 Results

6.3.1 Establishing murine xenograft model of human triple negative breast cancer (TNBC)

In order to investigate the influence of combining LD-Dox with adoptive NK cell transfer on the growth of TNBC *in vivo*, we established a murine orthotopic xenograft model using the human MDA-MB-231/LUC/RFP cells (obtained from AMSbio), which are transfected to stably express the firefly luciferase (Luc 3) reporter to enable optical bioluminescence imaging of the tumours following the administration of luciferin. Of note is that these cells also stably express red fluorescent protein (RFP), thereby enabling fluorescence-based imaging. MDA-MB-231 is a highly aggressive human breast cancer cell line and one of the most commonly used models for studying TNBC and its metastasis, as these cells metastasize to distant organs from primary tumours implanted in the mammary fat pad (Simmons et al. 2015; Minn et al. 2005). MDA-MB-231 cells also express features associated with Cancer Stem Cells (CSC) such as the $CD44^{+}CD24^{-/low}$ phenotype (Minn et al. 2005; Sheridan et al. 2006). The orthotopic model used in this study involved the direct subcutaneous injection of the cells into the fourth abdominal mammary fat pad.

The NSG (NOD-*scid IL2rγ*^{null}) mice strain was selected for this study, as it represents one of the most immunodeficient mice available and allows for the engraftment of human cells (Shultz et al. 2005; Shultz et al. 2007). They are void of mature T and B cells and, most importantly, lack functional NK cells (King et al. 2008).

The luciferase activity of MDA-MB-231/LUC/RFP cells and the relationship of the bioluminescence signal with cell number was first determined *in vitro* using with bioluminescent intensity (BLI) technique, as shown in Figure 6.1 (A). Cells titrated in the absence of luciferin (the substrate for luciferase) and PBS alone (no cells) acted as controls. As expected, the intensity of bioluminescence was highly correlated with the number of cells, with the linear correlation/determination coefficient (R^2=0.95).

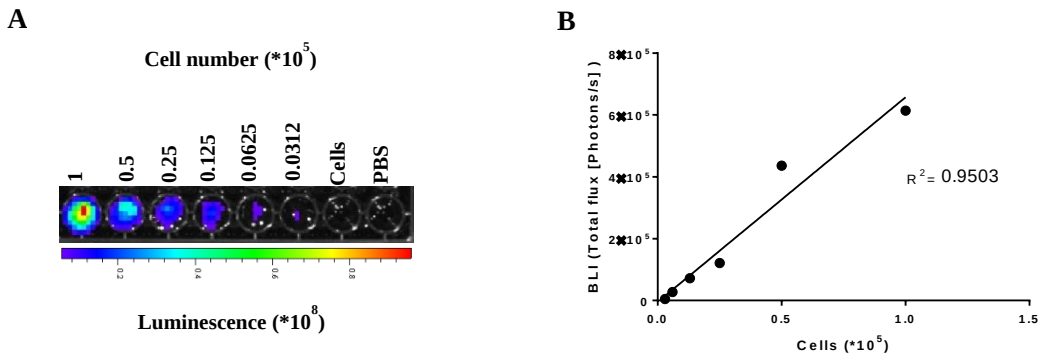

Figure 6.1. Relationship between MDA-MB-231/LUC/RFP cell number and bioluminescent intensity *in vitro*. A. *In vitro* bioluminescence of MDA-MB-231/LUC/RFP cells. **B.** linear correlation between cell number and BLI (R^2=0.95). (BLI: bioluminescent imaging). The figure is representative of one experiment, however the luminescent properties of cells was evaluated and confirmed prior to the start of each study.

To test the Tumourigenicity, as well as the capacity to image tumours using *in vivo* bioluminescent intensity (BLI) imaging, we performed a tumour growth kinetics study using 3 different cell doses ($0.5/2.5/5x10^6$) in order to determine the optimal cell number to use for tumour implantation. We also assessed the capacity of administering cells in the basement membrane extracellular (ECM) gel matrix (Matrigel™, 1:1 (v:v)) to improve tumour engraftment and growth using the $2.5x10^6$ cell dose.

Tumour growth was monitored using caliper measurements as well as BLI. As described in the methods chapter, the specific BLI signal was calculated in the region of interest. IVIS™ Spectrum images were used at the start of all studies to verify that the tumours were present in the correct location. Figure 6.2-A, shows tumour growth over time of the 4 cell doses assayed (tumour volume assessed by caliper measurements on the right and BLI on the left). The three cell doses generated a detectable luminescence signals *in vivo* which rose with increased tumour dosage. The quantification of the BLI correlated with the tumour load *in vivo* (R^2=0.99) (Figure 6.2-B).

Administering MDA-MB-231/LUC/RFP cells in Matrigel™ ($2.5x10^6$) increased tumour growth both in terms of tumour volume and luminescence signal. Figure 6.2- C shows a representative time course of the BLI, detected at different time points of the group of animals that received $2.5x10^6$ cells in Matrigel™. Based on these findings, and that a dose of $5x10^6$ MDA-MB-231/LUC/RFP cells generated robust tumour growth rates in the absence of Matrigel™, all subsequent *in vivo* studies implanted $5x10^6$ MDA-MB-231/LUC/RFP cells in Matrigel™ (1:1 v/v).

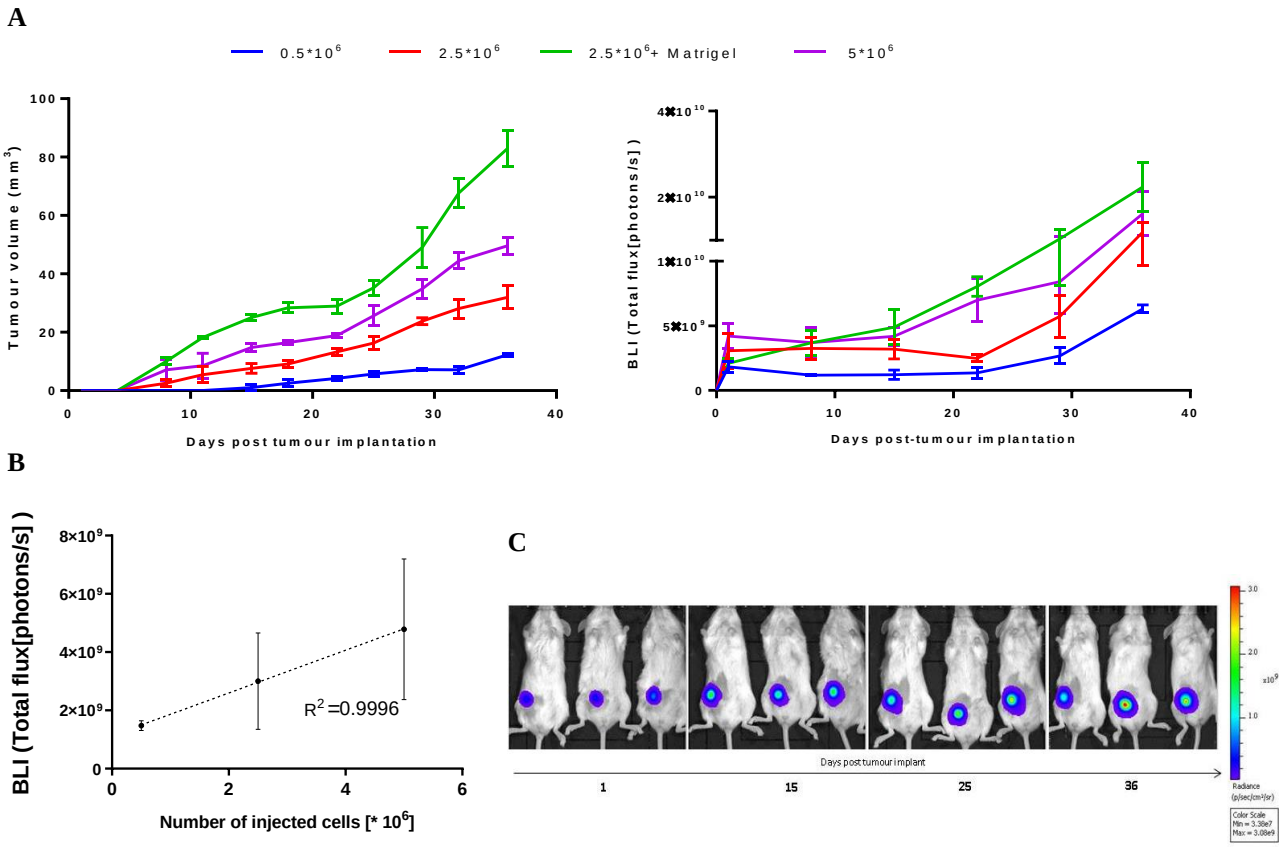

Figure 6.2 Establishment of xenograft mouse model of triple negative breast cancer (TNBC) using MDA-MB-231/LUC/RFP cells. (A) NSG mice (n=3/group) received s.c injections of the indicated doses of MDA-MB-231/LUC/RFP cells. **Left:** Tumour growth/volume measured by calipers; **Right:** Tumour growth/volume measured using BLI quantification. Data are mean ± SEM (n = 3) **(B)** correlation between BLI quantification and the tumour load in vivo at day 18 after MDA-MB-231/LUC/RFP cell implantation (R^2=0.99). Data are mean ± SEM (n = 3). **(C)** Respective time course of IVIS™ Spectrum images for animals implanted with 2.5x10^5 MDA-MB-231/LUC/RFP cells in Matrigel™ (1:1 v/v). (BLI= Bioluminescence intensity. Tumour volume = π/6 x (width x length^2)).

6.3.2 Influence of low-dose doxorubicin (LD-Dox) and/or adoptive NK cell transfer on the growth of MDA-MB-231/LUC/RFP cell-derived xenografts in immunedeficient NSG mice

6.3.2.1 Treatment schedule

Following the establishment of the tumour model and the validation of its suitability for BLI quantification *in vivo*, the Influence of low-dose doxorubicin (LD-Dox) and/or adoptive NK cell transfer on the growth of MDA-MB-231/LUC/RFP cell-derived xenografts in immunedeficient NSG mice was evaluated.

Although metronomic LD-Dox is currently used in the clinic, until now, very few studies have reported its use in combination with immunotherapy in preclinical models. The reported LD-50 of doxorubicin in the literature is 10.7 mg/kg body weight and the common single maximum tolerated dose (MTD) reported in murine models is 10mg/kg (Aston et al., 2017; Johansen, 1981). Our LD-Dox (LDM) mimetic regimen was based on a 20 times lower dose than the MTD for single doses, with a dosing regimen of doxorubicin at 0.5mg/kg three times a week. This represents a cumulative dose for 1 cycle of 1.5mg/kg and a whole treatment cumulative dose of 3mg/kg. This LDM mimetic regimen was previously evaluated by Mainetti et al (Mainetti et al. 2013) in combination with cyclophosphamide in a murine breast cancer model (0.5 mg/kg i.p. three times/week).

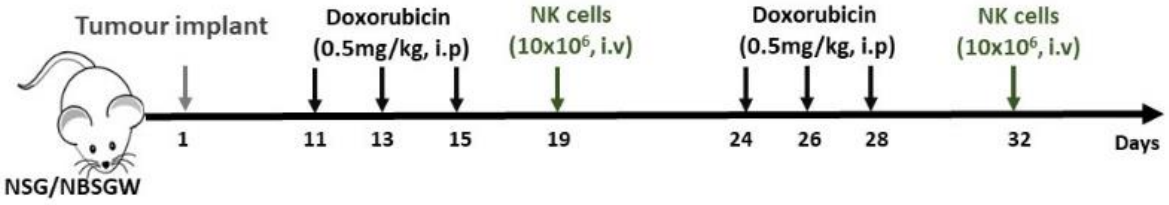

Figure 6.3. Treatment schedule for MDA-MB-231/LUC/RFP cell-based xenograft model. Animals received two cycles of the combination treatment, one cycle containing three doxorubicin injections (i.p) followed by one dose of $10x10^6$ NK cells (i.v. infusion). NK cells were purified from peripheral blood mononuclear cells (PBMCs) isolated from healthy volunteers, stimulated overnight with IL-2 (200U/ml) and then injected i.v via the tail vein into tumour-bearing animals. For some studies, NK cells were fluorescently labelled labeled with Vivotrack 680 near-infrared (NIR) fluorescent agent to enable tracking of NK cell infiltration using *in vivo* fluorescence imaging.

6.3.2.2 Tumour growth inhibition

LD-Dox treatment started 11 days after tumour implantation when tumours were palpable. In Study 1 shown in Figure 6.4-A (left panel) tumour growth was monitored using *in vivo* imaging during the treatment period and tumour volume (right panel) was assessed using caliper measurements at the indicated time points following the end of the treatment. The combination treatment group showed

smaller tumours than control groups starting from around day 25 following tumour cells injection as detected by the BLI signal. LD-Dox combined with adoptive transfer of donor-derived NK cells significantly slowed the growth of tumours compared to LD-dox alone and untreated controls (PBS only). In terms of tumour volume, a marked reduction in tumour growth rate was also observed in the combination treatment group, however a smaller difference (non-statistically significant) could be observed between LD-Dox alone and LD-Dox+NK cells. Treatment also had a significant impact on the weight of tumours obtained at the end of the study, thereby confirming an inhibition of tumour growth by LD-Dox+NK cells compared to LD-Dox alone and control groups. These differences are illustrated by representative images of harvested tumours (Figure 6.4-C.). For all experiments, animals were culled when evidence of metastasis was apparent from the IVIS™ Spectrum images.

