Pre-metastatic neutrophils directly support highly tumourigenic breast cancer cells during lung metastasis via a leukotriene-ERK1/2 axis

Stefanie Wculek

University College London
and
Cancer Research UK London Research Institute
PhD Supervisor: Ilaria Malanchi

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Declaration

I, Stefanie Kristin Wculek, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Evidence is mounting that cancer development and progression depends on a supporting microenvironment or stroma. Pro-tumourigenic and pro-metastatic properties of inflammation are long recognised and systemic or intra-tumoural presence of the cellular inflammatory mediators neutrophils shows strong associations with poor prognosis in the clinic. Thus, we were prompted to investigate the contribution of neutrophils to tumourigenesis and metastasis.

Taking advantage of mouse models for lung metastatic breast cancer, we find neutrophils to be the predominant inflammatory component to strongly accumulate in the pre-metastatic organ of tumour-bearing mice prior to metastatic colonisation by cancer cells. Preventing increased neutrophil presence in the lung during metastasis initiation resulted in a pronounced decrease of metastatic burden. In fact, we unravelled a novel function of mammary tumour-induced neutrophils at the distant site to directly aid proliferation and initiation of metastatic outgrowth of cancer cells. Neutrophils specifically promote the intrinsically highly potent metastasis-initiating subpopulation of mammary cancer cells during initiation of metastatic lung colonisation via secretion of Alox5 metabolites, the lipid mediators leukotrienes. Engagement of leukotriene receptors with their ligands induced cell proliferation by activation of ERK1/2 kinases in mammary cancer cells. Leukotriene receptor expression is strongly enriched on metastasis-initiating cells and makes them susceptible to the neutrophil-derived proliferation-inducing signals leading to their expansion at the metastatic site. In fact, leukotriene receptor expression itself might represent a novel marker to identify highly tumourigenic cancer stem cells. Interference with neutrophil-derived Alox5 metabolites/leukotrienes holds potential to weaken the highly potent cancer stem cell-like subpool, the main cellular cause of metastasis initiation and relapse. Importantly, genetic or pharmacologic block of the Alox5 enzyme prevents the proliferation and expansion of metastasis-initiating cells and subsequently the metastasis-promoting activity of neutrophils. The Alox5 inhibitor Zileuton, which is routinely used in the clinic to treat asthmatic patients, significantly reduced lung metastasis in three mouse models of breast cancer. This observation, together with expression of leukotriene receptors in the majority of

examined human breast cancers and lymph node metastases, suggests a promising novel therapeutic approach targeting the tumour stroma to limit metastatic progression.

In summary, we found neutrophils, an inflammatory component of the metastatic microenvironment, to act pro-metastatic. Neutrophils specifically promote early events of metastasis initiation at the distant site by providing a direct, proliferation-inducing signal to intrinsically highly potent metastasis-initiating cells. Interference with the neutrophil-leukotriene-ERK1/2 axis-dependent support might hold great potential to be exploited in the clinic.

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Abbreviations

Alox5	Arachidonate 5-lipoxygenase
AOM	Azoxymethane
APS	Ammonium Persulfate
Arg1	Arginase 1
BLT1/2	Leukotriene B4 receptor 1/2
BMN	Bone marrow neutrophil-conditioned medium
BrdU	5-bromo-2'-deoxyuridine
BSA	Bovine serum albumin
CAF	Cancer-associated fibroblast
Cat.Nr.	Catalogue number of the respective company
CCL2	Chemokine C-C motif ligand 2
CCL22	Chemokine C-C motif ligand 22
CCL3	Chemokine C-C motif ligand 3
CCL5	Chemokine C-C motif ligand 5
CCL6	Chemokine C-C motif ligand 6
CD11b	Cluster of differentiation 11b
CD31	Cluster of differentiation 31
CSC	Cancer stem cell
CXCL1/2	Chemokine C-X-C motif ligand 1 and 2
CXCR2	Chemokine C-X-C motif receptor 2
CysLT1/2	Cysteinyl leukotrienes C4, D4, E4 receptor ½
DAMPs	Danger-associated molecular pattern
DAPI	4,6-Diamidino-2-phenylindole dihydrochloride
DMBA	7,12-Dimethylbenz[a]anthracene
DMEM	Dulbecco's Modified Eagle's medium
DMEM/F12	DMEM with half Ham's F-12 Nutrient Mixture
DMEM/FBS	10% FBS in DMEM
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSS	Dextran sulfate sodium
DTA	Diphtheria toxin

ECM	Extracellular matrix
EGF	Epidermal growth factor
EIA	Enzyme immunoassay
Ela2	Neutrophil elastase 2
EMT	Epithelial to mesenchymal transition
ERK1/2	Extracellular signal- regulated kinase 1/2
EtOH	Ethyl alcohol
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FDA	US Food and Drug Administration
FGF	Fibroblast growth factor
Fig.	Figure
FSC	Forward scatter
G-CSF	Granulocyte colony stimulating factor
G-MDSC	Granulocytic myeloid-derived suppressor cell
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
HBSS	Hanks' balanced salt solution
HGF	Hepatocyte growth factor
HRP	Horseradish peroxidase
ICAM1	Intercellular Adhesion Molecule 1
IgG	Immunoglobulin G
IL-1	Interleukin 1
iNOS	Nitric oxide synthase
JAK	Janus kinase
L	Litre
LT	Leukotriene
LTB4	Leukotriene B4
LTC-D-E4	Cysteinyl leukotrienes C4, D4, E4
LTR	Leukotriene receptor
LuN	Lung neutrophil-conditioned medium
Ly6G	Lymphocyte antigen 6 complex, locus G
M-MDSC	Monocytic myeloid-derived suppressor cell

MACS	Magnetic-activated cell sorting
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ERK kinase
MHC	Major histocompatibility complex
MIC	Metastasis-initiating cell
Mmol	Millimolar
MMP9	Matrix metallopeptidase 9
MMTV	Mouse mammary tumour virus
mPGK	Mouse phosphoglycerate kinase 1
mRNA	Messenger ribonucleic acid
MRP14	Myeloid-related protein 14 (also known as S100A9)
NaCl	Sodium Chloride
NET	Neutrophil extracellular trap
NFkB	Nuclear factor kappa B
PAMPs	Pathogen-associated molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PG	Prostaglandin
PGE2	Prostaglandin E2
PI	Propidium iodide
PI3K	Phosphatidylinositol 3-kinase
PyMT	Polyoma middle T antigen
RIPA	Radioimmunoprecipitation assay buffer
Rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
SSC	Side scatter
STAT	Signal Transducer and Activator of Transcription
TEMED	Tetramethylethylenediamine
TGF-beta	Transforming growth factor beta
Th1/2	CD4+ T helper cells type 1 or 2
TIC	Tumour-initiating cell
TME	Tumour microenvironment
TNF-alpha	Tumour necrosis factor alpha

TPA	12-O-Tetradecanoylphorbol 13-acetate
TPA	12-O-Tetradecanoylphorbol 13-acetate
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VEGF	Vascular endothelial growth factor

Chapter 1. Introduction

Cancer research came a long way since the first proposals of a multi-stage model of cancer development in the late 1920s to the 1950s (Fisher, 1958, Frank, 2007). This model arose from the notion that cancer risk increases with age (Armitage and Doll, 1954) and the observations that multiple applications of chemicals or carcinogens are required for or enhancing cancer development (Deelman, 1927, Twort and Twort, 1928). The concept of a primary event within a tissue cell leading to cancer initiation followed by additional events to promote cancer development (in mice) took shape (Nordling, 1953, Friedewald and Rous, 1944) and this general principle is still directly reflected in widely used spontaneous carcinogenesis protocols for animals (De Robertis et al., 2011, Abel et al., 2009). By the year 2000, our knowledge of successful development of cancer was largely refined and detailed. Many key features intrinsically acquired by tumour cells were associated with specific mutations and it appeared to be the rare event of the right combination of those in the right place and at the right time driving the disease (Hanahan and Weinberg, 2000). Other, functional differences between cancer cell subpopulations were ascribed to variations in their epigenetic status and transcriptional signatures. Tumours emerged to contain a variety of cancer cells with different tumourigenic competence rather than being a homogeneous mass (Cabrera et al., 2015). This notion led to the proposal of a cellular hierarchy in cancer and the idea of special subpopulations of cancer cells that are exclusively responsible for tumour homeostasis and maintenance. These highly potent cancer cells where named “cancer stem cells” (CSCs) to draw a link with the hierarchical organisation in normal tissues (Nguyen et al., 2012). There, populations of undifferentiated, multi-potent adult stem cells persist long term and sustain tissue function by retaining the ability to replace the different cell types in the tissue when required (Tetteh et al., 2015). The very malignant subpopulations of CSCs are distinguished by their distinctive epigenetic state and also appear to sustain tumour growth, cause therapy-resistance and drive metastatic spread (Kreso and Dick, 2014).

Adding to the complexity and heterogeneity among cancer cells is the fact that a tumour usually undergoes some sort of evolution towards increasing aggressiveness over time (McGranahan and Swanton, 2015). This cancer evolution does not only involve acquisition of further genetic hits and intrinsic

features of cancer cells with increased tumourigenic potential, but also regulatory signals from the microenvironment (Lorusso and Ruegg, 2008). In fact, the microenvironment might directly shape the heterogeneity of cancer cells and cause additional mutations or contribute to epigenetic modulation and maintenance of CSC states (Plaks et al., 2015, Ronnov-Jessen and Bissell, 2009). We are beginning to understand aspects of the influence of the non-cancer cell compartment of tumours (stromal cells, vasculature, inflammatory cells and extracellular matrix) to cancer and metastasis development. Tumour-associated hosts become skewed in a cancer supporting fashion and, as such, evolve in close association with tumour cells. Many cancer types have also been shown to depend on a favourable environment, termed niche, which allows cancer initiation to progress, likely, by providing the essential “promoting” signals (Hanahan and Coussens, 2012, Borovski et al., 2011, Hu and Polyak, 2008, Polyak et al., 2009, Quail and Joyce, 2013). On the other hand, several immune cell types within the cancer microenvironment emerged to specifically recognise and eradicate growing cancer cells (Gajewski et al., 2013, Zitvogel et al., 2008).

Large efforts are focussed on understanding the unfolding degree of complexity within tumours and to elucidate strategies to therapeutically interfere. Likely, it will be a combination of approaches targeting multiple levels of cancer cell-acquired intrinsic features together with blocking the pro-tumourigenic stromal compartment and fostering cancer cell-destruction by immune cells that will provide the next leaps in improving cancer therapies.

1.1 Cancer and metastasis

1.1.1 Overview of cancer development with the example breast cancer

A tumour is abnormal growth of tissue or a neoplasm that can remain benign or become a malignant cancer invading adjacent tissue and causing metastasis. The main classified cancer types are distinguished by the type of initially transformed cells and consist of haematopoietic and lymphoid cancers like lymphomas and leukaemias and solid cancers including carcinomas, sarcomas, melanoma and gliomas or cancers of the nervous system. Lymphomas originate from lymphocytes, leukaemias from haematopoietic cells and sarcomas from non-haematopoietic mesenchymal cells. Carcinomas are by far the most common cancer type in humans and arise from mutations in epithelial cells (Cooper, 1993). Accumulation of somatic mutations in normal cells sparks cancer initiation and subsequent genetic, epigenetic and environmental events drive cancer progression. Initially, neoplasms undergo different stages with increasing malignancy as illustrated for invasive ductal carcinoma, the most frequent type of breast cancer (Fig. 1.1 a). The normal duct becomes hyperplastic up to atypical hyperplasia that constitutes of proliferating dysplastic, but benign ductal cells (Hartmann et al., 2015). Ductal carcinoma in situ (DCIS) is characterised by seemingly malignant proliferative growth of transformed ductal cells that does not extent beyond the ductal basement membrane and is usually removed by surgery with promising recovery rates. If untreated, a significant proportion (about 40%) of DCIS develop into invasive ductal carcinoma, where cancer cells breach the basement membrane and become invasive and metastatic (Fig. 1.1 b) causing significantly worse overall survival (Boughey et al., 2007, Cowell et al., 2013, Fulford et al., 2007). The intrinsic molecular subtypes of breast cancer include basal-like, human epidermal growth factor (HER2)-enriched, normal breast-like, luminal A, luminal B and claudin-low and are divided according to their gene expression (Eroles et al., 2012). Breast cancer types can also be classified according to the expression of receptors such as HER2 or the receptors for the hormones oestrogen and progesterone (Onitilo et al., 2009). Clinically, breast cancer is staged from stage 0 to IV based on primary tumour size, microscopic invasion of cancer cells and detection of distant metastases (Todd RF et al., 2015). In human patients, the principal sites of distant

metastasis by breast cancer cells include the bone, lung, liver and brain (Nguyen et al., 2009).

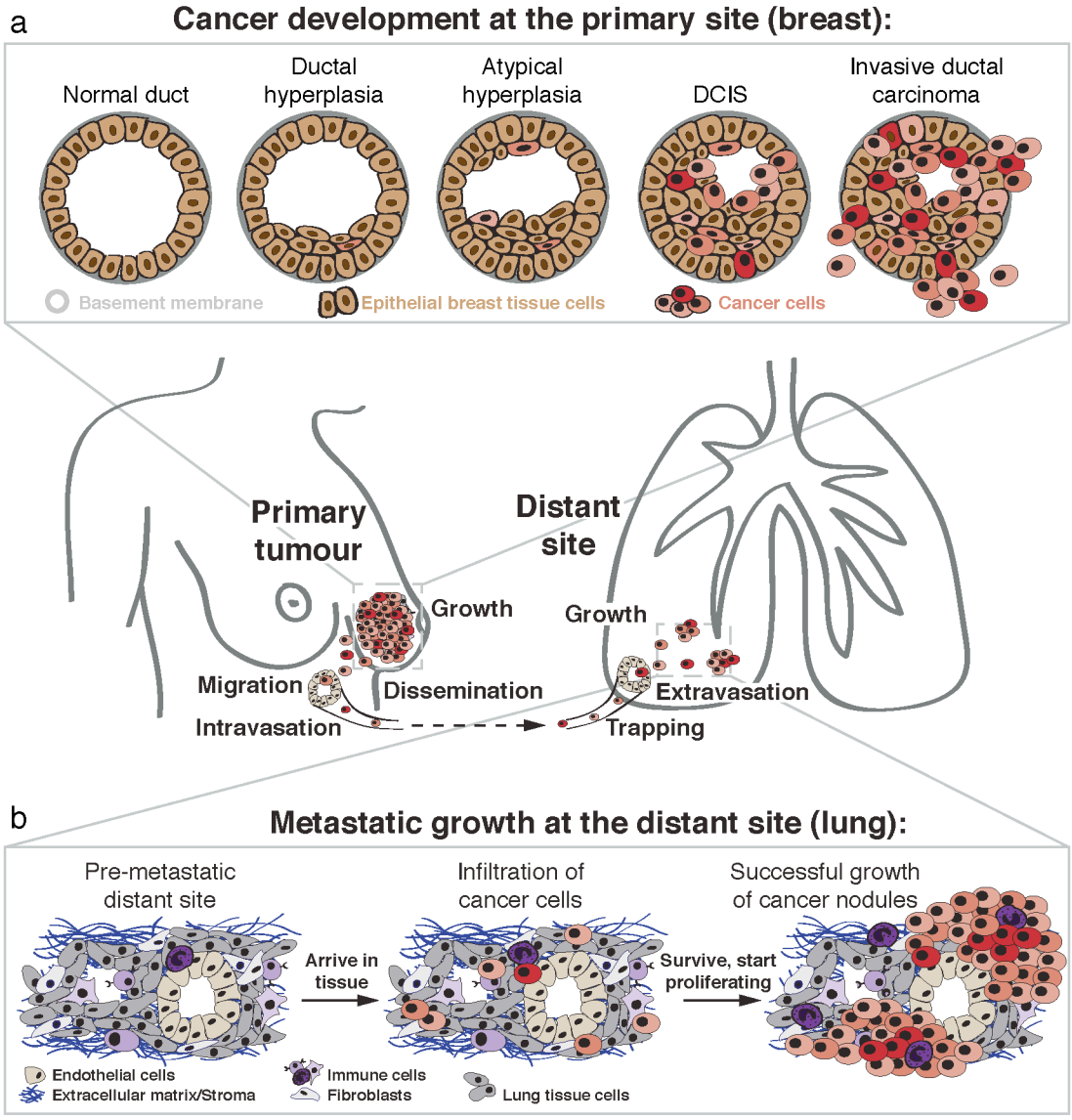


Figure 1-1 Breast cancer development and metastatic progression to the lung
(a) Mammary ductal carcinoma develops through pathologically distinct stages from the transformation of the normal duct to become hyperplastic up to an invasive carcinoma phenotype. Disseminated breast cancer cells then migrate through the vasculature to infiltrate secondary sites like the lung. (b) Arriving at the lung, these metastatic cancer cells have to survive, overcome dormancy and start proliferating in order to establish distant metastases. (a) adapted from (Brisken, 2013).

1.1.2 The principles underlying metastasis

Metastasis is the process when cancer cells leave the primary tumour, spread within the body and grow a secondary tumour usually at a distant site or another position within the same organ. To enable this process, individual carcinoma cells or cell clusters have to become migratory, disseminate from the epithelial primary tumour mass and intravasate into blood or lymphatic vessels. Cancer cells are then trapped or arrest within the vasculature at the target site, extravasate and start colonising the new tissue by proliferating to form distant metastases immediately or after a latency period (Steeg, 2006) (Fig.1.1 b). Metastatic spread is extraordinarily complex and very inefficient. Cancer cells are not only challenged to migrate through inhospitable environments away from the primary site, such as the blood stream, but also have to acquire the ability to grow in a foreign microenvironment (Luzzi et al., 1998). Several models were proposed to explain how cancer cells achieve metastatic spread. The most accepted model is the metastatic progression model based on a subpopulation of cancer cells gaining malignant and metastatic features over time. Other proposals suggest that all cancer cells retain metastatic potential, but are impaired due to their position (transient compartment model). Metastatic properties of a tumour might also be defined by certain mutations early during its development, rather than being acquired through progressive stages (Early oncogenesis model) (Hunter et al., 2008).

The importance of the tumour-associated stroma and microenvironment is extraordinarily highlighted in limiting or mediating the complex process of metastasis. Tumour-associated stromal cells were shown to be fundamental promoters of cancer cell invasion by multiple mechanisms. For example, the tumour microenvironment can foster single cell invasion of cancer cells by promoting their epithelial to mesenchymal transition (EMT) where epithelial carcinoma cells lose their polarity and cell-to-cell adhesion and gain migratory properties (Gao et al., 2012). Stromal cells at the primary tumour border were also shown to provide matrix-degrading enzymes or chemotactic signals that facilitate cancer cell invasion into the surroundings and intravasation into blood vessels (Quail and Joyce, 2013). Microenvironmental components can protect cancer cells from immune cell-mediated killing within the blood stream (Palumbo et al., 2005)

and aid their extravasation into distant tissue (Bendas and Borsig, 2012, Reymond et al., 2013). Cancer cells that accomplished metastatic spread and arrive at distant sites are then pressured to initialise metastatic colonisation, namely establishing metastatic colonies or micro-metastases that can grow to form macro-metastases. The enormous difficulty to achieve metastatic colonisation is best illustrated by the fact that numerous successfully disseminated, circulating cancer cells appear entirely unable to initiate distant metastases in many cancer types (Luzzi et al., 1998, Meng et al., 2004). Evidently, several of these disseminated cancer cells will not die, but enter in a viable state of reversible, long-term dormancy (Aguirre-Ghiso, 2007, Giancotti, 2013). In breast cancer, for example, these dormant metastatic cells can grow macro-metastases many years after surgical removal of the primary tumour (Marches et al., 2006), indicating the acquisition of additional properties of these cells over time to enable outgrowth. Intrinsic cancer cell dormancy is caused when metastatic cancer cells arrest in their cell cycle and become quiescent (Aguirre-Ghiso, 2007). Cancer cells can also find themselves in hostile surroundings during distant tissue colonisation where local inhibitory signals from the extracellular matrix or immune cells cause dormancy (Barkan et al., 2010, Teng et al., 2008), again stressing the importance of the microenvironment during metastatic colonisation. This idea and the ability of specific cancer cells to circumvent and benefit from a new microenvironment (Giancotti, 2013) explain many aspects of the tissue tropism of cancer cells for specific metastatic sites. The “seed-and-soil” hypothesis of preferential metastatic spread of certain cancer cell types to distinct secondary organs (Fidler, 2003) was clearly demonstrated by experiments isolating cancer cells from established macro-metastases. Upon re-inoculation, these metastasised cancer cells showed preferred and improved re-colonisation ability of the same organ where they originally grew metastases (Minn et al., 2005, Nguyen et al., 2009). These experiments also support the idea that metastatic cancer cells have to acquire additional, stable features to outgrow at secondary organs. In concert, transcriptional signatures correlating with the ability of metastatic cells to initiate distant organ colonisation were defined for many cancer types (Albini et al., 2008). Interestingly, these gene expression profiles also highlight the involvement of the metastatic microenvironment. Functional investigation of metastatic cancer cells with these signatures to give rise to metastases and their interaction with the microenvironment will hopefully improve

our mechanistic understanding of the dauntingly complex process of metastasis. Importantly, development of distant metastases poses an enormous risk in the clinic and remains the foremost cause for cancer-related mortality (Nguyen et al., 2009, Steeg, 2006), stressing the necessity to shed light onto it's complexity to develop effective treatments.

1.1.3 Molecular insights into genetic changes driving tumourigenesis

Cancers arise through a sequential accumulation of mutations in cancer cells in concert with a co-developing microenvironment. The ying and yang of intrinsic features necessary to be acquired by normal cells for successful cancer establishment comprise the ability to ensure continuous proliferation and cell cycle progression while avoiding cell death, apoptosis or senescence (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011). The best example how cancer cells manage to sustain proliferation is hijacking proliferation-inducing cellular pathways that are crucial during organogenesis, homeostasis and stress responses while circumventing their tight regulation. For example, very frequently human cancers display continuous stimulation or activation of mitogen-activated protein kinase (MAPK) pathways that control the cell cycle, cell survival as well as cell proliferation and allow integration of signals following extracellular stress (Samatar and Poulikakos, 2014). Ras guanosine triphosphate hydrolases (GTPases) have a central role in MAPK signalling as they cycle in an active GTP-bound state and inactive GDP (guanosine diphosphate)-bound state and trigger a phosphorylation cascade by activation of Raf kinases. Raf kinases subsequently phosphorylate MAPK/ERK (MEK) kinases that phosphorylate the MAPKs extracellular signal-regulated (ERK) kinases that translocate to the nucleus to activate target genes controlling growth-related mechanisms, predominantly cell proliferation (Dhillon et al., 2007) (Fig. 2.1). The phosphoinositide 3-kinase (PI3K)/ protein kinase B (Akt) pathway or the transcription factor c-Myc represent other frequently over-activated oncogenic features in cancer cells that promote continuous growth and proliferation (Fruman and Rommel, 2014, Gabay et al., 2014).

However, cell proliferation and survival are tightly controlled processes in normal physiology, raising the necessity for cancer cells to prevent concomitant induction

of apoptotic programmes or senescence. In fact, the master regulators of apoptosis and cell cycle arrest, p53, p21 and retinoblastoma (Rb) protein, are activated by growth-stimulatory or oncogenic signals, including Ras/MAPK signalling (Agarwal et al., 2001, Lavin and Gueven, 2006, Li et al., 2005, Ohtani et al., 2004). Loss-of-function of the tumour suppressor p53, a nuclear transcription factor is observed in over half of human cancers (Ozaki and Nakagawara, 2011). Thereby, its function to induce apoptosis in response to DNA damage or cellular stress via, for example, mitochondrial cytochrome C release and activation of caspases (Schuler et al., 2000) is lost, which allows malignant transformation. Additionally, mutant p53 proteins might also directly contribute to cancer progression (Muller and Vousden, 2014).

Cancer cells mostly ensure the continuation of their cell cycle by re-activation of telomerase to prevent telomere dysfunction-induced cellular senescence. Additionally, the energy metabolism is often altered in cancer cells to facilitate their high proliferative rates. Lastly, cancer cells frequently display features of genomic instability that is now an accepted hallmark of cancer (Hanahan and Weinberg, 2000, Hanahan and Weinberg, 2011).

In summary, accumulation of genetic features in cancer cells over time drives the initiation and many aspects of the progression of tumours from benign to malignant stages and different types of cancer cells utilise various approaches to ultimately achieve tumour growth.

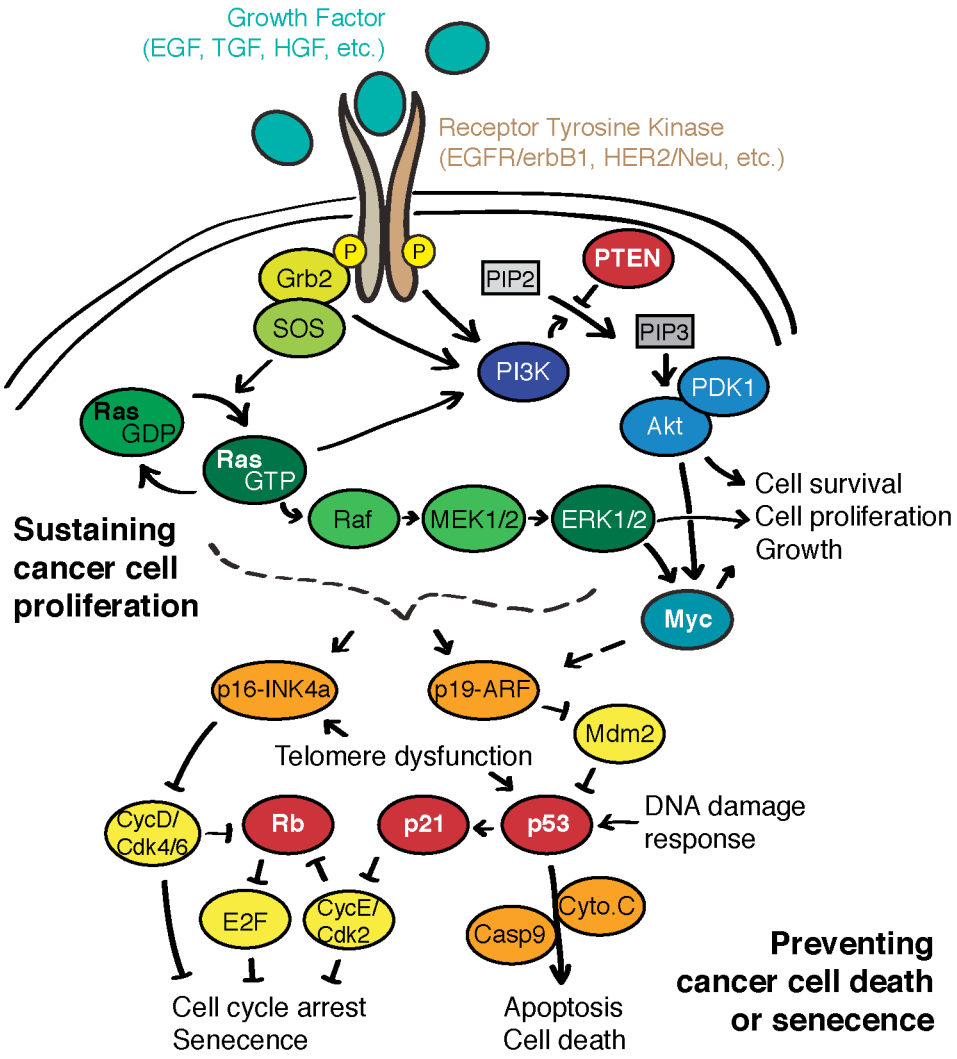


Figure 1-2 Cellular signalling cascades frequently altered in cancer cells
Overview of signalling molecules that are often deregulated in cancer cells. Growth factors bind to receptor tyrosine kinases that activate the Ras/MAPK signalling cascade via accessory molecules like Grb2 (Growth factor receptor-bound protein 2) and SOS (Son of sevenless). Alternatively, PI3K can be induced leading to phosphorylation of PIP (Phosphatidylinositol phosphates) and the activation of Akt by PDK1 (phosphoinositide dependent kinase 1). PI3K is counteracted by PTEN (Phosphatase and tensin homolog). Ras/MAPK and PI3K/Akt signalling often lead to activation of Myc and mechanisms to negatively control cell proliferation.
Telomere dysfunction, DNA damage, p16-INK4a (cyclin-dependent kinase inhibitor 2A, multiple tumour suppressor 1) and p19-ARF (ARF tumour suppressor) modulate the activity of the key regulators of cellular senescence: p53, p21, and Rb. This network involves factors such as Cyclins (Cyc), cyclin-dependent kinases (Cdk), E2F transcription factors, Mdm2 (Mouse Double Minute 2, p53 binding protein), caspase 9 (Casp9) and cytochrome C (Cyto.C). Figure adapted from (Fruman and Rommel, 2014, Gabay et al., 2014, Ohtani et al., 2004, Ozaki and Nakagawara, 2011, Samatar and Poulikakos, 2014, Schuler et al., 2000).

1.1.4 Cancer heterogeneity: Cancer stem cells and clonal evolution

1.1.4.1 The clonal evolution model in cancer

A key challenge for successful cancer therapy is the complexity of the disease comprised by the vast inter- and intra-tumoural heterogeneity. First reports of the existence of multiple, distinguishable cancer cell subpopulations within the same tumour mass date back to the 1960s and 70s (Dexter et al., 1978, Henderson and Rous, 1962, Heppner, 1984). Studies on functional intra-tumoural heterogeneity followed quickly reporting, for example, different proliferative capacity (Danielson et al., 1980, Saunders et al., 1967), resistance to chemotherapy (Heppner et al., 1978, Yung et al., 1982) and varying metastatic competence (Fidler and Kripke, 1977, Raz et al., 1980) among cancer cell subpools originating from the same cancer cell population.

Advanced sequencing techniques offered a possible explanation for this heterogeneity. The mutational landscape is highly variable between not only different tumour types but also within the same tumour, which might account for the observed inter- and intra-tumoural heterogeneity. This idea fuelled the historical clonal genetic evolution model of cancer development consistent with sequential accumulation of pro-tumourigenic mutations yielding malignant growth (Fig. 1.3 a). This acquisition of additional mutations in the initial genetic cancer cell clone would lead to sub-clonal genetic diversification that can be detected in different regions of the same tumour (Burrell et al., 2013, Gerlinger et al., 2012). According to the clonal evolution model, more tumourigenic subclones would outcompete other populations and even cause their disappearance over time while additional subclones arise (Greaves and Maley, 2012, Nowell, 1976). The nature of the acquired mutations can be essential for the tumourigenic properties of the cancer cell, a driver mutation, or a rather harmless passenger mutation probably a side-effect of genomic instability and stochastic mutations (Kreso and Dick, 2014, Stephens et al., 2012). Common driver mutations leading to cell expansion are usually found in the majority of cancer cells within the same tumour, despite considerable variation of further driver mutations. This observation suggests a primary genetic hit in a cancer-initiating cell clone, clonal expansion, and its maintenance in subclonal lineages (Gerlinger et al., 2012, Kreso and Dick, 2014).

Lineage mapping analysis of breast cancer development confirmed not only the presence of a dominant clonal lineage with its cytogenetic driver mutations being represented in more than half of the tumour cells, but also that the “most recent common ancestor” of this cell lineage always emerged at very early stages of development of every tested tumour (Nik-Zainal et al., 2012). Genetic cancer subclones were further shown to possess distinct metastatic organ tropism and cancer cells from metastatic lesions displayed additional mutations to the initial parental clone (Campbell et al., 2010, Yachida et al., 2010). However, the functional benefits of individual mutational landscapes of different genetic cancer cell subclones to their tumourigenicity remains largely elusive, probably due to sheer number of acquired mutations and difficulty to distinguish between driver and passenger mutations (Garraway and Lander, 2013). Nevertheless, the clinic faces the challenge of moderate efficacy of therapy, highly variable responses within very similar tumour types and, especially, high resistance and relapse probably due to cancer heterogeneity (McGranahan and Swanton, 2015). Indeed, rigid and mostly irreversible somatic mutations might form the essential basis for cancer initiation and influence progression. However, recent studies showed that developmental, epigenetic and microenvironment-associated determinants strongly contribute to cancer progression, suggesting that there is more to the tumourigenic abilities of individual cancer cells and functional cancer heterogeneity.

1.1.4.2 Concepts of cancer stem cells, tumour- and metastasis-initiating cells

Another likely explanation for the pronounced intra-tumoural heterogeneity also initiated in the 1970s and 80s with studies on haematological and solid cancers based on observations that cancers appear to have a hierarchical organisation highly reminiscent of a normal organ (Bennett et al., 1978, Clarkson et al., 1967, Kreso and Dick, 2014, Pierce and Speers, 1988). Tissues are tightly controlled structures organised into functionally different entities or cell types with usually very infrequent tissue stem cells residing at the top of a hierarchy. Their self-renewal ability ensures the maintenance of the organ by differentiation of its daughter cells into all tissue cell lineages present within the organ. Additionally, activation and differentiation of tissue stem cells lies at the base of tissue regeneration after injury

(Clevers et al., 2014, Nelson and Bissell, 2006, Roh and Lyle, 2006). Cell fate, differentiation and self-renewal abilities are not controlled by genetic mutations, but by epigenetic mechanisms like histone and DNA modifications or post-transcriptional regulation by, for example, microRNAs. Epigenetic deregulation and thereby non-genetic diversification of cancer cell subsets appears to constitute a similar organisation in tumours with accordingly termed “cancer stem cells” (CSCs) being responsible for its maintenance (Baylin and Jones, 2011, Easwaran et al., 2014, Iorio and Croce, 2012, Kreso and Dick, 2014). Additionally, mutations regulating epigenetic programmes were found in human cancers with high prognostic value and these epigenetic programmes do not only underlie the function of normal stem cells, but also “cancer stemness” in liquid cancers (Kreso and Dick, 2014, Abdel-Wahab and Levine, 2010, Shah and Licht, 2011). Indeed, rare subpopulations of cancer cells within a plethora of tumour types like acute myeloid leukaemia (Bonnet and Dick, 1997), breast (Al-Hajj et al., 2003) and pancreatic cancer (Hermann et al., 2007) can be distinguished by their extraordinary degree of malignant, tumourigenic competence compared to more differentiated cancer cells. This notion draws a picture of an organised hierarchy of malignancy within cancer cells, despite having lost the tight control observed in normal tissues (Fig. 1.3 c). Their abilities include the initiation potential for a primary tumour upon transplantation that is completely reminiscent of the original tumour. This ability suggests a self-renewal and differentiation competence of CSCs to give rise to all cell types to entirely reconstitute their organ of origin, the tumour (Kreso and Dick, 2014, Nguyen et al., 2012). For example, 100-200 CD44+/CD24- primary breast cancer cells formed tumours with phenotypic and histologic diversity comparable to the parent tumour upon grafting onto immunodeficient mice, while about 100-fold more CD44+/CD24+ cells were necessary (Al-Hajj et al., 2003). This profound capability for tumour initiation of only a small subset of cancer cells (about 15% in the mentioned study of breast cancer) highly reflects the maintenance and regeneration potential of normal stem cells within a tissue. Further, CSCs appear to be resistant to many types of anti-cancer therapies, which target highly proliferative cells due to their rather quiescent nature (Chen et al., 2012, Dean et al., 2005). They have also been shown to drive metastatic spread, being especially well equipped to initiate a secondary tumour at a distant site (Oskarsson et al., 2014).