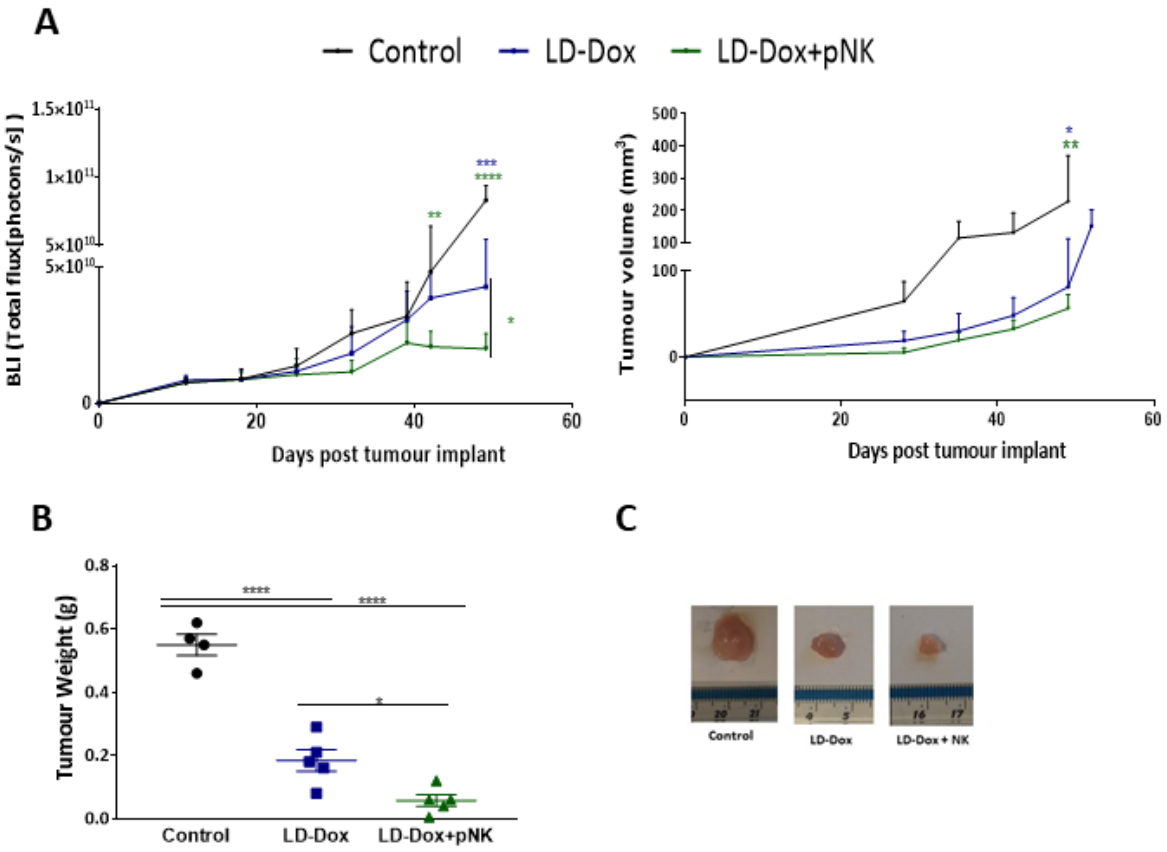

Figure 6.4. Low-dose doxorubicin combined with adoptive transfer of donor-derived NK cells inhibits the growth of MDA-MB-231/LUC/RFP cell-derived xenografts. A. Left: Tumour growth determined by bioluminescence intensity using *in vivo* imaging. **Right:** Tumour volume assessed by caliper measurements. Data are mean+SEM (n=4-5 per group) Statistical significance was calculated by two-way ANOVA (* p≤0.0332, **p≤0.0021, ***p≤0.0002, **** p≤0.0001). **B.** Tumour weights, data are mean±SD (two-way ANOVA, *p≤0.0332, ****p≤0.0001) . **C.** Representative images of harvested tumours.

Studies also evaluated the therapeutic potential of combining LD-Dox treatment with the adoptive transfer of the NK-92™ MI cell line and compared this to that using donor-derived NK cells. This study (study 2) was carried out using NOD,B6.SCID Il2rg$^{-/-}$ Kit$^{W41/W41}$ (NBSGW) mice, which support engraftment of hematopeoetic cells without requiring myeloablative irradiation due to a mutant Kit^{W41} allele. The use of this mouse strain was necessary as it was not possible to irradiate mice at Nottingham Trent University. Previous studies have reported similar levels of engraftment in irradiated NSG and NBSGW mice (McIntosh et al. 2015). For the studies reported in Figure 6.5, the frequency of *in vivo* imaging was reduced due to the reported effect of anesthetics such as isoflurane used in this case on NK cell viability and function (Stollings et al. 2016; Tazawa et al. 2017). One BLI measurement was taken in order to randomize animals before the start of the treatment (day 8) and BLI measurements were taken at the indicated time points following the end of the two cycles of treatment (Figure 6.5-A right). In addition, tumour volume was measured by calipers twice weekly (Figure 6.5-A left).

Although LD-Dox treatment alone delayed tumour growth relative to untreated controls, combining this with adoptive transfer of NK cells enhanced the anti-tumour effect, this was true for both primary NK cells and the NK92™-MI cell line. For the group that received the combination of LD-Dox with primary NK cells, two of five animals (40%) remained free of measurable tumours at the end of the study (day 47). Animals that received the combination treatment had significantly smaller tumours compared to both untreated animals and animals treated with LD-Dox alone, as shown by tumour weights (Figure 6.5-B) and representative images of harvested tumours (Figure 6.5-C).
However, GvHD-like clinical signs became apparent following the second administration of NK-92™ MI cells to mice treated with LD-Dox, requiring these animals to be culled before the end of the study (day 36). Post-mortem examination revealed an apparent accumulation of cells (presumably NK-92™ MI cells as detected by fluorescence imaging) in the liver. The same symptoms were also apparent in animals that received NK cells from healthy donors, though the effect here was delayed by ~ 12 days.

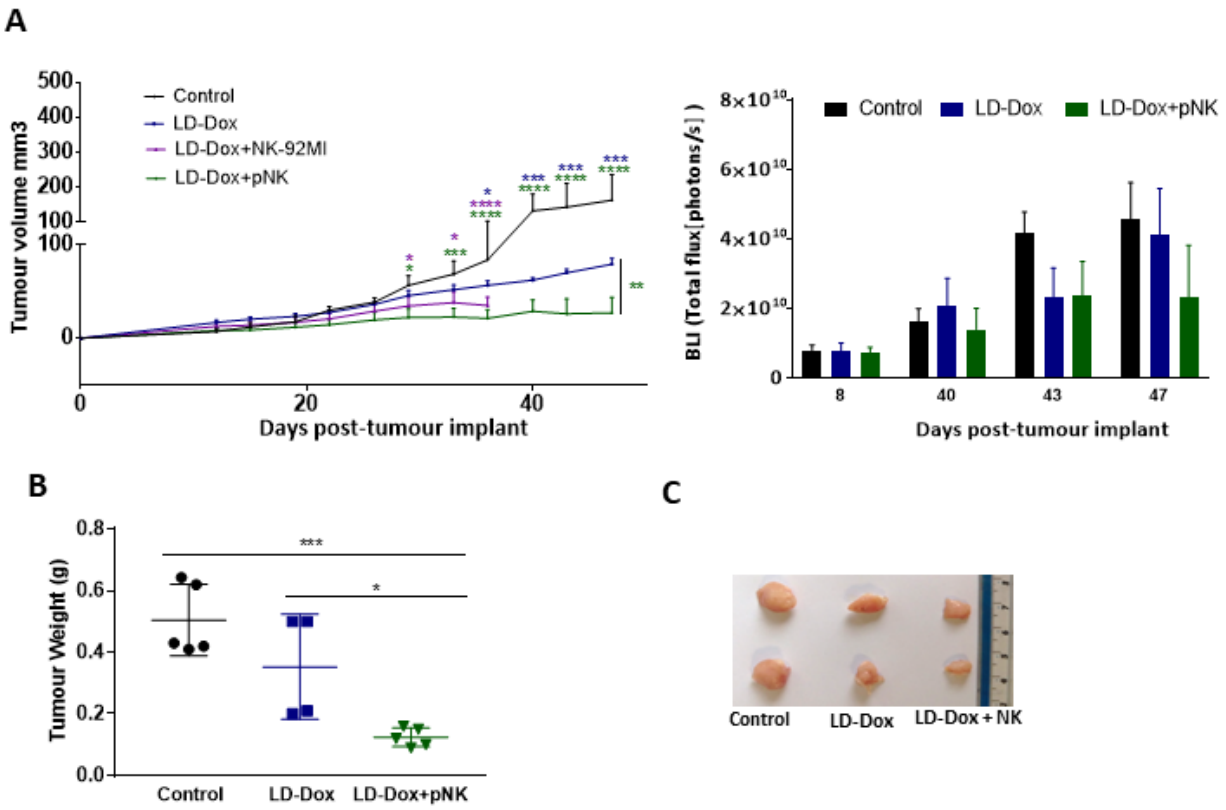

Figure 6.5. Low-dose doxorubicin combined with adoptive transfer of NK-92™ MI cells inhibits the growth of MDA-MB-231/LUC/RFP cell-derived xenografts. A. Left: Tumour volume assessed by caliper measurements. **Right:** Bioluminescence intensity of tumours determined by *in vivo* imaging. Data are mean+SEM (n=4-5 per group). Statistical significance was calculated by two-way ANOVA (* p≤0.0332, **p≤0.0021, ***p≤0.0002, **** p≤0.0001). **B.** Tumour weights, data are mean±SD (One-way ANOVA,* p≤0.0332, ***p≤0.0002). **C.** Representative images of tumours.

Does irradiation of NK-92™ MI cells prevent the development of GvHD-like side-effects?

In order to counteract the GvHD-like side-effects that we observed when using the NK-92™ MI cell line, we assessed whether irradiating NK92™ MI cells with sub-lethal doses of gamma irradiation prior to administration would lower their proliferation rate and thereby avoid these GvHD-like symptoms. For this, NK-92™ MI cells were irradiated at 10Gy, a dose which has been reported to decrease proliferation, but not the cytotoxicity of NK-92™ cells (Cheng et al. 2011). The viability of irradiated NK-92™ MI cells was determined 4,24 and 48 hours following irradiation and their cytotoxicity 24 hours after irradiation and compared to control (non-irradiated NK-92™ MI cells) and transport control cells (NK-92™ MI cells that were transported to the irradiation facility at City Hospital, Nottingham, but not irradiated). Although the viability of irradiated NK-92™ MI cells progressively decreased over 48 hours, their cytotoxic potential 24 hours after irradiation was comparable to that of non-irradiated and transport control NK-92™ MI cells (Figure 6.6). However, adoptive transfer of irradiated cells did not prevent the appearance of GvHD-like symptoms, but did delay their onset by 12 days (data not shown).

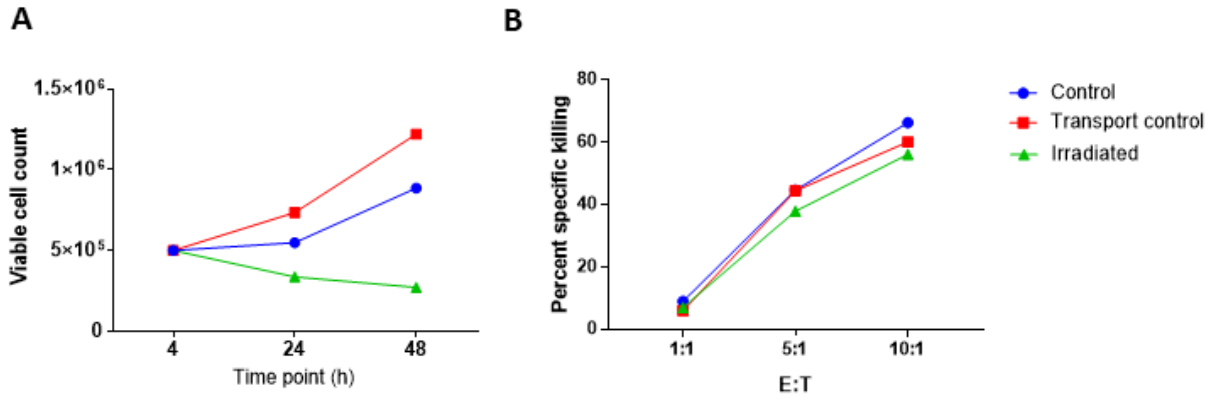

Figure 6.6. Effect of irradiation on the viability and cytotoxicity of NK-92™ MI cells. A. Viability of irradiated, non-irradiated and transport control NK-92™ MI cells over 4,24 and 48 hours. **B.** Cytotoxicity of irradiated, non-irradiated and transport control NK-92™ MI cells against K562 cells at different effector to target ratios. Irradiated cells were used 24h post irradiation.

Does a lower dose of doxorubicin further potentiate anti-tumour effects?

To assess whether a lower dose of doxorubicin would potentiate the anti-tumour effects of adoptive NK cell transfer, experiments in NSG mice were repeated using 0.25mg/kg doxorubicin (study 3). Although 0.25mg/kg doxorubicin alone inhibited tumour growth, as detected by luminescence, but not so clearly using caliper measurement, this effect was smaller than that seen when using 0.5 mg/kg doxorubicin. The adoptive transfer of donor-derived NK cells into animals treated with 0.25 mg/kg doxorubicin resulted in a significant delay in tumour growth ($p<0.0001$, Figure 6.7).

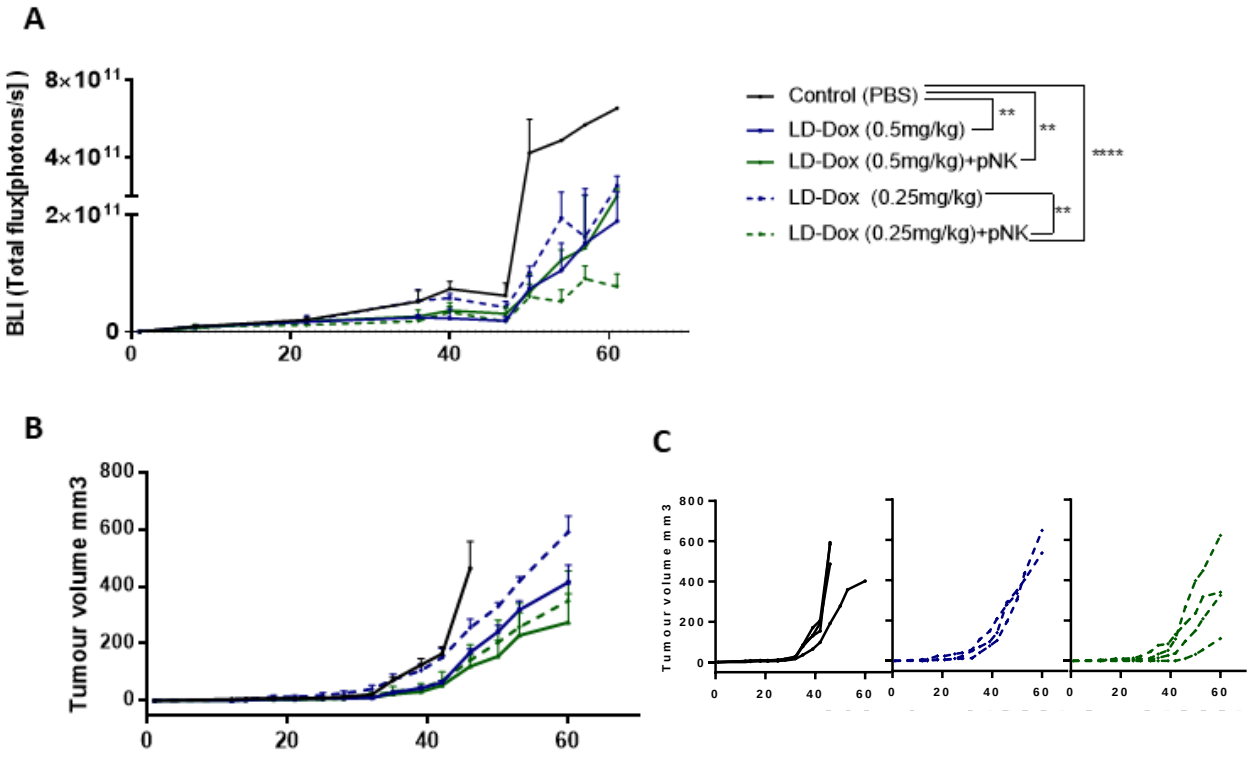

Figure 6.7. Effect of 0.25 mg/kg doxorubicin alone and in combination with the adoptive transfer of donor-derived NK cells on the growth of MDA-MB-231/LUC/RFP cell-derived xenografts. A. Bioluminescence intensity as determined by *in vivo* imaging. Data are mean+SEM (n=4-5 per group). Statistical significance was calculated by two-way ANOVA (**p≤0.0021, **** p≤0.0001). **B.** Tumour volume growth determined by caliper measurements mean+SEM (n=4-5 per group). **C.** Tumour volume of individual mice.

Can the adoptive transfer of donor-derived, primary NK cells alone influence the growth of MDA-MB-231/LUC/RFP cell-derived xenografts?

We next determined whether the adoptive transfer of donor-derived primary NK cells alone could influence the growth of MDA-MB-231/LUC/RFP cell-derived xenografts in NSG mice (study 4). Here, the treatment started at day 7 following tumour implantation (when tumours were palpable) (Figure 6.8.A). Although the findings were not of statistical significance, caliper measurements indicated that tumour growth was slower in animals that received LD-Dox in combination with NK cells, than that in animals treated with LD-Dox or adoptively transferred NK cells alone (Figure 6.8 left). However, no difference was observed using BLI imaging (Figure 6.8-B right).Tumour weight on the other hand was consistent with previous finding, as tumours derived from animals treated with LD-Dox and primary NK cells combination were of a lower weight compared to controls.

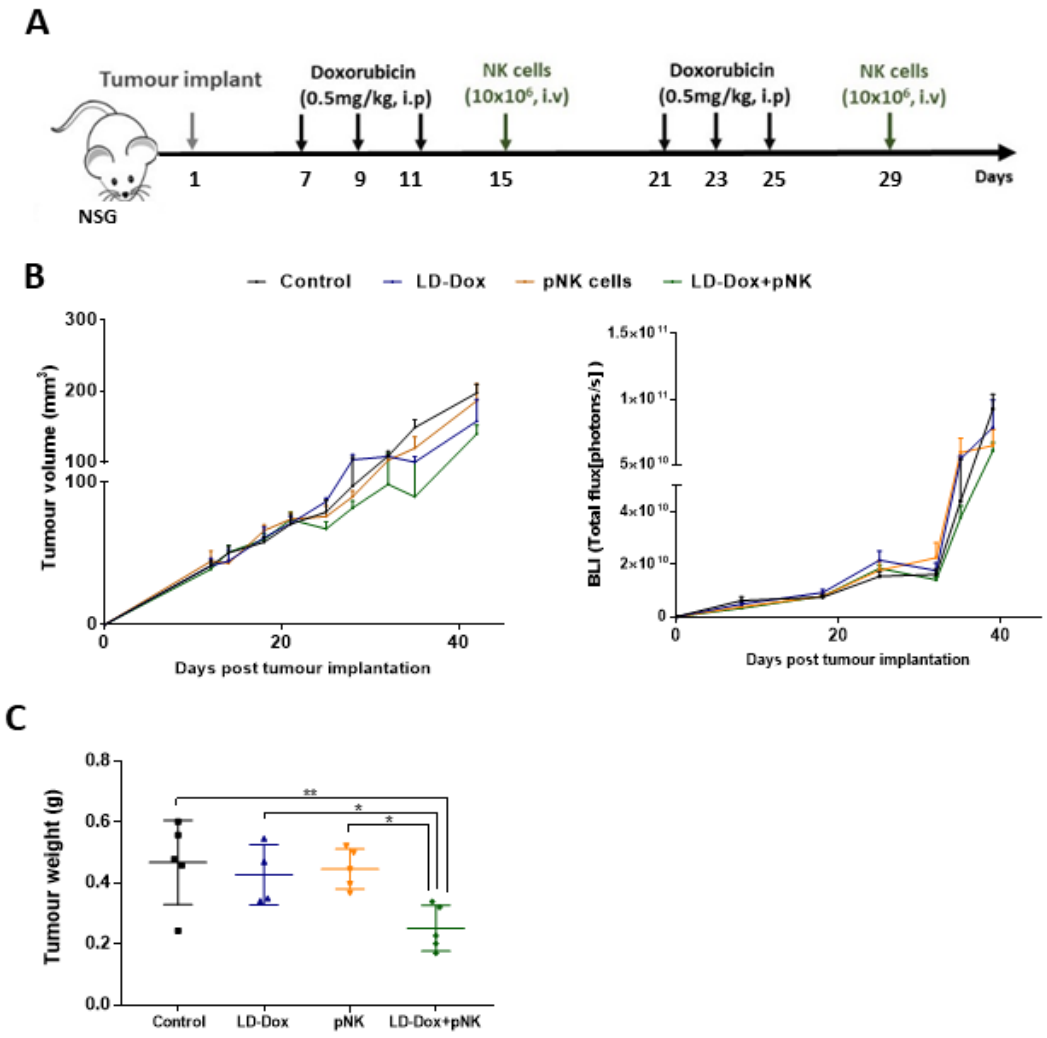

Figure 6.8. Influence of adoptive transfer of donor-derived, primary NK cells alone on the growth of MDA-MB-231/LUC/RFP cell-derived xenografts. A. Treatment schedule. **B.** Tumour volume growth determined by caliper measurements (Left). Bioluminescence intensity as determined by in vivo imaging (Right). Data are mean+SEM (n=4-5 per group). **C.** Tumour weights, data are mean±SD (One-way ANOVA,* p≤0.0332, **p≤0.0021).

6.3.3 Influence of low-dose doxorubicin (LD-Dox) and/or adoptive NK cell transfer on the metastasis of MDA-MB-231/LUC/RFP cell-derived xenografts in immunedeficient NSG mice

As *in vivo* imaging allows for the monitoring of the growth of the primary tumours, it also allows for the detection of metastasis if/when tumour cells travel to distant organs. As depicted in Figure 6.10-A, in Study 1, metastasis was delayed by 7 days in animals treated with LD-Dox) alone compared to untreated animals, and no evidence of metastasis was apparent in animals that received LD-Dox and adoptively transferred donor-derived primary NK cells at the termination of the study on day 50.

The first evidence of metastasis was observed in control group between day 35 and day 42 after the implantation of MB-231/LUC/RFP cells and between day 46 and 56 for animals treated with LD-dox alone. Animals in receiving the combination therapy were excluded from the study/terminated at day 50 because of GvHD-like symptoms described above. Figure 6.10-B shows representative *in vivo* images at day 49 post tumour implantation. Metastasis was primarily apparent in the right axillary region and the liver and lungs, with some distant metastases also being observed in the spleens and left inguinal area for some animals, as shown by *ex vivo* whole body images that were generated following the removal of the primary tumour (Figure 6.10-C).

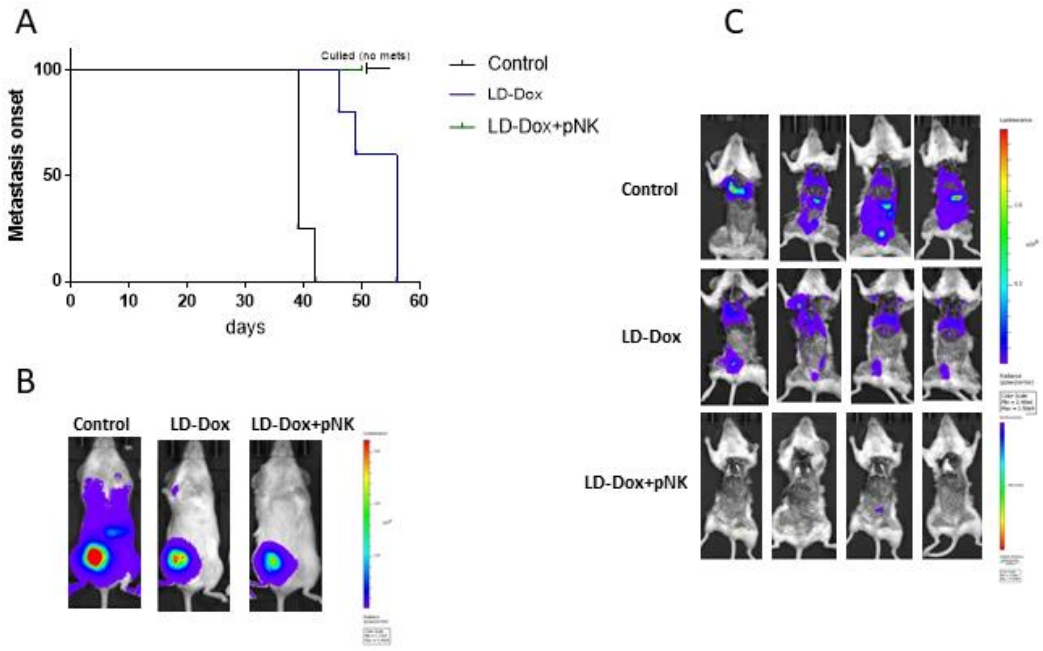

Figure 6.9. Influence of low-dose doxorubicin (LD-Dox) and/or adoptive NK cell transfer on the metastasis of MDA-MB-231/LUC/RFP cell-derived xenografts in immunedeficient NSG mice. A. Onset of metastasis presented as a Kaplan-Meier curve showing changes in percentage of metastasis-free animals by elapsed time after tumour cell implantation (n=4-5 animals/group). **B.** Representative in vivo images of animals at day 49 post tumour cell implantation. **C.** *Ex vivo* whole body images of metastases following removal of the primary tumour.

A delay in the onset of metastasis in animals receiving 0.25 mg/kg and 0.5 mg/kg doxorubicin in combination with adoptively transferred donor-derived, primary NK cells was also observed, with a longer delay being observed in animals treated with 0.25mg/kg (Figure 6.11). Because of the latter, 0.25mg/kg was used in subsequent studies.

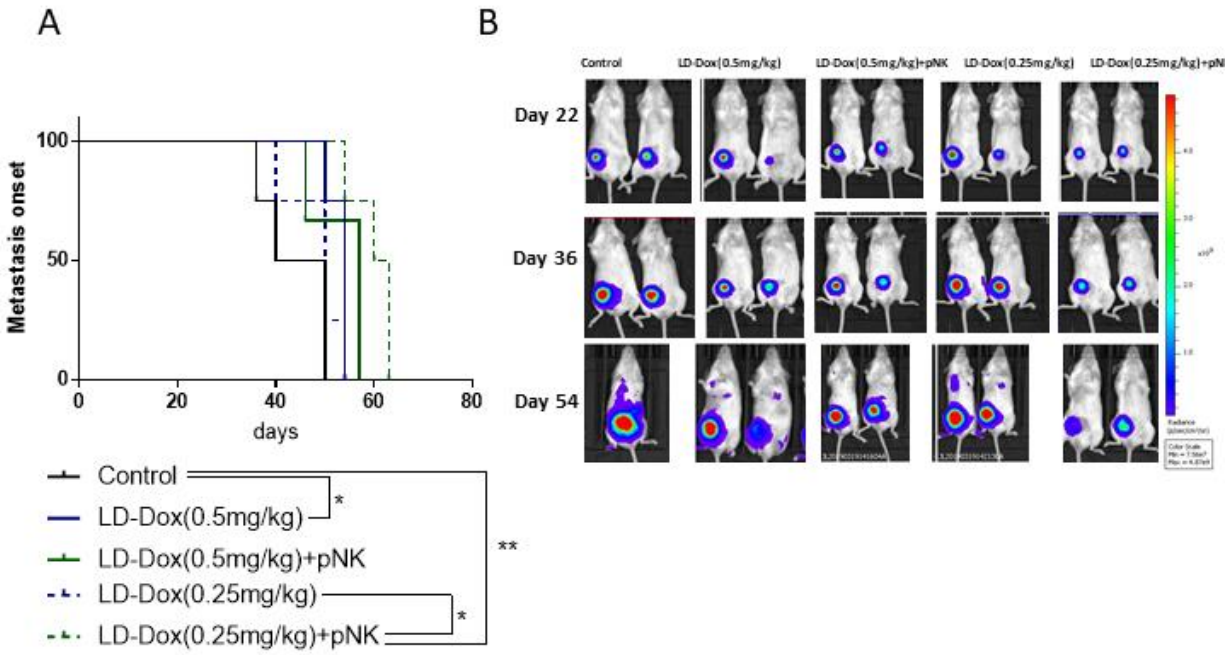

Figure 6.10. Influence of low-dose doxorubicin (LD-Dox) at 0.25 mg/kg and 0.5 mg/kg and/or adoptive NK cell transfer on the metastasis of MDA-MB-231/LUC/RFP cell-derived xenografts in immunedeficient NSG mice. A. Onset of metastasis presented as a Kaplan-Meier curve showing changes in percentage of metastasis-free animals by elapsed time after tumour cell implantation (n=4 animals/group). Statistical comparison: Log-rank (Mantel-Cox) test (control vs LD-dox(0.5mg/kg) * p=0.0285, LD-Dox (0.25mg/kg) vs LD-Dox(0.25mg/kg)+ pNK * p= 0.0180, **p= 0.0091).**B**. Representative *in vivo* images of metastasis.

In regards to the effect of adoptive transfer of NK cells alone, although there was no significant delay in metastasis onset between the groups, at the end of the study (day 45),4 out of 5 and 4 out of 4 animals had *in vivo* detectable metastatic signal in control and LD-dox alone groups respectively. in the group of animals that received NK cell adoptive transfer alone 5/5 animals had a detectable metastatic signal by day 45. While in the combination groups 2 out of 5 animals had metastasis signals detectable by *in vivo* imaging. Whole body *ex-vivo* IVIS images and BLI quantification, indicate a difference in terms of metastasis spread, with the combination group presenting lower metastatic spread compared to control groups although here again it was not statistically significant (Figure 6.12).