The variation of cellular states that correlate with the functional ability of a cell within a tumour can be expressed by the analysis of gene expression profiles that reflect genetic mutational as well as epigenetic modifications. For example, one study described colon tumours to display a very similar heterogeneous diversity in transcriptional activity and cell morphology compared to normal colon tissue. There, maintained signatures of stem/progenitor cells and other cell types were found in colon tumours that indicate differentiation into multiple lineages (Dalerba et al., 2011). This transcriptomic diversity was maintained even when the tumour originated from a single genetic subclone (Kreso et al., 2013). Moreover, transcriptional signatures or properties of normal stem cells or CSCs are highly predictive of poor prognosis for a wide range of cancer types (Eppert et al., 2011, Gentles et al., 2010, Merlos-Suarez et al., 2011, Pece et al., 2010). Also, the activation of the same pathways is frequently associated with stemness potential as well as self-renewal of cancer cells, such as Wnt, NFkB and Notch signalling (Holland et al., 2013, Shostak and Chariot, 2011, Takebe et al., 2011, Wang et al., 2012). This raises the interesting possibility that the functional features ascribed to CSCs, including enhanced tumour initiation potential, might represent the ultimate goal of varying driver mutations in different contexts.

However, there is growing confusion and controversy about the term “cancer stem cells” and their precise abilities. This issue is largely a caveat of varying definitions of CSCs and subsequent differences in testing tumourigenic stemness potential among laboratories and the use of simple, but non-sufficient surrogate assays. Stemness defines the capability of a cell to initiate multi-lineage differentiation while maintaining its own state of potency (self-renewal) mostly by some sort of asymmetric cell division generating daughter cells of different cell fate (Kreso and Dick, 2014). The underlying, principal molecular mechanisms for stemness are based on expression and regulation of transcription-controlling factors like Oct4, Nanog and Sox2 (embryonic stem cells) (Martello and Smith, 2014), activation of stemness-associated signalling pathways like Wnt, NFkB and Notch pathways (Holland et al., 2013, Reya and Clevers, 2005, Shostak and Chariot, 2011, Wang et al., 2012), the post-transcriptional regulation of gene expression by, for example, microRNAs (Liu and Tang, 2011, Mathieu and Ruohola-Baker, 2013) and, especially, the epigenetic landscape of a cell (Easwaran et al., 2014, Lunyak and Rosenfeld, 2008, Yamada and Watanabe, 2010). However, these molecular

stemness programmes remain rather poorly understood and frequently their contributions to the tumourigenic potential of a cell have yet to be experimentally proven. Hence, the assessment of tumourigenic stemness potential has to be a functional test of the ability of individual cells (Kreso and Dick, 2014). It is a technical challenge (if not currently impossible) to monitor cancer cell self-renewal or the functions of non-CSCs and thereby cell differentiation within the tumour mass. In part, this might be due to the fact that a tumour does not serve a particular purpose within an organism like a normal organ and that “cancer stemness” appears to be a state of “stemness potential” that a cancer cell can find itself in rather than a stable cell population within a tumour. Hence, individual cells stemming from a tumour have to be identified and their individual potential tested at a given time to establish differential tumour initiation competence among cancer cells and thereby a hierarchy within tumours. The gold standard consists of assessment of clonal reconstitution or repopulation ability of single cells ideally in long-term serial passaging *in vivo*. However, this xenograft assay only determines clonal tumour initiation ability and does not formally test for self-renewing potential. Consequently, together with a frequently plastic nature of highly potent tumour cells, terming these cancer cells “tumour-initiating cells (TICs)” and thereby with the actual function that is being tested for rather than CSCs appears more appropriate and avoids confusion (Kreso and Dick, 2014).

Cytometric cell sorting technology based on fluorescent antibody-mediated labelling of cell surface markers on individual cells allowed assessment of functional properties of individual tumour cells (Bonner et al., 1972). Cells with tumour initiation potential upon serial transplantation (TICs) and, importantly, cells without this ability (nonTICs) where identified in many human and mouse tumour types (Al-Hajj et al., 2003, Bonnet and Dick, 1997, Cho et al., 2008, Hermann et al., 2007, Malanchi et al., 2008, O'Brien et al., 2007, Singh et al., 2014). Other ways to identify TICs are constantly developed such as cell sorting based on microRNA levels (Amendola et al., 2013), cellular reporters for stem cell-associated signalling like Wnt (Vermeulen et al., 2010) or activity of efflux transporters and detoxifying enzymes like aldehyde dehydrogenase 1 (ALDH1) (Ginestier et al., 2007, van den Hoogen et al., 2010). However, the xenograft assay is accompanied by its own technical limitations including the tough conditions to obtain single cell suspensions, different tissue microenvironments and milieu of secreted factors especially upon

ectopic cell grafting or transplantation of human cells. Large efforts went into improving the conditions of this transplantation assay, which is of central importance for the CSC concept of cancer heterogeneity (Kreso and Dick, 2014, Rongvaux et al., 2013).

Importantly, there are studies using xenotransplantation that suggest a rather homogeneous potential of the total cancer cell population to reconstitute a tumour under certain conditions (Joo et al., 2008, Quintana et al., 2010, Quintana et al., 2008). These observations raise the semantic question of the actual nature of tumour initiation and maintenance potential. How much is it a purely intrinsic feature of a cell to self-renew and differentiate or how much should tumour initiation potential also depend on the microenvironment that every tumour finds itself in. This idea is highlighted by the importance of the local microenvironment or niche in maintaining and influencing (cancer) stem cell populations even in patients (Borovski et al., 2011, Li et al., 2013, Sneddon and Werb, 2007, Ye et al., 2014). Hence, it is under debate if providing a less restrictive environment for cancer cells to grow in a new host is actually of benefit for testing tumour initiation potential or prevents the functional differences seen between cell populations under more stringent conditions (Malanchi, 2013).

All these described observations point towards the notion that not all CSCs within a tumour have equal competence to initiate and maintain a tumour at the site of origin, cause metastatic spread and initiation of metastases at secondary organs and chemotherapy resistance. More likely appears a scenario with heterogeneity among CSC populations especially for the ability to grow in very different microenvironments. It is therefore important to move away from the general “cancer stem cell” term towards a classification and nomenclature according to the actual tested activity of a cell like tumour-initiating, drug-resistant or metastasis-initiating cell (Valent et al., 2012).

The metastasis-initiating ability ascribed to CSC-like cells and the nature of these cells is by far less well characterised compared to their tumour initiation potential, which appears surprising given the high clinical relevance of metastatic progression. The existence of small cell populations within the total tumour mass with notable metastatic competence was already reported more than 30 years ago (Fidler and Kripke, 1977, Raz et al., 1980). The apparent clonal origin of metastases

(Talmadge et al., 1982) suggested individual cancer cells with enhanced metastatic activity to initiate distant metastases. However, this clonality of individual metastases would not exclude the existence of different metastasis-initiating cell (MIC) subpopulations within the same heterogeneous tumour (Campbell et al., 2010, Yachida et al., 2010). MICs have been functionally identified by grafting cancer cells from a primary tumour onto a secondary site and determination of their ability to outgrow. They can be distinguished from nonMICs in several cancer types in mouse models and human primary cancer or cancer cell lines including breast (Liu et al., 2010a, Malanchi et al., 2012), prostate (Hermann et al., 2007, van den Hoogen et al., 2010), renal (Khan et al., 2014) and colorectal cancer (Pang et al., 2010). Overall, MICs appear to be a rarer population among total cancer cells than TICs (Hermann et al., 2007, Oskarsson et al., 2014), suggesting the acquisition of additional driver mutations or a distinct epigenetic state which might also dictate the tissue affinity or organ tropism of MICs (Albini et al., 2008, Campbell et al., 2010, Minn et al., 2005, Yachida et al., 2010). Metastatic dissemination has been shown to commence at very early stages of tumourigenesis in a “parallel progression model” (Klein, 2009, Rhim et al., 2012), arguing against the clonal evolution theory and suggesting presence of CSC-like cells with enhanced metastatic competence in less evolved tumours. Nevertheless, indications of a late emergence of metastatic cells even within the same tumour types exist (Yachida et al., 2010). Moreover, MICs might have to acquire further competences after dissemination especially with regard to the ability to overcome cell dormancy and grow within the distant microenvironment (Marches et al., 2006). In fact, compelling evidence for an intrinsically highly potent metastasis-initiating cell subpopulation within a primary tumour mass comes from “stemness” or “metastatic” transcriptional signatures that correlate with poor prognosis, metastatic progression and relapse in the clinic (Albini et al., 2008, Eppert et al., 2011, Merlos-Suarez et al., 2011, Patsialou and Condeelis, 2014, Pece et al., 2010, Ramaswamy et al., 2003, van den Hoogen et al., 2010, Weigelt et al., 2005). These functionally relevant signatures are present in limited numbers of cells within the primary tumour, but are enriched among circulating and metastatic cancer cells and correlate with poor prognosis and metastatic incidence (Aktas et al., 2009, Yu et al., 2013).

Collectively, all this evidence strongly suggests the existence of intrinsically highly metastatic cancer cell subpopulations – MICs – within the primary tumour or among

circulating tumour cells. In fact, these small cell populations were identified, isolated and functionally tested in metastasis initiation assays, proving their formidable metastatic competence compared to nonMICs (Baccelli et al., 2013, Charafe-Jauffret et al., 2010, Khan et al., 2014, Malanchi et al., 2012, Pang et al., 2010, van den Hoogen et al., 2010).

1.1.4.3 Merging cancer stem cells with clonal evolution

The clonal evolution model and the hierarchical organisation/cancer stem cell model appear insufficient to explain all aspects of scientific evidence addressing the significant heterogeneity among cancer cells and its functional consequences (Fig. 1.3 a+c). There was historically little overlap between the two points-of-view and only rarely an incorporation of genetic, epigenetic and functional analysis within the same study or system (Kreso and Dick, 2014). Genetically identical subclones have to be tested for their diversity in function and transcriptional signature and CSCs (thereby long-term repopulating cells) should be analysed for their genetic landscape. Several studies report the existence of different genetic subclones within functionally defined TICs in acute lymphoblastic leukaemia (Anderson et al., 2011, Clappier et al., 2011, Notta et al., 2011) and genetic diversity was described among cancer cells that initiated metastasis (Campbell et al., 2010, Yachida et al., 2010). Importantly, certain mutations even correlated with altered functional competences of cancer cells and frequency of TICs. In fact, genetic mutations of epigenetic regulators were shown to increase cancer stemness, initiation and self-renewal abilities (Kreso and Dick, 2014, Notta et al., 2011). Also the phenotype of TICs appears to be influenced by genetic diversity. For example, mouse lung tumours or human colon cancers with a different genotype or driver mutation show variations in the phenotype of their TICs as observed by different surface marker expression (Curtis et al., 2010, Sahlberg et al., 2014). These observations suggest a possible clonal evolution of CSCs or cells in an epigenetic “stemness” state capable of self-renewal and raise the question of the cancer cell of origin. A few reports point towards an initial genetic hit within a normal stem cell (Barker et al., 2010, Malanchi et al., 2008, Shlush et al., 2014, Visvader, 2011, Woll et al., 2014). Also, transcriptional signatures of normal stem

cells, TICs and MICs appear highly similar and integrated into a common signature of “stemness” that showed potent prognostic value (Albini et al., 2008, Eppert et al., 2011, Gentles et al., 2010, Merlos-Suarez et al., 2011, Patsialou and Condeelis, 2014, Pece et al., 2010, Ramaswamy et al., 2003, Weigelt et al., 2005). However, stochastic accumulation of mutations leading to continuous clonal evolution of cancer cells towards increased tumourigenicity can occur in TICs and nonTICs as well as MICs and nonMICs (Kreso and Dick, 2014, Oskarsson et al., 2014). Hence, genetic mutations or other epigenetic alterations might provide nonTICs with self-renewal and repopulation competences and this notion would explain the plasticity of the CSC state rather than a rigid cell population. For example, forced Wnt activation in normal colon cells caused their dedifferentiation and acquisition of TIC competences (Schwitalla et al., 2013). Recently, an attempt was made to integrate the concept of clonal evolution and hierarchical organisation on the basis of these observations by combining genetic and functional properties of cancer cells (Kreso and Dick, 2014) (Fig. 1.3 b). The authors proposed a dynamic model with a clearly defined functional hierarchy among cancer cells similar to the normal organ at early stages of tumourigenesis. Increasing acquisition of mutations in TICs that contain long-term repopulation ability might enhance their self-renewing potential with tumour progression leading to the expansion of the TIC pool within the tumour. In a similar fashion, nonTICs might gain functional characteristics of TICs by accumulation of somatic mutations and thereby contribute to increased TIC frequencies within the tumour. Moreover, TICs might acquire further malignant competences. The expansion of cell pools with TIC function within the tumour (that might be genetically different) would indicate enhanced malignancy, aggressiveness and therapy resistance, consistent with published evidence. Also, increased frequency of TIC subpopulations suggests a progressively shallow hierarchical organisation of a tumour while it advances and goes hand in hand with cancer evolution towards malignancy. This cancer cell evolution might yield very advanced TIC clones with ultimate self-renewing ability resulting in such a high TIC frequency that basically constitutes a functionally homogeneous, highly potent and malignant tumour (Kreso and Dick, 2014). In the same manner, this hypothesis might also be true for MICs and nonMICs, especially since increased MIC frequency among (circulating) cancer cells correlated with increased metastatic incidence (Baccelli et al., 2013, Charafe-Jauffret et al., 2010). However, the

development and evolution of MICs is very likely strongly influenced by the metastatic microenvironment (Borovski et al., 2011, Sneddon and Werb, 2007, Ye et al., 2014).
In summary, for a convincing linkage of the cancer stem cell and clonal evolution models, technically extremely challenging experiments will be necessary (Kreso and Dick, 2014). Further research should determine the frequencies of TICs and MICs in tumours from early to advanced stages as well as their genetic and functional evolution in combination with lineage tracing approaches to monitor nonTIC to TIC or nonMIC to MIC conversion.

Ultimately, it emerges impossible to ignore the scientific evidence for genetic branching evolution of cancer cell subclones by genetic mutations during tumour progression. Also, the existence of genotype-independent functional diversity among cancer cells has to be acknowledged, with certain cell subsets retaining unique tumour or metastasis initiation and reconstitution potential reminiscent of the abilities of normal stem cells. Hence, more sophisticated approaches will be necessary to increase our understanding of the emergence and consequences of functional cancer heterogeneity to allow therapeutic targeting of the cancer cell subpopulations that matter.

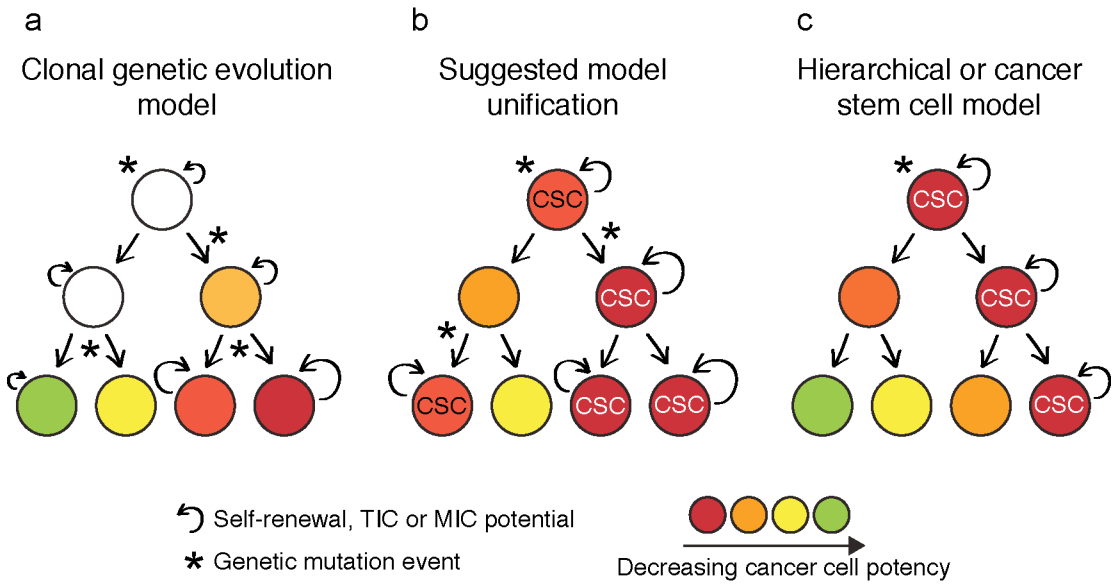


Figure 1-3 Models explaining heterogeneity among cancer cells
Schematic representation of the “clonal genetic evolution” model (a), the “hierarchical or cancer stem cell” model (c) and the proposed “unified evolution of cancer stem cells” model (b). Figure adapted from (Kreso and Dick, 2014).

1.2 Tumour microenvironment, angiogenesis, cancer-associated inflammation and immunity

Cancer arises in host tissues and, as such, is not only composed of tumour cells but lies within a cancer-specific and complex environment that reacts to the oncogenic disturbance and evolves with the growing tumour to form a structure reminiscent of normal organs (Hanahan and Coussens, 2012). This tumour microenvironment (TME) is the usually genetically unaltered, non-cancer cell part of the tumour consisting of numerous cell types like stromal, endothelial, inflammatory or other immune cells as well as extracellular matrix (ECM) components, soluble and matrix-bound signalling factors like cytokines, chemokines, developmental and growth factors. The composition and activation of the TME varies remarkably between tumour types and even within the same tumour reflecting the dynamic nature of the TME (Quail and Joyce, 2013). Cancer progression is characterised by continuous cross talk between the tumour cells and TME components, which is known to favourably influence almost all hallmarks of cancer. The TME even entirely constitutes some of these hallmarks or enabling characteristics like neo-angiogenesis and tumour-promoting inflammation (Hanahan and Coussens, 2012, Hanahan and Weinberg, 2011). Moreover, the TME is involved in inducing and regulating stemness of cancer cells in specialised environments accordingly termed cancer stem cell niches (Plaks et al., 2015). The dependency of cancer cells on a promoting stroma is especially highlighted during metastatic spread and colonisation, which is unlikely to be achieved solely in a cell-autonomous fashion (Quail and Joyce, 2013). In fact, primary tumours even educate distant tissue to form permissive pre-metastatic niches for the arrival of disseminated cancer cells which contributes to organ tropism together with cancer cell-intrinsic factors and pre-existing features of specific tissues (Sceneay et al., 2013).

The increasing understanding of intrinsic alterations in cancer cells, including genetic mutations or epigenetic regulation, allowed the development of a plethora of therapeutic approaches targeting key players in tumourigenicity like the Ras/MEK/ERK or PI3K/AKT pathway (Fruman and Rommel, 2014, Samatar and

Poulikakos, 2014). Despite these achievements in the development of drugs efficiently targeting cancer cell-intrinsic features, treatments are often ineffective or fail due to resistance and relapse. We know today that the TME plays a crucial role not only in promoting cancer progression, but also susceptibility to therapy and can cause resistance (Junttila and de Sauvage, 2013, Olson and Joyce, 2013). Hence, the pro-tumourigenic and malignancy-promoting functions of the TME provide a promising target for therapeutic intervention due to the lack of genetic instability. However, the TME can also have normalising and adverse effects for a growing tumour leading to suppression of its growth and eradication. Enhancing these features of the TME or re-education of a permissive TME to an anti-tumourigenic function offers another line of developing cancer treatments that is creating global excitement (Quail and Joyce, 2013).

1.2.1 Cells and factors involved in angiogenesis, hypoxia and coagulation

A tumour is dependent on supply of nutrients, oxygen, mitogenic and other factors from the bloodstream to sustain its growth. The dependence of cancer on angiogenesis or vascularisation was first proposed in 1971 (Folkman, 1971). An angiogenic switch is necessary for tumour nodules to reach sizes larger than 1-2mm in diameter (Naumov et al., 2006) because of the limited diffusion of oxygen within tissues (MacDougall and McCabe, 1967).

Blood vessels are formed by tube-like structures of a vascular endothelial cell layer held together by tight cell-cell and cell-ECM interactions to ensure vessel integrity. These channels are coated by pericytes (or vascular smooth muscle cells) that control stability, vessel maturity and perfusion. A basement membrane between endothelial cells and pericytes provides additional structural support (Carmeliet and Jain, 2011). Existing vasculature is activated to form new vessels by sprouting angiogenesis and vascular branching or other mechanisms. These activating signals include a plethora of soluble and membrane-bound factors and their receptors on endothelial cells, such as vascular-endothelial growth factors (VEGFs), platelet-derived growth factor (PDGF), fibroblast growth factors (FGFs), angiopoietins and Tyrosine kinase with immunoglobulin-like and EGF-like domains (TIE) receptors, transforming growth factor (TGF)-beta, Notch/Delta and Wnt

signalling. Endogenous inhibitors of neovascularisation like thrombospondins (TSPs) or the statins angiostatin and endostatin also bind transmembrane receptors on endothelial cells and prevent unnecessary vessel sprouting (Bergers and Benjamin, 2003, Bornstein, 2009, Carmeliet and Jain, 2011). Some tumours co-opt existing blood vessels to allow initial growth (Holash et al., 1999), but predominantly tumours imbalance the control of angiogenesis to permanent and aberrant activation. This process is called the angiogenic switch and leads to the formation of tumour blood vessels that remain quite distinct from their normal counterparts. Tumour neo-vasculature contains irregular, distorted, dilated and pre-mature vessels that show excessive branching with dead ends. The interactions between endothelial cells and pericytes become interrupted leading to increased permeability, haemorrhaging and irregular blood flow (Bergers and Benjamin, 2003, Carmeliet and Jain, 2011, Naumov et al., 2006). Moreover, newly recruited bone marrow-derived endothelial precursor cells as well as tumour cells “mimicking” endothelial cells were reported to form part of the wall of tumour vessels or even entire channels (Folberg et al., 2000, Lyden et al., 2001). The poorly organised tumour vasculature appears very dynamic, but with limited functionality and often hinders supply of anti-cancer drugs or infiltration of anti-tumour immune cells (Chen et al., 2003a, Jain, 2005). Likely it is the tumour-individual balance between angiogenesis-stimulating or inhibiting signals that accounts for the variations in integrity of the tumour vasculature and its aberrant nature (Hanahan and Coussens, 2012, Hanahan and Weinberg, 2011). A consequence of the uncontrolled tumour growth and predominantly insufficient tumour vasculature is the creation of different microenvironments with varying levels of hypoxia and oxygen availability within the tumour. In general, the core of a tumour is associated with increased hypoxia that strongly attracts stromal and inflammatory cells (Quail and Joyce, 2013).

The inhibition of neo-angiogenesis during tumour progression by blocking VEGF or a normalisation of the tumour vasculature to improve drug delivery were the basis for the development of several anti-cancer therapies that showed clinical success (Chung et al., 2010, Gasparini et al., 2005, Jain, 2005), however not without its pitfalls. Frequently, the effects of blocking angiogenesis appear temporal or transitory and might even drive tumours into enhanced invasiveness and metastatic spread (Bergers and Hanahan, 2008, Ebos et al., 2009).

1.2.1.1 Endothelial cells

Endothelial cells are not only the structural building blocks of blood vessels, but directly influence cancer cell proliferation by secretion of growth-stimulating factors and provide cancer cell supportive niches (Butler et al., 2010). For example, endothelial cells emerge as important niches maintaining cancer cell "stemness" via cell-cell contacts, soluble factors and deposition of distinct ECM components (Butler et al., 2010, Plaks et al., 2015). However, endothelial cells can also limit cancer cell proliferation, invasion and metastatic potential in a partially perlencan-dependent manner (Franses et al., 2011). Another study showed that endothelial cells in mature, organotypic blood vessels kept metastatic breast cancer cells quiescent via secretion of TSP-1. In sprouting neo-vessels, endothelial cell-derived TSP-1 was lost, which led to enhanced metastatic cancer cell outgrowth associated with TGF-beta1 and periostin (Ghajar et al., 2013). Interestingly, hypoxia-induced expression of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha isoforms in endothelial cells has metastasis-promoting or inhibiting effects, respectively. This opposing role of HIFs is due to differential nitric oxide production that influences endothelial transmigration by tumour cells (Branco-Price et al., 2012). Hence, the pleiotropic pro- or anti-tumourigenic roles of endothelial cells might be microenvironment and context dependent.

1.2.1.2 Platelets and coagulation factors

As non-nucleated products of cytoplasm of megakaryocytes, platelets are the smallest haematopoietic cells in the circulation. Platelets are covered with integrins and glycoproteins essential for adhesion and platelet aggregation. They are activated by numerous stimuli *in vivo*, for example thrombin and collagen during thrombosis, and are essential mediators of coagulation and haemostasis (Bambace and Holmes, 2011).

Tumour cells have been shown to directly activate platelets in the circulation via cell-to-cell contact or secretion of paracrine factors like tissue factor (TF) causing tumour cell-induced platelet aggregation (Gay and Felding-Habermann, 2011). Tumour cell-derived TF generates thrombin that recruits platelets by engaging their surface receptor protease-activated receptor 4 (PAR4) (Ruf et al., 2011). Through

tumour cell-platelet aggregation, platelets provide a permissive environment for disseminated cancer cells in the blood steam and, for example, protect tumour cells from being recognised and eliminated by natural killer (NK) cells via a physical platelet-composed shield and secretion of fibrinogen (Gay and Felding-Habermann, 2011, Palumbo et al., 2005). Platelets also drive metastasis by directly promoting cancer cells and were shown to favour their survival by activation of NFkB signalling and cancer cell EMT features by secretion of TGF-beta (Labelle et al., 2011). Additionally, cancer cell extravasation is facilitated by platelets that become activated at sites of endothelial retraction via interaction if their integrins with the exposed collagen of the basement membrane. Platelet activation causes stimulation of coagulation and fibrin clot formation that facilitates association of tumour cells (Gay and Felding-Habermann, 2011).

In fact, high platelet count correlates with poor patient prognosis in various cancer types (Gay and Felding-Habermann, 2011) and coagulation factors including TF are strongly activated in cancer patients (Kakkar et al., 1995). These observations highlight the pro-tumourigenic functions of platelets especially in providing a niche for metastatic cells in the circulation.

1.2.2 Mesenchymal cells and structural components of the tumour microenvironment

1.2.2.1 Cancer-associated fibroblasts

Fibroblasts are spindle-shaped stromal cells within connective tissue that show pronounced diversity depending on their tissue location. They have multiple functions like secretion and deposition of ECM and basement membranes, controlling epithelial cell differentiation as well as regulation of immune responses and frequently show anti-tumourigenic functions (Kalluri and Zeisberg, 2006). In the context of cancer, fibroblasts display an aberrantly activated phenotype and exhibit characteristics of myofibroblasts, such as exacerbated proliferation, increased secretion of ECM components and growth factors. These altered transcriptional signatures of cancer-associated fibroblasts (CAFs) show high prognostic value in the clinic (Herrera et al., 2013, Madar et al., 2013, Navab et al., 2011) and CAFs

are present in very high numbers in the TME of, for example, breast cancer (Kalluri and Zeisberg, 2006, Sappino et al., 1988).

The origins of CAFs are not restricted to alternative activation of fibroblasts by tumour cells, despite this being demonstrated for example via the fibroblast activation mediators TGF-beta, PDGF and FGF2 (Elenbaas and Weinberg, 2001, Mueller et al., 2007, Ronnov-Jessen and Petersen, 1993). Several studies suggest subpopulations of CAFs to emerge from TGF-beta-induced EMT of endothelial, epithelial and even tumour cells. Other reported CAF sources include mesenchymal stem cells, smooth muscle cells, pericytes and adipose tissue-derived stem cells. The difficulty to define specific surface markers for CAFs is a likely consequence of the heterogeneous origins of CAFs, adding another level of complexity in studying these cells (Kalluri and Zeisberg, 2006, Madar et al., 2013).

The activation state and behaviour of CAFs are strongly influenced by microenvironmental factors. For example, cancer cell-derived tumour necrosis factor (TNF)-alpha, interleukin (IL)-1 and epidermal growth factor (EGF) modulate the activation status and secretome of CAFs (Madar et al., 2013) and increased matrix stiffness enhances pro-tumourigenic activities of CAFs via Yap/Taz transcription factor activation (Calvo et al., 2013). Moreover, although stromal cells are usually associated with genomic stability, fibroblastic-cells in the TME were reported to carry diverse somatic mutations (Patocs et al., 2007, Wernert et al., 2001).

In the TME, CAFs have been shown to have multiple, pro-tumourigenic features likely affecting cancer initiation and progression. CAFs are an important source of ECM components and remodel the tumour-associated matrix. Moreover, they induce EMT in cancer cells, regulate cancer “stemness” and promote invasion and metastasis. Also, CAFs produce tumourigenesis-promoting growth factors and modulate the metabolism of cancer cells, induce angiogenesis via production of VEGF and secrete pro-inflammatory mediators that enhance tumourigenesis and inhibit anti-tumour immunity (Hanahan and Coussens, 2012, Kalluri and Zeisberg, 2006, Ohlund et al., 2014, Quail and Joyce, 2013). For example, CAFs produce several mitogenic growth factors including hepatocyte growth factor (HGF), EGF, and FGFs with the ability to promote cancer cell proliferation (Ohlund et al., 2014). CAF-derived HGF also induces chemotherapy resistance via induction of PI3K signalling in lung cancer cells (Ying et al., 2015) or alternatively, CAF-dependent

therapy insensitivity of prostate cancer cells was caused by the CXCL12/CXCR4 axis (Domanska et al., 2012).
Collectively, the complexity of the origin and heterogeneity among CAFs is only outperformed by the sheer avalanche of CAF-associated functions to exacerbate tumourigenesis. CAFs directly influence cancer cells, other stromal cells in the TME or the ECM and thereby the entire environment. In fact, CAFs were shown to potently affect virtually every hallmark of cancer (Hanahan and Coussens, 2012), highlighting their importance in the context of cancer.

1.2.2.2 Mesenchymal stem cells

Mesenchymal stem cells (MSCs) reside in the bone marrow and can differentiate into mesenchymal cells including adipocytes, chondrocytes or osteoblasts. Interestingly, they are mobilised to tumour and metastatic tissues where they play a controversial role and can both, promote and suppress cancer progression (Yagi and Kitagawa, 2013).
Foetal and adult bone marrow-derived MSCs facilitated growth of subcutaneously transplanted cell lines (Zhu et al., 2006) and, in breast cancer, MSCs in the TME were shown to increase cancer cell motility and metastatic competence via a direct paracrine CCL5/CCR5 axis (Karnoub et al., 2007). In contrast, MSCs co-grafted with glioma cells reduced tumour growth by limiting angiogenesis, likely via reduction of PDGF (Ho et al., 2013). MSCs also suppressed Kaposi's sarcoma growth by inhibition of AKT activation (Khakoo et al., 2006). Interestingly, MSCs also appear to influence cancer "stemness" and increased the frequency of CD133+ cancer stem cells-like cells in a gastric carcinoma cell line *in vivo* via induction of Wnt signalling (Nishimura et al., 2012).

1.2.2.3 Adipocytes and adipose tissue

Dysfunctional adipose tissue and adipocyte-derived cytokines such as Lectin and Plasminogen activator inhibitor-1 (PAI-1) promote cancer cell proliferation. For example, they can induce ERK1/2 or PI3K signalling in cancer cells and were shown to promote tumour growth and counteract apoptosis in various cancer types

(Prieto-Hontoria et al., 2011). Cancer-associated adipocytes show lipolysis, phenotypically exhibit a fibroblastic shape, remodel the ECM, induce inflammation and appear to promote progression of breast cancer via direct cross talk with cancer cells (Tan et al., 2011). Importantly, adipose tissue invasion of ductal breast carcinoma cells associated with poor outcome and increased metastasis in the clinic (Yamaguchi et al., 2008). Moreover, adipocytes confer radioresistance of breast cancer cells (Bochet et al., 2011). In ovarian cancer, adipocytes facilitate invasion and homing of cancer cells to the omentum via adipokine secretion, induce cancer cell growth by direct transfer of lipids and activate beta-oxidation in cancer cells altering their energy metabolism. These pro-tumourigenic and pro-metastatic functions of adipocytes were at least partially dependent on adipocyte fatty acid-binding protein 4 (FABP4) (Nieman et al., 2011). Lastly, adipocytes also prove their plasticity during cancer progression. Fluorescent-labelling suggested the integration of adipose stromal cells into tumour blood vessels as pericyte-like cells in an obesity-dependent fashion (Zhang et al., 2012). Taken together, this evidence sheds light on the correlation of obesity and cancer, however the investigation of the contribution of adipocytes appears to be it's infancies (Vucenik and Stains, 2012).

1.2.2.4 Extracellular matrix and mechanical forces/tissue stiffness

The extracellular matrix (ECM) is a complex network of macromolecular components like proteins, glycoproteins, polysaccharides and proteoglycans with varying physical, biomechanical and biochemical characteristics that assemble basement membranes and the interstitial matrix of tissues. The ECM was traditionally considered as rigid entity providing structural support and anchorage, but emerged as highly dynamic structure that is constantly remodelled by stromal cells, provides a storage of signalling factors and influences cellular behaviour (Lu et al., 2012). The individual composition of the ECM varies between different tissue types resulting in various elasticity levels and is largely deregulated in cancer. The cancer-associated ECM is aberrantly deposited and remodelled, which leads to increasing stiffness and reduced material elasticity during the course of cancer progression from a neoplasm, carcinoma in situ to an invasive carcinoma with

notable cellular responses (Yu et al., 2011). In fact, the ECM has prognostic value in breast cancer with high presence of protease inhibitors correlating with good clinical outcome in contrast to an ECM rich in integrins or matrix metallopeptidases (MMPs) (Bergamaschi et al., 2008).

Interestingly, the ECM and matrix stiffness can directly activate the main pathways associated with proliferation and transformation in cancer via integrins. Cell surface integrins are important mechano-sensors and cluster upon matrix stiffening by, for example, collagen cross-linking through enhanced Lysyl oxidase (LOX) production of breast cancer cells. Integrins activate focal adhesion kinase (FAK), which results in increased PI3K/Akt or Ras/ERK signalling, oncogenic transformation and invasion (Levental et al., 2009, Paszek et al., 2005). Cancer accompanied changes in the ECM composition and elasticity also influence stromal cells like CAFs (section 1.2.2.1). ECM tension at focal adhesions induces Src tyrosine kinases and leads to activation of Yap/Taz transcription factors that are crucial for pro-tumourigenic functions of CAFs such as promotion of matrix stiffening, angiogenesis and cancer cell invasion. Yap activation results in stabilisation of the actin cytoskeleton facilitating CAF-mediated ECM remodelling that increases stiffness and, in turn, enhances Yap/Taz levels in CAFs to foster tumourigenesis (Calvo et al., 2013).

The composition of the ECM influences multiple processes during cancer development such as cancer cell invasion, immune cell infiltration and activation as well as angiogenesis. Thereby, an aberrant ECM aids the formation of a tumour-supportive microenvironment (Lu et al., 2012). Hence, improving our understanding of ECM components, their composition, deregulation and consequent effects on cancer progression might provide a so far unexploited direction for intervention.