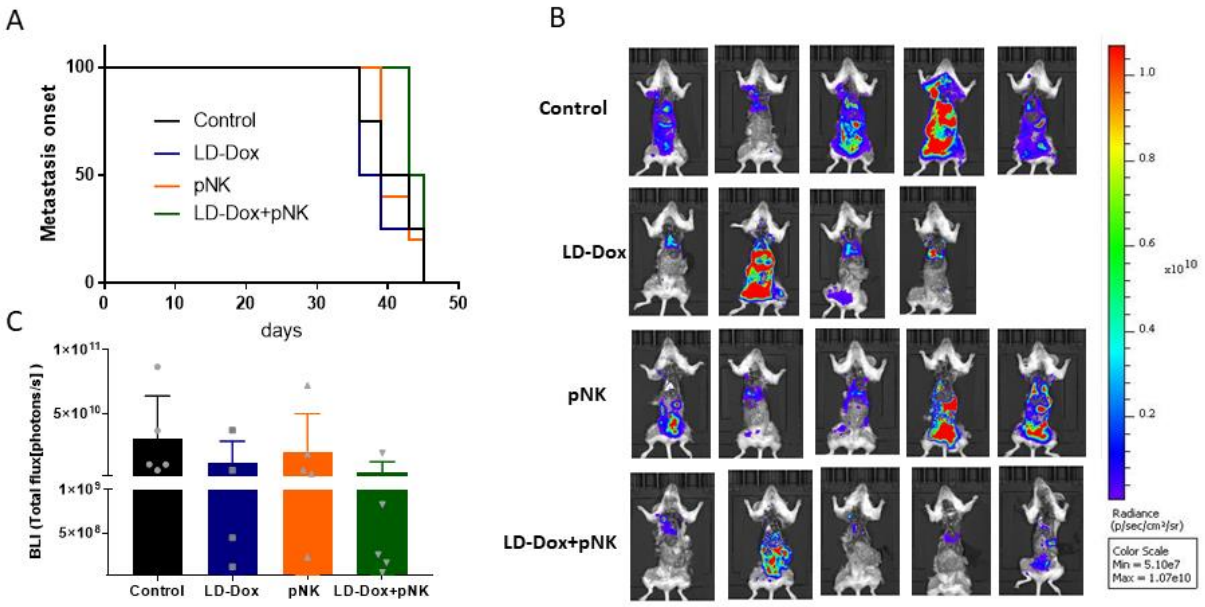

Figure 6.11. Influence of adoptive transfer of donor-derived, primary NK cells alone on the metastasis of MDA-MB-231/LUC/RFP cell-derived xenografts in immunedeficient NSG mice. A. Onset of metastasis presented as a Kaplan-Meier curve showing changes in percentage of metastasis-free animals by elapsed time after tumour cell implantation (n=4-5 animals/group **B**. Representative *ex vivo* images of metastasis at the end of the study, following removal of the primary tumours.

6.3.4 *In vivo* infiltration of injected NK cells to subcutaneous Tumours

One of the major limiting factors of NK cell-based therapies is the effective infiltration and homing of these cells in/to solid tumours, as the tissue presents a barrier preventing NK cells from reaching the core of the tumour (Gajewski et al. 2013). Recent studies have shown a positive association between NK cell infiltration and the prognosis of colorectal, gastric and lung cancer (Krasnova et al. 2017; Souza-Fonseca-Guimaraes 2016). Tracking NK cell trafficking is therefore important for assessing the potential efficacy of their transfer. We have used direct fluorescence cell labeling to track NK cells using *in vivo* imaging and to evaluate the effect of LD-Dox treatment on their recruitment and infiltration into tumours.

NK cells were labeled with VivoTrack 680 near infrared (NIR) fluorescent imaging agent prior to adoptive transfer. At the end of the study, NK cell infiltration in tumours was also assessed using flow cytometric analysis of isolated cell populations and infiltration patterns between animals that received NK cells alone and animals that received the combination treatment comparted. Figure 6.12-B shows NK cell infiltration as measured by epi-fluorescence *in vivo*. At day 25 post tumour cell implantation, corresponding to 10 days following the first NK cell infusion, NK cell infiltration in tumours was significantly higher in animals that were pre-treated with LD-Dox. The same was observed for day 32 which represent 3 days following the second NK cell infusion. Although the difference was not of statistically significant for the other time-point, it was slightly higher in LD-Dox+pNK combination group. In line with these results, flow cytometric analysis of NK cell infiltration at the end of the study (Figure 6.12-C) also showed higher percentages of NK cells in tumours from animals that had received the combination treatment.

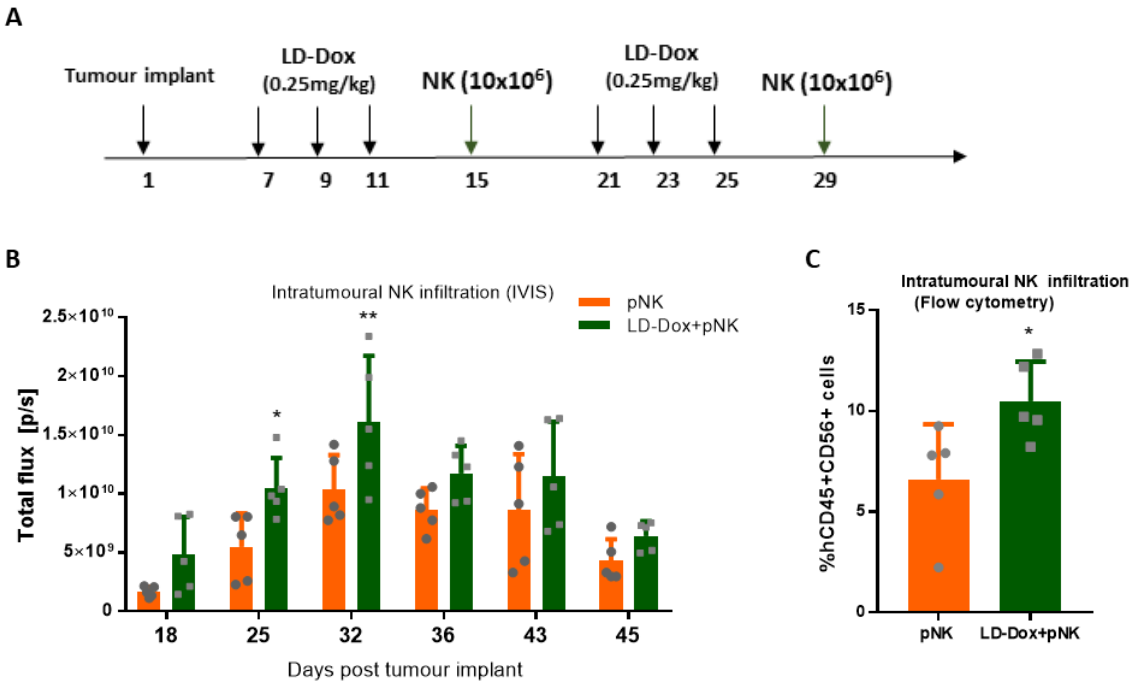

Figure 6.12. *In vivo* infiltration of intravenously injected NK cells to subcutaneous Tumours. A. Treatment Schedule. **B.** Epi-fluorescence measurements of NK cell infiltration in tumours at different time points following NK infusions (n=5, Two-way ANOVA *p<0.0332 **p<0.0021). **C.** Flow cytometry measurement of NK cell infiltration in tumours (n=5. Unpaired T-test * p=0.0317).

6.3.5 Influence of low-dose doxorubicin (LD-Dox) and/or adoptive NK cell transfer on tumour cell proliferation, expression of MICA/B ligand and sensitivity to NK-92™ MI cell cytotoxicity

To further evaluate the effects of treatment, we assessed the expression of the proliferation marker Ki-67 and the ligand MICA/B on tumours following their dissociation into single cell suspensions. As shown in Figure 6.8-A, Ki-67 expression was lower in the tumours from animals that had received LD-

Dox (0.5 mg/kg) in combination with adoptively transferred donor-derived primary NK cells. Expression of MICA/B ligands on cells isolated from the tumours of mice treated with the combination treatment was also higher than that on cells isolated from the tumours of control animals (Figure 6.9-B). Furthermore, cells isolated from the tumours of mice treated with the combination therapy were more sensitive to NK-92™ MI cytotoxicity, as assessed using an *in vitro* cytotoxicity assay (E:T 1:5, Figure 6.8-C).

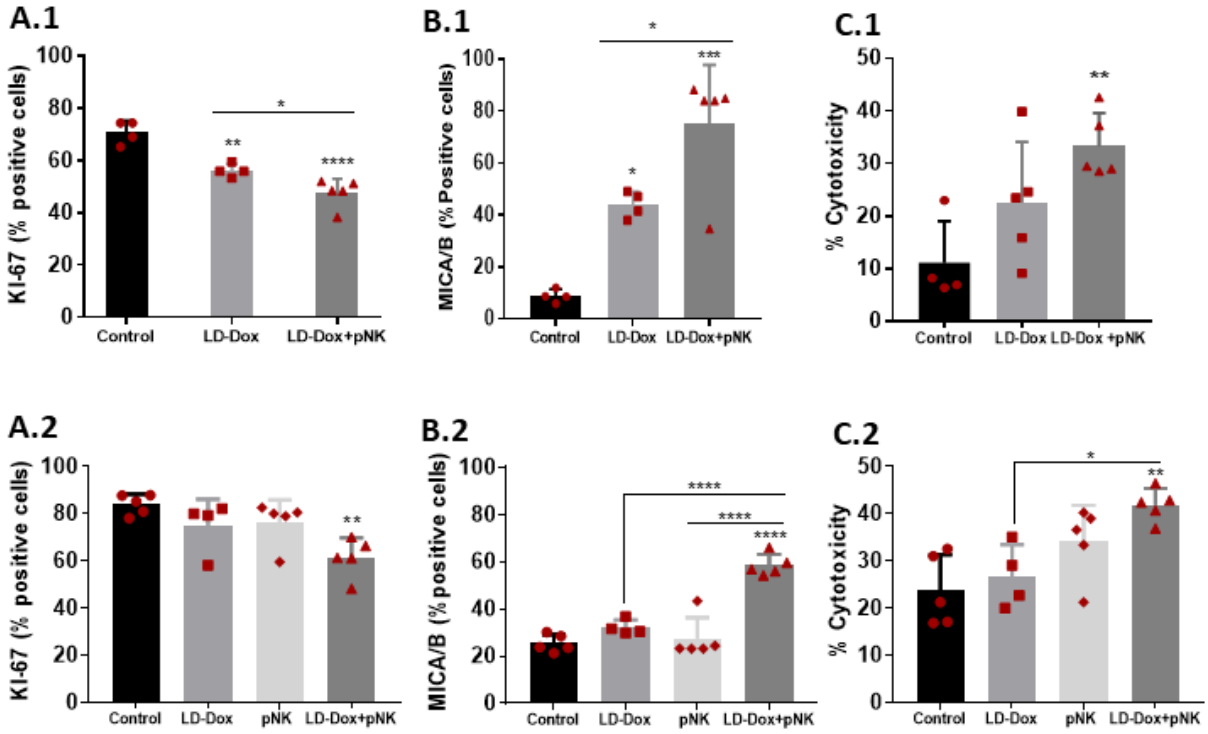

Figure 6.13. Influence of low-dose doxorubicin (LD-Dox) and/or adoptive NK cell transfer on tumour cell proliferation, expression of MICA/B ligand and sensitivity to NK-92™ MI cell cytotoxicity A. Expression of the proliferation marker Ki-67 by tumour-derived cells: data are mean + S.D from 4-5 animals (One-way ANOVA,*P≤0.05, **P≤0.01, ****P≤0.0001). **B.** Surface expression of MICA/B by tumour-derived cells: data are mean + S.D from 4-5 animals (One-way ANOVA,*P≤0.05, ***P≤0.001). **C.** Killing of tumour-derived cells by NK-92™ MI cells (E:T 1:5): data are mean + S.D from 4-5 animals (Two-way ANOVA,**P≤0.01).(**1:**study 2, **2:** study 4/**.** Top Panel: study 2. Bottom Panel: study 4).

6.3.6 Influence of low-dose doxorubicin (LD-Dox) and/or adoptive NK cell transfer on cancer initiating cells (CICs) in tumours

Gene expression profiling studies have revealed that most primary tumours contain a subset of cells with a cancer stem cell (CSC) or cancer initiating cell (CIC) phenotype (Reya et al. 2001). In breast cancer, such cells are defined on the basis of their expression of CD44, CD24 and EpCAM (CD326) (Al-Hajj et al. 2003; Fillmore, Kuperwasser 2008). Many studies have shown that higher proportions of CICs in tumours are associated with therapeutic resistance and development of metastasis (Lawson et al. 2009). We therefore determined the proportion of $EpCAM^{+}/CD44^{high}$ CICs in MDA-MB-231/LUC/RFP

cell-derived xenografts following low-dose doxorubicin (LD-Dox) and/or adoptive NK cell transfer. MDA-MB-231 cells are negative for CD24, thus this marker has not been used. Lower proportions of $EpCAM^{+}/CD44^{high}$ CICs were found in tumours from animals that received LD-dox and LD-dox combined with primary NK cells, as compared to controls (Figure 6.13).

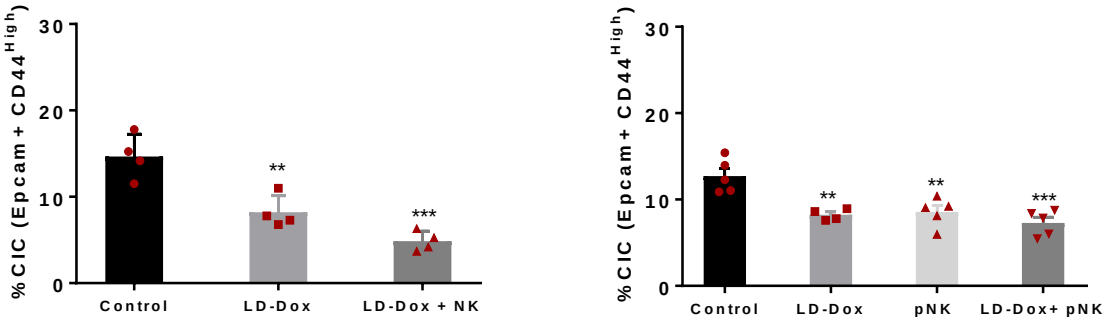

Figure 6.14. Proportion of $EpCAM^{+}CD44^{high}$ cancer initiating cells (CICs) in tumours from control tumour-bearing animals and tumour-bearing animals treated with low-dose doxorubicin (LD-Dox) and/or adoptive NK cell transfer. Following dissociation of tumours, single cell suspensions were assessed for EpCAM/CD44 surface expression using flow cytometry. Data are as mean+SD (Two-way ANOVA, **P≤0.01).

6.3.7 Immune Checkpoint inhibitors

Tumours can evade and avoid immune recognition and surveillance by expressing inhibitory receptors and so called 'immune checkpoint' molecules. Immune checkpoint inhibitors have been shown to increase the effectiveness of cytotoxic lymphocytes and to increase patient survival in a number of studies and tumour entities (Seidel et al. 2018). Pairing immune checkpoints inhibitors with immunotherapy approaches therefore has the potential to enhance anti-tumour responses and therapeutic outcomes. In line with this, the expression of three main checkpoints molecules, PD1, TIGIT and TIM3, on the surface of tumour infiltrating NK cells was assessed using flow cytometry in order to evaluate the potential usefulness of including checkpoint inhibition in the therapeutic protocol. However, no significant differences in the expression of these molecules between animals that received NK cells alone and animals that were treated with the combination approach were observed (Figure 6.14).

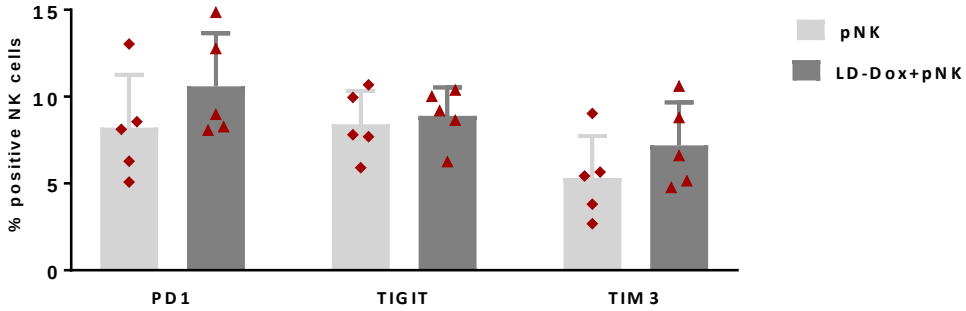

Figure 6.15. Expression of immune checkpoint inhibitors by tumour infiltrating NK cells in untreated tumour-bearing animals and tumour-bearing animals treated with LD-Dox. Flow cytometry analysis of surface expression of TIM3, PD1 and TIGIT in tumour infiltrating NK cells (n=5).

6.4 Summary

Following proof from the *in vitro* studies showing that LD-Dox induce higher expression of ligands for activatory NK cell receptors, and enhance the sensitivity to NK lysis, the next step was to test this combination *in vivo*. We have established an orthotropic TNBC model, using the human cell line MDA-MB-231/LUC/RFP. TNBC is an aggressive metastatic disease, it was then important to select a cell line that would spontaneously mimic these critical features of the disease. Multiple studies demonstrated the suitability of MDA-MB-231 cells for application in preclinical models (Simmons et al. 2015; Minn et al. 2005; Arroyo-Crespo et al. 2019). Moreover, luciferase activity in the cells, allow for *in vivo* monitoring and tracking of tumour progression and metastasis by bioluminescence imaging using the IVIS® system.

Cells were allowed to grow until tumours were palpable, from that point animals received a two-cycle treatment, with LD-Dox three times a week and a single NK cell infusion the following week. Initial studies showed that LD-Dox combined with adoptive transfer of donor-derived NK cells significantly slowed the growth of tumours compared control animals that received either LD-dox alone or were left untreated. A significant impact on tumour weight was also observed for all studies, in animals receiving the combination treatment. The adoptive transfer of donor-derived NK cells into animals treated with an even lower dose of doxorubicin, 0.25 mg/kg also resulted in a significant delay in tumour growth.

We next sought to determine the effect of adoptive transfer of donor-derived primary NK cells alone. Although the findings were not consistent with previous results, in regard to the overall tumour growth profile, there was an indication that tumour growth was slower in animals that received LD-Dox in combination with NK cells, than that in animals treated LD-Dox monotherapy or adoptively transferred

NK cells alone. Overall, with the exception of the last study presented, results indicated a synergistic effect of LD-Dox with NK cells in inhibiting tumour growth.

In order to evaluate the therapeutic potential of combining LD-Dox with the adoptive transfer of the NK-92™ MI cell line and compare this to that using donor-derived NK cells. We used the NOD,B6.SCID Il2rg. A/A KitW41/W41 (NBSGW) mice, as it support engraftment of hematopeoetic cells without requiring myeloablative irradiation. However, some problems were encountered with this model as GvHD-like clinical symptoms were observed following the last NK-92™MI infusion, a possible indication of the high proliferation rate of the cells. Sub-lethal Irradiation of NK-92™MI cells was tested as a way to counteract the GvHD-like side-effects observed, by lowering their proliferation rate. NK-92™MI viability and cytotoxicity was tested after irradiation. The viability of irradiated NK-92™MI cells progressively decreased over 48 hours, on the other hand, their lysis potential 24 hours after irradiation was not affected. However, adoptive transfer of irradiated cells did not prevent the appearance of GvHD-like symptoms, but did delay their onset by 12 days (data not shown). One potential reason for this could be due to expansion of other cells from the PBMC, although NK cells injected were always >94% pure ($CD3^-/CD56^+$). There are some alternative models to prevent the appearance of the symptoms, such as the NSG B2m, which has an MHC class I molecule (beta-2-microglobulin) deficiency. However these could still require pre-conditioning irradiation (King et al. 2009).

As mentioned earlier, MDA-MB-231 tumours have the ability to spontaneously metastasize to distant organs. In Study 1, metastasis was delayed by 7 days in animals treated with LD-Dox alone compared to untreated animals, and no evidence of metastasis was apparent in animals that received LD-Dox and adoptively transferred donor-derived primary NK cells at the termination of the study on day 50.
A delay in the onset of metastasis in animals receiving 0.25 mg/kg and 0.5 mg/kg doxorubicin in combination with adoptively transferred donor-derived, primary NK cells was also observed, with a longer delay being observed in animals treated with 0.25mg/kg doxorubicin. In regards to the effect of adoptive transfer of NK cells alone, although there was no significant delay in metastasis onset between the groups, *ex vivo* IVIS images and BLI quantification, indicate a difference in terms of the extent of metastasis spread, with the combination group presenting lower metastatic spread compared to control groups, even though here again it was not statistically significant. The difference was also more pronounced in terms of tumours weights.

Labelling NK cells with VivoTrack 680 near infrared (NIR) fluorescent imaging agent prior to adoptive transfer allowed their tracking and comparison of their tumour infiltration with and without LD-Dox pre-treatment. Analysis of NK tumour infiltration using IVIS and flow cytometry showed that NK cell infiltration in tumours was higher in animals that were pre-treated with LD-Dox.

Tumours were used for further mechanistic studies. First, the proliferation of tumour cells was assessed using the proliferation marker Ki67, tumours derived from the combination treatment group were found to express lower levels of Ki67 compared to control and LD-Dox alone groups. Expression of MICA/B ligands on tumour cells was also significantly higher than that on cells isolated from the tumours of control and LD-Dox alone animals. Furthermore, these cells were more sensitive to NK-92™ MI cell-mediated cytotoxicity *in vitro*. LD-Dox alone and the combination treatment also reduced the number of $EpCAM^{+}/CD44^{High}$ Cancer Initiating Cells (CICs) in tumours derived from treated mice.

With the aim of evaluating the potential addition of checkpoint inhibitors to the combination treatment, the expression of PD1, TIGIT and TIM3, on the surface of tumour infiltrating NK cells was assessed using flow. However, there was no significant difference in the expression of these molecules between animals that received NK cells alone and animals that were treated with the combination approach.

Chapter 7: Discussion

Triple negative breast cancer (TNBC) is the most fatal form of breast cancer, due to its aggressive clinical features, very poor prognosis and the lack of targeted therapies (Palma et al. 2015). Although immunotherapy has shown great promise recently for various cancer types, breast cancer has been considered as being difficult to treat with immunotherapy due to its perceived poor immunogenicity and immunologically "cold" features. However, emerging evidence suggests that TNBC is the most immunogenic subtype of all breast cancers, because of chromosomal instability and increased mutational burden (Disis, Stanton 2015; Chokr, Chokr 2018). This enhanced immunogenicity means that patients with TNBC could benefit substantially from immunotherapy strategies and recent clinical studies with checkpoint inhibitors showed encouraging results for patients with TNBC. However, response rates remain very modest (Adams et al. 2017; S Adams et al. 2019; Schmid et al. 2018).

Until recently, most immunotherapeutic approaches focused on adaptive immune cells, mainly T cells, leaving the potential of the innate arm of the immune system not fully addressed. NK cells are major effectors in tumour immunosurveillance. Their role and contribution in tumour control has been demonstrated in multiple studies. Indeed, in various cancers, the presence of tumour-infiltrating NK cells is recognised as being a good prognostic marker (Souza-Fonseca-Guimaraes 2016; Krasnova et al. 2017; Pasero et al. 2015; Hu et al. 2019b). To date, NK cell-based therapeutic approaches as monotherapies have delivered limited clinical success, particularly in the case of solid tumours, partly due to the profound immunosuppressive nature of the tumour microenvironment (TME) (Wagner et al. 2017; Kim et al. 2019; Chiossone et al. 2018b). On the other hand, it is now widely accepted that the most promising and effective cancer treatments are combinatorial strategies that integrate different treatment modalities and the aim of which is the induction of a synergistic tumour response by targeting distinct elements of the tumour (Bayat Mokhtari et al., 2017; Esteva et al., 2019; Schmidt, 2019). Therefore, it is crucial to identify and develop strategies that would improve NK cell-based therapy.

Furthermore, there is a growing evidence that low-dose chemotherapy can activate the immune response, and chemo-immunotherapy treatment strategies aimed at targeting multiple aspects of tumour development and progression are under development (Muthusamy, 2016; Chen, Chang and Cheng, 2017; Kerbel and Shaked, 2017; Simsek, Esin and Yalcin, 2019).

In this thesis, the effect of low-dose doxorubicin on the phenotype of breast cancer cells is described and the contribution of this treatment regime to the modulation of the sensitivity to NK cell lysis *in vitro* and *in vivo* is detailed. The results presented herein demonstrate that low-dose

doxorubicin impairs the proliferation of breast cancer cells and induces a more permissive phenotype to NK cell lysis. This supports the rationale for combining low-dose chemotherapy with NK cell based immunotherapy for TNBC.

7.1 Low-dose doxorubicin as an approach to enhance the susceptibility of TNBC cells to NK cell lysis

The activity of different chemotherapeutics at lower doses that the standard MTD used in the clinic has been investigated for cancer treatment, with the aim of reducing toxicities and immunosuppressive effects (Muthusamy 2016; Cazzaniga et al. 2017). Along with their well reported anti-angiogenic activity, multiple studies suggest that the administration of some chemotherapeutics at sub-lethal doses could trigger anti-tumour immunity by enhancing tumour cells immunogenicity and eliminating immunosuppressive cell populations (Zitvogel et al. 2008; Lake, Robinson 2005; Apetoh et al. 2008). These studies provide a rationale for using low-dose chemotherapy as a standalone or pre-conditioning treatment, as well as in combination with other agents. However, few studies have investigated the effects of combining low-dose metronomic doxorubicin with an adoptive NK cell-based approach. As mentioned previously, one of the main studies reporting the effect of low-dose doxorubicin on enhancing the sensitivity of cancer cells NK cell killing was carried out by Wennerberg et al (Wennerberg et al. 2013). However, this study did not investigate this in the context of TNBC.

7.1.1 Low-dose doxorubicin impairs the proliferation of breast cancer cells

Clinical doses of doxorubicin lead to DNA damage as a consequence of its intercalation into DNA, which causes double-strand breaks and apoptosis in various tumour cells (Tacar et al. 2013b). However, these molecular mechanisms may vary depending on multiple factors such as tumour type and drug dose. Anti-tumour mechanisms of low-dose doxorubicin have been shown to include inhibition of tumour growth and spread through anti-angiogenic activity rather than apoptosis (Damber et al. 2006b). These inhibitory effects have also been shown to be associated with a state of senescence in some tumours (Rebbaa et al. 2003). Notably, as tumour progression is usually accompanied by resistance to apoptosis-based therapies due to accumulation of different defects in the apoptotic signaling pathways (Mori et al. 2004), approaches looking at alternatives to apoptosis-induction, such as anti-proliferative/senescence-targeted, could be more beneficial.

The data presented in this thesis showed that low-dose doxorubicin treatment impaired the proliferation of breast cancer cells, as indicated by cell cycle arrest and lower Ki67 expression, in association with other features of senescence. Senescence is a state of terminal growth arrest which is thought to be a process of response to stress that could be triggered by different stimuli, including DNA damage, oxidative stress and chemotherapeutic drugs. Multiple anti-cancer

therapeutic strategies have been shown to induce cancer cell senescence, such as targeted strategies based on cyclin dependent kinases 4/6 inhibitors (Rader et al. 2013), but also immunotherapy (Braumüller et al. 2013b). Features of senescence observed here included the distinct, large and flattened morphology which has previously been described following treatment with low-dose doxorubicin, and which was reported to be associated to focal adhesion kinase activity (Cho et al. 2004) and upregulation of cell cycle regulators p16, p21, p53 along with SA-β-gal expression which have also been used as indicators of senescence (Wang, Dreesen 2018). Senescent cells also accumulate DNA damage markers. In this study, the DNA double-strand breaks biomarker γ-H2AX was used to detect DNA damage, and was only found to be upregulated in LD-Dox treated TNBC cells (Kuo and Yang, 2008).

Overall, these findings are in line with previous studies that reported that low-doses of doxorubicin induce cell cycle arrest and features of senescence (Kim et al. 2009; Bar-On et al. 2007; Lüpertz et al. 2010). However, it is important to note that the expression of these markers following LD-Dox treatment varied across the different cell lines tested, thereby indicating that response to low-dose doxorubicin could be cell line/subtype dependent. In this regard, multiple studies have revealed that different subtypes of breast cancer have very distinct gene expression profiles, different survival prognosis (Sotiriou et al. 2003; Sorlie et al. 2001) and respond differently to therapy. Rouzier et al, as well as Kim et al, reported that the basal-like TNBC and HER+ subtypes were more sensitive to neoadjuvant chemotherapy, including doxorubicin (Rouzier et al. 2005; Kim et al. 2010). Therefore, these observed differential effects of low-dose doxorubicin across the different cell lines, and the higher extent of response in TNBC cells, could be explained by mechanistic differences in sensitivity to chemotherapy across the subtypes.

It was crucial to verify that the growth inhibitory effects of low-dose doxorubicin do not induce resistance to the MTD of doxorubicin. Results showed that cells remained sensitive to killing by higher doses of doxorubicin following low-dose doxorubicin treatment.

7.1.2 Low-dose doxorubicin treatment enhances NK cell ligand expression by TNBC cells and their sensitivity to NK cell-mediated cytotoxicity

NK cells play an important role in tumour surveillance and control. However, as reviewed in the introduction chapter, their potential is hindered by multiple immune escape mechanisms developed by tumour cells and features of NK cell dysfunction have been observed in multiple studies of cancer patients (Waldhauer and Steinle, 2008; Krneta *et al.*, 2015; Vitale Gobbi *et al.*, 2015). Therefore, developing strategies that would circumvent NK immunosuppression could be game-changing for the development of effective NK cell-based therapeutics.