1.2.3 Inflammation and immunity – tumour promotion and immune-mediated destruction

Inflammation is a physiological response of the body to infection and injury in order to remove pathogens or other destructive factors and restore normal tissue homeostasis and function during wound healing. The normal inflammatory response is divided into an acute phase characterised by redness, heat, swelling

and pain that are mainly caused by an influx of leukocytes. These leukocytes include granulocytes followed by macrophages, the activation of tissue-resident as well as adaptive immune cells and cause local and systemic amplification of the inflammatory response by secreting cytokines and other inflammatory mediators. Importantly, the inflammation has to be dampened after elimination of the initial insult during a resolution phase. Leukocyte numbers and activation status are normalised, which is usually mediated by immunosuppressive soluble signals and cells like regulatory T cells (Nathan, 2002). Importantly, inflammation associated with tumour development and progression is significantly different to an acute inflammatory response following infection and often termed “smouldering inflammation” because of its low grade (Balkwill et al., 2005, Grivennikov et al., 2010). Cancer-related inflammation is characterised by the presence of inflammatory cells and inflammatory mediators such as cytokines as well as chemokines in concert with tissue remodelling and neo-angiogenesis. This pro-tumourigenic microenvironmental change is observed in the vast majority of malignant cancers and appears independent of the tumour-initiating cause (Mantovani et al., 2008).

The presence of inflammatory cells within tumour tissue was first noted in 1863 by Rudolf Virchow (Balkwill and Mantovani, 2001), in 1986 Harold Dvorak proposed tumours to resemble “wounds that do not heal” (Dvorak, 1986) and, by today, cancer-associated inflammation is a recognised enabling characteristic of cancer (Hanahan and Weinberg, 2011). Numerous types of infections and chronic inflammatory conditions pre-dispose to cancer development, for example *Heliobacter pylori* infection to gastric cancer, *Haemophilus influenza* infection to lung cancer, hepatitis virus infection or liver cirrhosis to hepatocellular carcinoma and inflammatory bowels disease to colorectal cancer (Balkwill et al., 2005, de Martel and Franceschi, 2009). The accumulation of inflammatory cells is suggested to create a mutagenic environment that fosters tumour initiation. For example, immune cell-derived generation of reactive oxygen species (ROS), nitric oxide (NO) and other highly reactive compounds cause mutations in surrounding epithelial cells. Moreover, inflammation also promotes genetic instability in normal and transformed cells triggering further malignant progression and inflammation (Colotta et al., 2009, Meira et al., 2008, Pang et al., 2007). Activation of the Ras/ERK signalling pathway in cancer cells, that is crucial for cancer cell

expansion, also induces cytokine/chemokine expression leading to an inflammatory state (Guerra et al., 2007, Mantovani et al., 2008, Sparmann and Bar-Sagi, 2004). Moreover, we know from mouse carcinogenesis models that induction of chronic inflammation is necessary to drive tumour development after an otherwise non-effective initial genetic hit, like carcinogen-induced DMBA/TPA or AOM/DSS-induced multi-stage carcinogenesis protocols (Abel et al., 2009, De Robertis et al., 2011). In fact, inflammatory cells and mediators were experimentally shown to directly promote all stages of tumourigenesis, from cancer initiation, growth, angiogenesis, cell invasion, migration, intra- and extravasation as well as metastatic growth, which shows promising potential for therapeutic interference (Coussens and Werb, 2002, Hanahan and Coussens, 2012).

However, not all immune cells and aspects of inflammation are necessarily pro-tumourigenic. For example, clinically induced fever and bacterial infections are proposed treatments for some cancer types. For example, William Coley developed a bacteria-based anti-cancer treatment by mixing toxins from heat-inactivated *Streptococcus pneumoniae* and *Serratia marcescens* in 1893, the “Coley toxin” (Coley, 1893), that was later shown to contain lipopolysaccharide which stimulates Toll-like receptor 4 (Rakoff-Nahoum and Medzhitov, 2009). In 1976, Morales, Eidinger and Bruce used bacillus Calmette-Guerin for the efficient treatment of bladder cancer, which remains an applied therapy for advanced bladder cancer (Wei et al., 2008). Moreover, impaired immunity was shown to correlate with increased incidence of certain cancer types. Patients under immunosuppressive treatment after organ transplantation displayed increased incidence of several cancer types such as sarcomas and carcinomas, however reduced risk of breast and rectal cancer (Stewart et al., 1997, Stewart et al., 1995, Vajdic and van Leeuwen, 2009). A large proportion of these anti-tumourigenic effects of the immune system might be attributed to cancer immunosurveillance and the action of effector cells of the innate and adaptive immune system that recognise and eradicate tumour cells (Igney and Krammer, 2002, Finn, 2012, Quezada et al., 2011). Moreover, the same inflammatory cell types, like macrophages and neutrophils, are known to have opposing roles in cancer progression, depending on the environment and context (Piccard et al., 2012, Sica and Mantovani, 2012).

In summary, these observations highlight the complexity and context-dependence of the functions of inflammation and inflammatory cells in cancer and we are only beginning to understand the exact mechanisms.

1.2.3.1 Anti-cancer immunity and cancer cell elimination

The immune system has an important protective role against cancer. It clears virus-infections and thereby limits virus-induced tumours, prevents the generation of chronic tumour-supportive milieus through eradication of bacterial infections followed by quick resolution of inflammation and, importantly, many immune cells directly eliminate transformed cells (Schreiber et al., 2011). This fact is best illustrated by the observations that impaired immunity of patients showed to be a risk factor for several types of cancer (Stewart et al., 1997, Vajdic and van Leeuwen, 2009). In concert, immunocompromised mice have a greater spontaneous incidence for cancer development and are more susceptible to experimental tumour induction by carcinogens or tumour cell transplantation. These genotypes include mice genetically deficient for interferon-gamma (IFN-gamma), recombining-activating gene (Rag) 1 or 2 and the non-obese diabetic (NOD)/Prkdc-scid/IL-2rg (NSG) mice (Kanaji et al., 2014, Quezada et al., 2011). IFN-gamma is a key cytokine that activates JAK/STAT signalling and, thereby induces Type 1 CD4+ T helper cells (Th1). Th1 cells contribute to pro-inflammatory responses by stimulating cell-mediated immunity and CD8+ cytotoxic T cells (Zaidi and Merlino, 2011). Rag1 and Rag2 proteins are essential for V(D)J rearrangement of B and T cell receptors (BCRs and TCRs) during lymphocyte generation and their loss causes severe combined immunodeficiency (scid), the absence of functional B and T lymphocytes (Mombaerts et al., 1992, Shinkai et al., 1992). In a similar fashion, NSG mice lack lymphocytes as a result of the scid mutation of Prkdc (protein kinase, DNA-activated, catalytic polypeptide), a protein crucial for DNA double-strand break repair during V(D)J rearrangement of BCRs and TCRs. The NOD background of these mice causes loss of the complement system and limited macrophage activity (Shultz et al., 1995) and the deficiency for the interleukin-2 receptor subunit gamma (IL-2rg) results in lack of functional NK cells (Shultz et al., 2012). Moreover, the presence of specific immune cell subsets like natural killer

(NK) cells, cytotoxic CD8+ T cells and Th1 cells in the TME correlates with significantly improved prognosis for many different cancer types (Fridman et al., 2012).

From these studies, it emerged that anti-cancer immunity is mediated by collaboration between arms of the innate and adaptive immune system and has proven to be very cancer type and context dependent (Dunn et al., 2002, Dunn et al., 2004, Schreiber et al., 2011) (Fig. 1.4). The main innate effector cells involved in cancer cell elimination are NK cells that mainly recognise cells that lack major histocompatibility complex (MHC)-I expression via their NKG2D receptor – a common feature of emerging neoplasms. NK cell cytotoxicity against tumour cells is predominantly mediated via secretion of perforin and granzyme that break tumour cell membranes and engagement of apoptosis-inducing receptors like tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) receptor and Fas on tumour cells (Waldhauer and Steinle, 2008). Other types of innate immune cells have also been shown to directly kill cancer cells, like macrophages and neutrophils (Piccard et al., 2012, Sica and Mantovani, 2012).

Many cancer cells express and present so-called tumour antigens via MHC-I or MHC-II that can mediate their clearance (Coulie et al., 2014). Dendritic cells and other antigen-presenting cells take up particles from dying cancer cells – including tumour antigens – and present these to cytotoxic CD8+ T cells. CD8+ T cells with specific TCRs recognising tumour antigens are then induced to expand and will specifically detect and lyse emerging tumour cells (Dunn et al., 2002, Dunn et al., 2004, Schreiber et al., 2011). Moreover, CD4+ T cells can also display direct anti-cancer cytotoxicity against, for example, melanoma cells (Quezada et al., 2010). If successful, innate and adaptive immunosurveillance can completely eliminate neoplastic cells and prevent any clinical significance. Sadly, these anti-tumour immunity mechanisms are not bulletproof, but can be circumvented by cancer cells by the emergence of poorly immunogenic cancer cells. These cancer cells are thought to initially enter an equilibrium phase were adaptive immune cells together with cytokines like IL-12 and IFN-gamma keep them in a dormant state and prevent outgrowth (Quezada et al., 2011, Schreiber et al., 2011, Dunn et al., 2004, Dunn et al., 2002, Aguirre-Ghiso, 2007). During this equilibrium phase, cancer cells are under pronounced immune-mediated pressure leading to “immunoediting” – the process where anti-cancer immunity is proposed to influence cancer malignancy by

forcing cancer cells to alter their immunogenicity. Consequently, cancer cells can acquire additional pro-tumourigenic transformations and become immunoevasive, for example by loss of tumour antigens or MHC molecules presenting these, and escape this equilibrium with adaptive immunity (Dunn et al., 2002, Dunn et al., 2004, Schreiber et al., 2011). These escapers of immune surveillance and elimination can actively suppress NK cells, cytotoxic CD8+ T cells and anti-tumourigenic macrophages or indirectly create an immunosuppressive microenvironment by recruitment of stromal cells. This tumour-supportive milieu inhibits anti-cancer immunity and allows cancer growth to clinically relevant stages (Dunn et al., 2002, Dunn et al., 2004, Schreiber et al., 2011) (Fig. 1.4). Studies on immunomodulatory molecules that control CD8+ T cell activation not only highlight their anti-tumourigenic functions, but also provide very promising approaches to re-initiate and maintain anti-tumour immunity (Quail and Joyce, 2013) (Quail and Joyce, 2013, Quezada et al., 2011).

1.2.3.2 Immune suppression – regulatory T cells, myeloid-derived suppressor and dendritic cells

Development and growth of tumours is often associated with the infiltration of several T cell subsets, the anti-tumourigenic cytotoxic CD8+ T cells and CD4+ Th1 cells (section 1.2.3.1) as well as immunosuppressive regulatory T cells (Treg). These Treg cells in concert with myeloid-derived suppressor cells (MDSCs) and dendritic cells comprise the main cellular components inhibiting or preventing immune-mediated eradication of tumours. Moreover, tumour cells and tumour-educated stromal cells release soluble mediators suppressing anti-cancer immunity like IL-10, TGF-beta, VEGF and galectin that create a cancer growth permissive milieu (Quezada et al., 2011, Schreiber et al., 2011).

Treg cells are important negative regulators of lymphocyte activation and thereby are crucial to prevent chronic inflammation and autoimmune diseases by dampening mainly adaptive immune responses. They suppress the effector functions and proliferation of an array for leukocytes, most notably antigen-presenting cells (APCs), CD8+ T cells, NK and Th1 cells globally by release of anti-

inflammatory cytokines including TGF-beta and IL10 (von Boehmer and Daniel, 2013). For example, TGF-beta causes downregulation of the NKG2D receptor on NK cells limiting their cytotoxicity towards MHC-I-deficient (cancer) cells and IL-10 prevents T cell proliferation (Ralainirina et al., 2007, Taga et al., 1993). Also, Tregs were shown to prevent antigen-presentation to T cells via contact-inhibition of APCs such as macrophages or dendritic cells and impede cytotoxic granule release by cytotoxic CD8+ T cells (von Boehmer and Daniel, 2013, Quezada et al., 2011) (Fig. 1.4). Nevertheless, tumour-associated Treg cell functions and phenotypes appear quite heterogeneous, as their infiltration into the TME correlates with poor clinical prognosis for breast and liver cancer patients and with improved survival in many other cancer types (Quail and Joyce, 2013).

MDSCs are a heterogeneous population of immature, multipotent myeloid progenitor cells with varying degrees of maturity, plasticity and differentiation potential into mature cells that display pronounced immunosuppressive function. They are thought to be the result of abnormal myelopoiesis and differentiation in the bone marrow occurring in tumour bearing hosts. This altered myelopoiesis is mediated by a complex array of often tumour-derived cytokines and growth factors including granulocyte-colony stimulating factor (G-CSF), granulocyte-monocyte (GM)-CSF, macrophage (M)-CSF, VEGF, IL-6, IL-1-beta, stem cell factor (SCF), Prostaglandin E2 (PGE2) and TNF-alpha (Gabrilovich and Nagaraj, 2009, Wesolowski et al., 2013). MDSCs often systemically accumulate with increased tumour burden and are recruited to tumour sites via the chemokines C-C motif ligand (CCL) 2, C-X-C motif ligand (CXCL) 12 and CXCL5 involving selectins and integrins (Talmadge and Gabrilovich, 2013, Wesolowski et al., 2013). In mice MDSCs comprise at least two clearly different subpopulations that have differential gene expression patterns, monocytic CD11b+ Ly6G-/Ly-6C+ M-MDSCs with monocytic morphology and granulocytic CD11b+ Ly6G+/Ly6C-low G-MDSC with granulocyte-like morphology. M-MDSC display high levels of inducible nitric oxide synthase (iNOS) with enhanced T cell suppressive activity and can differentiate into mature macrophages, granulocytes and dendritic cells while G-MDSCs have increased Arginase 1 (Arg1) production and no known differentiation ability (Gabrilovich and Nagaraj, 2009, Talmadge and Gabrilovich, 2013, Youn et al., 2008). In humans, M-MDSCs are associated with HLA-DR- CD11b+ CD33+ CD14+

and G-MDSCs with HLA-DR- CD11b+ CD33+ CD15+ expression (Wesolowski et al., 2013). The phenotypic identification of MDSCs is challenging and M-MDSCs and G-MDSCs are very difficult to be distinguished from mature macrophages and neutrophils or granulocytes by expression of surface markers, respectively (Talmadge and Gabrilovich, 2013, Youn et al., 2008). Hence, it was suggested to define this mixed and complex immature myeloid cell population by their function to suppress (anti-cancer) immune responses instead of their phenotypes. This functional definition would argue for a functional "MDSC" state that is associated with particular myeloid cell populations and also let to their nomenclature (Gabrilovich et al., 2007, Talmadge and Gabrilovich, 2013, Youn et al., 2008, Youn and Gabrilovich, 2010).

MDSCs trigger this functional inhibition of anti-cancer immune responses via various mechanisms. For example, MDSCs produce NO via iNOS and withdraw the available non-essential amino acid L-arginine by metabolism with the enzyme Arg1. NO inhibits T cell function by suppression of JAK/STAT signalling and MHC-II expression and triggers T cell apoptosis. MDSC-mediated reduction of L-arginine quantities causes suppression of T cell proliferation via, for example, downregulation of cell cycle genes. Moreover, MDSCs were shown to foster the development of immunosuppressive Treg cells from naïve CD4+ T cells that was dependent on IFN-gamma, IL-10, Arg1 or CTLA-4 in a context specific fashion (Gabrilovich and Nagaraj, 2009, Gabrilovich et al., 2012, Gabrilovich et al., 2001). MDSCs affected NK cell cytotoxicity predominantly by decreasing perforin but not granzyme B production and depended on cell-to-cell contact as well as STAT5 (Liu et al., 2007). Importantly, circulating MDSC frequencies positively correlated with tumour burden as well as inversely with T cell frequencies and, probably as a consequence, high MDSC numbers associated with worse clinical outcome for many different cancer types (Gabrilovich et al., 2012, Talmadge and Gabrilovich, 2013) (Fig. 1.4).

Dendritic cells are mature, bone marrow derived myeloid cells usually residing in tissues that are professional APCs dedicated to processing and presentation of antigens to lymphocytes. They actively collect cellular material from their environment and become potent antigen presenters upon stimulation and activation by pathogen-associated molecular patters (PAMPs) or danger-associated

molecular patters (DAMPs) (Gabrilovich et al., 2012). Dendritic cells are under special attention for the development of anti-cancer therapies to due to their natural property to generate potent antigen-specific T cell immune responses. Dendritic cell-based immunotherapeutic strategies or “anti-cancer vaccines” aim to specifically deliver tumour-antigens and induce anti-tumour T cell activation via transfer of dendritic cells pre-incubated with tumour material (Palucka and Banchereau, 2012).

1.2.3.2.1 Immune checkpoint blockade in cancer therapy

Recent evidence points towards a crucial role of immune checkpoint signals – co-stimulatory or co-inhibitory molecules complementing antigen-recognition by the TCR or BCR of lymphocytes – as mechanisms for evasion of immune destruction by cancer cells (Postow et al., 2015). Cytotoxic T-lymphocyte antigen (CTLA)-4 is a co-inhibitory receptor that is upregulated on CD4+ and CD8+ T cells upon activation and constitutes a homeostatic mechanism to contain T cell activation. CLTA-4 competes with the T cell-expressed activating molecule CD28 for binding of co-stimulatory CD80 and CD86 ligands present on APCs that usually facilitate T cell activation and proliferation upon TCR engagement. In contrast, binding of the higher affinity CTLA-4 causes T cell cycle arrest and reduction of cytokine release leading to T cell exhaustion (Quezada et al., 2011, Topalian et al., 2015). Interestingly, CTLA-4 is also expressed on Treg cells and its block limits their immunosuppressive function (von Boehmer and Daniel, 2013). PD-1, the receptor for programmed cell death ligand (PD-L) 1 and 2, is another activated CD4+ and CD8+ T cell-expressed inhibitory molecule that causes effector T cell inactivation and exhaustion. PD-L1 is expressed by APCs, Tregs, other cell types and, importantly, by many tumour cells and directly correlates with worse clinical outcome. Hence, the PD-L1/PD-1 axis provides another promising target to sustain cytotoxic CD8+ T cell responses in the context of cancer (Quezada et al., 2011, Topalian et al., 2015). In fact, inhibition of CTLA-4, PD-1 or PD-L1 proved very effective in the treatment of pre-clinical cancer mouse models and cancer patients leading to international excitement and a “revolution” of care for cancer patients (Postow et al., 2015, Pardoll, 2012, Allison, 2015). For example, Ipilimumab and

Nivolumab, monoclonal antibodies targeting CTLA-4 or PD-1 respectively, significantly improved the overall survival of patients with metastatic melanoma, a cancer type with very limited therapeutic options (Camacho, 2015, Ascierto and Marincola, 2015). In detail, Ipilimumab administration raised the one-year survival rate to about 45% compared to about 25% of the patient group treated with gp100 (melanoma antigen glycoprotein 100) that has also been reported to stimulate cytotoxic T cell responses (Hodi et al., 2010). Nivolumab therapy showed a high one-year patient survival rate of about 73% while overall survival was only about 42% upon dacarbazine (an antineoplastic chemotherapy drug) treatment (Robert et al., 2015). Moreover, the one-year survival rate of previously–treated advanced non-small-cell lung cancer patients was up to 56% (dose-dependent) following nivolumab treatment compared to about 31% of current second-line therapies in a phase I clinical trial (Gettinger et al., 2015). CTLA-4, PD-1 or PD-L1 inhibition also showed promising therapeutic benefits in phase I clinical trials in, among others, triple-negative breast cancer, urothelial bladder cancer, pancreatic cancer, prostate cancer, colon cancer, renal cancer mesothelioma and Hodgkin's lymphoma. In fact, advanced melanoma was the first cancer type to be granted US Food and Drug Administration (FDA) approval for the use of drugs targeting CTLA-4, PD-1 or PD-L1 followed recently by non-small cell lung cancer and the list is likely to be extended (Ascierto and Marincola, 2015, Postow et al., 2015, Pardoll, 2012, Allison, 2015, Topalian et al., 2015, Swaika et al., 2015) and (http://www.fda.gov).

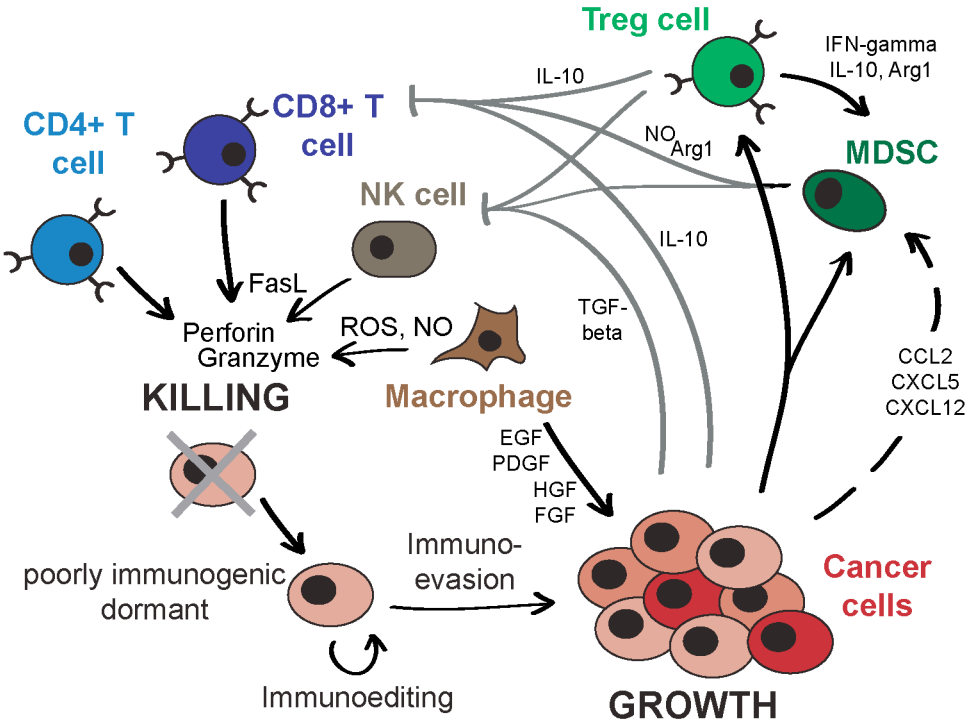


Figure 1-4 Mechanisms of immune cell-mediated cancer cell killing and immunosuppression
Schematic representation of cells and molecules involved in mediating cancer cell lysis, immunoediting, immunoevasion and growth of cancer cells as well as their interplay. Further, cells and molecules are depicted that suppress anti-cancer immunity.

1.2.3.3 Cancer-promoting inflammation – macrophages, neutrophils, mast cells and gut microbiota

Pro-tumourigenic effects of inflammation go beyond the suppression of anti-cancer immunity and especially macrophages and neutrophils (section 1.3.5) have been ascribed with potent direct pro-tumourigenic functions. Macrophages are terminally differentiated myeloid cells closely related to dendritic cells and their density at tumour sites correlates with poor prognosis for patients in 80% of conducted studies. Additionally, the macrophage transcriptome as well as expression of factors involved in regulating macrophage differentiation and recruitment show prognostic value (Qian and Pollard, 2010). The main functions of macrophages include the protection from infections and maintenance of tissue homeostasis by direct phagocytosis of microbes or dead cells and antigen presentation to T cells. Moreover, they release a plethora of growth factors, cytokines, chemokines, scavenger receptors and proteolytic enzymes that control tissue morphogenesis and growth as well as inflammation and wound healing. During steady state, tissue resident macrophage populations such as Kupffer cells in the liver or interstitial and alveolar macrophages in the lung play essential roles in tissue homeostasis while, during inflammatory responses, bone marrow-derived macrophages that differentiate from monocytes infiltrate tissues (De Palma and Lewis, 2013). The phenotype, activation state and functions of macrophages are strongly influenced by the microenvironment leading to pronounced macrophage plasticity. The classically activated M1 state of macrophages is induced by engagement of Toll-like receptors (TLRs) and Th1 cytokines like IFN-gamma via STAT1 and NFkB activation to stimulate their fighting abilities against invading pathogens and is associated with anti-tumourigenic properties. M1 macrophages are characterised by upregulation of inflammatory type I cytokines such as TNF-alpha, IL-1-beta and IL-12 and generation of ROS and NO that foster their phagocytic activity as well as by enhanced expression of MHC-II molecules increasing their participation in antigen presentation to T cells (Qian and Pollard, 2010, Sica and Mantovani, 2012). In contrast, Th2 cytokines like IL-4 and IL-13 cause an alternatively activated M2 state of macrophages via STAT3 and STAT6 activity to participate in wound healing and humoural immunity and is recognised as pro-tumourigenic

macrophage polarisation. M2-associated features are intended to foster resolution of inflammation after infection and promote tissue regeneration, for example via stimulation of angiogenesis and ECM remodelling (De Palma and Lewis, 2013, Sica and Mantovani, 2012). In cancer, numerous mechanisms induce the pro-tumourigenic functions of macrophages and facilitate M2 features apart from Th2 cells, including B cells, fibroblasts and tumour cells themselves by release of ECM components, IL-10, M-CSF and chemokines like CCL2 (Sica and Mantovani, 2012).

Tumour cells directly recruit macrophages through secretion of M-CSF, VEGF as well as chemokines like CCL2-5 and CCL8 and macrophage presence is associated with cancer cell invasiveness and angiogenesis (Lewis and Pollard, 2006). Active infiltration of macrophages was also reported to occur at early stages of tumourigenesis (Qian and Pollard, 2010). In fact, macrophages within the TME secrete a variety of factors fostering proliferation and survival of cancer cells, such as EGF, PDGF, TGF-beta, HGF and FGF (Lewis and Pollard, 2006). Moreover, tumour-associated macrophages have been shown to strongly promote cancer cell invasion by multiple mechanisms. For example, macrophages secrete various matrix-degrading enzymes, MMPs and cathepsin proteases that facilitate cancer cell migration through the ECM (Joyce and Pollard, 2009). A considerable amount of evidence also indicates that macrophages contribute an important part to tumour angiogenesis. M-CSF release, and thereby macrophage presence in the TME, is required for the angiogenic switch and M-CSF overexpression caused acceleration of blood vessel formation and tumour progression. Moreover, macrophage depletion suppressed angiogenesis in various transplanted tumour models (Qian and Pollard, 2010). Macrophages secrete an array of pro-angiogenic cytokines including VEGF, angiopoietin, TNF-alpha, FGF as well as IL-8 and ECM-remodelling enzymes facilitating blood vessel formation (Lewis and Pollard, 2006).

Importantly, genetic and pharmacologic depletion or reduction of macrophage numbers limits tumour growth, malignancy and metastasis while accelerated macrophage recruitment promoted tumour progression. These observations were made in various mouse and human models for, among others, melanoma, rhabdomyosarcoma, breast, ovarian, lung, colon and prostate cancer (Qian and Pollard, 2010). Hence, macrophages appear to have predominantly pro-tumourigenic roles within the TME and a re-education of M2-like to M1-like

macrophages, for example via M-CSFR block, might provide a promising therapeutic approach (Guiducci et al., 2005, Pyonteck et al., 2013).

Mast cells are a heterogeneous population of bone marrow-derived cells that are present in many human tissues in steady state and infiltrate tumour sites. The role of mast cells in cancer is rudimentary understood compared to macrophages, but they have been shown to directly influence cancer cell proliferation and invasion, tumour angiogenesis, TME/ECM remodelling and orchestrate inflammation and immune responses (Khazaie et al., 2011). In mice, generation of tumour blood vessels and ECM reorganisation was dependent on mast cells (Coussens et al., 1999) and blocking of mast cell degranulation caused hypoxia, inhibited angiogenesis and tumour progression (Samoszuk and Corwin, 2003, Soucek et al., 2007). During intestinal polyp formation, genetic or pharmacologic mast cell reduction caused cancer cell apoptosis, hypoxia and angiogenesis (Gounaris et al., 2007). Moreover, mast cells recruited to thyroid and breast tumours induced their invasion as well as survival (Melillo et al., 2010, Xiang et al., 2010). These pro-tumourigenic features of mast cells were partially ascribed to mast cell granule-derived tryptases, histamines, CXCL1 and CXCL10. Additionally, mast cells have important immunomodulatory properties and are a source of prostaglandins and leukotrienes that recruit macrophages and neutrophils. Also, they secrete considerable amounts of TGF-beta and IL-10 that suppress anti-cancer immunity and can positively as well as negatively control immunosuppressive properties of Treg cells (Gounaris et al., 2009, Khazaie et al., 2011). In the clinic, mast cells correlate with cancer progression and poor prognosis for example in different types of lymphoma, Merkel cell carcinoma, liver and prostrate cancer. However, other studies report contrasting findings and a better prognosis with increased mast cell infiltration even in the same cancer types including lymphomas, lung and colorectal cancer (Khazaie et al., 2011, Marichal et al., 2013).
In summary, evidences from pre-clinical models and clinical correlation studies suggest promoting as well as protective roles of mast cells in cancer and further research will be required to shed light on these controversies.

In colorectal cancer, a significant tumour-promoting function is recognised for gut microbiota-elicited inflammation. In fact, inhibition of the bacterial flora by treatment

with antibiotic agents ameliorated colorectal cancer-associated inflammation, suppressed tumour growth and improved outcome (Zitvogel et al., 2015). Microbes can, for example, directly cause DNA damage and facilitate mutations in endothelial cells by secretion of toxins and they can also trigger Wnt signalling by injection of effector molecules leading to aberrant cell growth. Moreover, breaching of the mucosal barrier by microbes during tumour growth activates pro-inflammatory pathways through engagement of pattern recognition receptors like TLRs and may result in NFkB- and STAT3-mediated inflammatory responses fostering tumour progression (Garrett, 2015). Antibiotics are suspected to alter the composition of the gut microbiota rather than their eradication and thereby create a non-permissive environment for tumour growth. Hence, the FDA initiated the characterisation of the human microbiome in health and disease (Quail and Joyce, 2013).

1.2.3.4 Examples for cytokines and chemokines regulating cancer-related inflammation

Soluble mediators like cytokines and chemokines are frequently secreted by cancer cells and many stromal and immune cell types likely all influencing the generation of an inflammatory microenvironment (Balkwill and Mantovani, 2012). Here a few important examples of potent inflammatory mediators and their effects on cancer development and progression are summarised:

Tumour necrosis factor (TNF)-alpha is a central cytokine in the cancer-associated inflammatory network and was ascribed with multiple roles mainly acting via its receptor TNFR1 rather than TNFR2 activating the c-Jun-N-terminal kinase (JNK) pathway. TNF-alpha supports cancer growth and metastatic spread in numerous cancer types likely via its promoting effects on inflammation. However, consistent with the context-dependence of an inflammatory response in cancer, TNF-alpha also shows anti-tumourigenic activity especially in high doses (Balkwill, 2009).

Interleukin-6 (IL-6) is a direct downstream target of oncogenic Ras/ERK signalling and its levels in plasma correlate with worse outcome in advanced cancer stages. It stimulates cancer growth, angiogenesis, cell survival and therapy resistance predominantly by orchestrating leukocyte infiltration and the inflammatory reaction

via stimulation of the JAK/STAT pathway (Balkwill and Mantovani, 2012). Interestingly, IL-6 from various cellular sources is involved in expanding cancer stem cell subpopulations in hepatocellular carcinoma, colitis-associated and breast cancer (Grivennikov et al., 2009, Korkaya et al., 2011b, Korkaya et al., 2011a, Wan et al., 2014).

The chemokine (C-C motif) ligand 2 is produced by cancer cells and various types of stromal cells and acts as a key regulator of the inter-cellular cross talk. CCL2 binds the cell surface receptors CCR2 as well as CCR7 and displays many pro-tumourigenic properties in a plethora of cancers stimulating tumour cell growth, metastatic spread, angiogenesis, attraction of innate immune cells such as tumour-associated macrophages and matrix remodelling (Lu et al., 2006, Tsuyada et al., 2012, Zhang et al., 2010). Therapeutic approaches neutralising CCL2 showed promising outcomes in pre-cancer models and are now evaluated in clinical trials (Zhang et al., 2010).

In summary, the various roles of cytokines and chemokines affecting multiple targets suggests that targeting secreted signalling factors within the inflammatory TME might be more effective and specific in the clinic than depletion of respective cell types or functions, as demonstrated for TNF-alpha inhibition in rheumatoid arthritis and psoriasis (Feldmann and Maini, 2001, Gisondi and Girolomoni, 2007).

1.2.3.5 The bioactive lipid signalling factors eicosanoids in cancer

Eicosanoids are a group of secreted, bioactive pro-inflammatory lipids like prostaglandins (PGs) and leukotrienes (LTs) that play important roles in numerous pathologies including cancer (Wang and Dubois, 2010). They are synthesised from arachidonic acid via a cascade of enzymes producing the different variants of eicosanoids with cyclooxygenase (COX) 1 and 2 being essential for synthesis of PGs by production of the PGH2 intermediate. PGH2 is further metabolised by the respective PG or TX synthase to yield the secreted prostaglandins PGE2, PGD2, PGF2, PGI2 and TXA2. Alox5 is the key enzyme for LT synthesis and produces the unstable LTA4 metabolite from arachidonic acid when associated with Alox5-activating protein (FLAP). LTA4 is either converted to LTB4 by LTA4 hydrolase or by LTC4 synthase to the cysteinyl leukotriene LTC4 that can be further modified to

LTD4 and LTE4 (Homaidan et al., 2002, Peters-Golden and Henderson, 2007) (Fig. 4.1). PGs and LTs are subsequently secreted via multidrug resistance-associated protein (MRP) efflux transporters. COX-1 is constitutively expressed at low levels in almost all human tissues producing homeostatic levels of PGs, while COX2 is an early response gene mainly induced upon inflammatory stimuli and constitutes the predominant enzymatic source for PGE2 in cancer (Dubois et al., 1998). Alox5 presence is normally restricted to leukocytes, predominantly neutrophils, eosinophils, macrophages and mast cells. However, inflammatory stimuli can induce Alox5 expression in epithelial cells, some cancer cells have been shown to produce Alox5 and epithelial as well as endothelial cells can synthesise leukotrienes from leukocyte-secreted LTA4 (Peters-Golden and Henderson, 2007, Pidgeon et al., 2007, Wang and Dubois, 2010). COX2-derived PGE2 is the most frequent prostaglandin and often elevated at inflammatory sites or within the TME of human cancers and binds to its cognate G-protein coupled rhodopsin-type receptors EP1-4 as well as peroxisome proliferator-activated receptors (PPARs). The less abundant PGD2 binds DP1-2 and TXA2 to the TP receptor (Wang and Dubois, 2006). LTB4 receptors include the Leukotriene B4 receptor (BLT) 1 and BLT2 and cysteinyl leukotrienes (CysLTs) LTC4, LTD4 and LTE4 bind to their receptors CysLT1 and CysLT2 (Fig. 4.1). BLT1 and CysLT1 are high affinity receptors that are virtually restricted to leukocytes and the low affinity receptors BLT2 and CysLT2 are more ubiquitously expressed on various cell types including cancer cells (Kanaoka and Boyce, 2004, Peters-Golden and Henderson, 2007, Tager and Luster, 2003). Importantly, altered metabolism of arachidonic acid by COX1/2 and Alox5 enzymes is commonly observed in carcinomas with significant effects on cancer progression (Wang and Dubois, 2010).