The first important/key aspect of NK cell cytotoxicity is the recognition of tumour cells, which is mediated by activatory receptors on NK cells that stimulate NK cell effector function upon interaction with cognate ligands on target cells. Results from the research programme described in this thesis show that low-dose doxorubicin treatment increased the expression of ligands for major NK cell activatory receptors (NKG2D, DNAM-1) in breast cancer cells, namely MICA/B, ULBP1/2/3 and CD155. These findings were consistent with previous research showing that responses to cellular stress, such as that caused by metronomic-/low-dose chemotherapy, modulate the expression of NK cell ligands (Alessandra Soriani et al. 2009; Zingoni et al. 2017; Khallouf, Märten, Serba, Teichgräber, Markus W Büchler, et al. 2012). Sagiv et al have shown that the NKG2D ligands, MICA/B and ULBP2, mediated immunosurveillance of fibroblasts following the induction of senescence using low-dose doxorubicin (Sagiv et al. 2016). It has also been shown that metronomic chemotherapy upregulates the expression of NKG2D ligands on melanoma cells (Hervieu et al., 2013). Although the exact mechanisms which lead to this upregulation remain largely unknown, much evidence points toward the central role of the DNA damage response pathway (Gasser et al. 2005b; Zingoni et al. 2017).

The NKG2D receptor-ligand interaction is a key activating pathway for NK cell function (Morisaki et al. 2012a). Thus, MICA and MICB are at the center of treatment approaches designed to enhance NK cell targeting towards tumour cells (Cheng et al. 2011; Morisaki et al. 2012b; Fionda et al. 2015b; Lupo, Matosevic 2019). These ligands are normally of low abundance and their shedding represents one of the main escape mechanisms to NK cell immunosurveillance (Bryceson et al. 2009; Malmberg et al. 2017b). Studies have sought approaches to prevent tumours from shedding these ligands and fix them onto the surface of tumour cells, thereby enabling NK cells to identify target cells for elimination. In this regard, in contrast to enhancing their expression at the transcriptional level, a recent creative approach to tackle this was presented by Ferrari de Andrade et al, using an engineered monoclonal antibody (mAb) directed against the specific region of their extracellular domain subject to proteolytic shedding by MMPs and ADAMs cleavage, without disturbing NKG2D receptor binding. The application of this mAb greatly reduced tumour burden in a mouse model of metastatic melanoma after the administration of human NK cells (Ferrari de Andrade et al. 2018).

The upregulation of activatory ligands expression demonstrated in this thesis resulted in an increased sensitivity to NK cell-mediated killing. Indeed, low-dose doxorubicin treatment rendered cells significantly more sensitive to killing by primary donor-derived NK cells as well as NK-92™ MI cell line when compared to untreated cells. This was true for all cell lines tested, with the exception of SKBR3 cells for which, although ligands for activatory NK cells were upregulated, killing efficiency was not significantly enhanced. These results pointed again to the differential response across breast cancer subtypes. SKBR3 represent the HER2 subtype of breast cancer, as it overexpresses the Her2 gene product. Our findings were particularly surprising as previous research has suggested

the permissive behaviour of HER+ breast cancer to NK cell lysis (Finak et al. 2008). However, it is likely that for these cells the upregulation of this ligand combination was not sufficient for inducing NK cell adhesion and subsequent activation. Indeed, adhesion and synapse formation are central to NK cell lysis. This level of NK cell regulation that precedes lysis of target cells was not explored here, but deserves further investigation. Expression levels and signalling by LFA1 and ICAM-1 could help further characterise the effect of low dose doxorubicin treatment on NK-target cell contact and synapse formation (Bryceson et al. 2009; Long et al. 2013). Of note, in their study, Wennerberg et al, pointed towards a TRAIL-dependent mechanism for the enhanced NK cell lysis, as opposed to an NKG2D or DNAM-1 pathway. However, as mentioned earlier this has not been tested on the same cell lines used for this study (Wennerberg et al. 2013).

Additionally, NK cell activation and proliferation are influenced by the local cytokine milieu. When NK cells are pre-activated with cytokines, such as IL-2, IL-12, IL-15, IL-18, and IL-21 which are commonly used in immunotherapeutic approaches, they show enhanced cytolytic activity and cytokine production (Guillerey et al. 2016b; Morvan, Lanier 2016). In line with that, IL-2 stimulation greatly enhanced NK cell-mediated cytotoxicity against cells treated with low-dose doxorubicin. Further functional analysis can be achieved by measuring degranulation and cytokine production. Upon contact with target cells, cytolytic granules, containing perforin and granzyme are secreted onto the surface through the cytolytic synapse, this process is highly focused and tightly regulated towards target cells. In degranulation and intracellular cytokine experiments, the exposure of NK-92™ MI cells to MDA-MB-231/LUC treated with low-dose doxorubicin increased their expression of the degranulation marker CD107a, granzyme B, perforin and IFN-γ. Another commonly reported characteristic of dysfunctional NK cells in cancer is the reduced secretion of effector cytokines and degranulation (Fang et al. 2018). Thus, these results further support the proposition that low-dose doxorubicin positively modulates NK cells toward an enhanced targeting of tumour cells.

From a therapeutic perspective, one of the questions that needed answering was the impact that doxorubicin might have on NK cells. In this context, we demonstrated that exposure of NK-92™ MI cells to the low-doses of doxorubicin had no effect on their viability or expression of activation receptors (NKG2D and NKp30). This was also shown by Soriani et al., in the case of multiple myeloma (Soriani et al. 2014b).

7.2 Low-dose doxorubicin modulates important cancer-relevant pathways in TNBC cells

The tumourigenesis of breast cancer is a complex, multi-step process. Understanding factors of tumour cell proliferation, tumourigenesis and aggressiveness is crucial in designing therapeutic strategies. In order to gain a better understanding of the effect of the signaling molecules and

pathways that are affected by low-dose doxorubicin treatment, and define the mechanisms underlying the acquired susceptibility of breast cancer cells to NK cell killing as a result of low-dose doxorubicin treatment, we have performed gene expression analysis comparing low-dose doxorubicin treated and untreated cells. As the focus of this study is TNBC, the two cell lines MDA-MB-231 and MDA-MB-468 were used, added to which was the luminal A cell line MCF7 in order to compare any differential effect of the treatment between the two different cancer subtypes. This is important given that the immunogenicity of breast cancer is subtype dependent, as previously mentioned, with TNBC being the most immunogenic and luminal A the least immunogenic (Bates et al. 2018; Hammerl et al. 2018).

An initial interrogation of gene expression signatures defined the deregulated pathways in response to treatment. Relatively similar results were found for the two TNBC cell lines, for which the most affected pathways were DNA-damage repair and cell cycle apoptosis. This was expected because of the previously described mode of action of doxorubicin. Indeed, regulation of the cell cycle is one of the main mechanisms behind anti-proliferation effects of many chemotherapeutic drugs in cancer cells (Vermeulen et al. 2003a). In response to DNA damage, the cell cycle is delayed or arrested by DNA damage checkpoints (Knudsen et al. 2019a; Cai, Liu 2017; Vermeulen et al. 2003b).

These mechanisms involve the activation of the p16-Rb (retinoblastoma) and/or p53-p21 pathways. Although ATM and ATR were both downregulated, an upregulation of p53 transcriptional target p21 was observed, as was observed using flow cytometry. p21 inhibits cyclin-dependent kinase 2 (CDK2) and activates Rb to prevent entry into cell cycle (El-Deiry 2016; Knudsen et al. 2019b). Recently early p21 expression was reported to control the proliferation versus senescence decision of cells following chemotherapy (Hsu et al. 2019) .On the other hand, p16 was also found upregulated in TNBC cells, which is reflected in these data by the downregulation of CDK4 (Sherr et al. 2016). Cyclins are an important inhibitory factor at the G2/M phase; (Pyo et al., 2013). Our data show that low dose doxorubicin treatment downregulated the expression of different Cyclins, along with upregulation of p21 consequently inducing cell cycle arrest. This was in line with the cell cycle analysis (Chapter 3) results indicating a G2/M cell cycle arrest. This also concords with the data demonstrating NKG2D ligand upregulation (Chapter 4), as previous studies reported that the DNA damage pathway played a central, direct role in the process of NKG2D ligand upregulation (Gasser et al. 2005b; Alessandra Soriani et al. 2009).

The lack of targeted therapies for TNBC motivated multiple genomic studies to identify the central signaling pathways to TNBC progression, and thus potential targeted therapies. The main pathways investigated for TNBC therapies include VEGF, EGFR, NOTCH, CDK, mTOR and PI3K (Mayer et al. 2014; Bayraktar, Glück 2013). However, it is worth noting that the exact mechanisms by which breast cancer arises, as well as the related clinical responses, remain poorly defined/largely unclear.

Interestingly, NOTCH and EGFR pathways were among the highly deregulated pathways, as indicated by the advanced analysis carried out using Metcore software. An overview of the enrichment analysis for the NOTCH pathway is presented in Figure 6.1.

There is ample evidence that Notch signalling plays a critical role in breast cancer growth, Epithelial to Mesenchymal Transition (EMT), invasion and metastasis. In TNBC, Notch signalling is hyper-activated as a result of combination of elevated levels NOTCH1 receptor protein, NOTCH1 precursor mRNA and NOTCH2 protein along with their ligands Jagged1 and Jagged2 (Giuli et al. 2019; Sethi et al. 2011; L. Li et al. 2014). This high Notch activity has been reported to be associated with chemoresistance, aggressive disease and decreased overall survival rate of patients with TNBC (Leontovich et al. 2018). Therefore, inhibition of Notch signalling has been considered for TNBC treatment and multiple pan-Notch inhibitors are currently under clinical trials (Bellavia et al. 2018; McGowan et al. 2011; Giuli et al. 2019).

An overall downregulation of these effectors can be observed in treated cells. Although Notch3 was found upregulated in MDA-MB-231 cells (Chapter 3), this was associated with cell cycle arrest in these cells, and is linked with its role as a tumour suppressor, as a significant decreased of Notch3 was reported in breast tumour tissues compared to normal tissues (Cui et al. 2013; C.-F. Chen et al. 2016). Indeed, previous research also pointed toward the individuality of Notch receptors function, as they can have opposite roles in the context of TNBC (Giuli et al. 2019).It is mainly for this reason that experimental studies looking at Notch-based therapies are challenging (Giuli et al. 2019).

These results also support the data generated from the most differentially expressed genes following low-dose doxorubicin treatment (Chapter 5) which shows that several genes involved in tumour survival, progression and metastasis were downregulated in treated TNBC cells. Of note, VEGF is also downregulated in both TNBC treated cells (Figure 7.1) along with beta-catenin, thereby further confirming the capacity of low dose doxorubicin treatment to inhibit EMT and invasion.

Some differences between the two TNBC cell lines were observed. However, this was not unexpected as even within the same subtype, the response of TNBC to chemotherapy may vary (Gautam et al. 2016). It is also crucial to keep in mind here the seminal work of Lehmann et al., which showed that TNBC can be clustered into six types based on gene expression, as previously described in the introduction chapter (Lehmann et al. 2011b). As previously mentioned, MCF7 cells showed contrasting results compared to TNBC cells, thereby endorsing the differential responses between breast cancer subtypes. Future studies should also analyse pathway deregulation and gene expression changes in low dose doxorubicin treated SKBR3 cells, as these were found to be more resistant to NK cell killing. This analysis could shed light on mechanisms of resistance of these cells.

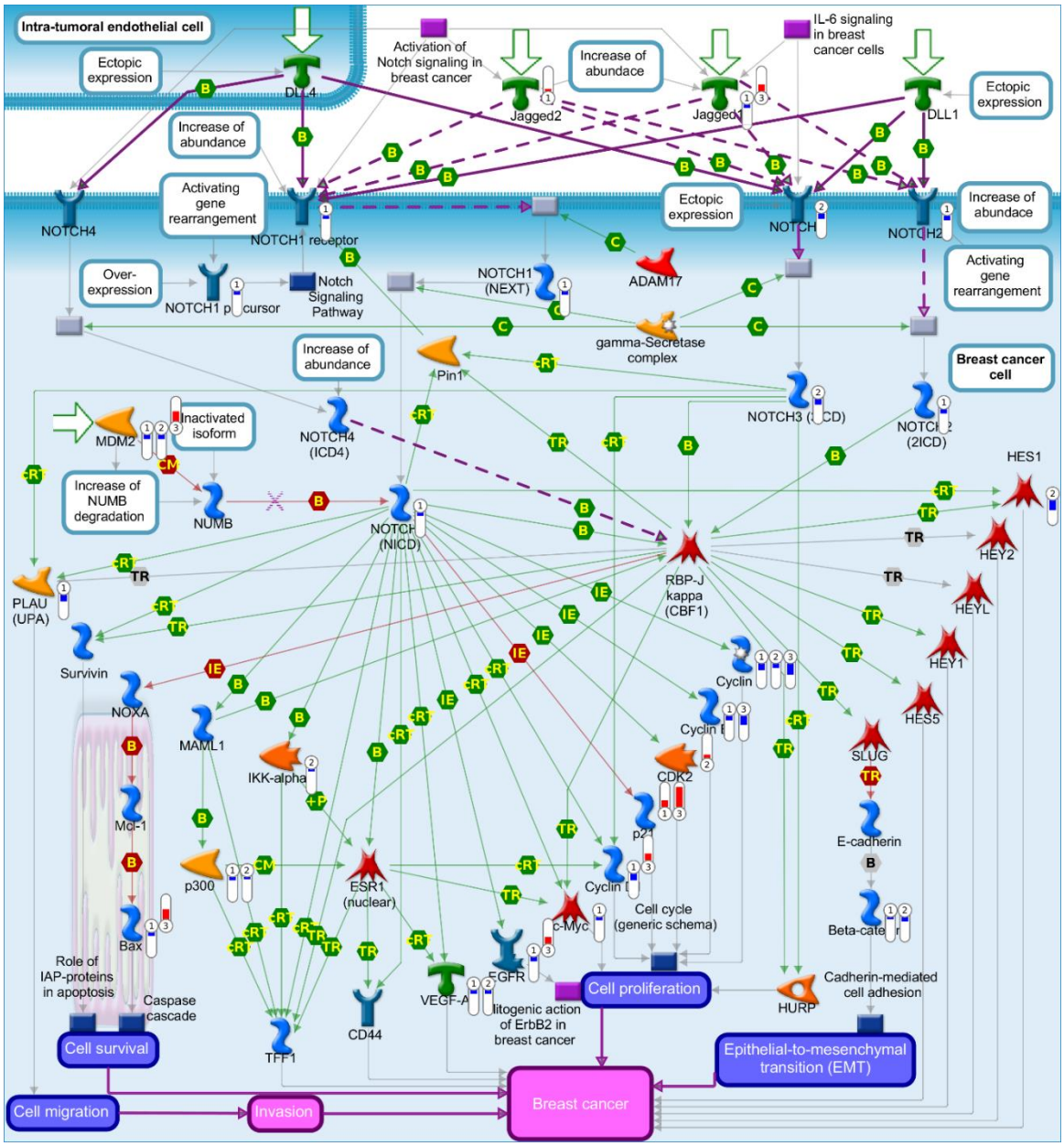

Figure 7.1. Enrichment analysis of mRNA for NOTCH Signaling Pathway. Enrichment analysis of NOTCH pathway, as analysed using MetaCore software. Upward thermometers indicate up-regulated signals (red) and downward (blue) indicate down-regulated expression levels of genes. 1: MDA-MB-231. 2: MDA-MB-468. 3: MCF7. A full legend is available in the Appendix.

7.3 Therapeutic potential of combining low dose doxorubicin and adoptive transfer NK cells for TNBC

The data presented in Chapter 4 demonstrated the immunomodulating effects of a single low-dose of doxorubicin *in vitro* in terms of upregulating NK cell ligands and sensitising breast cancer cells to NK cell-mediated cytotoxicity. Next, it was crucial to investigate the anti-tumour effect of metronomic doxorubicin in combination with NK cell adoptive transfer *in vivo.*

Establishing the tumour model and effects on tumour growth

Preclinical studies are important and essential for translation of therapeutic approaches into the clinic, and experimental mouse models represent a key tool to test and validate anticancer therapeutics. In order to perform *in vivo* experiments that would be relevant to the treatment strategy proposed herein, we established a TNBC xenograft model that would enable the analysis of adoptively transferred NK cells. As mentioned previously, in this study, NSG mice represented a suitable strain to carry out these experiments. However, we reported issues encountered when using the NK-92™ MI cells with both the NSG and NBSGW mice models. Also, sub-lethal irradiation of the cells only delayed the onset of the reported GvHD-like symptoms, this highlights the necessity for preconditioning irradiation of the animals prior to NK cell infusion. Along with inhibition of GvHD-like symptoms, a better engraftment has been reported when mice are given a low dose total body preconditioning irradiation (250cGy) prior to NK cell infusion (Jeffrey S. Miller et al. 2014).

Overall, studies provided evidence of the synergistic effect *in vivo* of NK cells with low-dose doxorubicin treatment in terms of tumour growth inhibition (with the exception of Study 4). Of note, variations in NK cell cytotoxicity across individual donors which have been previously observed and reported (Nagel et al. 1981; Pasero et al. 2015) could partly explain the variability between studies. However. one of the limitations of this model is the lack of the adaptive immunity component and future studies would benefit from investigating the effects on a model including both, especially given that low-dose doxorubicin treatment has been shown to influence the susceptibility of tumour cells to T cells (Wennerberg et al. 2013). The use of an immunocompetent model would also deliver useful information to evaluate the efficacy of the proposed combination therapy. The use of patient-derived xenografts would also enhance this model, as although cell line derived tumour models provide a useful tool for testing immunotherapies for cancer, patient-derived xenograft model are superior since they more closely mimic the clinical setting (Murayama, Gotoh 2019).

One challenge of NK cell therapy is how to optimize the effector-to-target ratio dose that is critical for cytotoxicity and ultimately clinical efficacy. One of the limitations encountered in this study was the availability of healthy blood donors. It is technically challenging to gather a sufficient amount

of NK cells from the blood of healthy donors, as they only make up to 10% of lymphocytes. The dose of $10x10^6$ NK cells was given twice in total for the whole duration of treatment. Further studies would benefit from optimizing the cell dose of NK cells, but also from the use of blood cones from NHS Blood and Transplant or *in vivo* expansion strategies to increase NK cell numbers and/or infusion frequency.

Indeed, preclinical studies have suggested that the prolonged treatment with NK cells was important, moreover, IL-2 activation has also been reported to be essential for optimizing cytotoxic effects (Jeffrey S. Miller et al. 2014; Miller, Lanier 2019; Lehmann et al. 2001). Although the idea is to provide competent, activated NK cells to the patient through NK adoptive transfer therapy, it is important to consider the immunosuppression that these NK cells will be subjected to in the tumour microenvironment (TME). To encounter this, NK cells may require further activation with cytokines following infusion. In this regard, the use of the NK-92™ MI cell line is very attractive. NK-92™ MI cells constitutively secrete low levels of IL-2, which allows for better activation of the cells without the toxicity that may be associated with a systemic administration of IL-2 (Tam et al. 1999; Zhu, Li, et al. 2017). Furthermore, the suitability and safety of the adoptive transfer of these cells with regard to viral safety aspects is not an issue, as their transfection was based on a non-viral gene transfer (i.e. particle-mediated gene gun) (Tam et al. 1999; Zhu, Li, et al. 2017).

It is worth noting, that the timing of NK cell infusion is also important, as in pre-clinical studies using the aggressive melanoma cell line (WM1341) it has been reported that infusion of NK cells was only efficient when they were administered shortly before, simultaneously with or shortly after tumour cells (~24h) (Hölsken et al. 2015). In the present study, it was important to us to treat established tumours, rather than start the treatment right after injection of tumour cells, however, as reported in previous studies, the doses and timings of treatments might not have been optimal to generate the maximum NK cell response and thus clear tumour regression in such a complex experimental setting.

Effect on metastasis

The ability to prevent and/or treat metastasis is crucial as it is the leading cause of death in TNBC. Our results suggested that treatment delayed metastasis on the basis of whole body imaging. Another indicator of metastatic potential is the presence of circulating tumour cells (CTCs) in the blood, and for one of the studies, blood from mice that received the combination treatment contained lower proportions of circulating tumour cells ($hCD45^-/HLA^+$) (Data not shown).

Murine models for cancer therapies have been dominated by treating established primary tumours or early stage, low volume microscopic disease. Although this is clearly important for the development of therapeutics, models for treating advanced metastatic disease also have immense

value, especially gibven that ~90% of cancer-related deaths are a consequence of advanced, aggressive and metastatic forms of disease (Francia et al. 2011). In this study, we tried to more closely mimic the clinical setting by resecting the primary tumours, however we encountered technical difficulties in doing so. Notwithstanding this, future studies should optimise this approach, as demonstrating the effect of the combination treatments on metastatic disease would be of immense value for their clinical translation.

Effect on tumour Infiltration

Another important indication of the potential of this combination is the infiltration of NK cells in tumours, as mentioned earlier, NK cell trafficking and infiltration into solid tumours is one of the main challenges of NK cell cancer immunosurveillance and NK adoptive cell transfer (Nayyar, Chu, Mitchell S Cairo 2019; Gras Navarro et al. 2015). Results showed that low-dose doxorubicin treatment increased NK cell infiltration into tumours, and is consistent with previous studies which have shown metronomic chemotherapy to enhance immune cell infiltration (Moschella et al. 2011; Sistigu et al. 2011; Nars, Kaneno n.d.; Wu, Waxman 2018; Simsek et al. 2019a).

In this study, NK cell infiltration was assessed by IVIS imaging and flow cytometry, staining of tumour sections would have also been useful to have a detailed information about the localisation and density of NK cell infiltrates. However, although there was evidence of enhanced NK infiltration in animals pre-treated with low-dose doxorubicin, along with higher expression of MICA/B ligands on the surface of tumours, in this particular study (Study 4) it did not translate into significant tumour control. This suggests that NK cells infiltrating tumours are not fully activated or functional. Of note is that although NK cells were pre-activated with IL-2 prior to their infusion, they did not receive any cytokines while they were *in vivo*, this could have translated into enhanced activation and function of NK cells, as reported by multiple studies (Lim et al. 2015; Zwirner, Domaica 2010b; Rezvani, Rouce 2015). Furthermore, it has previously been reported that although infusion of *ex vivo* activated NK cells by IL-2 generated high numbers of circulating NK cells, no effect was observed, as the transferred cells rapidly lost their activation *in vivo*, however, re-activation of the cells with IL-2 led to clinical benefit in a cohort of patients with metastatic melanoma or renal cell carcinoma (Parkhurst et al. 2011b).

Impaired proliferation of tumours

One of the most reliable, and clinically used indicators of the proliferative status of tumours is the expression of the proliferation marker Ki67. Various studies have reported its close association with the growth and invasion of breast cancer (F. Li et al. 2014; Scholzen, Gerdes 2000; Dowsett et al. 2011).

Tumours from mice that received low-dose doxorubicin alone or the combination treatment showed significantly lower expression levels of Ki67. This was in line with our *in vitro* data, but also with previous preclinical studies using low-dose metronomic chemotherapy. In a study by Di Desidero et al., the number of Ki67 positive cells in tumours obtained from LDM topotecan plus pazopanib-treated mice was 48% lower than that found in the control group (Di Desidero et al. 2015). Similarly for clinical studies, the expression of Ki67 after treatment was decreased by 41%, and 91% of the patients had complete pathological response (Cancello et al. 2015).

Effect on Cancer Initiating Cells (CICs)

One interesting aspect of both metronomic therapy and NK cell therapy is their potential to target Cancer Initiating Cells/Cancer Stem Cells (CICs/CSCs) (Murphy Urayama et al. 2019; Chan et al. 2016). Indeed, a recent study demonstrated the capacity of adoptively transferred NK cells to reduce intra-tumoural CSCs and tumour burden in a pancreatic carcinoma xenograft model (Murphy Urayama et al. 2019). This led us to investigate the effect on the proportion of CSC/CICs in tumours following treatment. Results showed that both LD-Dox and LD-Dox+pNK cell combination treatment reduced the proportions of CICs in tumours. Of note, *in vitro* studies also confirmed the effect of low-dose doxorubicin treatment on MDA-MB-231/LUC sphere formation ability (data not shown), this is particularly interesting in the context of TNBC, as TNBC tumours are known to be enriched for CICs (O'Conor et al. 2018), and a strategy able of eliminating this population would have immense potential in inhibiting tumour progression and metastasis.

7.4 Aspects and considerations for clinical translation

The clinical translation of anti-cancer therapeutics is a complex and challenging process, particularly when it comes to combination strategies that involve and affect different aspects of the tumour and its microenvironment. In this section, the main aspects and considerations related to the potential implementation of the therapeutic strategy proposed in this thesis, namely the combination of LD-Dox metronomic chemotherapy and adoptive transfer of NK cells will be discussed.

7.4.1 The choice of the chemotherapeutic agent, route of administration and schedule

With regards to the first arm of this combination (LD-Dox), essential design criteria need to be taken into consideration, such as the choice of the chemotherapeutic agent, route of administration and schedule. Although multiple chemotherapeutics have been investigated for their immunomodulatory potential, until now no 'ideal' chemotherapeutic partner for immunotherapy has been identified (Shurin et al. 2012; Wu, Waxman 2018; Sheng Sow, Mattarollo 2013). With this in mind, and although the use of doxorubicin has many advantages in this setting, as previously

described, better agents may be available and their use in this context should be taken into consideration. One of the main challenges with clinical translation is the length of time that it might take to get from preclinical testing to the clinic, and this is in part affected by the choice of the drug. For example, using chemotherapeutics that have been FDA-approved already would much likely have a faster timeline to get to the clinic compared to novel, complex drugs that are yet to gain regulatory approval. In this regard and as previously discussed, the advantage of doxorubicin is that is already has a broad spectrum of applications in the clinic. As metronomic chemotherapy is based on the frequent administration of the drug, generally daily or weekly, the most convenient route of delivery would be oral administration. Although in the present study, we have used doxorubicin as an injectable solution (i.p injections) it does exist in an oral form, and different nanocarriers have been used to improve its absorption and efficacy. Among these, liposomes have shown encouraging results for doxorubicin delivery, such as the Pegylated liposomal doxorubicin (Doxil® or European trademark Caelyx®) which prolongs circulation time and has a very low toxicity profile (Gabizon 2001). Multiple liposomal doxorubicin formulations, including given in a metronomic fashion have shown encouraging results in terms of safety and efficacy (Munzone et al. 2010; Manso et al. 2013; Masci et al. 2013). Thus, future studies in the context of this combination treatment, should consider the use of liposomal doxorubicin.

Very few data regarding the right dose and schedule of the different drugs to be used in the metronomic administration are available. This is mainly due to the lack of predictive biomarkers for response to metronomic chemotherapy. However, several preclinical studies have highlighted the dose and schedule dependency of the immune modulatory effects associated with metronomic chemotherapy. For instance, Cyclophosphamide, one of the most commonly used chemotherapeutics in metronomic clinical trials (K. Lien et al. 2013; Simsek et al. 2019a), has been shown to induce transient innate immune responses in a model of glioma when given on an MTD schedule, whereas sustained immune responses and regression of tumours were observed when using a metronomic schedule (Chen et al. 2014; Wu, David J Waxman 2014). In their seminal paper, Shaked et al, looked at the determination of the optimal biologic dose (OBD), which was defined as the dose able to cause maximum reduction in tumour volume with no or minimal toxicity, as determined for 4 chemotherapeutics, and this OBD was associated with an enhanced anti-angiogenic activity (Shaked et al. 2005). Some studies rely on mathematical models to determine the optimal dose and schedule for administering the drug (Ledzewicz, Schättler 2017). Overall, for a combination of low-dose chemotherapy and immunotherapy to be effective, the dose of the chemotherapeutic agent needs to be high enough for inducing the immunomodulation effects and lower than the threshold dose that induces toxicity. As mentioned previously, the dose and schedule of doxorubicin used in this study was based on encouraging results reporting its use as

monotherapy or combination with other chemotherapeutics. Interestingly, a lower dose of doxorubicin assayed was also able to inhibit tumour growth. However, as it is in the context of combination with immunotherapy, pre-clinical studies looking at the optimisation of the dose and schedule of doxorubicin should be carried out, and these should be based on responsiveness to NK cell anti-tumour immunity.

7.4.2 NK cell-adoptive transfer

The second element of the proposed combination treatment is the adoptive transfer of NK cells. Essential considerations relating to the adoptive transfer of NK cells in patients with TNBC include source, manufacture and activation/enhancement of NK cells.