Prostaglandins are important regulators of homeostasis and involved in many pathologies, including inflammatory malignancies and cancer. They have a key function in initiating the acute phase of an inflammatory response and are significantly increased at inflammatory sites, however their role during resolution of inflammation is more debated. PGE2 directly affects a plethora of leukocytes such as granulocytes, macrophages, dendritic cells, B and T lymphocytes by binding to its 4 different receptors E1, E2, E3 and E4 and exerts a dual role with pro- and anti-inflammatory properties. In concert with the role of PGE2, other PGs also exert

both, stimulatory and antagonistic functions on inflammatory processes (Ricciotti and FitzGerald, 2011).

Leukotrienes are powerful inducers of acute inflammation with essential functions in immune defence. LTB4 is mainly produced by neutrophils and lower amounts by macrophages and dendritic cells and cysteinyl leukotrienes LTC4, LTD4 and LTE4 are predominantly secreted by eosinophils, basophils and mast cells, with smaller contributions from macrophages, dendritic cells and neutrophils. Virtually all innate and adaptive leukocytes express receptors for LTB4 and cysteinyl leukotrienes. This directly points towards the essential role of leukotrienes to stimulate and amplify immune responses and inflammation (Peters-Golden and Henderson, 2007).

Many therapeutic approaches blocking leukotriene generation or signalling are proving successfully in the clinics, for example pulmonary function and many other features of asthma are improved in patients by Alox5 blockade with the inhibitor Zileuton or inhibition of CysLT1 by Montelukast and Zafirlukast (Barnes et al., 2005, Wenzel and Kamada, 1996). LTB4 receptor antagonists, for example, also showed promising effects in the treatment of rheumatoid arthritis (Diaz-Gonzalez et al., 2007).

In summary, prostaglandins and leukotrienes play pivotal physiological roles in regulating immune responses and beyond. While the functions of prostaglandins appear context-dependent and controversial, leukotrienes appear clearly pro-inflammatory, maintain normal host defence and are drivers of chronic inflammatory diseases.

1.2.3.5.1 The role of eicosanoids in cancer development and progression – with a focus on leukotrienes

Prostaglandins in cancer

Prostaglandins and leukotrienes were shown to directly affect tumourigenesis by binding their receptors expressed on epithelial cancer cells resulting in direct induction of pro-tumourigenic properties like increased proliferation or invasion. Alternatively, similar to infection processes, eicosanoids are strongly implicated in promoting a pro-inflammatory and tumour growth-permissive TME by stimulating

secretion of growth, angiogenic and pro-cancer inflammatory factors by cancer or stromal cells as well as signals suppressing anti-cancer immunity (Wang and Dubois, 2010).

In the TME, PGE2 appears to have an overall pro-tumourigenic role and targets angiogenic, anti-apoptotic and immunosuppressive factors as well as chemokines/chemokine receptors via induction of several pathways including Ras/ERK, PI3K/AKT, NFkB, Wnt and PPAR pathways and thereby promotes tumour growth, angiogenesis, invasion and metastasis (Menter and Dubois, 2012, Wang and Dubois, 2006, Wang and Dubois, 2010).

Leukotrienes and their receptors in cancer (Fig. 1.5)

Leukotrienes and their role in cancer is far less well understood compared to prostaglandins (Wang and Dubois, 2010). Human prostate and colon cancers show increased levels of LTB4, Alox5 expression is elevated in neuroblastoma, colon and oesophageal cancer and LTB4 receptor expression, especially BLT2, is enhanced in many different cancer types like pancreatic, skin, oesophagus, breast, colon, renal, bladder, ovarian and lung cancer (Chen et al., 2004, Dreyling et al., 1986, Hennig et al., 2002, Larre et al., 2008, Melstrom et al., 2008, Sveinbjornsson et al., 2008, Yoo et al., 2004). However, it remains to be identified if the increased LTB4, Alox5 or BLT2 levels are a reflection of an inflammatory infiltrate, cancer cell derived or both. Blocking LTB4 synthesis or signalling via BLT1 effectively limited tumour burden and progression of human colorectal and pancreatic cancer cell lines in mice as well as spontaneous oesophageal adenocarcinoma in rats (Chen et al., 2003b, Gunning et al., 2002, Hennig et al., 2004, Hennig et al., 2005, Melstrom et al., 2008). Further, Alox5 inhibition prevented cell proliferation and resulted in apoptosis while LTB4 treatment induced proliferation in neuroblastoma and colon cancer cell lines (Bortuzzo et al., 1996, Ihara et al., 2007, Sveinbjornsson et al., 2008). BLT1 was shown to be upregulated in neutroblastoma (BLT2 was not determined) (Sveinbjornsson et al., 2008) and BLT2 was elevated in breast cancer cells were BLT2 inhibition resulted in apoptosis via loss of a ROS-dependent survival signalling (Choi et al., 2010). Several studies also examined the dual inhibition of COX2 and Alox5 and showed individually and combined efficacy in limiting rat spontaneous oesophageal adenocarcinoma and mouse lung cancer as well as growth of a cigarette-smoke promoted colon cancer line and a human

skin squamous cell carcinoma cell line in mice (Chen et al., 2004, Fegn and Wang, 2009, Rioux and Castonguay, 1998, Ye et al., 2005).

More mechanistic studies revealed that LTB4 directly induces MEK/ERK1/2 as well as Akt/PI3K activation that results in accelerated proliferation of pancreatic cancer cell lines and was prevented by LTB4 receptor inhibition (Tong et al., 2002, Tong et al., 2005). In a similar fashion, LTB4 stimulated ROS production and a Rac/ERK cascade that facilitated proliferation and chemotaxis of rat fibroblasts (Woo et al., 2002) and the LTB4/BLT2 axis activated ERK1/2, caused keratin reorganisation and induced migration of pancreatic cancer cells (Park et al., 2012). The functions of LTB4 in tumour angiogenesis are very rudimentary characterised, however activation of endothelial cell-expressed BLT2 was required for VEGF-stimulated angiogenesis and LTB4-mediated BLT2 activation induced endothelial cell migration and was sufficient to induce angiogenesis *in vivo* (Kim et al., 2009). Additionally, LTB4 appeared to mediate ROS generation and leukocyte adherence to the endothelium during hypoxia, a key inducer of angiogenesis (Steiner et al., 2001).

Analysis of cysteinyl LT receptor expression was conducted in non-hodgkin lymphomas, breast and colorectal cancer with the result that high CysLT1 expression correlated with worse prognosis and high CysLT2 presence with better outcome (Magnusson et al., 2011a, Magnusson et al., 2011b, Magnusson et al., 2010, Schain et al., 2008). In prostate and bladder cancer, CysLT1 expression increased with cancer progression and CysLT1 inhibition decreased tumour growth and even caused cancer cell apoptosis (Matsuyama et al., 2009, Matsuyama et al., 2007). CysLT1 antagonists also limited tumour growth of colon cancer xenografts (Savari et al., 2013) and metastatic spread of a lung and colon cancer cell line by reducing the extravasation efficiency through brain and peripheral capillaries (Nozaki et al., 2010). The CysLT2 receptor was shown to correlate and directly be involved in the differentiation of colorectal cancer cells (Bengtsson et al., 2013, Magnusson et al., 2007). LTD4 treatment caused activation of Wnt signalling and induced migration of colon cancer cells (Salim et al., 2014) as well as proliferation and survival of neuroblastoma cells (Sveinbjornsson et al., 2008). A series of studies from the same group reported that LTD4-stimulation of intestinal epithelial cells caused ERK1/2, PI3K and Wnt signalling activation mainly via CysLT1 leading to increased proliferation, survival and migration – a likely cancer-related

mechanism in light of inflammatory bowel disease being a clear risk factor for neoplastic transformation (Mezhybovska et al., 2006, Ohd et al., 2000, Paruchuri et al., 2005, Paruchuri et al., 2002, Paruchuri et al., 2006). Lastly, cysteinyl LTs might influence tumour angiogenesis as LTC4 and LTD4 promoted endothelial cells to produce inflammatory cytokines via CysLT2/Rho kinase and proliferation via CysLT1/ERK (Duah et al., 2013, Modat et al., 1987). Overall, these studies suggested a pro-tumourigenic role of the LTD4/CysLT1 axis and rather anti-tumourigenic properties of CysLT2 in colorectal and other cancer types (Savari et al., 2014).

Inhibitors for COX and Alox5 enzymes or receptors for prostaglandins or leukotrienes showed promise in some respects in the clinics in treatment of cancer (Wang and Dubois, 2010). Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit COX1 and 2 and reduce the risk and incidence of many different cancer types such as colorectal, breast and lung. However, severe side effects of NSAIDs in regard to the cardiovascular system prevent a standardised use (Wang and Dubois, 2006). Nevertheless, Celecoxib, a selective COX2 inhibitor, is FDA-approved for treatment of patients with elevated colorectal cancer risk (Wang and Dubois, 2010). Moreover, long- and short-term use of in particular acetylsalicylic acid (Aspirin) at low doses shows promising efficacy to diminish the risk of developing numerous types of cancer and metastasis with only minor and dose-dependent side effects of gastrointestinal bleeding (Huang et al., 2011, Rothwell et al., 2012a, Rothwell et al., 2012b). Alox5 and leukotrienes are important mediators during inflammatory asthma disease and targeted in the clinic by the specific Alox5 inhibitor Zileuton since its initial FDA approval in 1996. Zileuton proved save in long-term use without significant side effects, for example in regard to liver injury (Lazarus et al., 1998, Watkins et al., 2007, Wenzel and Kamada, 1996). Moreover, Zileuton was employed in two cancer-related clinical trials for the prevention or treatment of lung cancer (Szabo et al., 2013). The clinical trial by the Alliance for Clinical Trials in Oncology tested the combination of the chemotherapeutic agents Carboplatin and Gemcitabine with Celecoxib and Zileuton in treating advanced non-small cell lung cancer. Additional Zileuton administration did not alter the efficacy of chemotherapy alone, despite the improvement of Celecoxib administration for the outcome of high COX2 expressing patients (Edelman et al., 2008). The clinical trial run by the Barbara Ann Karmanos Cancer Institute is currently evaluating the impact of

Zileuton to limit lung cancer development in patients with bronchial dysplasia (*U.S. National Institutes of Health, clinicaltrials.gov identifier NCT00056004*), because lung leukotriene levels appear strongly increased in this disease (Mirro et al., 1990, Rupprecht et al., 2014). The results of this second clinical trial are pending (Szabo et al., 2013).

Regulation of "cancer stemness" by eicosanoids (Fig. 1.5)

An elegant study showed that BLT2 is required for Ras-mediated transformation of rat fibroblast-like cells – a key oncogenic event (Hanahan and Weinberg, 2011, Samatar and Poulikakos, 2014). Ras-transformation in turn increased LTB4 secretion by cancer cells and BLT2 inhibition decreased tumour initiation potential (Yoo et al., 2004). BLT2 and Ras collaborated with TGF-beta to induce EMT, that is associated with cellular plasticity and stem cell-like properties (Mani et al., 2008), in human immortalised mammary epithelial cells via induction of reactive oxygen species (ROS) and NFkB signalling (Kim et al., 2014). Also, BLT2 is upregulated upon detachment of prostate cancer cells and resulted essential for anoikis resistance via mechanisms involving ROS and NFkB (Lee and Kim, 2013). These observations suggest a functional role for the LTB4/BLT2 cascade in tumour initiation, metastasis and cancer "stemness".

Interestingly, LTB4, LTD4 and PGE2 were implicated in modulation of stem cell homeostasis. In zebrafish, stimulation of PGE2 synthesis elevates haematopoietic stem cell (HSC) numbers and COX enzymes were necessary for HSC formation. PGE2 was demonstrated to be essential for zebrafish and murine HSC maintenance and function by activation of the Wnt signalling cascade (Goessling et al., 2009, North et al., 2007). Also, PGE2 prevented apoptosis of mouse embryonic stem cells (Liou et al., 2007).

LTB4 acted anti-apoptotic and induced proliferation and ex vivo expansion of human CD34+ cord-blood HSCs via BLT2 as well as of mouse neural stem cells via BLT1 (Chung et al., 2005, Wada et al., 2006). LTD4 stimulated adhesion, retention and proliferation of human primary CD34+ hematopoietic progenitor cells and cell lines via activation of ERK1/2 through CysLT1 (Boehmler et al., 2009). Also, LTD4 stimulated mouse embryonic stem cell proliferation and migration by activation of STAT3, PI3K/Akt and Wnt signalling via CysLT1 and CysLT2 (Kim et al., 2010b).

In summary, the important roles of eicosanoids like PGE2, LTB4 and cysteinyl leukotrienes in promoting cancer progression are beginning to emerge. In order to develop effective treatments to be used in the clinics, we have to improve our understanding of their functions in the specific contexts of cancer growth and metastatic progression (Wang and Dubois, 2010).

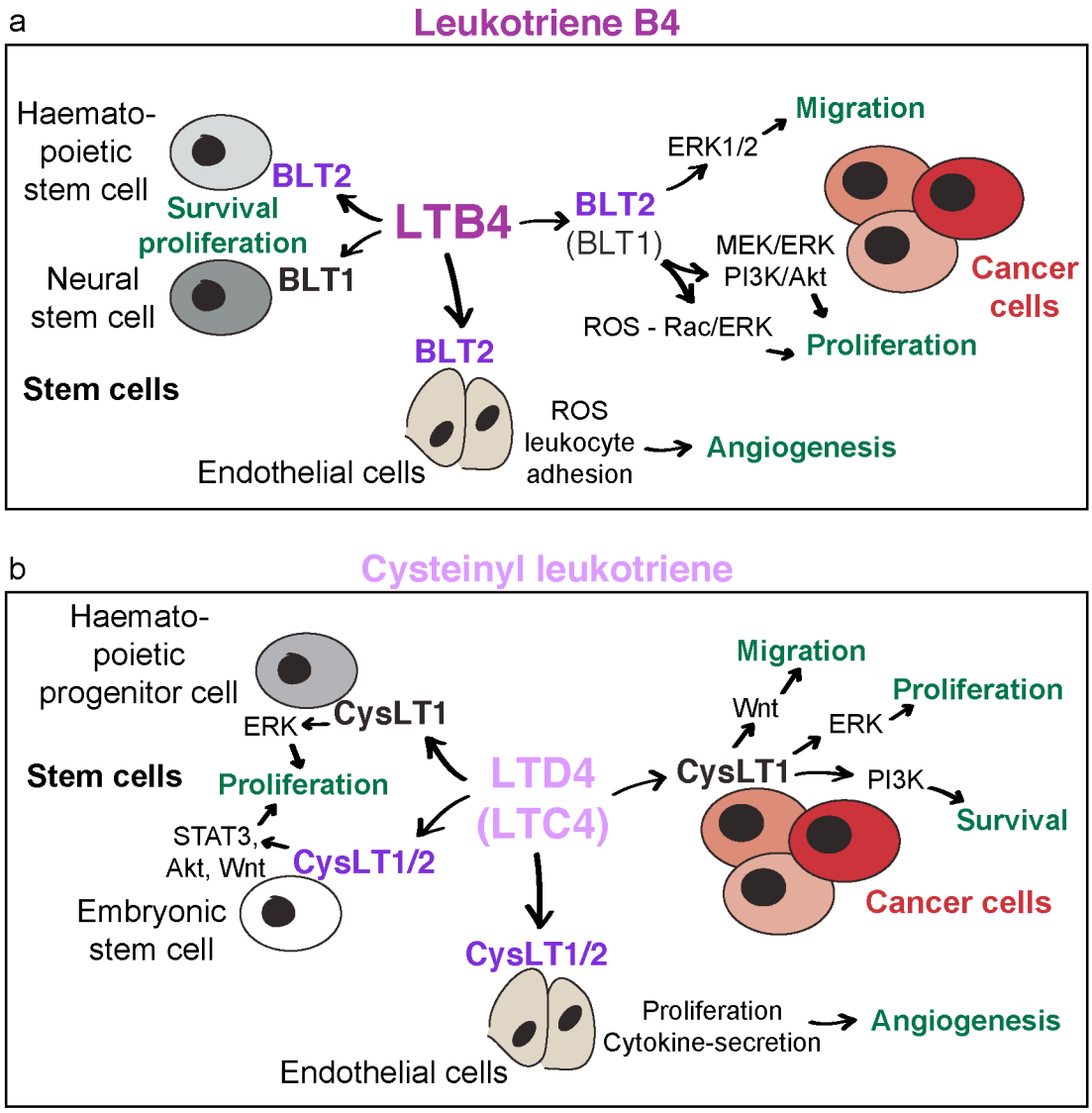


Figure 1-5 Actions of leukotriene B4 and cysteinyl leukotrienes D4 and C4 influencing stem cells and cancer cells
(a) Schematic of the signals and processes influenced by leukotriene B4 (LTB4) and (b) cysteinyl leukotrienes D4 and C4 via their receptors BLT1/2 and CysLT1/2, respectively.

1.2.4 Environmental regulation of cancer stem cells and their functions

Cancer stem cells (CSCs) or tumour- and metastasis-initiating cells (TICs and MICs) are thought to be supported and maintained by niches, just like normal adult tissue stem cells (Borovski et al., 2011, Sneddon and Werb, 2007). Niches are complex microenvironments controlling stem cell functions and fate by direct contact-mediated or soluble signals (Schepers et al., 2015). As part of the TME, CSC niches are made up by fibroblastic cells, endothelial cells, pericytes, immune cells and their respective progenitors embedded in an ECM and surrounded by a specific growth factor and cytokine milieu (Cabarcas et al., 2011, Ye et al., 2014). Additionally, nonCSCs are considered to take part in providing a permissive environment for CSCs or obtain CSC characteristics (Kreso and Dick, 2014, Schwitalla et al., 2013). Importantly, the concept of a CSC niche is only emerging and a CSC niche has not been defined for every cancer type often due to a lack of identification of a CSC population. Nevertheless, evidence is mounting that the TME has a crucial influence on cancer “stemness”, the competence of certain cancer cell populations to self-renew, initiate tumours and metastases and survive therapeutic interventions causing relapse (Plaks et al., 2015).

The best-described CSC niche is the leukaemia-induced stem cell niche in the bone marrow, which appears like an aberrantly activated haematopoietic stem cell (HSC) niche that directly provides instructive cues for leukaemia-initiating cells (Schepers et al., 2015). The HSC niche consists of endothelial cells, mesenchymal stromal cells, megakaryocytes, osteoblasts and nerve cells as well as a network of soluble factors and adhesion molecules. SCF, platelet factor 4 (CXCL4), TGF-beta, and angiopoietin 1 (Angpt1) control HSC quiescence; Notch signalling, interleukins and erythropoietin (EPO) regulate HSC cell fate and proliferation while the CXCL12/CXCR4 interaction, ECM components like fibronectin, selectins and vascular cell adhesion protein 1 (VCAM1) modulate HSC niche homing (Schepers et al., 2015, Wilson and Trumpp, 2006). As several human myeloid diseases such as acute myeloid leukaemia (AML) are know to arise from mutations in HSCs or progenitor cells (Shlush et al., 2014, Woll et al., 2014), it does not appear surprising that leukaemia-initiating cells also show a strong degree of interaction with their bone marrow niche. Leukaemia-initiating cells have an increased requirement for

niche-derived CD44-mediated signals and different selectins for adhesion to the ECM compared to HSCs in human and animal models. However, they show reduced dependency on environment-modulated Wnt signalling for homing and engraftment to the bone marrow (Jin et al., 2006, Krause et al., 2014, Krause et al., 2006, Lane et al., 2011).

Our knowledge of defined CSCs niches in solid cancers remains rather rudimentary, likely due to limited understanding of the regulation of normal tissue stem cells. Notably, a potential CSC niche was proposed in breast cancer as incorporation of several microenvironmental signals from different cellular sources (Korkaya et al., 2011b, Korkaya et al., 2011a). In fact, numerous stromal cells clearly collaborate to influence solid cancer cells with increased “stemness” potential. Activation of Wnt, Notch and NFkB signalling pathways as well as the EMT programme in cancer cells are closely related to increased stemness potential and strongly influenced by the microenvironment (Holland et al., 2013, Scheel and Weinberg, 2012, Shostak and Chariot, 2011, Takebe et al., 2011, Wang et al., 2012). For example, CAFs are important inducers of EMT that often goes hand in hand with the acquisition of stem cell properties of cancer cells (Giannoni et al., 2011, Giannoni et al., 2010, Kalluri and Zeisberg, 2006, Scheel and Weinberg, 2012). CAFs can directly induce Wnt activation and thereby enhance stemness potential of cancer cells (Malanchi et al., 2012, Ohlund et al., 2014, Vermeulen et al., 2010). Other cancer cell stemness-promoting signals are derived from MSCs, for example PGE2 and other cytokines that induce Wnt signalling and stemness in colorectal cancer (Li et al., 2012). Endothelial cells were also shown to mediate stem cell-like characteristics in a contact-dependent manner by NO-induced Notch signalling activation on glioma cells (Charles et al., 2010). Also, platelets were suggested to protect and maintain colon carcinoma cells in the circulation and promote their stemness via induction of NFkB signalling and an EMT programme (Labelle et al., 2011). Tumour-associated macrophages (TAMs) were shown to physically interact with breast CSCs via Ephrin A4/Ephrin A4 receptor and promote their stem cell state via activation of NFkB signalling (Lu et al., 2014). Also, TGF-beta secretion by TAMs induced an EMT programme in hepatocellular carcinoma cells and conferred cancer stem cell-like properties (Fan et al., 2014) and TAM-derived IL-6 expanded the hepatocellular carcinoma CSC pool (Wan et al., 2014). Additionally, TAM-derived milk-fat globule-epidermal growth factor-VIII (MFG-E8)

supported tumourigenicity and drug resistance of lung CSCs via activation of STAT3 and Hedgehog pathways (Jinushi et al., 2011). In pancreatic cancer, TAMs directly promoted tumour initiation capability and chemoresistance partially by induction of STAT3 (Marusyk et al., 2014, Mitchem et al., 2013). Very recently, glioblastoma stem cells were shown to recruit TAMs via periostin that in turn exacerbate tumour growth (Zhou et al., 2015).

Collectively, this evidence supports the dependence of stem cell properties of cancer cell subpopulations on their environment and suggests an additional CSC feature, namely the ability to create and interact with their niche (Malanchi, 2013). However, the important aspect of the TME controlling tumour initiation potential of cancer cells and vice versa is yet underappreciated when using xenograft transplantation assays, probably due to technical limitations and current standards (Plaks et al., 2015).

1.2.5 The pre-metastatic niche, cancer cell dormancy and the microenvironmental roles during metastatic colonisation

In 1889, Stephen Paget proposed in his “seed-and-soil” hypothesis that metastatic spread in cancer patients is not random, but shows clear preferences of cancer cells to colonise certain organs, a feature nowadays often referred to as organ tropism (Fidler, 2003). The exact reasons explaining these preferences of cancer cells are slowly beginning to unfold. Intrinsically, genome sequencing did not reveal specific mutations in cancer cells associated with metastatic traits or organ tropism, but alterations of the epigenetic landscape and the translational machinery are emerging to be important factors for directing metastatic dissemination. Certain metastatic traits are suspected to be present in the primary cancer as gene expression signatures show prognostic value for metastasis, however our current knowledge on organ tropism establishment remains largely limited (Oskarsson et al., 2014). One recent study in mammary cancer reported a mechanism how CAFs in the primary TME determine the preference of metastatic cancer cells to colonise the bone. CAF-derived CXCL12 and insulin growth factor (IGF) at the primary site selected for cancer cells with highly active Src kinase that boosts CXCL12/IGF-mediated PI3K signalling. Thereby, these cancer cells were favoured to survive in

CXCL12-rich microenvironments like the bone marrow and show preferential metastasis to bone (Zhang et al., 2013). This observation is already suggesting features of the important role of the distant tissue environment in enabling metastatic seeding. It is widely accepted that the lack of survival and proliferation signals from a supportive environment together with suppressive effects of immune surveillance are the challenges to be overcome during distant tissue colonisation, making it a remarkably inefficient process. However, metastases do pose the highest risk for cancer-associated death, despite the dramatic experiences and challenges metastatic cancer cells have to master (Oskarsson et al., 2014, Plaks et al., 2015, Psaila and Lyden, 2009, Quail and Joyce, 2013).

In concert with Paget's "seed-and-soil" hypothesis, it is becoming increasingly clear that primary tumours provide more to facilitate the metastatic process than the disseminated cancer cell (the seed). In fact, a cancer and its TME are emerging to actively "fertilise" the distant secondary site (the soil) and thereby set the stage for metastatic colonisation to occur in specific locations. Evidence is accumulating that endocrine-like mechanisms originating from the primary cancer can educate secondary sites to be permissive for arriving cancer cells by creating a so-called pre-metastatic niche characterised by infiltration of stromal cells and presence of secreted cytokines and oncoproteins (Oskarsson et al., 2014, Plaks et al., 2015, Psaila and Lyden, 2009, Quail and Joyce, 2013). Pioneering studies implicated the influx of myeloid cells in the pre-metastatic site to favour and direct metastatic seeding. Primary tumours were shown to upregulate MMP9 in endothelial cells and macrophages in the pre-metastatic lung in a VEGFR1-dependent fashion and prevention of MMP9 induction ameliorated metastasis (Hiratsuka et al., 2002). Further, primary mammary tumour-derived factors such as VEGF, TNF-alpha and TGF-beta caused induction of S100A8 and S100A9 in pre-metastatic lung endothelial and myeloid cells that directed migration and homing of metastatic cancer cells to the lung (Hiratsuka et al., 2006). S100A8 appeared to stimulate Serum Amyloid A3 production in the pre-metastatic lung that triggered TLR4/NFkB signalling activation in macrophages, which in turn supported cancer cell migration and metastasis (Hiratsuka et al., 2008). Macrophages in the pre-metastatic lung of mammary cancer-bearing mice were also shown to express integrin-alpha4-beta1 that serves as a docking site and survival signal for arriving cancer cells via VCAM-1 and downstream PI3K signalling (Chen et al., 2011). Other reports described the

infiltration of bone marrow-derived VEGFR1+ haematopoietic progenitor cells (HPCs) into pre-metastatic sites, which was induced by activation of fibroblasts at the secondary site prior to cancer cell arrival. These VEGFR1+ HPCs directed metastatic cancer cell organ tropism via mechanisms involving integrins and matrix remodelling enzymes (Kaplan et al., 2005). Moreover, hypoxia-induced cancer cell expression of lysyl oxidase (LOX), a target gene of HIF transcription factors, fostered recruitment of myeloid cells to the lung of mammary cancer-bearing mice hand in hand with mediating metastatic efficiency (Erler et al., 2006). Bone marrow-derived myeloid progenitors in the pre-metastatic lung were shown to aid reversion of an EMT state in disseminated cancer cells, that was previously described to be necessary for metastatic outgrowth (Ocana et al., 2012, Tsai et al., 2012), via secretion of the ECM component versican. Myeloid cell-derived versican induced mesenchymal-to-epithelial transition of metastatic cancer cells by preventing activation of Smad2 and thereby allowed proliferation and metastatic outgrowth (Gao et al., 2012).

Recently, mechanisms of pre-metastatic niche formation were described involving material transported by exosomes, small membrane-formed nanovesicles that are released into the blood or lymphatic fluids. Tumour cell-derived exosomes containing the receptor tyrosine kinase Met fostered a permissive metastatic niche by influencing bone marrow-derived (progenitor) cells to promote metastatic seeding by facilitating angiogenesis. Interestingly, exosomes affected the organ tropism of metastatic cancer cell lines as exosomes originating from different melanoma cells redirected transplanted melanoma cells to colonise usually no-typical organs. The study also determined a signature of exosomes with high prognostic value in patients (Peinado et al., 2012).

These observations suggest a pro-metastatic activity of immature myeloid cells that accumulate in pre-metastatic tissues via their potential immunosuppressive function, however there is little direct *in vivo* evidence to prove this notion at the moment (Plaks et al., 2015).

One of the key challenges that metastatic cancer cells have to master is overcoming dormancy at the distant site. As described in section 1.1.2, cancer cell dormancy in hostile environments is induced by nutrient limitation through insufficient supply by the vasculature (tumour-mass dormancy), a quiescent cell

cycle arrest in G0-G1 (cellular dormancy) or immunosurveillance-mediated dormancy (Aguirre-Ghiso, 2007, Marches et al., 2006, Quail and Joyce, 2013). Tumour-mass dormancy is characterised by a balance between proliferation and apoptosis that can be broken by induction of angiogenesis. Hence, it is perhaps not surprising that several metastasising cancer cell types, including melanoma, breast and lung cancer, placed themselves around capillaries – the perivascular niche – when infiltrating the brain (Carbonell et al., 2009). Pro-angiogenic molecules like VEGF and FGF play an important role in neoangiogenesis of metastases (Naumov et al., 2006) and endothelial progenitor cells mobilised from the bone marrow were shown to induce the angiogenic switch at metastatic sites (Gao et al., 2008). The immune system also keeps cancer cells in a dormant state at metastatic sites. Adaptive immune cells prevent metastatic cell outgrowth by mechanisms involving Th1 cytokines such as IL-12 and IFN-gamma keeping equilibrium. Further cancer cell transformations like loss of MHC-I molecules (Khong and Restifo, 2002) as well as the creation of an immunosuppressive environment by MDSCs and Treg cells were shown to overcome this dormant state. Similar mechanisms are thought to occur during metastatic colonisation (Aguirre-Ghiso, 2007, Dunn et al., 2004, Schreiber et al., 2011).

Cellular dormancy or metastatic cancer cells that arrested their cell cycle were reported in breast and ovarian cancer patients and significantly increased the risk of relapse (Braun et al., 2000, Braun et al., 2001, Pierga et al., 2003). The arrested state of disseminated cancer cells was proven in mouse models by absence of proliferation markers implying a G0-G1 cell cycle arrest (Naumov et al., 2002). Later studies showed this cancer cell cycle arrest to be at least partially induced by the microenvironment through, for example, ECM-integrin interactions or activation of stress signalling pathways, independent of angiogenesis or immunosurveillance (Quail and Joyce, 2013). For example, the urokinase plasminogen activator receptor (uPAR) was reported to be expressed by carcinoma cell lines and triggered *in vivo* growth by activating fibronectin-integrin and ligand-independent EGFR-ERK signalling to induce cancer cell proliferation (Liu et al., 2002). Absence of uPAR signalling caused a shift favouring p38 signalling that suppresses cell transformation and growth over ERK1/2 activation and proliferation resulting in cancer cell dormancy (Ranganathan et al., 2006). However, mouse models only rarely exhibit a comparable degree of dormancy of metastatic cells compared with

human patients what hinders the investigation of many aspects of these processes (Plaks et al., 2015).

Nevertheless, it is crucial to understand how cancer cells exit the state of dormancy at the metastatic site for therapeutic intervention and CSCs are especially suspected to play an important role due to their quiescent nature (Plaks et al., 2015). Interestingly, the transcriptional signatures of grown metastases show similar pathway activation compared to adult stem cell niches (Oskarsson et al., 2014). Moreover, metastatic cancer cells hijack normal tissue stem cell niches in the host to establish distant metastases. For example, human prostate cancer cells were shown to infiltrate the HSC niche in the bone marrow in order to profit from an environment rich in CXCL12, TGF-beta and hedgehog signals that maintain stemness (Shiozawa et al., 2011). Moreover, cancer cells with high CXCR4 (the receptor for CXCL12) expression, such as subsets of breast cancer cells, might directly be recruited to the HSC niche and, in fact, have highest metastatic affinity to the bone (Zhang et al., 2013). Additionally, perivascular niches appear to support CSCs or MICs at metastatic sites by supplying paracrine signals such as Notch signalling (Butler et al., 2010). Arriving CSCs or metastasis-initiating cells were also shown to contribute themselves to create a permissive niche. Mammary CSC-derived TGF-beta in the metastatic lung induced resident fibroblasts to release and deposit periostin, an ECM component. Periostin, in turn, bound stromal Wnt ligands and thereby triggered upregulation of Wnt signalling in CSCs that proved essential for lung colonisation and metastatic growth (Malanchi et al., 2012). In accordance, successful metastatic colonisation was associated with periostin, TGF-beta and generation of neo-vessels (Ghajar et al., 2013). Tenascin C (TNC) is another ECM component regulating CSCs and their metastatic potential via augmenting Wnt and Notch signalling. Hence, TNC-expressing breast cancer cells showed higher potential for metastatic initiation and outgrowth (Oskarsson et al., 2011).

After initiation of metastatic growth and escaping from dormancy, several further signalling circuits were reported to sustain metastatic colonisation, for example the interaction between EGFR and Met kinase, NFkB signalling and JAK/STAT signalling (Oskarsson et al., 2014). Hence, disseminating cancer cells face multiple obstacles from arriving at the distant site, finding a hospitable environment, evading growth inhibitory signals and dormancy, induce angiogenesis and establish growing macro-metastases. All these processes are heavily influenced by the

microenvironment and the cancer cells' ability to modulate or interact with it. Considering that CSC-like or metastasis-initiating cells were shown to be the most potent cells to spark successful metastatic colonisation, it is intriguing to think that the competence to hijack or respond to signals from the metastatic niche might be an important hallmark of metastasis-initiating cells (Malanchi, 2013).

In summary, the tumour and metastases-associated microenvironment is as complex as the mutational landscape of cancer cells and cannot be ignored as important factor and driver of tumourigenesis. Numerous cellular players, soluble or bound factors emerged to regulate all stages of carcinogenesis in promoting or suppressing manners. In fact, cancer cells evolve together with their microenvironment under continuous cross-influences that dictate tumour progression and their dynamics. These observations provide tempting targets to clinically tackle the supportive basis of the TME or enhance and re-activate its suppressed anti-tumourigenic properties. Further research efforts are needed to intensify our knowledge on the composition and functions of the TME to design effective combinatorial therapeutic strategies targeting both, the cancer cell and the stroma, to fight such a multi-facetted disease like cancer.

1.3 Neutrophil granulocytes and their role in cancer

Neutrophils are myeloid, polymorphonuclear leukocytes physiologically present in the bone marrow, blood, spleen, liver and lung. They are part of the granulocyte lineage generated in the bone marrow from HSCs and myeloid precursors that are released into the blood stream upon maturation. In the blood, they are thought to live for about 1.5 to 12.5 hours in mice and up to several days in humans before returning to the bone marrow to be phagocytised by macrophages. Interestingly, the lung seems to be particularly enriched in mature neutrophils that appear to sit within the tissue or patrol the vasculature (Kolaczkowska and Kubes, 2013, Kruger et al., 2015).

Neutrophils are the first responders to insults by rapidly infiltrating into target tissue from the circulation. Diverse stimuli such as bacterial infection or tissue damage cause the immediate recruitment of neutrophils to peripheral tissues where they mainly participate in fighting pathogens. Neutrophils can directly kill microorganisms by various mechanisms like phagocytosis, degranulation and release of an arsenal of anti-microbial factors or by deposition of neutrophil extracellular traps (NETs) (Borregaard, 2010, Kruger et al., 2015). Additionally, neutrophils play important roles in the induction and activation of a complete inflammatory immune response. Neutrophils can be stimulated to express key inflammatory mediators and be polarised towards distinct phenotypes by the microenvironment. Thereby, they modulate innate and adaptive immunity and play pivotal roles in promoting immune responses against pathogens and during diseases like autoimmune diseases and cancer (Mantovani et al., 2011). Hence, it is crucial to tightly control neutrophil production and activity to avoid chronic inflammation (Borregaard, 2010, Kolaczkowska and Kubes, 2013).