Source and manufacture of NK cells

As reviewed in the introduction chapter, there are different source options for NK cells. Along with autologous or allogeneic PBMC-derived NK cells, which are usually used for adoptive transfer of NK cells, induced pluripotent stem cells (iPSCs), cord blood (UCB) and NK cell lines are also important cell sources for NK immunotherapy. Infusion of the human NK-92 cell line shows remarkable anti-tumour effects in preclinical studies and clinical studies have demonstrated its safety in cancer patients (Klingemann et al. 2016; Tonn et al. 2013c). Although multiple studies have reported a superior cytotoxicity of NK-92™ cells towards various tumour targets compared to primary NK cells (Tonn et al. 2001; Suck et al. 2016), our *in vitro* and *in vivo* data did not necessarily reflect that, and because of the issues encountered with the use of NK-92™ MI cells, we focused on the use of primary NK cells. However, we still think that NK-92™ cells offer a viable option for use in this combination treatment and should be further investigated in appropriate pre-clinical models.

As discussed earlier, the variable efficacy/cytotoxicity of NK cells across individual donors need to be taken into account. For this, some studies proposed the use of predicted donor selection models, considering KIR-MHC class I mismatches (W. Wang et al. 2017). However, further characterisation of response biomarkers are needed. Moreover, expansion methods should be evaluated to provide sufficient numbers of NK cells.

Improving target recognition, NK cell homing and tumour infiltration

In addition to enhancing tumour target recognition, one of the main desired outcomes of this combination treatment was to better target NK cells to tumours. In fact, the migration and homing of NK cells to the tumour site is critical and essential to its success in inducing tumour control upon adoptive transfer. Tumour infiltration of NK cells in cancer patients is associated with good prognosis in multiple tumours (Coca et al. 1997; Ishigami et al. 2000; Jeffrey S. Miller et al. 2014). It also represents one of the main challenges NK adoptive transfer therapy has been encountering in

its clinical application to solid tumours (Jeffrey S Miller et al. 2014; Fang et al. 2018). Over the years, different approaches have been used to enhance tumour targeting by NK cells and overcome immunosuppression, such as administration of cytokines or antibodies, enhancement of tumour-specific chemokine receptor expression on NK cells or genetic engineering (Zhang et al. 2019; Wennerberg et al. 2015; Nayyar, Chu, Mitchell S Cairo 2019). In this context, CAR NK-92™ MI cells could further enhance tumour targeting. Indeed, pre-clinical studies have already shown the efficacy of CAR NK-92™ cells (Romanski et al. 2016), and multiple clinical studies are ongoing (Zhang et al. 2019; Zhang et al. 2017).

The overall efficacy of this combination could also be enhanced by introducing an immune checkpoint blockade. There was no differential expression in PD1, TIM3 and TIGIT expression on NK cells from tumours treated with the combination, however the expression of other immune checkpoint molecules could be investigated. Additional combination strategies could involve the blockade of inhibitory pathways, such as KIR receptors or NKG2A. Indeed, blocking of NKG2A has previously been shown to enhance both NK cell and $CD8^+$ T cell effector functions (André et al. 2018). Interestingly, low-dose chemotherapy has also been shown to overcome tumour resistance to immune checkpoint inhibitors (Dosset et al. 2018; Pfirschke et al. 2016; Rivera Vargas, Apetoh 2019).

7.4.3 Effects and interaction with other components of the immune system and the tumour microenvironment

Low-dose metronomic chemotherapy is a multi-targeted therapy, and although the focus of the research programme described in thesis and treatment combination proposed is on NK cell-based immunotherapy, one important aspect to consider is the effect of low-dose doxorubicin on the adaptive immune response and the tumour microenvironment. Some chemotherapeutic drugs, including doxorubicin and cyclophosphamide, can contribute to the induction of T cell-dependent anti-tumour responses and modulation of the tumour microenvironment. (Nars and Kaneno, 2011; Sistigu *et al.*, 2011; Lien *et al.*, 2013; Hao *et al.*, 2014; Wu, Jordan and Waxman, 2016; Wu and Waxman, 2018; Simsek, Esin and Yalcin, 2019b). For instance, a combination of doxorubicin and lapatinib has been shown to enhance the infiltration of IFN-γ secreting $CD4^+$ and $CD8^+$ T cells into the mammary tumours of mice bearing HER2+ breast cancer and this to result in improved anti-tumour responses (Hannesdóttir et al. 2013). On the other hand, as reviewed in the Introduction chapter, although NK cells are a part of innate immunity, they contribute to adaptive immune responses either indirectly by influencing adaptive cells or directly by the release of cytokines. In addition, they can adopt a memory phenotype which allows a stronger response to a previously encountered stimulus (Cerwenka, Lanier 2016; Capuano et al. 2019b).

One possible hurdle that could be encountered is the possibility of tumours to develop resistant clones to low-dose doxorubicin. Although *in vitro* studies showed that low-dose doxorubicin did not induce resistance to MTD, this should also be tested *in vivo*. With this in mind, the different strategies proposed earlier to enhance the anti-tumour effects should be taken into consideration in order to minimise the risk of resistance. Additionally, another aspect that needs to be considered is the effect on the host microbiota, as chemotherapy was shown to impact gut microbiota and with the ever-growing body of evidence linking the microbiome to the efficacy of cancer therapies (Zitvogel et al. 2015; Viaud et al. 2013; Pouncey et al. 2018).

7.5 Therapeutic implications and concluding remarks

Despite its general sensitivity to standard chemotherapy, TNBC is considered as the most "aggressive" form of breast cancer. Unresponsiveness to current treatment makes the development of novel therapeutic strategies an urgent priority. In recent years, the encouraging outcome of immunotherapy and the emergence of synergistic combination strategies generated high expectations. Although identifying clinically successful combinations of immunotherapy with low-dose metronomic chemotherapy is a challenging task, it has tremendous potential for cancer treatment. The ultimate aim is the development of a strategy capable of increasing tumour immunogenicity and NK cell homing in the tumour bed, while reducing tumour burden and minimising toxicities and side effects.

In this study, we report for the first time to our knowledge, the potential for combining low-dose doxorubicin with the adoptive transfer NK cell as a treatment for TNBC treatment. *In vitro* studies demonstrated that low-dose doxorubicin impairs the proliferation of TNBC cells, enhances their sensitivity to NK cells and modulates important cancer-relevant pathways. *In vivo* studies demonstrated the capacity of the combined treatment to control tumour growth, enhance NK cell infiltration into tumours as well as delay/inhibit metastasis and reduce the prevalence of cancer initiating cells. The optimal timing and dosing, along with potential combinations with other agents to achieve therapeutic efficacy must be carefully considered in future studies.

These findings expand upon the potential of NK cell-based immunotherapeutic approaches currently in clinical trials and offer a rationale for a new clinically translatable approach based on a combination with low-dose chemotherapy for patients with TNBC and, potentially, other solid malignancies.

8. Appendix

8.1 Cell culture media, reagents and products

Product	Source
Media	
alpha-MEM	ThermoFicher scientific
EMEM	SLS (Lonza)
Leibovitz media (L-15)	SLS (Lonza)
McCoy's 5	SLS (Lonza)
RPMI 1640	SLS (Lonza)
RPMI1640	SLS (Lonza)
Supplements and reagents	
2-mercaptoethanol	Sigma Aldrich
Acetic acid	Fisher Scientific
Anhydrous ethanol	Sigma Aldrich
Dimethyl sulfoxide (DMSO)	Insight Biotechnology
Dulbecco's phosphate buffered saline (DPBS)	SLS (Lonza)
EDTA 0.5M	Ambion
Foetal calf serum (FCS)	Fisher (GE Healthcare)
Folic acid	Sigma Aldrich
Human IL-2	Peprotech
Inositol	Sigma Aldrich
L-Glutamine	SLS (Lonza)
Matrigel ECM	Sigma Aldrich
Pen/strep antibiotic solution	SLS (Lonza)
Phosphate buffer saline (PBS)	BioWhittaker Europe
Trypan Blue solution 0.4%	Sigma Aldrich
Trypsin/Versene	SLS (Lonza)
Products	
0.22 µm syringe filter	Sartorius
0.45 µm syringe filter	Sartorius
24-well flat-bottom with lid – Standard TC	Sarstedt
5 ml round-bottom polypropylene test tubes	Tyco healthcare group
6-well flat-bottom with lid – Standard TC	Sarstedt
70 µm nylon strainer	Greiner
96-well flat-bottom with lid – Standard TC	Sarstedt
Cell culture flasks (T25,T75,T175)	Sarstedt
Coverslips	SLS
Cryovial, 2 ml sterile	Sarstedt
Cryovials	TPP
Eppendorf tubes (0.5 ml, 1.5 ml, 2 ml)	Sarstedt
Falcon tubes (15 ml,50 ml)	Sarstedt
Filter tips (10, 20, 100,200, 1000 µl)	Greiner bio-one
Leucosep® tubes	Greiner Bio-One
Micro tips (0.5-10 µL, 20-200 µL, 200-1000 µL)	Sarstedt
Multichannel pipette	Sartorius
Pasteur pipettes	Sarstedt
Petri dishes	Sarstedt
Pipettes (5mL, 10mL, 25mL)	Sarstedt
Serological pipettes	Sarstedt
Syringes (10ml,20ml)	Becton Dickenson
Universal tubes (20ml)	Greiner

8.2 Kits

Product	Source
EasySep™ Human NK-Cell Isolation Kit	StemCell technologies
RNAqueous™-micro total RNA-isolation kit	Ambion™, Life Technologies
Nanostring PanCancer Pathways Panel kit	Nanostring Technologies
FcR Blocking Reagent	Miltenyi Biotec
LIVE/DEAD™ Fixable Violet Dead Cell Reagent (1µ in 1ml)	ThermoFisher Scientific
LIVE/DEAD™ Fixable Yellow Dead Cell Reagent (1µ in 1ml)	ThermoFisher Scientific
OneComp eBeads Compensation Beads	ThermoFisher Scientific
Intracellular Fixation & Permeabilization Buffer Set	eBioscience™
PerFix-nc assay kit	Beckman Coulter
Vivotrack680	Perkinelmer
CellVue™ NIR780 cell labelling kit	Affymetrix-eBioscience

8.3 Antibodies

Antibody	Fluorochrome	Volume µL/tube	Clone	Manufacturer
Senescence DNA-Damage markers				
p21	Alexa Fluor 700	5 µl	WA-1 (HJ21)	Novus Biological
p16	Alexa Fluor 647	5 µl	EPR1473	Abcam
P53	FITC	5 µl	DO-7	Biolegend
H2A.X	PerCP/Cy5.5	5 µl	2F3	Biolegend
Ki-67	PE/Cy7	5 µl	Ki-67	Biolegend
Notch 3	PE	5 µl	MHN3-21	Biolegend
NK cell ligands				
CD48	Alexa Fluor 700	2.5 µl	HM48-1	Biolegend
CD112	PE/Cy7	5 µl	TX31	Biolegend
CD155	PE	2.5 µl	4.24.1	Biolegend
E-Cadherin (CD324)	APC	2 µl		Biolegend
HLA A-B-C	PerCP/Cy5.5	2.5 µl	W6/32	Biolegend
HLA-E	PE/Cy7	2.5 µl	3D12	Biolegend
ULPB-1	Alexa Fluor 488	5 µl	170818	R&D
ULPB-2	PE	5 µl	165903	R&D
ULPB-3	APC	5 µl	166510	R&D
MICA/B	PE	5 µl	6D4	Biolegend
NK cell markers				
CD3	FITC	2.5 µl	HIT3a	Biolegend
CD56	APC	2.5 µl	HCD56	Biolegend
CD16	PerCP	5 µl	3G8	Biolegend
NKp30	Alexa Fluor 647	5 µl	P30-15	Biolegend
NKG2D	PE	5 µl	1D11	Biolegend
CIC markers				
CD44	APC	5 µl	IM7	Biolegend
CD24	PE	5 µl	ML5	Biolegend
CD326 (EpCam)	FITC	5 µl	9C4	Biolegend
Intracellular cytokines and checkpoint antibodies				
CD107a(LAMP-1)	Pacific blue		H4A3	Biolegend
IFN-gamma	PE/Cy7	5 µl	4S.B3	Biolegend
TNF-alpha	Alexa Fluor700	5 µl	MAb11	Biolegend
Peforin	FITC	5 µl	dG9	Biolegend
Granzyme B	PE	5 µl	QA16A02	Biolegend
PD1	Alexa Fluor 700	5 µl	EH12.2H7	Biolegend
TIGIT (VSTM3)	Brilliant Violet 421™	5 µl	A15153G	Biolegend
CD366 (Tim-3)	APC/Cy7	5 µl	F38-2E2	Biolegend
Other				
anti-mouse CD16/32	Purified		/	Biolegend
CD45	FITC		HI30	Biolegend

8.4 Chemical and biological reagents

Product	Source
Bafilomycin-A1	Sigma Aldrich
Bovine serum albumin (BSA)	Merck
Brefeldin A (BFA) solution	Biolegend
C12FDG	Sigma Aldrich
Collagenase IV	Sigma Aldrich
DNAse I	Sigma Aldrich
Doxorubicin	Sigma Aldrich
Ficoll-Paque density gradient medium	GE Healthcare
Monensin solution	Biolegend
Heparin	Sigma Aldrich
ISOTON™ sheath fluid	Beckman Coulter
Liquid nitrogen	BOC
Xenolight RediJect D-Luciferin	Perkinelmer
Matrigel™	Corning
ECM Gel from Engelbreth-Holm-Swarm murine sarcoma	Sigma Aldrich
Propidium iodide	Sigma Aldrich
Ribonuclease A	Sigma Aldrich
Sodium chloride (NaCl)	Calbiochem
Sodium hydroxide (NaOH)	Fisher Scientific
Solution 18 AO.DAPI	Chemometec

8.5 Laboratory equipment and software

Equipment	Supplier
Autoclave	Rodwell
Cell culture incubator 37°C (without CO_2)	LEEC
Cell culture incubator 37°C, 5% CO_2	Sanyo, Binder
Centrifuges	Sanyo, Eppendorf
Class II safety cabinets	Walker
Fridges	Lec
Freezers -20°/-80°	Lec/Revco/ Sanyo
Gallios Flow cytometer	Beckman Coulter
Haemocytometers	SLS
Light microscope	Nikon/Olympus
NucleoCounter®® NC-250™	ChemoMetec
Microcentrifuge	MSE
MoFlo™ cell sorter	Beckman Coulter
Nanodrop 8000 Spectrophotometer	Thermo scientific
Vortex	Scientific industries
Water baths	Clifton
IVIS Lumina III Imaging machine	Perkin Elmer
Gulmay RS225 Ionising Radiation Cabinet (City hospital)	Xstrahl
NanoString nCounter™ FLEX platform	NanoString Technologies
Softwares	
NucleoView™ software	Chemometec
Kaluza 1.3 version	Beckman Coulter
Nanostring nSolver™ version 3.0/ Advanced Analysis module version2.0	NanoString Technologies
MetaCore version 19.2	Thomson Reuters
Living Image Software (IVIS Imaging Systems)	Perkin Elmer
GraphPad Prism 7	Graph Pad software

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