1.3.1 Generation and life of neutrophils

The production of neutrophils consists of 60% of haematopoiesis and is considered to be the major workload in the bone marrow leading to the generation of 1-2 x 10^{11} cells per day in adults under normal conditions (Borregaard, 2010). In steady state, bone marrow HSCs give rise to myeloid precursors – myeloblasts – controlled by

the activity of the transcription factor PU.1 (PU-box binding 1). A balance between PU.1 and CCAAT/enhancer-binding protein (C/EBP)-alpha determines monocytic or granulocytic commitment, respectively. Neutrophils are generated through myeloblast, promyelocyte, (meta-)myelocyte, band cell to polymorphonuclear granulocyte stages. The transcription factors Gfi-1, C/EBP-alpha, C/EBP-beta, C/EBP-gamma, C/EBP-delta, C/EBP-epsilon, C/EBP-zeta and, at later stages, PU.1 are required for neutrophil maturation and regulate repression of M-CSF to block monocyte maturation, transcription of granule proteins and the exit from the cell cycle (Fiedler and Brunner, 2012). Neutrophil granules, the name-giving hallmarks of granulocytes, are formed step-wise and filled with temporally synthesised factors such as lysosymes and defensins at precise stages during neutrophil maturation. Primary azurophil granules containing myeloperoxidase and neutrophil elastase (ela2) are formed in promyelocytes, secondary specific granules filled with lactoferrin and MMP8 in (meta-)myelocytes, tertiary gelatinase granules with gelatinases and MMP9 in band cells and secretory vesicles storing organelles and membrane proteins arise in polymorphonuclear granulocytes by endocytosis. Proteins produced by neutrophils after release from the bone marrow are no longer packed into granules, but directly secreted. Upon neutrophil activation, neutrophil granules appear to be sequentially mobilised and released inversely to their generation. Secretory vesicles fuse with the cell membrane to allow interaction with the endothelium during transmigration and gelatinase, specific and azurophilic granules are subsequently exocytosed with decreasing efficiency (Borregaard, 2010, Faurschou and Borregaard, 2003, Fiedler and Brunner, 2012).

Neutrophil production is stimulated by, but not dependent on, granulocyte-colony stimulating factor (G-CSF) that can be released by various cells in the body such as fibroblasts, mesenchymal cells, endothelial cells and resident-tissue macrophages in response to insults and activation via, for example, TNF-alpha, lipopolysaccharides and IL-1. G-CSF stimulates the proliferation of HSCs and all non-cell cycle arrested cell types of the granulocytic lineage up to myelocytes. The effect of G-CSF of HSCs depends on co-stimulatory signals, however more committed neutrophil progenitor cells are stimulated to divide by solely G-CSF (Lieschke and Burgess, 1992a, Lieschke and Burgess, 1992b, Roberts, 2005). Release of mature neutrophils from the bone marrow is mainly controlled by their

surface receptors CXCR4 as well as CXCR2, G-CSF receptor (G-CSFR) and Toll-like receptors (TLRs). CXCL12 is constitutively secreted by stromal cells in the bone marrow niche and engages its receptor CXCR4 expressed on neutrophils resulting in neutrophil retention. CXCR2, G-CSFR and TLRs are expressed late during neutrophil maturation and mediate neutrophil release from the bone marrow. The ligands for CXCR2, CXCL1 and CXCL2, are produced by bone marrow endothelial cells as well as other cell types in peripheral tissues, TLRs respond to pathogen-associated molecular patterns (PAMPs) originating, for example, from bacteria and G-CSFR engages with G-CSF. G-CSF facilitates neutrophil release by downregulating CXCL12 and increasing CXCL1 and 2 secretion by bone marrow endothelial cells (Borregaard, 2010, Eash et al., 2010).

Mature neutrophils are granular and relatively large cells that constitute about 50-70% of circulating leukocytes in humans and 10-25% in mice. In the blood, neutrophils were shown to age leading to upregulation of CXCR4 and re-home to the bone marrow to be cleared by macrophages, a process that in turn modulates the haematopoietic niche and HSCs (Casanova-Acebes et al., 2013). Alternatively, Kupffer cells clear neutrophils that die in the vasculature and dendritic cells also contribute to removing apoptotic neutrophils in tissues (Kolaczkowska and Kubes, 2013). A well understood mechanism to modulate neutrophil production is initiated by macrophages and dendritic cells that decrease IL-23 production upon neutrophil phagocytosis. IL-23 would otherwise stimulate regulatory T cells, mainly gamma-delta T cells, to produce IL-17A that in turn enhances G-CSF production in stromal and immune cells (Borregaard, 2010).

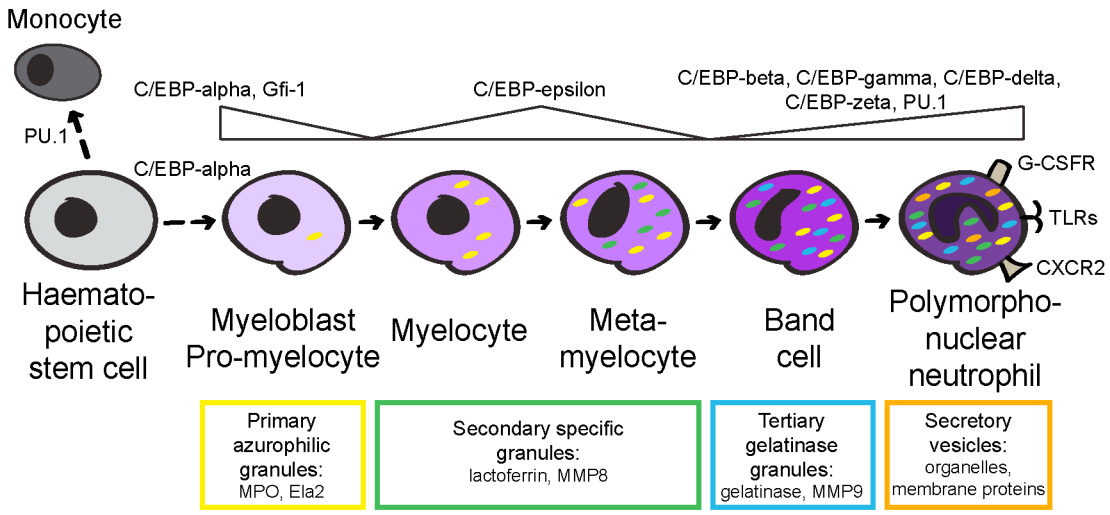


Figure 1-6 Development and maturation of neutrophils in the bone marrow
Overview of the stages of neutrophil maturation, the involved transcription factors and the generated neutrophil granules including examples of their content. Figure adapted from (Borregaard, 2010, Fiedler and Brunner, 2012).

1.3.2 Neutrophil recruitment to sites of inflammation or tissue damage

In order to infiltrate peripheral tissues upon inflammatory stimuli, neutrophils have to interact with the endothelium and transmigrate, a process that usually takes place at postcapillary venules. There, the vessel wall is thin enough and the vessel diameter allows neutrophil contact with endothelial cells without occluding the blood flow. Endothelial cells are activated by inflammatory or infection-induced stimuli such as TNF-alpha, IL-1-beta or IL-17 and upregulate selectins and integrins like ICAMs and VCAMs on the luminal side. Neutrophils bind ICAM and VCAM to cross the endothelial layer by either penetrating directly through an endothelial cell or squeezing past in-between two endothelial cells, with the latter usually accounting for 80% of neutrophil transendothelial migrations (Borregaard, 2010, Kolaczkowska and Kubes, 2013, Kruger et al., 2015).

Initial recruitment of neutrophils to peripheral tissues is controlled by tissue-resident macrophages and mast cells that serve as sensors for insults and detect PAMPs and DAMPs corresponding to infection or tissue damage as well as fibroblasts, endothelial cells, pericytes and epithelial cells (Kim and Luster, 2015). Upon stimulation, these resident cells influence blood vessel permeability and release of neutrophil-attracting chemokines from stromal cells. Platelets subsequently aid endothelial transmigration of neutrophils. IL-17-producing T cells also favour the recruitment and activation of neutrophils to inflammatory sites by secretion of IL-17, CXCL8, IFN-gamma, TNF-alpha and GM-CSF. Additionally, neutrophils are potent attractors of neutrophils by directly releasing the potent chemoattractant LTB4 or via the production of IL-17 that causes release of pro-inflammatory factors by the stroma (Ford-Hutchinson, 1981, Kim et al., 2006, Kolaczkowska and Kubes, 2013, Mantovani et al., 2011, Oyoshi et al., 2012, Saiwai et al., 2010).

1.3.3 Finding and eliminating danger in peripheral tissues

Inflammation is usually initiated by PAMPs during infection and DAMPs during sterile injury such as chemical exposure or burn. PAMPs include lipopolysaccharides, CpG (C-phosphate-G DNA regions) and other viral and bacterial products, while DAMPs are host-derived molecules like ATP (adenosine

triphosphate) and HMGB1 (high-mobility group box 1) or structurally altered molecules such as collagen. These danger signals bind to the vast repertoire of PPRs (pattern recognition receptors) expressed on the surface of neutrophils, such as almost all members of the TLR family, C-type lectin receptors as well as cytoplasmic sensors of ribonucleic acids. The sensing of PAMPs or DAMPs and thereby infection or tissue damage together with lymphocyte-derived signals such as IFN-gamma, TNF-alpha and GM-CSF activates neutrophil effector functions (Kolaczkowska and Kubes, 2013, Mantovani et al., 2011).

These effector functions of neutrophils are mainly targeted to kill invading microbes or infected cells and consist of direct phagocytosis, lysis by degranulation or deposition of toxic NETs. Activated neutrophils that infiltrated tissues have usually higher phagocytic activity than blood neutrophils. They efficiently encapsulate pathogens in phagosomes followed by degranulation into the phagosome (Nauseef, 2007). Neutrophil granules, especially azurophilic granules, contain an arsenal of anti-microbial substances like defensins, azurocidin and bacterial permeability-increasing protein, proteases such as cathepsins and neutrophil elastase as well as the peroxidase myeloperoxidase. These granules can fuse with the phagosome, attack and kill the contained microbe (Faurschou and Borregaard, 2003). In concert, phagocytosis triggers a respiratory burst, the rapid generation and release of ROS like superoxide radicals and hydrogen peroxide generated by the enzyme complex NADPH oxidase. Likely, the oxidants and granule proteins collaborate within the phagosome to maximise microbial killing (Nauseef, 2007). Alternatively, neutrophils degranulate and release the content of their granules into the environment to kill extracellular microorganisms and remodel the ECM, as many granule proteins include factors like collagenases, gelatinases and MMPs (Faurschou and Borregaard, 2003, Kolaczkowska and Kubes, 2013). NETs are formed during netosis, a form of cell death distinct from apoptosis or necrosis, were nuclei swell and chromatin dissolves. This process leads to the generation of large decondensed DNA strands associated with cytosolic and granule proteins as well as histones that are deposited in the ECM. These NETs contain proteins like defensins, neutrophil elastase, lactoferrin, myeloperoxidase and S100A8 as well as A9 and retain extracellular microbial killing activity long after neutrophil death (Brinkmann and Zychlinsky, 2012).

The crucial role of neutrophils as first line defence against bacterial and fungal infections is highlighted by the detrimental effects of impaired neutrophil functionality and neutropenia on the host (Kruger et al., 2015). Neutropenia is a disorder characterised by chronically low neutrophil count in the circulation that can be congenital or acquired. A large proportion of congenial neutropenias is caused by a mutation in the gene encoding neutrophil elastase and leads to loss of neutrophils from the myelocyte stage onwards or a loss of function of the G-CSF receptor. Congenital neutropenias can be permanent or irregular with cyclic phases of deficiency and sufficiency (Donadieu et al., 2011). A variety of factors can lead to acquired neutropenia that is not necessarily reversible, these include certain infections, drugs (antibiotics or chemotherapeutic agents), diet (copper or vitamin B12 deficiency) as well as neutropenia accompanying autoimmune and haematopoietic diseases (including leukaemias, lymphomas and myelomas) (Gibson and Berliner, 2014). Neutropenia clearly predisposes for susceptibility to acute or chronic and life-threatening infections like pneumonia. Moreover, neutropenia can lead to febrile neutropenia characterised by high fever and presence of bacteria in the circulation as well as largely enhances the risk of lethal sepsis (Berliner et al., 2004, Donadieu et al., 2011, Gibson and Berliner, 2014, Klastersky et al., 2011). Administration of recombinant G-CSF is routinely used to treat congenital and acute neutropenia as it largely normalises neutrophil counts, reduces fever and the use of antibiotics (Lieschke and Burgess, 1992a, Lieschke and Burgess, 1992b, Smith et al., 2015).

However, neutrophil presence and activation also has to be tightly controlled, because the plethora of toxic and ECM-remodelling enzymes released by neutrophils can cause severe tissue damage and chronic inflammation. The resolution phase of an inflammation is usually associated with upregulation of anti-inflammatory cytokines like IL-10 or chemokine receptor antagonists and overall downregulation of NFkB signalling. Soluble scavenger receptors such as IL-1R2, IL-1Ra, CCR5 or CC-chemokine receptor D6 are secreted by neutrophils and either sequester pro-inflammatory cytokines and chemokines or block their signalling function (Mantovani et al., 2011). Also, there is a switch in lipid production of leukocytes (including neutrophils) from pro-inflammatory arachidonic acid products such as LTB4 and prostaglandins to pro-resolving lipids like lipotoxin A4 (LXA4) as well as omega-3 polysaturated fatty acid-derived resolvins and protectins. For

example, LXA4 blocks neutrophil migration, resolvin E1 inhibits BLT1-LTB4 interaction-mediated neutrophil recruitment and resolvin D1 dampens neutrophil activation by inhibiting actin polymerisation and beta2-integrin activation (Kolaczkowska and Kubes, 2013). Other negative feedback mechanisms include, mobilisation of inhibitory receptors to the surface of activated neutrophils and downregulation of G-CSF signalling. Tissue resident macrophages are stimulated by peptides produced by neutrophil-derived proteases via the receptor for chemerin and resolvin E1 (ChemR23). Thereby, phagocytic activity of macrophages is enhanced leading to clearance of neutrophils (Borregaard, 2010). Ineffective resolution of inflammation and continued presence and activation of neutrophils can lead to chronic inflammation, extended tissue damage and scarring (Mantovani et al., 2011).

Overall, the sheer quantity and speed of neutrophils being recruited to sites of inflammation together with their arsenal of antimicrobial and tissue remodelling factors makes these innate immune cells a central basis for successful host immunity containing infections and tissue damage.

1.3.4 Neutrophils as modulators of immunity

Historically, neutrophils have been considered to be very short-lived cells with limited biosynthetic activity, but crucial for host defence against invading pathogens. However, the improvements in genomics, proteomics and transcriptomics within the last 20 years together with the notion that microenvironmental factors can extend neutrophil survival in tissues supported more complex functions of activated neutrophils in various inflammatory settings. In fact, neutrophils newly express an arsenal of immune- and microenvironment-modulatory factors either spontaneously or upon proper activation by microbial or tissue-damage-associated moieties combined with stimulation by G-CSF, GM-CSF, TNF-alpha and type I or II IFNs (Colotta et al., 2009, Kruger et al., 2015, Mantovani et al., 2011). These neutrophil-derived factors include CXC-chemokines, CC-chemokines, pro- and anti-inflammatory cytokines, colony-stimulating factors, angiogenic and fibrogenic mediators. However, there might be differences between the cytokine repertoire of

human and mouse neutrophils for example regarding IL-6, IL-10, IL-17A, IL-17F and IFN-gamma production (Wright et al., 2010).

Neutrophils have been shown to crosstalk with numerous cell types during inflammatory responses including macrophages, dendritic cells, NK cells, B and T lymphocytes as well as mesenchymal stem cells (Kruger et al., 2015). For example, *Toxoplasma gondii*-stimulated neutrophils induce bone marrow-derived dendritic cell maturation towards a Th1 immunity-promoting phenotype through contact-dependent interaction (Megiovanni et al., 2006). However, neutrophils can also limit the stimulatory activity of monocyte-derived dendritic cells via neutrophil elastase (Maffia et al., 2007). Neutrophil-derived ROS, prostaglandins and granule proteins also influence NK cell proliferation, survival and functions such as IFN-gamma production or cytotoxic activity. In turn, NK cells support neutrophil survival, activation and cytokine biosynthesis by signals like GM-CSF and IFN-gamma (Costantini and Cassatella, 2011). Furthermore, T cells and neutrophils engage in bi-directional interactions influencing both, each other's recruitment to sites of inflammation and cellular functions. Neutrophils are a source of CCL2, CXCL9 and CXCL10 to attract CD4+ Th1 and Th17 cells while Treg and Th17 cells release CXCL8 to directly mediate neutrophil infiltration. Alternatively, Th17 cells induce endothelial cells to express neutrophil chemoattractants and stimulatory molecules (Mantovani et al., 2011). Interestingly, antigen-loaded neutrophils were shown to re-circulate to lymph nodes where they modulate T cell priming by dendritic cells, suggesting a complex regulatory role of neutrophils in adaptive immunity (Chtanova et al., 2008, Yang et al., 2010).

These various predominantly pro-inflammatory functions of neutrophils make them important contributors to pathologic disorders like chronic inflammations and autoimmune diseases. For example, cigarette smoke-induced inhibition of LTA4 hydrolase causes accumulation of LTB4 and the proline-glycine-proline tripeptide (PGP) that strongly trigger neutrophil infiltration and chronic lung inflammation leading to chronic obstructive pulmonary disease. A similar chronic neutrophilic inflammation is also associated with cystic fibrosis (Snelgrove et al., 2010, Weathington et al., 2006). Moreover, crucial roles for neutrophils and neutrophil-derived products have been described in systemic lupus erythematosus, rheumatoid arthritis and multiple sclerosis (Chakravarti et al., 2009, Chou et al., 2010, Liu et al., 2010b, Mantovani et al., 2011).

In summary, the functions of neutrophils extend beyond their crucial role as first line host defence against infection and they emerge as important regulators of immunity that can initiate and sustain inflammatory responses.

1.3.5 Pleiotropic roles of neutrophils in cancer and metastasis

1.3.5.1 Neutrophils in cancer

Opposing roles for neutrophils have been reported in cancer and range from direct cytotoxicity towards tumour cells and blocking of tumour growth as well as angiogenesis to the suppression of anti-tumour immunity and direct proliferative, angiogenic, pro-invasive and pro-metastatic functions in animal models (Piccard et al., 2012). In the clinic, a majority of correlation reports suggests a worse prognosis of patients with elevated blood neutrophil count, increased ratio of neutrophils vs. lymphocytes in blood or the presence of tumour-infiltrating neutrophils. Most intensely examined where melanoma, renal cell carcinoma, hepatocellular carcinoma, colorectal cancer, cholangio-carcinoma, gastrointestinal stromal tumours, glioblastoma, gastric, oesophageal, lung, ovarian and head and neck cancer (Donskov, 2013) as well as recently breast cancer (Chen et al., 2015, Koh et al., 2015, Ozyalvacli et al., 2014). Importantly, high blood or tumour neutrophil count was a strong, independent risk factor for poor outcome in multivariate analyses and patients with low neutrophil baseline showed greatest response to therapy (Donskov, 2013). Neutropenia is a common side-effect of chemotherapy in many cancer types and, interestingly, several studies showed that moderate chemotherapy-induced neutropenia correlated with improved survival, for example for ovarian, cervical, breast, colorectal and lung cancer patients (Cameron et al., 2003, Di Maio et al., 2005, Shitara et al., 2009, Eskander and Tewari, 2012, Tewari et al., 2014). Recombinant G-CSF treatment is frequently used in the clinic to prevent the detrimental effects of neutropenia and is usually well tolerated. Nevertheless, the use of G-CSF was associated with adverse effects and can promote tumourigenesis in cancer patients and pre-clinical models (Aliper et al., 2014, Kowanetz et al., 2010, Voloshin et al., 2011), while other reports suggest the opposite (Souto et al., 2011, Ghalaut et al., 2008, Morstyn et al., 1988). In fact, beneficial tumour-suppressive features of bacterial product-mediated or drug-

induced, sustained neutrophilia are reported in pre-clinical models. Conceptually, these bacterial products are thought to mimic an acute, rather than a chronic, inflammation and enhance cytotoxic effects of neutrophils. Moreover, several reports on the anti-tumourigenic effects of neutrophils in the clinic have been published (Souto et al., 2011).

Interestingly, many types of tumour cells can directly secrete chemoattractants for neutrophils such as CXC chemokines, stressing a functional role of neutrophils in tumourigenesis. For example, the murine neutrophil chemoattractants CXCL1, CXCL2 and human CXCL8/IL-8 are direct transcriptional targets of oncogenic Ras signalling via PI3K and ERK effector pathways in transformed lung and HeLa cells. CXCL1, 2 and 8 induced neutrophil infiltration and supported tumour cell survival and angiogenesis (Jin et al., 2006, Sparmann and Bar-Sagi, 2004). Signals from tumour cells or the TME might influence neutrophils, which in turn secrete factors like ROS, degrading enzymes and growth factors that might directly affect tumour cells (Borregaard, 2010), thereby creating a potential cell-to-cell crosstalk. Also, neutrophils are a major source of chemokines and cytokines that stimulate other leukocytes and boost inflammatory reactions (Mantovani et al., 2011), which can impact on cancerogenesis indirectly. These multiple properties of neutrophils represent an important challenge in understanding their role in tumourigenesis.

Early studies in rodents in the 1990s suggested a tumour-influenced pro-tumourigenic role of neutrophils. Blood neutrophils from mammary carcinoma-bearing rats enhanced invasiveness *in vitro* and metastatic potential upon co-injection *in vivo* of benign and malignant cancer cell lines in contrast to neutrophils from tumour-free controls (Welch et al., 1989). Furthermore, the depletion of granulocytes was shown to inhibit sarcoma growth (Pekarek et al., 1995). The promoting contribution of neutrophil granulocytes to genetic instability, angiogenesis, tumour growth and metastasis was then corroborated in many other mouse models (Gregory and Houghton, 2011, Piccard et al., 2012). For instance, blocking or genetic deletion of CXCR2 receptors, that went hand in hand with decreased neutrophil infiltration, inhibited tumour growth and angiogenesis in mouse lung, intestinal and pancreatic cancer models. Although, it has to be noted that these pro-tumourigenic effects of CXCR2 could also be mediated through other cells expressing this receptor such as endothelial or other stromal cells (Gong

et al., 2013, Ijichi et al., 2011, Jamieson et al., 2012, Keane et al., 2004). Also, non-tumourigenic primary melanoma cells were dependent on neutrophil influx to grow tumours *in vivo*. Non-metastatic melanoma cells gained metastatic competence through neutrophil presence and neutrophils also increased aggressiveness of malignant melanoma cells (Schaider et al., 2003).

Further research efforts elucidated mechanisms of pro-tumourigenic actions of neutrophils to promote tumour growth, angiogenesis, invasion, metastatic growth and suppression of anti-cancer immunity. First of all, neutrophils have to be considered as part of the TME and interact not only with cancer cells, but also the stroma and modify the ECM. A proposed ability of neutrophils to indirectly promote cancer growth and progression is by the suppression of anti-cancer immune responses mounted by NK and T cells. Neutrophils can render T cells inactive by secretion of Arg1 and ROS or by ROS in an immunological synapse leading to limited T cell proliferation by, for example, limitation of L-arginine availability or NFkB activation (Pillay et al., 2013). G-CSF was involved in the generation of immunosuppressive neutrophil-like cells in breast cancer mouse models that inhibited Th1 and CD8+ T cell proliferation *in vitro* when isolated from the spleen (Casbon et al., 2015). Despite not being directly cancer-related, a subset of human neutrophils were shown to form an immunological synapse with T cells and suppressed their proliferation in a hydrogen peroxide-dependent manner during acute inflammatory reactions (Pillay et al., 2012). Also, Arg1 exocytosis from neutrophil granules induced by TNF-alpha and ionomycin inhibited T cell proliferation by depletion of extracellular L-arginine *in vitro* in rheumatoid arthritis models (Rotondo et al., 2011). In glioblastoma patients, the expansion of a population of degranulated, circulating neutrophils was associated with enhanced blood Arg1 levels and limited T cell activation. *In vitro*, these neutrophils directly suppressed T cell function and pharmacologic inhibition of Arg1 restored T cell activation and function *in vitro* and *in vivo* (Sippel et al., 2011). In accordance, a subset of circulating granulocytes were found in cancer patients and correlated with decreased T cell activation and cytokine expression. These neutrophils showed altered density likely due to their activation status and directly suppressed cytokine release by T cells via release of the ROS hydrogen peroxide *in vitro* (Schmielau and Finn, 2001). Circulating, low-density neutrophils were also identified in another study to be the preferentially propagated neutrophil subpopulation in cancer-

bearing hosts (Sagiv et al., 2015). The immunosuppressive functions of neutrophils might share similarities and overlap with the functions of G-MDSCs, however neutrophils and G-MDSCs were shown to be phenotypically, morphologically and functionally different. Nevertheless, the field remains divided as the immature G-MDSCs have been proposed to be clearly distinct from neutrophils or a novel immunosuppressive neutrophil subset (Fridlender et al., 2012, Gabrilovich et al., 2012, Pillay et al., 2013, Sagiv et al., 2015, Youn et al., 2012, Youn and Gabrilovich, 2010).

Additionally to influencing stromal and immune cells, neutrophils are also an important source of matrix-remodelling enzymes in the TME. These neutrophil-derived proteases act on cytokines, chemokines, their receptors, integrins and ECM components and can mediate tumour cell proliferation, angiogenesis and metastatic dissemination (Gregory and Houghton, 2011). MMP9 is released from neutrophil secondary, specific granules and is an important component of the TME. MMP9 was shown to be upregulated early during epidermal carcinogenesis and haematopoietic cell deficiency of MMP9 reduced epidermal proliferation and tumour incidence (Coussens et al., 2000). Similar results were obtained using lung cancer cell lines, there haematopoietic cell-derived MMP9 supported survival of lung tumour cells at early stages after seeding in the lung (Acuff et al., 2006). Depletion of neutrophils in a pancreatic cancer mouse model also reduced incidence of dysplasias and prevented the angiogenic switch due to limited availability of bioactive VEGF which is known to be released from the ECM by MMP9 (Nozawa et al., 2006). Moreover, neutrophil-derived MMP9 also promoted angiogenesis and invasion of grafted tumours of human fibrosarcoma and prostate carcinoma cell lines (Bekes et al., 2011). In contrast, MMP8 is associated with better patient prognosis and MMP8 deficiency in mice protects from skin carcinogenesis. Despite the exact mechanisms remaining elusive, MMP8 loss causes enhanced neutrophil influx that is suggested to promote tumour growth (Gregory and Houghton, 2011). Neutrophil elastase is an extracellular protease that is almost exclusively produced and released by neutrophils and recognised as important mediator of neutrophil functions. Notably, low levels of neutrophil elastase were reported to enter lung adenocarcinoma cells via early endosomes where it caused hyperactivity of PI3K and uncontrolled cell proliferation by degradation of a binding partner of the PI3K regulatory subunit (Houghton et al.,

2010). Higher neutrophil elastase concentrations cause cancer cell death, highlighting the dose-dependency of many neutrophil-derived factors. Another study also reported that exaggerated presence of neutrophils has detrimental effects on tumourigenesis and metastasis, while moderate increase of neutrophil infiltration was pro-tumourigenic. Melanoma cells transduced to express IL-8 displayed neutrophil infiltration-dependent accelerated tumour growth, however very high IL-8 transduction levels caused massive neutrophil presence and tumour growth inhibition (Schaider et al., 2003). Moreover, the same dose- and context dependency holds true for neutrophil-derived ROS levels in the microenvironment. Reactive oxygen species (ROS) are produced by neutrophil NADPH (nicotinamide adenine dinucleotide phosphate) oxidase by reduction of molecular oxygen to superoxide radicals and further conversion by enzymes like myeloperoxidase. Neutrophil-derived ROS is associated with direct cytotoxic effects towards cancer cells causing tumour regression (Dallegri et al., 1991, Piccard et al., 2012), however low ROS levels may lead to the acquisition of tumour-promoting mutations (Knaapen et al., 2006). Co-culture of neutrophils with a variety of cell types showed their genotoxic properties to induce mutations, such as sister-chromatid exchanges, DNA strand breaks and DNA base modifications, that did not lead to cell death but malignant transformation. This carcinogenic function of neutrophils is largely attributed to their capacity to generate mutagenic ROS that induces DNA damage, which was confirmed by NADPH oxidase-deficient neutrophils or the use of ROS inhibitors (Knaapen et al., 2006). For example, activated neutrophils caused DNA single-strand breaks and DNA base modifications in several epithelial cell lines (Dizdaroglu et al., 1993, Shacter et al., 1988), moderate levels of neutrophil-derived HOCl were directly shown to cause DNA damage in epithelial lung cells *in vitro* (Gungor et al., 2010) and neutrophil-mediated sister chromatid exchanges in mammalian cells were prevented by agents like superoxide dismutase, catalase and radical scavengers (Weitberg et al., 1985). In fact, pre-treatment of normal mouse fibroblasts with activated human neutrophils or superoxides caused malignant transformation and tumour growth of these cells after transplantation (Weitzman et al., 1985). *In vivo*, a strong correlation with genotoxicity and neutrophil influx was reported upon lung exposure to several different stimuli that lead to tumour development or chronic inflammation including quartz, carbon black, diesel fumes or lipopolysaccharides (Knaapen et al., 2006). For instance,

intratracheal instillation of the carcinogen quartz led to neutrophil influx, mutations in p53 and subsequent lung tumour growth (Seiler et al., 2001) and neutrophils accumulating in high numbers in bronchioalveolar lavage fluid of quartz-induced lung tumour-bearing mice showed high mutagenicity to lung epithelial cell lines (Driscoll et al., 1997). Moreover, lung neutrophil influx or depletion correlated with accumulation or reduction of mutations in the HPRT (hypoxanthine guanine phosphoribosyl transferase) gene in lung cells *in vivo* after lipopolysaccharide exposure, respectively (Gungor et al., 2010). Moreover, independent from direct carcinogenic insults, cancer cell lines with various expression levels of the neutrophil chemoattractant IL-8 displayed different neutrophil frequencies in the TME that positively correlated with genomic instability (Haqqani et al., 2000). These observations suggest a contribution of neutrophils to tumour initiation processes in the lung by neutrophil-derived ROS-mediated mutagenesis.

In contrast, neutrophils were shown to mediate the lysis of cancer cells via oxidative burst, release of ROS and antibody-dependent cellular cytotoxicity already back in the 1980s and 1990s. These studies showed that activated neutrophils lysed a variety of cancer cells, but not fibroblasts, via ROS and oxygen radical release mainly *in vitro* and that these anti-tumourigenic neutrophils could be recruited to tumour sites *in vivo* by injection of bacterial-derived products (Dallegri et al., 1991, Dallegri et al., 1984, Fujimura and Torisu, 1987, Gerrard et al., 1981). For example, neutrophils mediated antibody-dependent cellular cytotoxicity against malignant B cells, melanoma and neuroblastoma cells through Fc receptor RI or RII and RIII (Elsasser et al., 1996, Kushner and Cheung, 1992). Also, treatment with IL-1beta increased neutrophil frequencies in the circulation that correlated with reduced melanoma growth and direct transfer of neutrophils into the tumour also inhibited tumour growth (Neville et al., 1990). Another melanoma study showed tumour-associated neutrophils in very high numbers preferentially kill primary over metastatic cancer cells *in vitro* (Schaider et al., 2003), suggesting cancer cell evolution and evasion from neutrophil cytotoxicity. Neutrophil recruitment, anti-tumourigenic and anti-metastatic properties of neutrophils are at least partially mediated by the receptor Met that is upregulated in neutrophils by tumour cell-derived signals such as TNF-alpha. Upon HGF engagement of MET, neutrophils can extravasate to the primary tumour or metastatic site and upregulate iNOS

leading to ROS-mediated cytotoxicity against cancer cells *in vitro* (Finisguerra et al., 2015). Neutrophils were also shown to cause tissue damage to stromal cells in the TME to limit tumour growth. VCAM1 and E-selectin-mediated leukocyte infiltration was associated with neutrophil cytotoxicity towards tumour blood vessels and hypoxia-induced tumour cell death (Colombo et al., 1996). This observation goes hand in hand with another report that suggested broader, context-dependent neutrophil cytotoxicity towards normal mammalian cells (Becker, 1988).

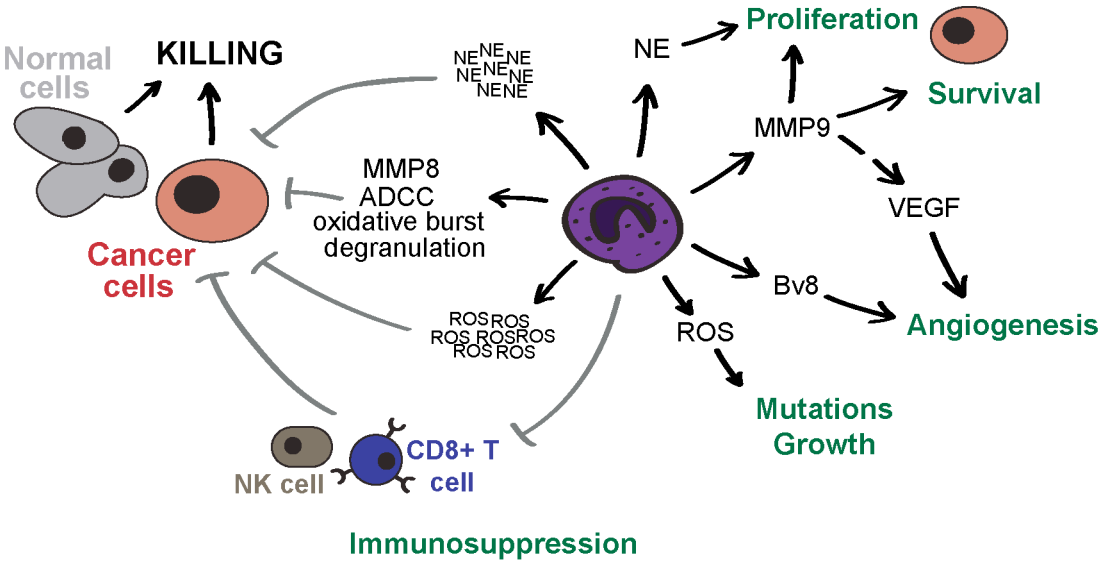


Figure 1-7 The pleiotropic roles of neutrophils in cancer
Neutrophils were shown to have both, pro- and anti-tumourigenic functions in the tumour microenvironment. Selected examples including the involved processes and neutrophil-released factors are depicted.

1.3.5.2 Neutrophils in metastasis

Neutrophils were described as important positive mediators of metastasis. Neutrophil recruitment to the primary tumour site was essential for the acquisition of metastatic traits by benign fibrosarcoma cells. Interestingly, neutrophil-ablation only at early tumour stages promoted metastatic ability, but was negligible at later time points (Tazawa et al., 2003), suggesting that the functions of neutrophils appear to be influenced by tumour stage. Elegant studies suggested the interaction of cancer cells with neutrophils to promote tumour cell invasiveness. Neutrophil-derived Bv8, a secreted signalling proteins, was identified as mediator of neutrophil-dependent angiogenesis in a pancreatic cancer mouse model (Shojaei et al., 2008) as well as inducer of cancer cell migration through its cancer cell expressed receptor PKR (Prokineticin receptor)-1 (Kowanetz et al., 2010). Moreover, breast cancer cell-derived GM-CSF stimulated neutrophils to produce oncostatin M that, in turn, promoted cancer cells to release VEGF and supported their invasiveness (Queen et al., 2005). Similarly, cholangiocellular or hepatocellular carcinoma cells induced HGF expression by neutrophils which also enhanced cancer cell invasion (Imai et al., 2005). Neutrophils were likewise shown to promote invasion of oral cell carcinoma cells (Glogauer et al., 2015) and melanoma cells. UV irradiation, a high risk factor for melanoma, caused release of HMGB (high-mobility group box) 1 by damaged keratinocytes and neutrophil recruitment via TLR4. These neutrophils stimulated angiogenesis and invasiveness of melanoma cells, especially their migration towards and along endothelial cells (Bald et al., 2014). Neutrophils were also shown to be involved in facilitating cancer cell trapping and extravasation. Melanoma cells entrapped in lung capillaries attracted neutrophils that enhanced their integrin-beta2 expression. Subsequently, melanoma cells piggybacked neutrophils that transmigrated through the endothelium by binding them via their ICAM1 (Huh et al., 2010). A similar mechanism of direct extravasation support of neutrophils to tumour cells was described for a lung cancer model that metastasises to the liver (Spicer et al., 2012). In the same model, NET deposition by neutrophils in the liver vasculature also aided trapping and subsequent hepatic metastasis formation (Cools-Lartigue et al., 2013). These studies clearly demonstrate a promoting effect of neutrophils

on cancer cell dissemination and arrival at the metastatic site, but there is little known about their function during initiation and outgrowth of metastases. In a breast cancer mouse model, tumour-derived IL-1-beta elicited a systemic inflammatory state by inducing gamma-delta T cells to express IL-17. In turn, IL-17 upregulated systemic G-CSF levels and caused neutrophilia, similar to it's function during infection or autoimmune diseases. Subsequently, circulating neutrophils were shown to inhibit cytotoxic CD8+ T cell activation and thereby facilitated metastatic growth in the lung (Coffelt et al., 2015). Another study corroborated these observations and involved mammary cancer cell-derived IL-6 and CCL20 in recruiting and activating T cells to upregulate IL-17 which, in turn, led to infiltration of pro-tumourigenic neutrophils (Benevides et al., 2015). Additionally, neutrophil-like cells have been proposed to have pro-metastatic effects in mammary cancer mouse models that are independent from immunosuppression. One study identifies neutrophil-like cells that are recruited to the mammary tumour and the metastatic lung in a CXCL1/2-dependent fashion and promote survival of mammary cancer cells, chemoresistance and metastasis via secretion of S100A8/A9 (Acharyya et al., 2012).

In contrast, anti-tumourigenic/anti-metastatic properties of neutrophils were observed in a xenografted metastatic breast cancer cell line model. There, neutrophils infiltrated the pre-metastatic and metastatic lung and their depletion promoted metastatic seeding. Circulating neutrophils from these tumour-bearing mice, but not G-CSF-stimulated naïve neutrophils, directly induced cancer cell death by hydrogen peroxide release *in vitro* (Granot et al., 2011). Another study described neutrophil cytotoxicity towards cancer cells at the metastatic site in two cancer cell line models that was dependent on Met receptor expression and iNOS upregulation (Finisguerra et al., 2015), suggesting a potential anti-metastatic role of neutrophils.

1.3.5.3 Modulation of neutrophil function by the tumour microenvironment

This conflicting evidence on the role of neutrophils in cancer and metastasis might be the result of the TME influencing the neutrophil secretome and functions. For instance, transplantation of a melanoma cell line onto IFN-beta-deficient mice

resulted in neutrophil recruitment and increased tumour growth, which is normalised upon neutrophil depletion. Interestingly, tumour-associated neutrophils of IFN-beta-deficient mice showed increased expression of CXCR4, VEGF and MMP9 that could be reverted by addition of recombinant IFN-beta (Jablonska et al., 2010). Moreover, neutrophil depletion resulted in differential effects on tumour progression depending on the treatment with a TGF-beta inhibitor (Fridlender et al., 2009). In non-TGF-beta inhibitor-receiving mice, neutrophil blockade resulted in increased CD8+ T cell activation and reduced tumourigenesis. Independent from neutrophils, TGF-beta inhibition reduced mesothelioma and lung cancer cell line-derived tumour growth via activation of cytotoxic CD8+ T cells and macrophages. Treatment with TGF-beta inhibitor also caused influx of neutrophils. Neutrophil-depletion in TGF-beta inhibitor-treated mice blunted the tumour-suppressive effect by the TGF-beta inhibitor and impaired CD8+ T cell activation as well as elevated tumour growth. The anti-tumourigenic neutrophils in a TME lacking TGF-beta expressed pro-inflammatory cytokines such as TNF-alpha, iNOS and ICAM1 and were cytotoxic towards tumour cells. In contrast, the pro-tumourigenic neutrophils under presence of TGF-beta produced Arg1, CCL2 and CCL5 (Fridlender et al., 2009). These observations suggest a possible polarisation of neutrophil phenotypes by IFN-beta and TGF-beta similar to macrophages. In concert, a follow up study identified three different circulating neutrophil populations that differed in their maturity, size and pro- or anti-tumourigenic function. These included granulocytic-MDSCs, mature low-density neutrophils that display pro-tumourigenic and immunosuppressive properties as well as mature high-density neutrophils that are anti-tumourigenic and cytotoxic. Interestingly, pro-tumourigenic neutrophils appear to be preferentially propagated in cancer bearing hosts, eventually via a TGF-beta-mediated transition from high-density neutrophils (Sagiv et al., 2015).

In conclusion, the various contributions of neutrophils to tumour onset and progression are only beginning to emerge. In particular, the investigation of the functions of neutrophils in metastatic colonisation is in its infancy (Mantovani, 2014, Gregory and Houghton, 2011, Houghton, 2010), despite metastasis posing the highest risk in the clinic. It appears that neutrophils can both, promote and limit tumourigenesis in experimental models. Hence neutrophil activity is very context-dependent as well as a question of the actual levels and activation status of

present neutrophils (Gregory and Houghton, 2011, Piccard et al., 2012). Nevertheless, clinical data strongly suggest a pro-tumourigenic role of neutrophils in many different cancer types and scenarios (Donskov, 2013), stressing their significance within the TME and therapeutic interest. We have to improve our understanding of the mechanisms behind the actions of tumour- and metastasis-associated neutrophils, because neutropenia comes with harmful side effects such as susceptibility to infection (Gibson and Berliner, 2014). Re-education of neutrophils to anti-tumourigenic, more acute inflammatory roles (Souto et al., 2011, Fridlender et al., 2009), however a lot of further research efforts will be necessary to improve our understanding of these processes. Hence, dissecting the role of neutrophils in cancer initiation and progression and finding targetable neutrophil-derived mediators is paramount for the development of new therapeutic approaches specifically targeting their pro-tumorigenic activity.

Chapter 2. Materials & Methods

2.1 Mouse strains

The MMTV-PyMT+ mice were a kind gift from Dr Erik Sahai (The Crick Institute, London), Actin-GFP (mice expressing GFP under the control of the Actin promoter), G-CSF-/-, CXCR2-/-, v-Ha-Ras transgene (TG.AC)+ and Rag1-/- mice were a kind gift from Dr Joerg Huelsken (École Polytechnique Fédérale de Lausanne, Lausanne), Actin-Luciferase (mice expressing Firefly Luciferase under the control of the Actin promoter) transgenic lines were a kind gift from Dr Dominique Bonnet (The Crick Institute, London), Rosa26R-EGFP-DTA mice were a kind gift from Dr Caetano Reis e Sousa (The Crick Institute, London), ela2-Cre knock in mice were purchased from the European Mouse Mutant Archive (EMMA), Alox5 null mice were purchased from Jackson Laboratory. All mouse strains have been used and described previously (Cacalano et al., 1994, Chen et al., 1994, Guy et al., 1992, Ivanova et al., 2005, Lassailly et al., 2013, Leder et al., 1990, Lieschke et al., 1994, Mombaerts et al., 1992, Okabe et al., 1997, Tkalcevic et al., 2000). We used wildtype, MMTV-PyMT+, Actin-GFP, Actin-Luciferase and Rag1-/- mice in pure genetic FVB/N background (Taketo et al., 1991) (more than 10 generations); wildtype, MMTV-PyMT+ and Alox5-/- mice in pure C57BL/6 background (Mekada et al., 2009); wildtype and CXCR2-/- mice in pure BALB/c background (Potter, 1985) as well as G-CSF-/-, v-Ha-Ras transgene (TG.AC)+, ela2-Cre and Rosa26R-EGFP-DTA mice in mixed genetic background with littermate controls. MMTV-PyMT+ mice in FVB/N background have a very high incidence of lung metastasis, while the same transgene in C57BL/6 background shows lung metastasis in only about 40-50% of mice at late stages (Roy et al., 2011, Fantozzi and Christofori, 2006, Guy et al., 1992).

All mice were bred at our own establishment and typically used at 6-8 weeks of age, excluding spontaneous tumour models. Breeding and all animal procedures were performed in accordance with United Kingdom regulations under project license PPL/80/2531.

2.2 Mouse experiments

Where applicable, mice were anaesthetised with IsoFlo® (Isoflurane, Abbott Animal Health) and temporally treated with the analgesics Vetergesic® (Alstoe Animal Health) and/or Rimadyl™ (Pfizer Animal Health).

2.2.1 Evaluation primary mammary tumour and lung metastasis burden in mice (Fig. 2.1)

Dissecting and weighing the entire mammary tumour (or multiple tumours if present) provided the measure for primary tumour burden and it is displayed in gram. Images were taken using a digital, handhold camera. Metastatic load was quantified by counting nodules on the surface of the lung using the Zeiss SteREO Lumar.V12 microscope and is shown as lung metastasis incidence for intravenously injected cancer cells or relative to primary tumour burden for spontaneous metastasis (Nodules/gram tumour). Alternatively, presence of experimental metastases was occasionally determined by sectioning the lung and staining with haematoxylin and eosin. Experimental lung metastases derived from GFP+ cancer cells were quantified by fluorescent microscopy (Zeiss SteREO Lumar.V12 microscope), by flow cytometric analysis of frequencies of GFP+ cells in total lung or sectioning the lung and immunohistochemically staining for GFP. Metastatic burden of Luciferase-expressing tumour cells was evaluated by measuring bioluminescence intensity (section 2.2.16).

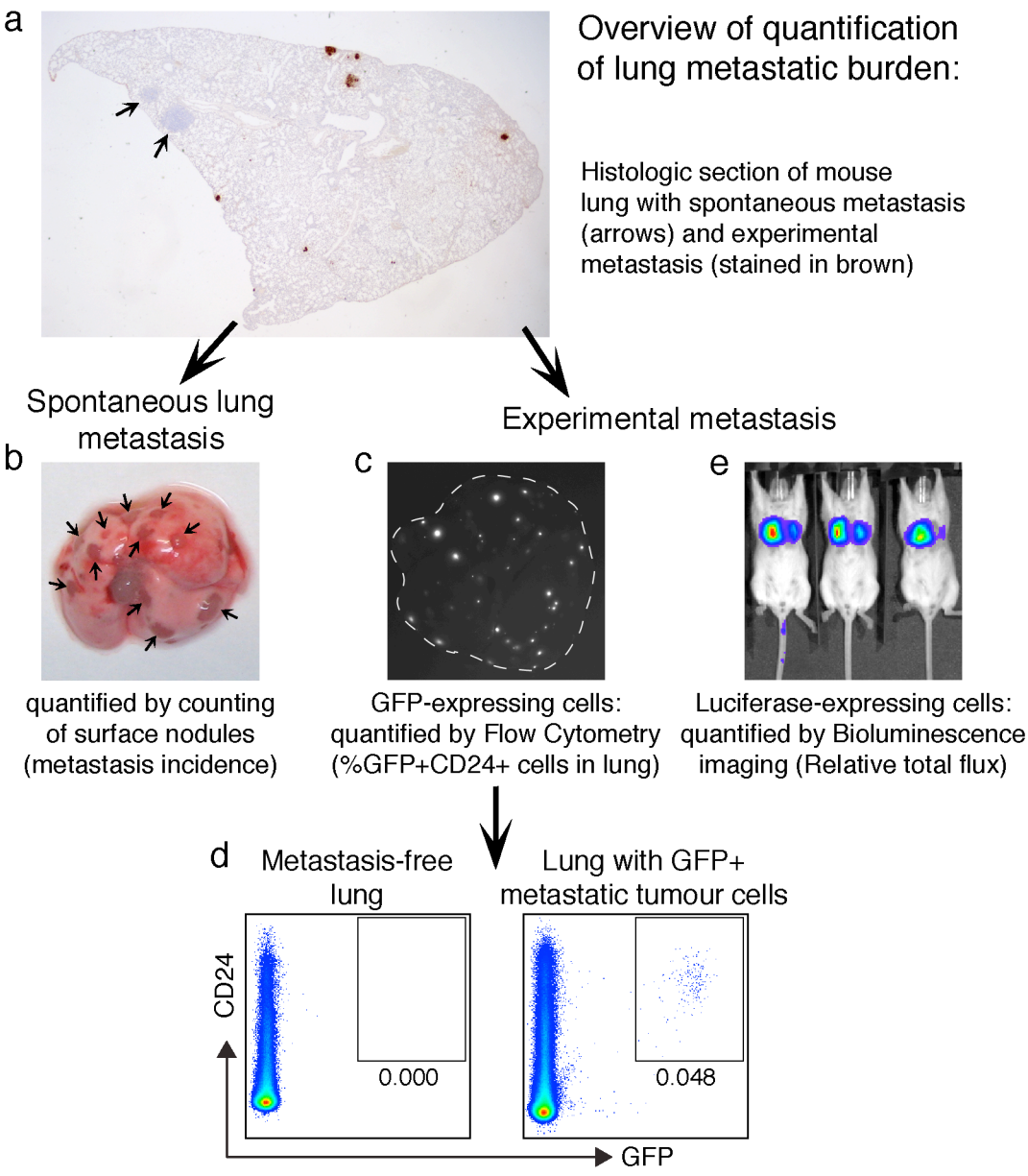


Figure 2-1 Quantification of lung metastasis burden
(a) Haematoxylin (blue) and GFP (brown, GFP+ cancer cells) stained histologic lung section. (b) Photograph of metastatic lung with spontaneous metastasis. (c) Stereomicroscopic image of green fluorescent channel of lung with GFP+ experimental metastasis and (d) representative flow cytometric analysis of frequencies of GFP+CD24+ metastatic MMTV-PyMT cancer cells in the lung, gated on CD45-negative CD31-negative cells. Insert in (d) shows relative frequencies of double-positive cells contained in the displayed gate. (e) Image of bioluminescence intensity emitted by Luciferase-expressing cancer cells (experimental metastasis) in the lung.

2.2.2 Tumour cell transplantations and induction of experimental metastasis

FVB/N wildtype mice were used for MMTV-PyMT tumour cell transplantations to isolate lung neutrophils and analysis of the pre-metastatic lung microenvironment. Rag1-/- mice were used for orthotopic transplantation testing tumour initiation potential of transplanted cancer cells or when using MMTV-PyMT G-CSF-/- cancer cells. Also, Rag1-/- mice were used for intravenous injection experiments involving human or mouse GFP- or Luciferase-expressing tumour cells. Primary MMTV-PyMT, MMTV-PyMT Actin-GFP or MMTV-PyMT Actin-Luciferase cells (10^5-10^6 cells per injection), the unmarked or stably mPGK-GFP-expressing mouse mammary cancer cell line 4T1 (10^5 cells per injection) and the unmarked or stably Actin-GFP-expressing human breast cancer cell line MDA-MB-231 (1-2x10^6 cells per injection) were used to orthotopically graft mammary tumours or to induce experimental metastasis by injection via the tail vein. For experimental metastasis, tumour cells were re-suspended in 100µl PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2PO_4 and 1.8 mmol/L KH_2PO_4 in distilled water) and tail vein injected. For orthotopic transplantations, tumour cells were re-suspended in 50µl growth-factor-reduced Matrigel (Costar, Cat.Nr. 356231) and transplanted into a small pocket within the fourth mammary fat pad on both flanks (MMTV-PyMT and MDA-MB-231 cells) or one flank only (4T1 cells).

2.2.3 Assessment of tumour and metastasis initiation capacity of MMTV-PyMT cancer cells

For determination of tumour initiation potential, 10^3 flow sorted BLT2+ and/or CysLT2+ MMTV-PyMT cancer cells, three-day neutrophil-conditioned medium (LuN-wt, LuN-Alox5ko or LuN-Zil) or LTB4 and LTC-D-E4 pre-treated MMTV-PyMT cells in adherent conditions and respective controls were grafted onto the mammary glands of Rag1-/- mice and tumour burden analysed five weeks thereafter.

For assessment of metastatic lung colonisation competence, 10^5 three-day neutrophil-conditioned medium (LuN) or LTB4 and LTC-D-E4 pre-treated MMTV-

PyMT cells and respective controls were injected into Rag1-/- mice and lung harvested and metastatic burden analysed five weeks thereafter.

2.2.4 Experiments analysing systemic neutrophil mobilisation and immune cell infiltration into pre-metastatic or metastatic lung of MMTV-PyMT tumour bearing mice

MMTV-PyMT+ mice in FVB/N background that spontaneously developed a primary tumour and had visible lung metastasis were used to determine immune cell presence in the lung and neutrophil presence in other organs (bone marrow, spleen, liver and mammary tumour) together with tumour-free littermate controls. For determination of timing and dynamics of lung infiltration by neutrophils and cancer cells, MMTV-PyMT+ mice carrying very small, spontaneously developed mammary tumours were used. Neutrophil infiltration was quantified by flow cytometry and cancer cell presence by examination of six histological lung sections (100µm apart) for Polyoma middle T antigen staining to confirm the pre-metastatic status of the lung of MMTV-PyMT+ tumour-bearing mice in these experiments. The timing of neutrophil infiltration into the pre-metastatic prior to cancer cells was confirmed in FVB/N wildtype mice carrying with two primary tumours originating from orthotopic injection of primary MMTV-PyMT cancer cells. These mice were used for analysis of pre-metastatic lung neutrophils and neutrophil-dependent immune cell presence in the pre-metastatic lung (daily treated with anti-Ly6G or control IgG-antibody starting 24 hours prior to tumour cell implantation).

2.2.5 Analysis of MMTV-PyMT+ G-CSF and MMTV-PyMT+ ela2-DTA mice

Mice were culled and analysed about six weeks after spontaneous primary tumour onset together with tumour-free control littermates. The analysed genotypes are described in detail in section 2.1. We did not observe any bias in penetrance of lung metastasis in these MMTV-PyMT+ mice in mixed background and all controls readily displayed lung metastasis at late tumourigenic stages.

2.2.6 *In vivo* treatments with neutrophil-blocking antibody anti-Ly6G or Zileuton

12.5 µg/mouse rat anti-Ly6G antibody (clone 1A8, BioXcell, Cat.Nr. BE0075-1) (Daley et al., 2008, Granot et al., 2011) or rat IgG isotype control (kindly provided by the Cell Services Unit of the London Research Institute of Cancer Research UK) in 100µl PBS were administered daily via intra-peritoneal injection. Zileuton (LKT Laboratories Inc., Cat.Nr. 111406-87-2) dissolved in DMSO (Dimethyl Sulfoxide, Sigma, Cat.Nr. 41640) or DMSO alone was fed to mice by pipetting on the back of the tongue once a day at a dosage of 4mg Zileuton / gram mouse weight.

2.2.7 Experiments analysing the effect of *in vivo* neutrophil depletion or Zileuton treatment on lung colonisation by cancer cells

Rag1-/- mice were orthotopically transplanted with unlabelled mammary tumour cells: 10^6 MMTV-PyMT cells or MDA-MB-231 cells into both fourth mammary glands and 10^5 4T1 cells into one mammary gland. Two or four weeks later, as indicated for individual experiments, labelled tumour cells were injected via the tail vein (intravenously): 10^5 GFP+ MMTV-PyMT cells or GFP+ 4T1 cells and 10^6 GFP+ MDA-MB-231 cells. Anti-Ly6G or Zileuton treatment for 10 days, two or four weeks, as indicated for individual experiments, started one day prior to intravenous injection of cancer cells. Then, total primary tumour burden, neutrophil presence in the lung, spontaneous lung metastasis incidence from the transplanted primary tumour and/or experimentally induced lung micro-metastasis originating from the intravenously injected cancer cells was analysed.

2.2.8 Experiments analysing the tumour and/or metastasis initiation potential of sorted leukotriene receptor-positive/negative or of *in vitro* pre-treated primary MMTV-PyMT cells

Primary MMTV-PyMT cells were either cell sorted for leukotriene receptor presence or absence, or treated for three days on collagen-coated dishes with either neutrophil-conditioned medium or LTB4 and LTC-D-E4. Subsequently, 10^3 cells were orthotopically transplanted into the mammary gland or 10^5-10^6 cells injected

via the tail vein into Rag1-/- mice and mammary tumour growth or lung metastasis incidence analysed about three weeks or five weeks thereafter, respectively.

2.2.9 Injections of neutrophils and neutrophil-conditioned medium and quantification of metastasis-initiating cells at early stages as well as metastatic burden *in vivo*

To analyse total cancer cells at early stages, Rag1-/- mice were injected with 10^6 GFP+ MMTV-PyMT cells via the tail vein followed 12 hours later by intravenous injection of $25x10^6$ neutrophils (freshly isolated from the lung of MMTV-PyMT tumour-transplanted mice) or 12, 24 and 36 hours later by intravenous injection of 200µl lung neutrophil-conditioned or control sphere medium. Cancer cells in the lung were analysed three days after intravenous tumour cell injection for frequencies of CD90+ metastasis-initiating cells (MICs) among GFP+CD24+ (nonMIC) cancer cells. For determination of effects of neutrophils or neutrophil-conditioned medium on metastatic burden at later stages, Rag1-/- mice were intravenously injected with $5x10^5$ GFP+ MMTV-PyMT or Luciferase-expressing MMTV-PyMT cells followed immediately, two and four days later by injection of $25x10^6$ neutrophils or five times every 12 hours by injection of 200µl lung neutrophil-conditioned medium. Metastatic burden was determined by Flow cytometric analysis of GFP+ cancer cells one week (neutrophil injections) or bioluminescence imaging of Luciferase-expression cancer cells 2.5 weeks (neutrophil-conditioned medium injections) thereafter.

2.2.10 Analysis of functional effects of G-CSF-deficiency of MMTV-PyMT cancer cells

Rag1-/- mice were transplanted with 10^6 G-CSF-/- primary MMTV-PyMT cancer cells or MMTV-PyMT cancer cells isolated from littermate controls into both fourth mammary glands and tumour growth, spontaneous metastatic incidence and neutrophil presence in the lung analysed four weeks thereafter.

2.2.11 Recruitment of neutrophils by metastatic cancer cells into the lung

Rag1-/- mice were seeded with 10^6 GFP+ MMTV-PyMT cancer cells into the lung by injection via the tail vein. Three days later mice were sacrificed, lungs harvested and analysed for the presence of CD11b+Ly6G+ neutrophils and GFP+ MMTV-PyMT cancer cells in the lung by flow cytometry.

2.2.12 Evaluation of MMTV-PyMT extravasation into lung tissue under presence or absence of neutrophils

Rag1-/- mice were grafted with two MMTV-PyMT mammary tumours and four weeks later treated with neutrophil-blocking anti-Ly6G or control IgG antibody. 24 hours thereafter, mice were intravenously injected with 10^6 GFP+ MMTV-PyMT cancer cells and treated a second time with antibodies. Mice were sacrificed and the lungs perfused with PBS 20 hours after intravenous cancer cell injection. Lungs were digested and the presence of CD11b+Ly6G+ neutrophils and GFP+ MMTV-PyMT cancer cells determined by flow cytometry.

2.2.13 Induction of benign skin papilloma growth

Papilloma development on eight weeks old v-Ha-Ras transgene (TG.AC)-expressing mice was achieved by topical application of 12.5 µg/mouse TPA (Sigma, Cat.Nr. P8139) dissolved in Acetone (Sigma, Cat.Nr. 650501) onto the back skin twice weekly for 3.5 months. At this time, the mice carried 4-6 skin papilloma of 0.5-1 cm in diameter that were superficial and did not penetrate the dermis. Wildtype littermate controls were simultaneously treated with TPA but did, as expected, not develop detectable skin papilloma. Mice were sacrificed, lungs harvested and analysed for the presence of CD11b+Ly6G+ neutrophils by flow cytometry.

2.2.14 Resection of grafted MMTV-PyMT mammary gland tumours

FVB/N wildtype mice were orthotopically transplanted with primary MMTV-PyMT cells into the fourth mammary fat pad on one side and the tumour grown to a size

of approximately 1x1x0.5cm. Surgical excision of the tumour was performed two weeks after tumour grafting by cutting the skin, sealing of blood vessels, removing the tumour and suturing of the skin. Mice recovered from surgery within hours and did not display any obvious adverse effects. Presence of CD11b+Ly6G+ neutrophils in the lung was analysed in untreated control mice, tumour-bearing mice at time of resection, and mice 24 hours or one week post-tumour removal.

2.2.15 Bone marrow transplantation of CXCR2-/- and Alox5-/- bone marrow-reconstituted mice

C57BL/6 wildtype mice were lethally irradiated (dosage: 2x 600rad, 4 hours apart) and 24 hours later injected via the tail vein with $2x10^6$ bone marrow cells freshly isolated from C57BL/6 or Alox5-/- donor mice. Alox5-/- bone marrow-reconstituted mice were orthotopically transplanted with 10^6 MMTV-PyMT cells into the fourth mammary fat pad on both sides eight weeks after bone marrow reconstitution and sacrificed for analysis of primary tumour burden, neutrophil infiltration into the lung and lung metastasis six weeks thereafter. Bone marrow-reconstituted mice where generated a pure C57BL/6 due to the background of Alox5-/- mice. Therefore, MMTV-PyMT cells from the same C57BL/6 background where used to generate primary tumours. In this lower tumourigenic background compared to FVB/N, metastasis only occurs in 50% of the mice (Roy et al., 2011, Das Roy et al., 2009, Fantozzi and Christofori, 2006, Guy et al., 1992). No alteration in this low penetrance was observed between wildtype and Alox5-/- bone marrow-reconstituted mice, therefore lung metastatic burden was only quantified in animals harbouring metastatic disease. Percentage of bone marrow-reconstitution was calculated by isolating total DNA from bone marrow of reconstituted mice and semi-quantitative PCR with a calibration curve from 100% wildtype DNA mixed at defined ratios with 100% Alox5 null DNA. Ratio between wildtype and Alox5-/- band was calculated for every mouse and percentage bone marrow reconstitution determined by comparison with calibration curve. Reconstitution was consistently between 80-96%.

BALB/c wildtype mice were irradiated (dosage: 2x 500rad, 4 hours apart) and also 24 hours later injected with $2x10^6$ bone marrow cells freshly isolated from BALB/c

or CXCR2-/- donor mice. 10^5 cells of the 4T1 mouse mammary cancer cell line that is in the syngeneic BALB/c background were grafted onto one mammary gland of reconstituted mice and mice analysed 2.5 weeks thereafter. CXCR2-/- mice are in BALB/c genetic background; hence bone marrow-reconstituted mice were generated in the same background and the syngeneic 4T1 cell line used. The efficacy of reconstitution of these mice was determined by flow cytometric analysis of lung neutrophils for the surface expression of CXCR2.

2.2.16 *In vivo* Luciferase-activity detection

Mice inoculated with Luciferase-expressing MMTV-PyMT cells were shaved around the chest area and injected with 3 mg XenoLight D-Luciferin Potassium Salt (PerkinElmer, Cat.Nr. 122799) dissolved in PBS into the peritoneum 5 minutes prior to imaging for at least 45 minutes using the IVIS® Spectrum Pre-clinical In Vivo Imaging System (PerkinElmer). The maximum bioluminescence intensity signal for the lung of every mouse was determined using Living Image 4.3.1 software (PerkinElmer) as total bioluminescence flux detected in the chest area.

2.2.17 *In vivo* BrdU incorporation assay

Rag-/- mice carrying MMTV-PyMT tumours were treated daily for three days with Zileuton and IV injected with 10^5 GFP+ MMTV-PyMT cancer cells. BrdU (1mg per mouse dissolved in PBS) was intraperitoneally injected 18 hours after GFP+ cancer cells and lungs harvested and digested six hours later. APC BrdU Flow Kit (BD Bioscience, Cat.Nr. 557892) was used for fixation/permeabilisation and anti-BrdU staining of single lung cells according to manufacturer's instructions followed by analysis by flow cytometry.

2.3 Analysis of mouse and human tissues

2.3.1 Tissue staining, immunohistochemistry and light microscopy

Mouse lung tissue was fixed in 4% paraformaldehyde (Sigma, Cat.Nr. P6148) in PBS for 24 hours, dehydrated for one hour each in 70% Ethanol (Sigma Cat.Nr. 459844), twice in 100% Ethanol and three times in 100% Xylene (Sigma, Cat.Nr. 247642) followed by three times infiltration with liquid paraffin wax (Sigma, Cat.Nr. 327204) at 65-70°C by a the Tissue Tek VIP Vaccum Infiltration processor followed by embedding into paraffin blocks on the Tissue Tek TEC embedding station. 4µm sections were cut with a Leica RM 2135 microtome, dried and re-hydrated with Xylene, Ethanol and water before antibody. Breast cancer tissue array paired with metastatic tumours, 96 samples (1.5mm) was purchased from Abcam (Cat.Nr. ab178118).

Lung sections were stained with Haematoxylin (Sigma, Cat.Nr. H3136) and Eosin (Sigma, Cat.Nr. 861006) with water and one 0.3% Acid alcohol solution (Sigma, Cat.Nr. 56694) washing steps in-between followed by dehydration with Ethanol and Xylene and mounted with CLARION^{TM} mounting medium (Sigma, Cat.Nr. C0487).

For immunohistochemistry, either secondary HRP-conjugated antibodies were used in combination with DAP Peroxidase substrate or the VECTASTAIN® ABC kit (all Vector Laboratories, Cat.Nrs. SK-4100 and PK-6105). Used primary antibodies included monoclonal rat anti-mouse S100A9 (clone 2B10, kindly provided by the Experimental Histopathology unit of the London Research Institute, Cancer Research UK), monoclonal rat anti-Polyoma virus-middle T antigen (PyMT) (Santa Cruz, clone and Cat.Nr. sc-53481), polyclonal goat anti-GFP (Abcam, Cat.Nr. ab6556), polyclonal rabbit anti-human BLT2 Receptor (Cayman Chemical, Cat.Nr. 120124) and polyclonal rabbit anti-human CysLTR2 antibody (Sigma, Cat.Nr. SAB2900039). Visualisation of cell nuclei was performed with haematoxylin and analysis employed the Nikon Eclipse 90i light microscope and NIS-elements software (Nikon).

2.3.2 Scoring of leukotriene receptor expression in human breast cancer tissue and paired lymph node metastasis (Fig. 2.2)

All breast cancer and lymph node samples of the Abcam tissue microarray (Cat.Nr. ab178118) stained for human BLT2 or human CysLT2 that contained sufficient tissue were sored as follows: Staining intensity was determined as negative (score 0), weak (score 1), moderate (score 2) or strong (score 3) and frequency of stained cells as 0% (score 0), <5% (score 1), 5-25% (score 2), 25-50% (score 3), 50-75% (score 4) and >75% (score 5). Staining intensity score and cell frequency score was added to a final score value for every sample.

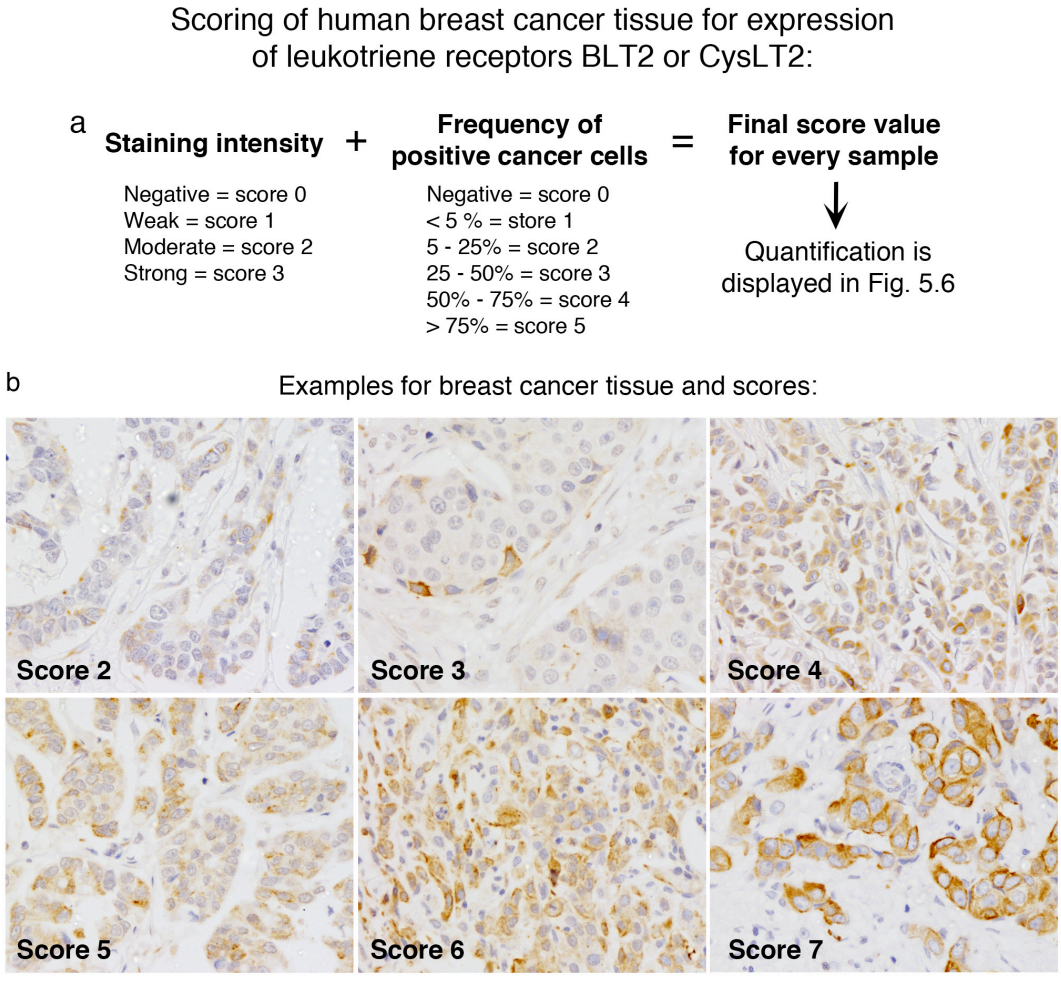


Figure 2-2 Scoring system for stained human breast cancer and lymph node metastasis tissue
(a) Overview on calculation of individual score for every sample: staining and frequency score are added to one final value. (b) Examples of breast cancer tissue stained for leukotriene receptors BLT2 or CysLT (brown) and haematoxylin to visualise nuclei (blue) with the indicated final score values.

2.3.3 Tissue digestion for cell isolation or analysis

Primary MMTV-PyMT cells, GFP+ MMTV-PyMT cells, Luciferase-expressing MTV-PyMT cells and G-CSF-/- MMTV-PyMT cells were isolated from spontaneously formed mammary gland carcinomas of MMTV-PyMT+, MMTV-PyMT+ Actin-GFP, MMTV-PyMT+ Actin-Luciferase or MMTV-PyMT G-CSF-/- mice, respectively. Primary MMTV-PyMT cells, GFP+ MMTV-PyMT cells and Luciferase-expressing MTV-PyMT cells were always used in genetic FVB/N background, with the exception of Alox5-/- bone marrow-reconstituted mice were C57BL/6 MMTV-PyMT cells were used. G-CSF-/- MMTV-PyMT cells were used in mixed genetic background.

Primary MMTV-PyMT tumours, liver, spleen and lung were dissected, minced, digested with Liberase TM and TH (Roche, Cat.Nrs. LIBTM-RO and LIBTH-RO) and DNaseI (Sigma, Cat.Nr. AMPD1) in HBSS (Sigma, Cat.Nr. H6648) and passed through a 100µm cell strainer. Some tumour cells were used for cell culture at this point. Bone marrow cells were isolated by crushing the femur and tibia and blood collected via bleeding from the tail vein with Heparin (Sigma, Cat.Nr. 84020) as a coagulant. For flow cytometric analysis or further purification, single cell suspensions of tumour, liver, spleen, lung, bone marrow and blood were subjected to hypotonic lysis (Red Blood Cell Lysis Solution, Miltenyi, Cat.Nr. 130-094-183) to remove erythrocytes and washed with MACS buffer in PBS, 2mM EDTA and 0.5% BSA (Sigma, Cat.Nr. A9418). Cells were immediately used for either re-injection into mice, flow cytometric analysis or cell culture.

2.3.4 Flow cytometry and cell sorting

Prepared single cell suspensions of mouse tissues and in vitro treated cancer cells were incubated with mouse FcR Blocking Reagent (Miltenyi, Cat.Nr. 130-092-575) followed by incubation with (a combination) of the following pre-labelled antibodies; TER119 (clone TER-119), CD24 (clone M1/69), CD31 (clone 390), CD45 (30-F11), CD90.1 (clone HIS51), CD11b (clone M1/70), CD19 (clone 1D3), CD49b (clone DX5), CD49f (clone GoH3), CD4 (clone GK1.5), CD8a (clone 53-6.7), CD44 (clone IM7), CD69 (clone H1.2F3), CD25 (clone PC61.5), CD115 (clone AFS98), CD86

(clone GL1), Fas (clone 15A7), MHC-II (clone M5/114.15.2), Ly6G (clone 1A8), CD11c (clone HL3), CD3 (145-2C11), Siglec-F (clone E50-244-), ICAM1 (clone 3E2), H-2kq (MHC-I, clone HK114), F4/80 (clone BM8), CXCR2 (clone 242216), polyclonal rabbit anti-BLT1 (Bioss Inc., Cat.Nr. bs-2654R) and polyclonal rabbit anti-BLT2 (Bioss Inc., Cat.Nr. bs-2655R); and/or unlabelled antibodies: polyclonal rabbit anti-CysLT1 (Santa Cruz, sc-25448) and polyclonal goat anti-CysLT2 (Santa Cruz, Cat.Nr. sc-27097) followed by incubation with fluorescently-labelled secondary antibodies (Invitrogen). For intracellular Foxp3 (antibody clone FJK-16s) staining, pre-stained cells were fixed, permeabilised and stained with the Foxp3 staining set APC (eBioscience, Cat.Nr. 77-5775-40).

Antibody fluorescently-labelled cells (including freshly isolated neutrophils for purity check) were additionally stained with DAPI (4,6-Diamidino-2-phenylindole dihydrochloride, Sigma, Cat.Nr. D9542) or PI (Propidium iodide, Sigma, Cat Nr. P4170) to exclude dead cells and analysed with an LSRFortessa™ cell analyser running FACSDiva™ software (BD Biosciences) and FlowJo software. Freshly isolated MMTV-PyMT tumour cells or 4T1 cancer cells fluorescently stained for BLT2 and CysLT2 or CD24 and CD90 were flow-sorted using the Influx™ cell sorter running FACS™ Sortware sorter software (BD Biosciences). MMTV-PyMT cells were used for *in vivo* experiments immediately after sorting and sorted 4T1 cells cultured in adherent conditions for three days prior to Western blot analysis.

2.4 Ex vivo experiments with primary cells and cell lines

2.4.1 Neutrophil isolation and neutrophil-conditioned medium

Freshly isolated lung cells from wildtype mice orthotopically transplanted with MMTV-PyMT tumours were incubated with mouse FcR Blocking Reagent and APC-coupled anti-Ly6G (clone 1A8) antibody followed by incubation with magnetic anti-APC MicroBeads (Miltenyi, Cat.Nr. 130-090-855). Magnetically labelled neutrophils were isolated using LS columns (Miltenyi, Cat.Nr. 130-042-401) and washed with MACS buffer according to manufacturers instructions. Neutrophil purity and viability was measured by flow cytometry. Some isolated Ly6G+ cells were smeared onto a glass slide and air-dried overnight followed by haematoxylin and eosin staining to evaluate cell morphology. Remaining neutrophils were kept in sphere medium at a concentration of 10^6 neutrophils per 150µl medium for 14 hours to allow conditioning. Neutrophils and cell debris were removed by centrifugation prior to downstream applications of neutrophil-conditioned medium. Lungs of MMTV-PyMT tumour-bearing Alox5-/- or Zileuton-treated wildtype mice were used for production of leukotriene-free neutrophil-conditioned medium LuN-Alox5ko and LuN-Zil, respectively.

2.4.2 Cell culture and *in vitro* cancer cell treatments

All used cell lines were kindly provided by the Cell Services Unit or Dr Erik Sahai of The Crick Institute, London and cultured in DMEM medium (Sigma, Cat.Nr. D5546) supplemented with 10% foetal bovine serum (DMEM/FBS, Invitrogen Cat.Nr. 12662-011). These included the unmarked or stably mPGK-GFP-expressing mouse mammary cancer cell line 4T1 and the unmarked or stably Actin-GFP-expressing human breast cancer cell line MDA-MB-231. Freshly isolated MMTV-PyMT cells were cultured overnight on PureCol® collagen (Advanced Biomatrix, Cat.Nr. 505B)-coated dishes in growth medium DMEM/F12 with 2% FBS, $20\,ng\,ml^{-1}$ EGF (Invitrogen, Cat.Nr. PHG0314) and $10\,\mu g\,ml^{-1}$ insulin (Sigma, Cat.Nr. I9278) before use in experiments. All cultured cells were detached using

treatment with 1mM ETDA in PBS for eight minutes followed by incubation with 0.05% trypsin (Sigma, Cat.Nr. T4799) and 1mM EDTA in PBS for five minutes.
Neutrophil-conditioned medium treatment *in vitro*: Primary MMTV-PyMT cells were cultured on collagen-coated dishes for three days in control or neutrophil-conditioned medium followed by further tests.
Leukotriene treatment *in vitro*: Primary MMTV-PyMT cells were cultured in sphere medium on collagen-coated dishes, 4T1 and MDA-MB-231 cells in DMEM/FBS on uncoated dishes for the indicated periods of time or in non-attachment conditions under presence of (as indicated for every experiment): 100% Ethanol control (EtOH), 1µM Leukotriene B4 (Cayman chemical, Cat.Nr. CAY20110), 100nM Leukotriene C4/D4/E4 (Cysteinyl Leukotriene HPLC Mixture I (Cayman chemical, Cat.Nr. CAY20001), 3µM BLT2 inhibitor LY255283 (Cayman chemical, Cat.Nr. CAY70715), 0.3µM CysLT2 inhibitor BAY-u9773 (Cayman chemical, Cat.Nr. CAY70770) and/or pan-MEK inhibitor PD0325901 (kindly provided by Dr Julian Downwards, The Crick Institute, London) followed by further tests.
Zileuton treatment *in vitro*: Primary MMTV-PyMT cells were cultured in sphere medium and 4T1 cells in DMEM/FBS under presence of 1μM Zileuton or control DMSO for the indicated periods of time.

2.4.3 Sphere formation assay

A single cell suspension of 10^4 MMTV-PyMT cells per well were plated in ultra low-attachment 96-well plates (Costar) in 100µl sphere medium DMEM/F12 (Invitrogen, Cat.Nr. 12634-010) supplemented with B-27® (Invitrogen, Cat.Nr. 10889-038), $20\,ng\,ml^{-1}$ EGF, $20\,ng\,ml^{-1}$ FGF (Invitrogen, Cat.Nr. 13256-029) and $4\,\mu g\,ml^{-1}$ Heparin or neutrophil-conditioned medium. After 7-10 days, if not otherwise indicated, all formed spheres were quantified from images taken with the inverted Leica DM IRBE light and fluorescence microscope. The area of the plane passing through the sphere-centre was measured for every sphere (sphere size) using ImageJ software and the areas of all formed spheres were summed up. The obtained number was divided by total number of plated cells. This value represents the sphere formation index (SFI) per cell for every experimental group and incorporates the number and size of all formed spheres.

Freshly isolated MMTV-PyMT cells were either only treated for three days in adherent conditions prior to sphere assay or directly treated during the sphere assay, as indicated for the individual experiments. Treatments included: neutrophil-conditioned media (control, LuN-wt, LuN-Alox5ko and LuN-Zil), LTB4 and/or LTC-D-E4, PGE2, Zileuton, recombinant CCL2, recombinant CCL6, recombinant CCL22 and recombinant MMP9 protein.

2.4.4 Assessment of toxicity after *in vitro* treatment with neutrophil-conditioned medium and TUNEL staining

Lung stromal cells were freshly isolated from the lung of wildtype mice and cultured in sphere medium in adherent culture on collagen-coated dishes for two days prior to treatment. Lung stromal cells and MMTV-PyMT were seeded in equal numbers and cultured in adherent conditions for three or five days in the following media: control sphere medium and media conditioned by neutrophils isolated from bone marrow or lung of MMTV-PyMT tumour-bearing untreated, DMSO-treated or Zileuton-treated wildtype mice or Alox5-/- mice, as indicated. Cells were washed with PBS, bright field images taken with the inverted Leica DM IRBE light and fluorescence microscope. Cell toxicity of the treatment was determined by detachment of cells and quantification of remaining alive cells using a Neubauer chamber. Presence of apoptotic cells was determined by fixation of adherent cells for ten minutes in ice-cold 100% Methanol (Sigma, Cat.Nr. 34860) and TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labelling) staining using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Cat.Nr. 11684795910) according to manufacturers' instructions and mounted with VECTASHIELD Mounting Medium with DAPI (Vector Laboratories, Cat.Nr. H-1200) followed by analysis with the inverted Leica DM IRBE light and fluorescence microscope.

2.4.5 Detection of intracellular reactive oxygen species (ROS) levels

Primary MMTV-PyMT cancer cells were detached, prepared in single cell suspensions and labelled with the DCF-DA dye that becomes fluorescent upon oxidation by ROS using the DCFDA - Cellular Reactive Oxygen Species Detection

Assay Kit (Abcam, Cat.Nr. ab113851) according to manufacturers' instructions. Ethanol (EtOH) control, 1µM LTB4 or 100nM LTC-D-E4 was added to cell suspensions and developing fluorescent signal in cells was immediately analysed by flow cytometry over time for every condition.

2.4.6 *In vitro* BrdU incorporation assay

Three-day MMTV-PyMT or 4T1 cells treated as indicated in adherent conditions were pulsed with 30µM BrdU for three hours and harvested. Cells were incubated with fluorescently labelled anti-CD24 and/or anti-CD90.1 antibody. BrdU Flow Kit (BD Bioscience) was used for fixation/permeabilisation and anti-BrdU staining according to manufacturer's instructions followed by analysis by flow cytometry.

2.4.7 Assays for *in vitro* quantification of metastasis-initiating MMTV-PyMT cells and highly tumourigenic sub-pools of cancer cell lines

Primary MMTV-PyMT cells were either directly used or cultured on collagen-coated dishes for three days supplemented with either LTB4 and LTC-D-E4 or Zileuton followed by incubation with fluorescently labelled anti-CD90.1 and anti-CD24 antibodies and/or anti-BLT2 and CysLT2 antibodies and analysis by flow cytometry. 4T1 and MDA-MB-231 cell lines were either directly used or cultured in DMEM/FBS supplemented with LTB4 and LTC-D-E4 for three days in adherent conditions followed by either staining with fluorescently labelled anti-CD49f, anti-BLT2, anti-CysLT2 and/or anti-CD44 antibodies and/or using the ALDEFLUOR^{TM} kit (Stemcell Technologies, Cat.Nr. 1700) according to manufacturers instructions and analysed by flow cytometry.

2.5 Molecular biology procedures

2.5.1 RNA expression analysis and quantitative real-time PCR

Neutrophils were freshly isolated from the lungs of wildtype or MMTV-PyMT tumour-bearing mice. RNA isolation was performed using MagMAX™-96 Total RNA Isolation Kit and cDNA synthesis using SuperScript® III Reverse Transcriptase. Quantitative PCR reactions were performed using EXPRESS SYBR® GreenERTM Supermix universal with the Applied Biosystems® 7500 Fast Real-Time PCR System (all Invitrogen, Cat.Nrs. AM1830, 18080-044 and 11784) and the primers TNF-alpha (ACCACGCTCTTCTGTCTACT and AGGAGGTTGACTTTCTCCTG), Arginase 1 (GATTGGCAAGGTGATGGAAG and TCAGTCCCTGGCTTATGGTT), VEGF-A (provided by PrimerDesign), CCL2 (CAGGTCCCTGTCATGCTTCT and GTCAGCACAGACCTCTCTCT), CCL3 (ACCATGACACTCTGCAACCA and TCAGGCATTCAGTTCCAGGT), iNOS (CCACCTCTATCAGGAAGAAA and CTGCACCGAAGATATCTTCA), CCL5 (ACCATGAAGATCTCTGCAGC and TGAACCCACTTCTTCTCTGG) and GAPDH (CGTGTTCCTACCCCCAATGT and TGTCATCATACTTGGCAGGTTTCT).

2.5.2 Semi-quantitative PCR of genomic DNA from Alox5-/- bone marrow-reconstituted mice

PCR was performed using Redtag® DNA Polymerase (Sigma, Cat.Nr. D4309), primers ATCGCCTTCTTGACGAGTTC, GCAGGAAGTGGCTACTGTGGA and TGCAACCCAGTACTCATCAAG and 25 amplification cycles on the T3000 thermocycler (Biometra). A 10% agarose (Sigma, Cat.Nr. A9539) containing GelRed Nucleic Acid Stain (Cambridge Bioscience, Cat.Nr. BT41003) was prepared with and run in Tris borate buffer (45 mmol/L Tris, 45 mmol/L boric acid and 1.25 mmol/L EDTA). Images of DNA gels were taken with the UVP High Performance UV transilluminator (UVP Ltd.).

2.5.3 EIA/Parameter ELISA for measurement of leukotrienes

Equal amounts of 100% Ethanol were used to precipitate protein from control or neutrophil-conditioned cell culture medium followed by 30 minutes centrifugation at 15,000 rpm on a table top centrifuge cooled to 4°C prior to analysis. Processed samples were analysed using either the EIAs (enzyme immuno-assays) Leukotriene C4/D4/E4 Biotrak EIA System (Amersham, Cat.Nr. RPN224) and the Leukotriene B4 EIA Kit (Cayman Chemical, Cat.Nr. RPN223) or the Prostaglandin E2 Parameter Assay Kit (R&D Systems, Cat.Nr. KGE004B) according to manufacturer's instructions. EIA reactions were read on a CLARIOstar® High Performance Monochromator Multimode Microplate Reader (BMG LABTECH).

2.5.4 Western blot analysis and protein detection (Fig. 2.3)

Primary MMTV-PyMT cells grown on collagen-coated dishes cultured overnight in DMEM/F12 with B-27®, and 4 $\mu g\,ml^{-1}$ Heparin before treatment with 1µM LTB4 or 100nM LTC-D-E4. Unsorted or sorted LTR-reduced 4T1 cells were stimulated with 1µM LTB4, 100nM LTC-D-E4, 3µM BLT2 inhibitor LY255283 and/or 0.3µM CysLT2 inhibitor BAY-u9773 as indicated. Cells were washed with PBS and protein isolated using RIPA buffer with supplements added freshly followed by centrifugation, addition of 2x Protein loading buffer and boiling at 95°C for five minutes. Protein separation, blotting and developing was performed as follows: SE260 Mighty Small II Deluxe Mini Vertical Electrophoresis Unit (Hofer Inc.) was used to run 10% Polyacrylamide protein gels and TE 22 Mighty Small™ Transphor Tank Transfer Unit (GE Healthcare) to transfer protein onto Hybond™-P Transfer Membranes (Amersham, Cat.Nr. RPN2020F). Protein membranes were blocked with 5% BSA in PBS with 0.5% Tween-20 (Sigma, Cat.Nr. P2287) and incubated with the following primary antibodies: polyclonal rabbit anti-Phospho-p44/42 MAPK (Thr202/Tyr204) (Erk1/2, Cell Signaling Cat.Nr. 9101S), monoclonal mouse anti-p44/42 MAPK (Erk1/2, clone 3A7, Cell Signaling Cat.Nr. 9107S) and monoclonal mouse anti-alpha-Vinculin (clone hVIN-1, Sigma, Cat.Nr. V9131). ECL Western Blotting System, Hyperfilm™ ECL (Amersham, Cat.Nrs. RPN2108 and 28906836) and JP-33 automatic X-ray film processor (JPI healthcare) were used according to manufacturers' instructions for protein blotting and development. Protein lysates of

three-hour LTB4-stimulated MDA-MB-231 cells were analysed using the Proteome ProfilerTM Human Phospho-Kinase Array Kit (R&D systems, Cat.Nr. ARY003B), which includes antibodies recognising phospho-ERK1/2. Proteins present in neutrophil-conditioned (LuN) medium were analysed using Proteome Profiler™ Arrays (Mouse Cytokine Array Kit, Panel A; Mouse Angiogenesis Array Kit; Mouse Adipokine Array Kit and Mouse Chemokine Array Kit; Cat.Nrs. ARY006, ARY013, ARY015 and ARY020) according to manufacturers' instructions. Western blot quantification was performed on scanned films using ImageJ software.

Western blot buffers and Gels	
RIPA buffer	25mM Tris-hydrogen chloride pH7.6, 50mM Sodium chloride, 1% NP-40, 1% Sodium deoxycholate, 0.1% Sodium dodecyl sulphate
RIPA buffer supplements	cOmplete ULTRA Tablets (Roche, Cat.Nr. 05-892-791-001), 1µM Sodium pyrophosphate, 1µM beta-glycerophosphate, 1µM Sodium vanadiumoxide, 1µM Sodium fluoride, 1µM Sodium Molybdate
	Dissolved in 1 L distilled water:
Separation gel buffer stock	181.65 g Tris base pH 8.8 (with HCl)
Stacking gel buffer stock	181.65 g Tris base pH 6.8 (with HCl)
Gel running buffer	3 g Tris base, 14.4 g Glycine, 1 g SDS
Membrane transfer buffer	3 g Tris base, 14.4 g Glycine
2x Protein loading buffer	50mL 1M Tris pH 7, 250mL 20% SDS, 200mL Glycerol, 20mg Bromphenol blue
Stacking gel composition	3.25 mL water, 1.25 mL stacking buffer, 0.5mL 30% Bis/Acrylamide, 20 µL 10% SDS, 20 µL 10% APS, 10 µL TEMED
Separation gel composition	3.1 mL water, 1.9 mL separation buffer, 2.5mL 30% Bis/Acrylamide, 20 µL 10% SDS, 20 µL 10% APS, 10 µL TEMED

Figure 2-3 Western blot reagents, buffers and protein gel compositions
Overview of the buffers used for protein isolation and analysis as well as the recipe for polyacrylamide gel composition for protein separation.

2.6 Statistical Analysis

The data are presented as mean ± standard error of the mean, individual values, “Tukey box&whiskers” or “floating bar” graphs and were analysed using Student's t tests for paired or unpaired experiments/experimental groups and adapted for experimental groups with unequal standard deviation. The exceptions are as follows: Column statistics test (value different to 1) for Fig. 3.16 c (mRNA expression data), Fig. 4.4 a-f, Fig. 4.6 a and Fig. 4.12 a; Column statistics test (value different to 0) for Fig. 3.25 e, Fig. 4.3 c and Fig. 5.1 g (Control vs. LuN-Zil); two-way ANOVA test for Fig. 3.21 b+e. Two-way ANOVA was performed when the control groups between experiments were significantly different. Differences were considered significant when $P<0.05$ and are indicated as: n.s. not significant, *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$ or the P value directly displayed.
Data were pooled from at least two experiments and the exceptions are indicated in the respective figure legends. The experiments were not randomized and there was no blinding as animals or samples were marked. No statistical methods were used to predetermine sample sizes, these were based on previous experience with the models (Malanchi et al., 2012, Qian et al., 2011). *n* values represent biological replicates, with the exception of the sphere assays, for which both technical and biological replicates are shown. All *in vitro* and *in vivo* experiments involving primary MMTV-PyMT cells were performed with at least two tumour cell preparations from different spontaneous MMTV-PyMT+ mice and each *in vitro* and *in vivo* experiment was performed with a different tumour cell preparation unless otherwise specified.

Chapter 3. Neutrophils support metastasis-initiating cells during lung colonisation in mammary cancer

3.1 Chapter Introduction

Metastasis is the leading cause for cancer-related mortality and the role of neutrophils during cancer progression appears rather pro-tumourigenic in experimental cancer models and in the clinic, but remains controversial (Donskov, 2013, Hunter et al., 2008, Piccard et al., 2012). Moreover, neutrophils with contradicting functions have been reported to accumulate in the lung, the site of metastasis, in mammary cancer-bearing mouse models (Acharyya et al., 2012, Casbon et al., 2015, Coffelt et al., 2015, Granot et al., 2011). Hence, we aimed to investigate how neutrophils affect distant tissue colonisation of metastatic cancer cells. To this end, we used mice expressing the Polyoma middle T oncogene (PyMT) under the control of the mouse mammary tumour virus promoter (MMTV) that spontaneously develop lung-metastatic mammary carcinoma at the age of 2-3 months (MMTV-PyMT+ mice) (Guy et al., 1992). PyMT is specifically expressed in the mouse mammary gland by the MMTV promoter, localises to cell membranes and causes activation of kinases like Src, PI3K and ERK1/2 by interacting with their regulators proteins. Thereby, PyMT mimics mitogenic signalling of activated tyrosine kinase-associated receptors for growth factors and allows malignant proliferation and cancer development (Zhou et al., 2011). The cancer progression of the MMTV-PyMT mouse model closely resembles human breast cancer development and can be divided into a hyperplasia, adenoma/mammary intra-epithelial neoplasia, early and late carcinoma stage that is characterised by downregulation of oestrogen and progesterone receptors, loss of integrin-beta1 and overexpression of ErbB2/Neu and Cyclin D1 (Lin et al., 2003). The highly potent metastasis-initiating cell population within primary tumour cells of this mouse model can be identified by the expression of the surface markers CD24 and CD90. This cancer cell subpopulation was functionally defined by their increased metastasis initiation ability *in vivo* (Malanchi et al., 2012) and is therefore termed metastasis-initiating cells (MICs). On occasions, we used the BALB/c background-syngeneic mouse mammary cancer cell line 4T1 (Pulaski and Ostrand-Rosenberg,

2001) or the triple-negative basal B subtype human breast cancer cell line MDA-MB-231 (Chavez et al., 2010, Subik et al., 2010).

Functionally, we determined different tumourigenic potential of total, heterogeneous cancer cell populations *in vitro* and *in vivo*. In *in vitro* non-adherent growth assays cancer cells are plated as single cell suspensions in ultra-low attachment plates were they are challenged to survive and grow anchorage independent. In this setting, cancer cell subpopulations are able to form and grow in spheres and sphere number and size were quantified. Enhanced *in vitro* sphere formation potential is commonly associated with highly potent CSC-like cancer cells, including breast cancer cells, due to the required anoikis resistance and self-renewal abilities (Grimshaw et al., 2008, Shaw et al., 2012) and was previously used to assess stemness properties of primary MMTV-PyMT cells (Malanchi et al., 2012) and human MDA-MB-231 cells (Grimshaw et al., 2008, Wang et al., 2014). *In vitro* sphere formation assays exclusively assess for intrinsic cancer cell potential and cross talk of cancer cell subpopulations, while the following *in vivo* tests determine cancer cell potentials together with their interaction ability with the microenvironment. We used two different assays to test the tumourigenic potential of cancer cells *in vivo*. Grafting of low dissociated cancer cell numbers orthotopically onto the mammary gland and the subsequent formation of tumours determines cancer cell tumour initiation and propagation competence and is measured by weighing of established tumours (Beck and Blanpain, 2013, Kreso and Dick, 2014). Lung colonisation and metastasis initiation potential of cancer cells is assessed by intravenous injection of limited cancer cell numbers as single-cell suspensions. These cancer cells are thereby challenged to survive and grow at the metastatic site, the lung, and their ability to do so is quantified by determination of lung metastatic burden as described in section 2.2.1 and (Malanchi et al., 2012).
Neutrophil-involvement in tumourigenic processes was studied taking advantage of genetically induced neutropenia or antibody-mediated neutrophil depletion. G-CSF (Granulocyte Colony-Stimulating Factor)-deficient mice show impaired mobilisation of neutrophils from the bone marrow resulting in strongly reduced neutrophil-counts in peripheral blood and tissues (Lieschke et al., 1994). In an alternative genetic strategy for genetic neutrophil-deficiency, we exploited mice with a knock-in of the

Cre-recombinase gene behind the endogenous neutrophil elastase (ela2) promoter (Heit et al., 2008, Thomas et al., 2004, Tkalcevic et al., 2000) and a ROSA-Flox-GFP-STOP-Flox diphtheria toxin (DTA) cassette (ela2-DTA) (Ivanova et al., 2005). The Cre gene was cloned into the endogenous ela2 promoter thereby causing ela2-deficiency. Here, Cre recombinase is predominantly expressed by neutrophils due to the ela2 promoter and leads to excision of the GFP-STOP sequences upstream of the DTA cassette and to ROSA promoter-mediated DTA production. DTA is a toxin that causes apoptosis by blockade of protein synthesis when intracellular and thereby ela2 promoter-mediated reduction of specifically neutrophil numbers in the lung of ela2-DTA mice (Fig. 3.6 and 3.7). Lastly, administration of the neutrophil-specific anti-Ly6G (clone 1A8) antibody (Borregaard, 2010, Daley et al., 2008, Granot et al., 2011, Kolaczkowska and Kubes, 2013) allowed efficient neutrophil depletion in a temporally controlled manner. We monitor neutrophil numbers predominantly by their well-described surface markers CD11b and Ly6G (Borregaard, 2010, Daley et al., 2008, Granot et al., 2011, Kolaczkowska and Kubes, 2013) as well as by immunohistochemistry for the cytosolic S100A9 (also known as MRP14) protein (antibody clone 2B10) that is mainly produced by neutrophils (Kohler et al., 2011, Stroncek et al., 2005) in the lung.

3.2 Results

3.2.1 Neutrophils accumulate in the metastatic lung and are critical for metastatic progression of mammary cancer

In accordance with previous reports of lung-metastatic mammary cancer mouse models (Casbon et al., 2015, Coffelt et al., 2015, Granot et al., 2011), we found CD11b+Ly6G+ neutrophils to be systemically mobilised in spontaneous MMTV-PyMT tumour-bearing mice and detected increased frequencies in the bone marrow, spleen, liver and predominantly the metastatic lung by flow cytometric analysis, despite comparably low neutrophil numbers within the primary tumour microenvironment (Fig. 3.1). Interestingly, neutrophils resulted to be the main immune cell type accumulating in metastatic lungs of MMTV-PyMT+ mice. We analysed the presence of total leukocytes (CD45+), total macrophages (CD11b+F4/80+), alveolar subpopulations of macrophages (CD11b-low F4/80-high), interstitial subpopulations of macrophages (CD11b-high F4/80-low), dendritic cells (CD45+CD11c+), B (CD45+CD19+) and T lymphocytes (CD45+CD3+) in wildtype control lung and late-stage metastatic lung of MMTV-PyMT mice without detecting any alterations (Fig. 3.2 a-e+g-h). Natural killer (NK) cell (CD45+CD49b+) frequencies showed a decrease in the MMTV-PyMT metastatic lung compared to wildtype mouse lungs (Fig. 3.2 f), suggesting a possible induction of an immunosuppressive environment when metastatic cancer cells are growing in the lung of MMTV-PyMT+ mice.

Next, we addressed the functional relevance of high numbers of tumour-induced CD11b+Ly6G+ neutrophils in the metastatic lung by analysing spontaneous metastatic progression of MMTV-PyMT+ crossed with G-CSF null mice (MMTV-PyMT+ G-CSF-/-). Indeed, these mice failed to specifically accumulate neutrophils in the lung when harbouring mammary tumours. This state of neutropenia results in a robust reduction of spontaneous lung metastasis incidence (Fig. 3.3 b-e). Primary tumour growth was not affected (Fig. 3.3 a), likely due to the low neutrophil presence in mammary tumours compared to the metastatic lung of MMTV-PyMT+ mice (Fig. 3.1 e). Neutropenia in MMTV-PyMT+ G-CSF-/- mice did not correlate with altered frequencies of total immune cells, macrophage populations, dendritic cells and T cells in the metastatic lung compared to MMTV-PyMT+ mice. Levels of

these leukocytes also remained unchanged in tumour-free G-CSF-/- versus wildtype mice (Fig. 3.4 a-d+f-g).

However, lung B lymphocyte frequencies increased in G-CSF-/- and MMTV-PyMT+ G-CSF-/- mice compared to wildtype control and MMTV-PyMT+ mice, respectively (Fig. 3.4 h). In fact, G-CSF was shown to actively inhibit B lymphopoiesis in mice (Day et al., 2015), which likely explains elevated B cell numbers in G-CSF-/- and MMTV-PyMT+ G-CSF-/- mice. Hence, accumulation of B cell upon G-CSF deficiency appears to be independent of mammary tumour growth and likely also not directly related to neutropenia. Interestingly, the reduced NK cell frequencies observed in late stage metastatic lungs of MMTV-PyMT+ mice compared to wildtype lungs (Fig. 3.2 f) are rescued in lungs of MMTV-PyMT+ G-CSF-/- mice while there is no alteration in tumour-free G-CSF-/- mice compared to controls (Fig. 3.4 e). NK cell activation in the lung was assessed by flow cytometry for their activation marker CD69 and did not reveal differences between the four tested genotypes (Fig. 3.4 e). Hence, also the presence of activated NK cells is reduced in the metastatic lung of MMTV-PyMT+ mice and restored to tumour-free wildtype lung levels upon G-CSF deficiency. This observation under neutrophil absence throughout the metastatic process strongly points towards a potential neutrophil-dependent suppression of total and activated NK cell frequencies in the lung at late stages of metastasis. NK cells are able to directly eliminate cancer cells and represent an important part of the anti-cancer immunity (section 1.2.3.1). Hence, suppression of (activated) NK cell recruitment to the metastatic site by neutrophils during advanced metastatic growth might contribute to the pro-metastatic functions of neutrophils observed in the MMTV-PyMT mammary cancer model.

G-CSF-deficiency might itself impair the metastatic ability of primary MMTV-PyMT cancer cells due to their pronounced secretion of this factor (Casbon et al., 2015). To exclude this possibility, we isolated primary MMTV-PyMT G-CSF-/- and MMTV-PyMT G-CSF+/+ cells from MMTV-PyMT+ G-CSF-/- mice and MMTV-PyMT+ littermate controls that spontaneously developed tumours and grafted them orthotopically onto the mammary gland of immunocompromised Rag1-/- mice (Mombaerts et al., 1992). We did not use wildtype FVB mice for this experiment because of the mixed MMTV-PyMT+ G-CSF genetic background and potential rejection of transplanted cancer cells. The lack of G-CSF expression by MMTV-PyMT cancer cells altered neither lung neutrophil accumulation, nor primary tumour

growth or spontaneous metastasis burden upon tumour engraftment (Fig. 3.5). This evidence indicates that genetically induced neutropenia due to systemic lack of G-CSF accounts for the reduced metastatic incidence in MMTV-PyMT+ G-CSF-/- mice compared to MMTV-PyMT+ controls and not G-CSF deficiency of MMTV-PyMT cancer cells.

We corroborated our findings using MMTV-PyMT+ mice crossed with ela2-Cre and ROSA-Flox-GFP-STOP-Flox-DTA (MMTV-PyMT+ ela2-DTA) mice. The analysed littermate controls included tumour-free wildtype controls and MMTV-PyMT+ ela2-Cre-negative and/or ROSA-DTA cassette-negative mice. The MMTV-PyMT+ ela2-DTA mice carried two copies of the Cre recombinase gene and therefore a deletion of the ela2 gene because one copy of the Cre recombinase did not result efficient enough to significantly reduce CD11b+Ly6G+ neutrophil frequencies in the lung (data not shown) and one copy of the ROSA-DTA cassette. In this genetic setting, MMTV-PyMT+ ela2-DTA mice displayed reduced CD11b+Ly6G+ neutrophil levels in the blood, bone marrow and lung at late carcinoma stages compared to MMTV-PyMT+ control mice (Fig. 3.6 a+e and Fig. 3.7 g). Notably, spontaneous metastatic progression was impaired in neutropenic MMTV-PyMT+ ela2-DTA mice without alteration of primary tumour growth (Fig. 3.6 b-d). Also, we analysed the presence of macrophages in the lung of MMTV-PyMT+ ela2-DTA mice as ela2-Cre has been reported to be expressed in some populations of macrophages (Thomas et al., 2004). Flow cytometric quantification of frequencies of total macrophages, alveolar and interstitial macrophage subpopulations in the lung did not show significant differences between MMTV-PyMT+ control and MMTV-PyMT+ ela2-DTA mice at advanced carcinoma stages (Fig. 3-6 f-h). Of note, the expression of the intracellular toxin DTA is causing neutrophil apoptosis and their ablation in the bone marrow in this model, which could potentially influence myelopoiesis or induce an immune response in the bone marrow. Hence, we carefully analysed the presence of myeloid cells and activated immune effector cells in the bone marrow and/or blood of MMTV-PyMT+ ela2-DTA and MMTV-PyMT+ control mice to exclude this possibility. Blood and bone marrow CD11b+F4/80+ macrophage and CD11b+CD115+ monocyte frequencies appeared to increase in advanced spontaneous MMTV-PyMT+ tumour-bearing compared to wildtype animals (Fig. 3.7 g). Also, MMTV-PyMT+ mice displayed elevated cytotoxic CD8+ T cell activation measured by CD44 or CD69 expression in the bone marrow, while the levels of

bone marrow NK cell activation remained unaltered in the three tested genotypes (Fig. 3.7 h). The increased frequency of activated CD44+ or CD69+ CD8+ T cells in the bone marrow might be a reflection of an anti-cancer immune response in advanced stage MMTV-PyMT+ tumour-bearing mice (section 1.2.3.1). The altered numbers of CD11b+F4/80+ and CD11b+CD115+ cells in the blood and bone marrow of MMTV-PyMT+ mice might result from the aberrant myelopoiesis induced by growing tumours (section 1.2.3.2). Alternatively, subsets of macrophage/monocyte-like cells with immunosuppressive character that accumulate in tumour-bearing hosts called monocytic myeloid-derived suppressor cells (M-MDSCs) were described to express CD11b, CD115 and F4/80 (Pereira et al., 2011, Umemura et al., 2008, Youn et al., 2008). Hence, the CD11b+F4/80+ and CD11b+CD115+ cells quantified in the blood and bone marrow might partially represent M-MDSCs. However, this hypothesis appears rather unlikely due to the potent ability of M-MDSCs to inhibit CD8+ T cells and frequencies of activated CD8+ T cells are actually increased. Besides, these observed tumour-dependent alterations of bone marrow and blood immune cell frequencies at late disease stages appeared to be independent of DTA expression or neutrophil presence. There was no difference in blood and bone marrow macrophage and monocyte frequencies or in CD8+ T cell activation levels between MMTV-PyMT+ control and MMTV-PyMT+ ela2-DTA mice (Fig. 3.7). Moreover, these leukocyte populations remained unaltered at the metastatic site in MMTV-PyMT+ mice with reduced neutrophil levels compared to controls (Fig. 3.4, Fig. 3.6 and 3.7). Importantly, ela2-mediated DTA expression in MMTV-PyMT+ mice lowered blood, bone marrow and lung neutrophil frequencies and did not affect presence of blood, bone marrow or lung macrophage populations as well as bone marrow and blood monocytes (Fig. 3.6 and 3.7). Hence, we excluded a significant contribution of these alterations in leukocyte numbers in the blood and bone marrow to the neutropenia-associated decrease in metastatic progression. Lastly, the unaltered numbers of macrophages, monocytes as well as activated NK and CD8+ T cells in the bone marrow of MMTV-PyMT+ control and MMTV-PyMT ela2-DTA mice (Fig. 3.7) indicate that the expression of the toxin DTA and associated apoptosis by neutrophils does not cause an non-physiological immune response.

In summary, spontaneous MMTV-PyMT+ tumour-bearing mice develop neutrophilia that is most pronounced at the metastatic site, the lung. These CD11b+Ly6G+

neutrophils appear to have pro-metastatic properties, as their constitutive depletion throughout the metastatic process by two independent genetic strategies results in reduced metastatic incidence. Interestingly, neutrophilia in MMTV-PyMT+ mice caused a decrease of NK cell presence in the lung harbouring established metastases, suggesting a potential immunosuppressive activity of neutrophils at advanced metastatic stages.

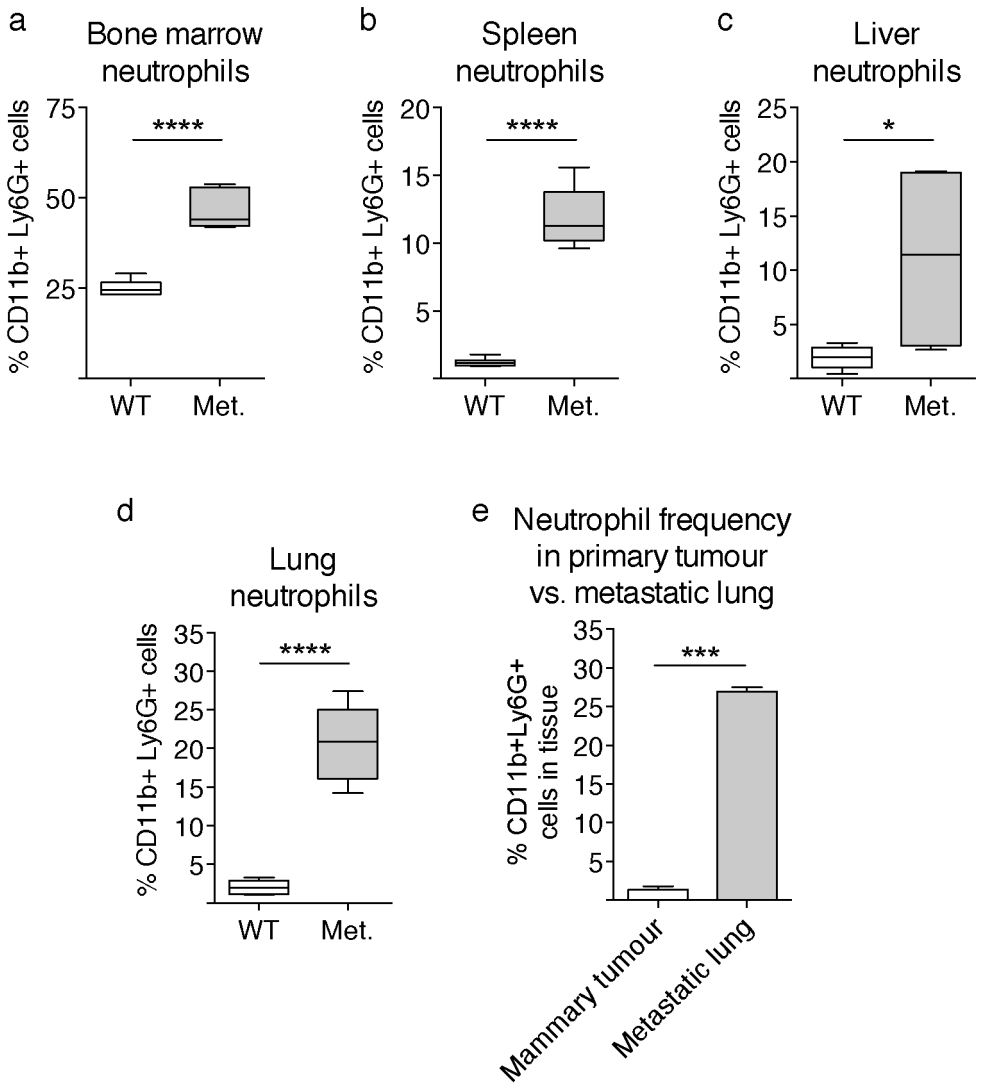


Figure 3-1 Systemic increase of neutrophils in MMTV-PyMT mice with late stage carcinoma
(a-d) Flow cytometric quantification of CD11b+Ly6G+ neutrophils in the bone marrow (a) spleen (b), liver (c) and lung (d) of wildtype and MMTV-PyMT+ mice at advanced disease stages (n=4 per group pooled from 4 different litters), gated on alive single cells. (e) Comparison of CD11b+Ly6G+ neutrophil frequencies present in primary mammary carcinoma and metastatic lung of MMTV-PyMT+ mice (n≥3 per group pooled from 3 different litters), gated on alive single cells. WT: wildtype littermate control. Met.: MMTV-PyMT+ mice with late stage lung metastases.

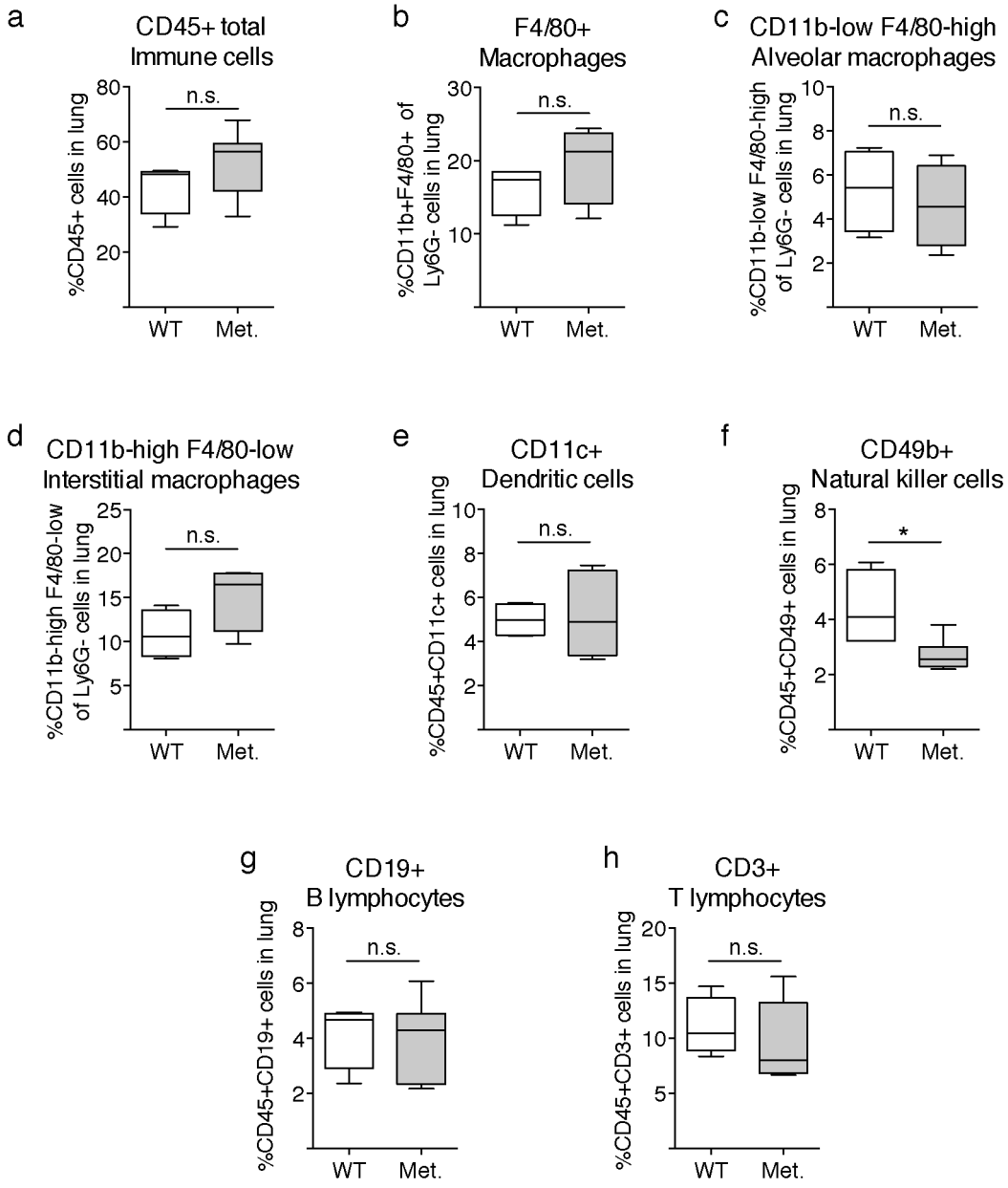


Figure 3-2 Characterisation of immune cell presence in the lung of MMTV-PyMT+ mice at advanced stages

(a-h) Flow cytometric quantification of immune cell frequencies in wildtype and metastatic lungs of MMTV PyMT+ mice (n≥4 per group pooled from 4 different litters) including CD45+ total immune cells (a), total CD11b+ F4/80+ macrophages (b), the CD11b-low F4/80-high alveolar macrophage subpopulation (c), the CD11b-high F4/80-low interstitial macrophage subpopulation (d), CD45+CD11c+ dendritic cells (e), CD45+CD49b+ natural killer cells (f), CD45+CD19+ B lymphocytes (g) and CD45+CD3+ T lymphocytes (h). (a+e-h) gated on alive single cells, (b-d) gated on Ly6G-negative cells. WT: wildtype littermate control. Met.: MMTV-PyMT+ mice with late stage lung metastases.

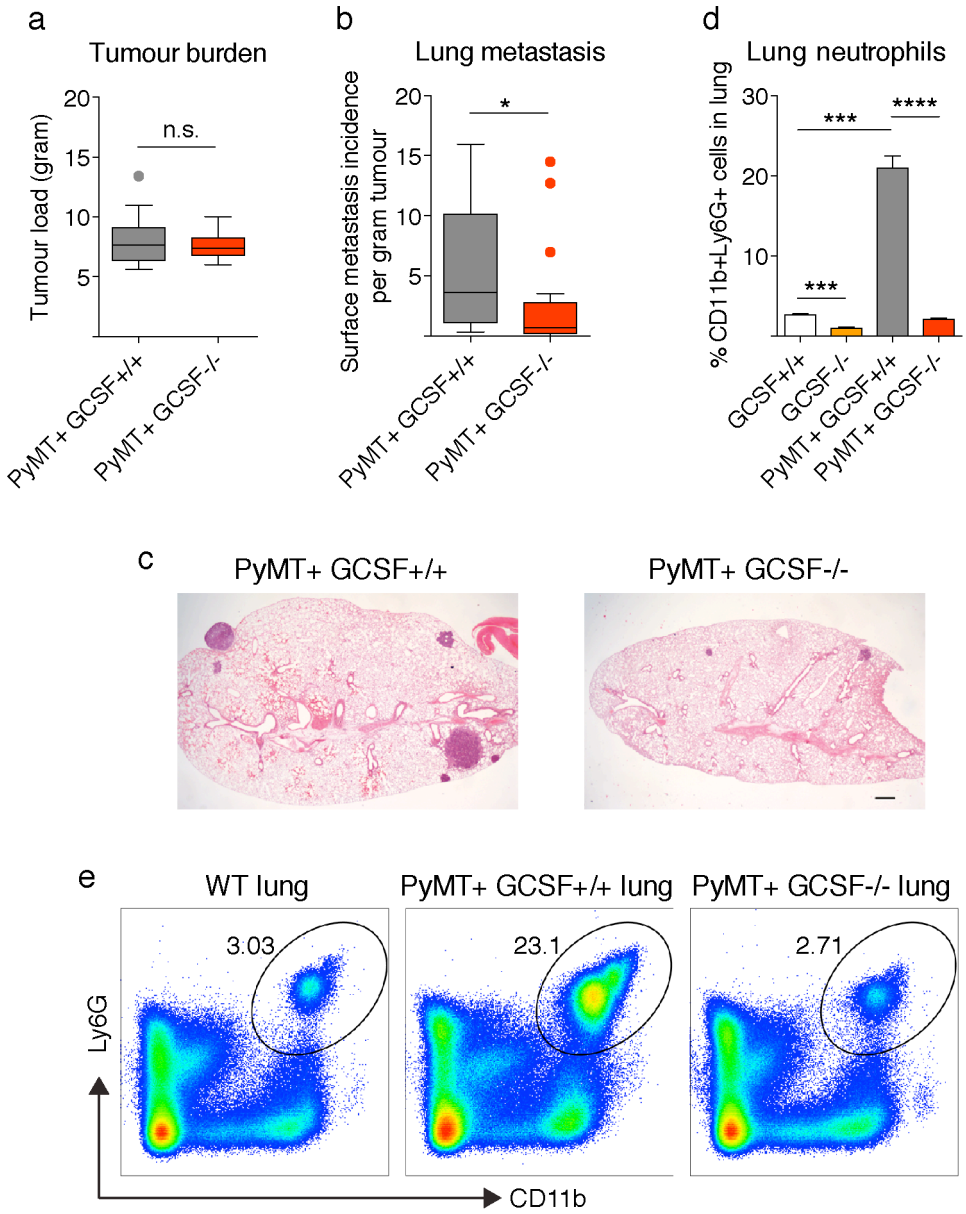


Figure 3-3 G-CSF deficiency of MMTV-PyMT+ mice causes lung neutrophil reduction and significantly ameliorates metastatic burden
(a-c) Analysis of advanced stage tumour-bearing MMTV-PyMT+ wildtype (MMTV-PyMT+ G-CSF+/+) and MMTV-PyMT+ G-CSF-/- mice for primary mammary tumour burden by resection of all tumours per mouse and weighing (a; n≥12 per group pooled from 11 different litters), spontaneous metastasis incidence by quantification of visible surface lung metastases relative to tumour load (b; n≥12 per group pooled from 11 different litters) as well as frequencies of CD11b+Ly6G+ neutrophils in the lung together with of tumour-free wildtype (G-CSF +/+) and G-CSF-/- mice by flow cytometric quantification (c; n≥5 per group pooled from 5 different litters), gated on alive single cells. (d) Representative haematoxylin and eosin-stained histological lung sections, scale bar is 500µm. (e) Representative flow cytometric analysis of CD11b+Ly6G+ neutrophils in the lung of wildtype, advanced stage tumour-bearing MMTV-PyMT+ G-CSF+/+ and MMTV-PyMT+ G-CSF-/- mice of quantification shown in (d). Insert shows relative frequencies of double-positive cells in the displayed gate.

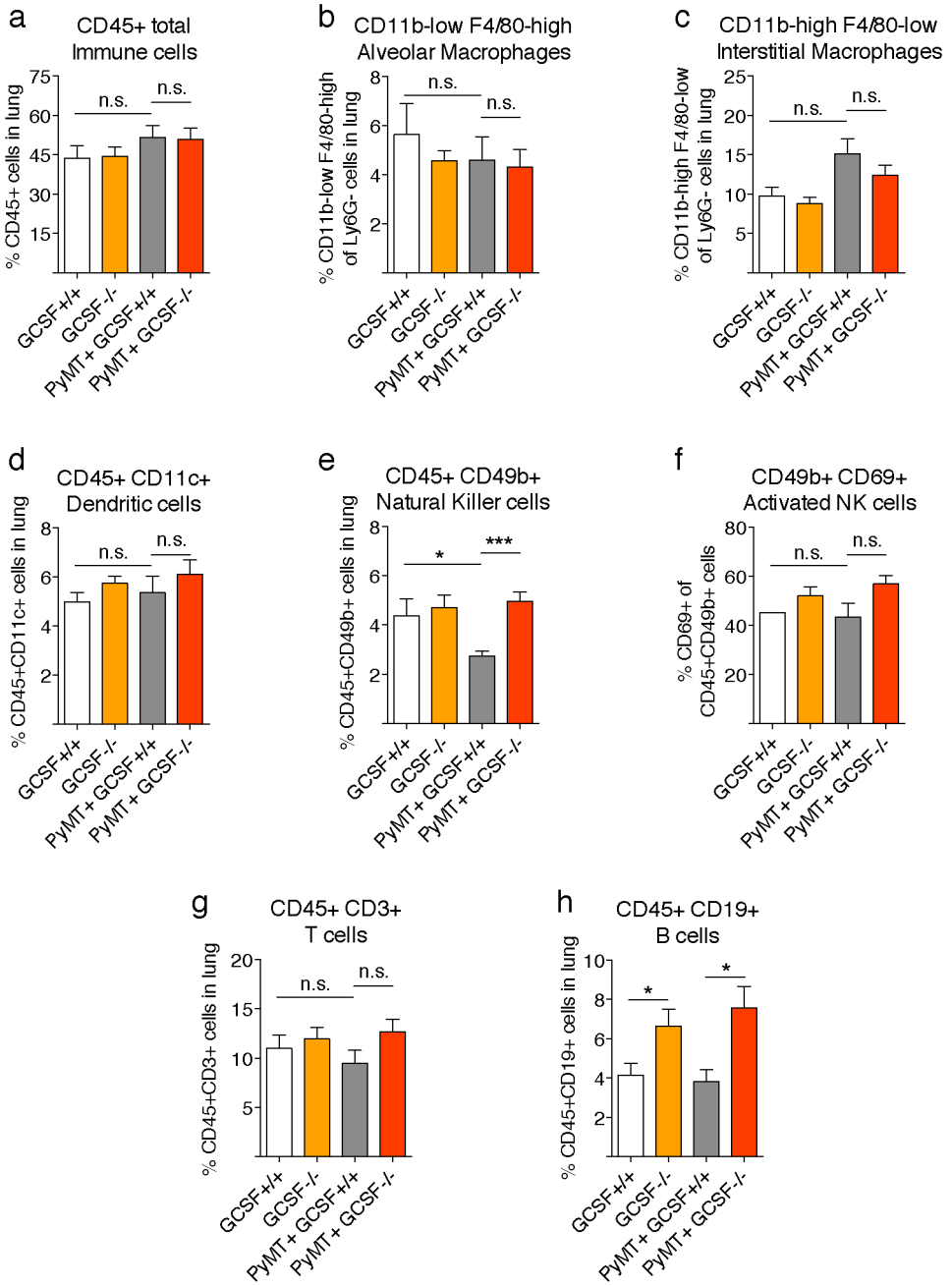


Figure 3-4 Effect of G-CSF loss on the immune cell presence in wildtype and MMTV-PyMT+ mice with advanced carcinoma

(a-e and g-h) Flow cytometric quantification of frequencies of CD45+ total immune cells (a), the CD11b-low F4/80-high alveolar macrophage subpopulation (b), the CD11b-high F4/80-low interstitial macrophage subpopulation (c), CD45+CD11c+ dendritic cells (d), CD45+CD49b+ natural killer cells (e), CD45+CD3+ T lymphocytes (g) and CD45+CD19+ B lymphocytes (h) in the lung of tumour-free wildtype (G-CSF+/+) and G-CSF-/- mice as well as advanced tumour-bearing MMTV-PyMT+ wildtype (MMTV-PyMT+ G-CSF+/+) and MMTV-PyMT+ G-CSF-/- mice. (f) Flow cytometric quantification of activated CD69+ NK cells in the lung of the same four genotypes. (a+d-h) gated on alive single cells, (b-c) gated on Ly6G-negative cells and (f) gated on CD45+CD49b+ cells. N≥5 per group pooled from 5 different litters.

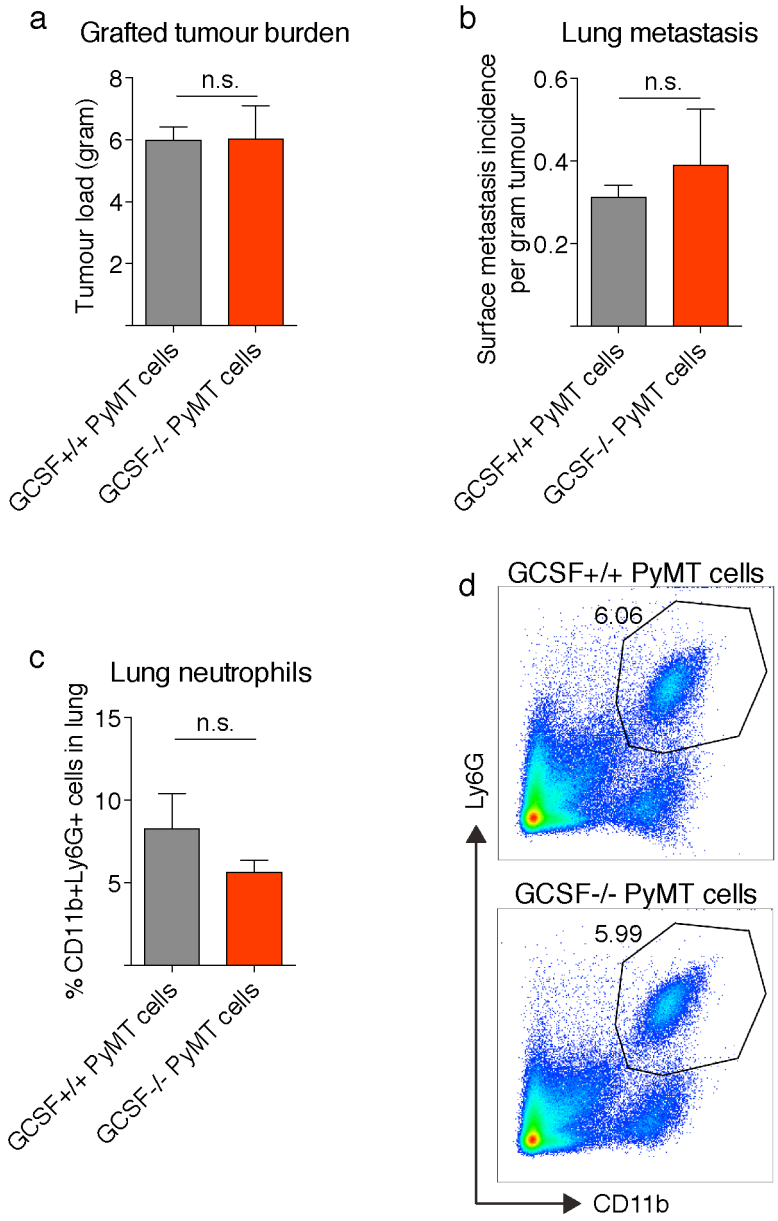


Figure 3-5 GCSF-deficiency of MMTV-PyMT cancer cells does not affect lung neutrophil accumulation, mammary tumour growth or metastatic efficiency
MMTV-PyMT wildtype (G-CSF+/+) and MMTV-PyMT GCSF-/- primary cancer cells (n≥3 biological replicates (≥3 independent primary MMTV-PyMT cancer cell preparations)) were freshly isolated from tumour-bearing mice, grafted onto the mammary gland of Rag1-/- mice (one mouse (technical replicate) per cancer cell preparation) and analysed 5 weeks later. (a) Primary tumour burden was determined by weighing of dissected tumours. (b) Spontaneous lung metastasis incidence was quantification by counting of visible surface lung metastases and is displayed relative to tumour load. (c) CD11b+Ly6G+ neutrophil frequencies in the lung were assessed by flow cytometry, gated on alive single cells. (d) Representative flow cytometric analysis of CD11b+Ly6G+ neutrophils in the lung of quantification shown in (c). Insert shows relative frequencies of double-positive cells contained in the displayed gate.

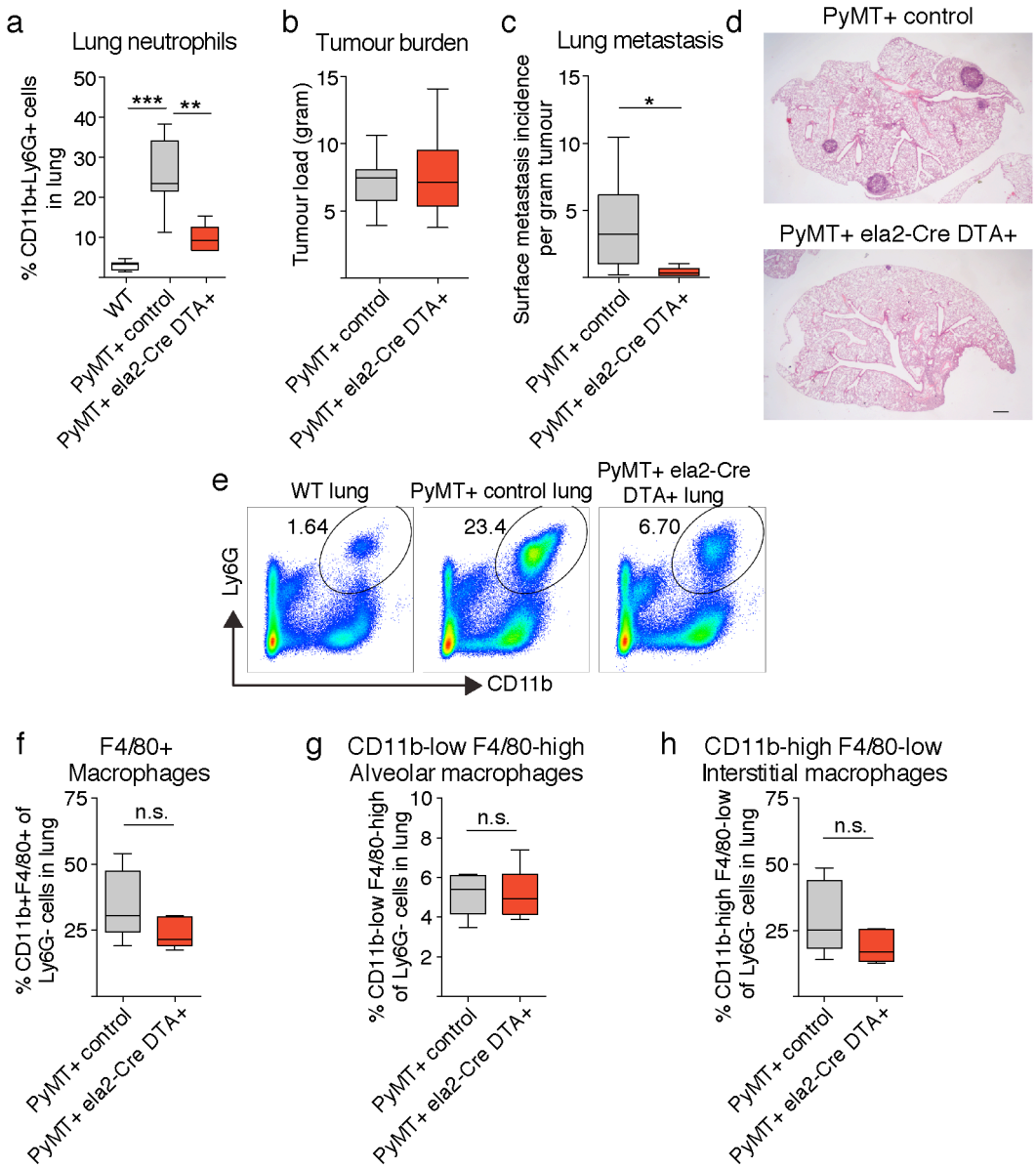


Figure 3-6 MMTV-PyMT+ ela2-Cre mice show reduced lung neutrophil presence hand in hand with a marked decrease in lung metastatic burden
(a-c) Analysis of tumour-bearing MMTV-PyMT+ control and MMTV-PyMT+ ela2-DTA mice for presence of CD11b+Ly6G+ neutrophils in the lung by flow cytometric quantification and gating on alive single cells (a), primary mammary tumour burden by weighing (b) and spontaneous metastasis incidence by quantification of visible surface lung metastases relative to tumour load (c); n≥5 per group pooled from 5 different litters. (d) Representative haematoxylin and eosin-stained histological lung sections, scale bar is 500µm. (e) Representative flow cytometric analysis of CD11b+Ly6G+ neutrophils in the lung of quantification shown in (a). Insert shows relative frequencies of double-positive cells contained in the displayed gate. (f-h) Flow cytometric quantification of frequencies of total CD11b+ F4/80+ macrophages (f), the CD11b-low F4/80-high alveolar macrophage subpopulation (g) and the CD11b-high F4/80-low interstitial macrophage subpopulation (h) in the lung of tumour-bearing MMTV-PyMT+ control and MMTV-PyMT+ ela2-Cre DTA+ mice, gated on alive single Ly6G-negative cells (n≥5 per group pooled from 5 different litters).

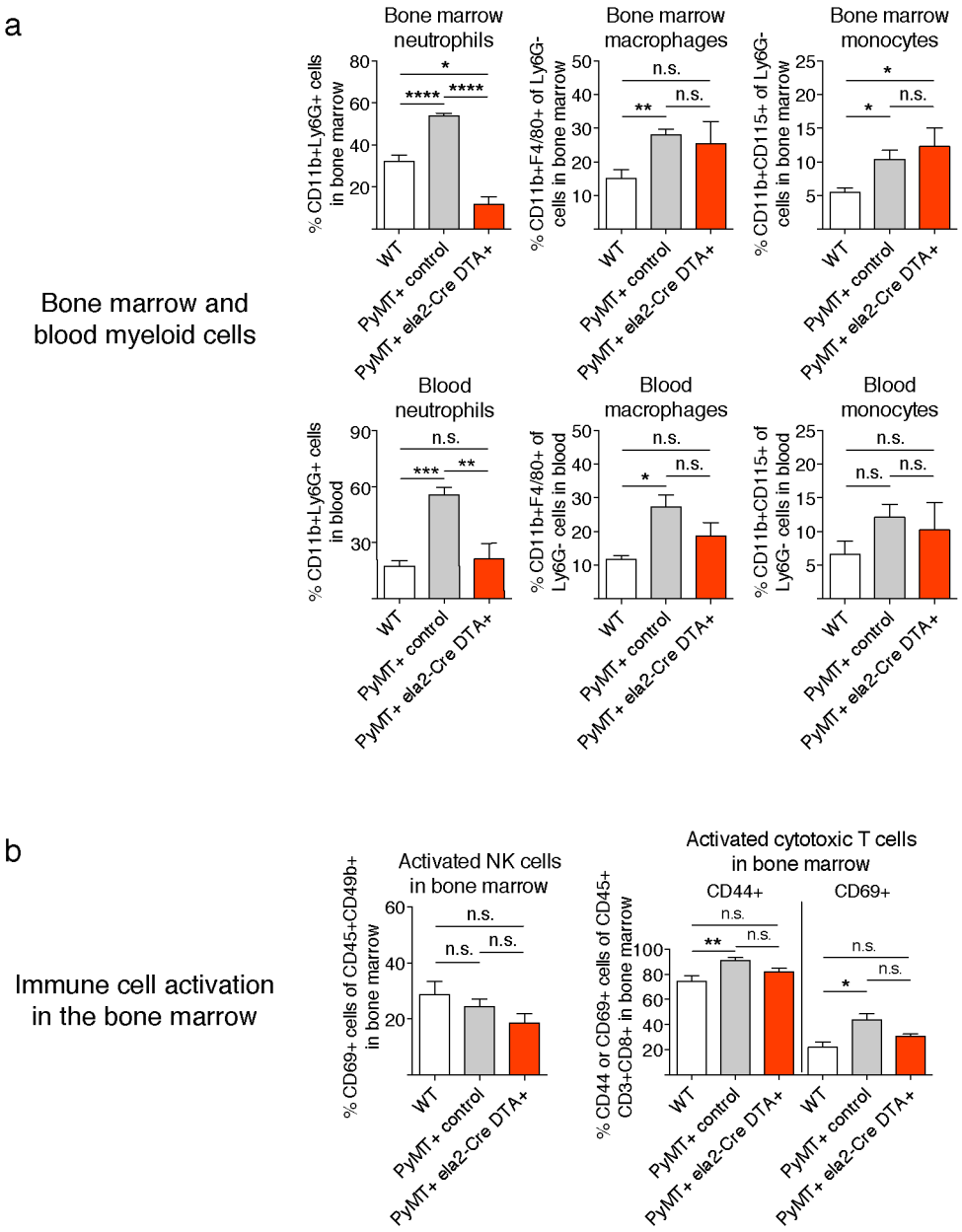


Figure 3-7 Determination of selected leukocyte frequencies and activation in the blood and bone marrow of tumour-free wildtype, MMTV-PyMT+ control and MMTV-PyMT ela2-DTA mice

(a) Frequencies of bone marrow and blood CD11b+Ly6G+ neutrophils, CD11b+F4/80+ macrophages and CD11b+CD115+ monocytes in wildtype, MMTV-PyMT+ control and MMTV-PyMT+ ela2-DTA mice analysed by flow cytometry, gated on alive single cells (neutrophils) or Ly6G-negative cells (macrophages and monocytes) (n≥2 per group pooled from ≥2 different litters). (b) Exclusion of immune responses against DTA expression in the bone marrow by analysis of NK cell and cytotoxic CD8+ T cell activation. Flow cytometric quantification of activated CD69+ among total CD45+CD49b+ NK cells as well as activated CD44+ or CD69+ among total CD45+CD3+CD8+ cytotoxic T cells in the bone marrow of wildtype, MMTV-PyMT+ control and MMTV-PyMT+ ela2-DTA mice (n≥2 per group pooled from ≥2 different litters).

3.2.2 Addressing the mechanisms of tumour-induced systemic neutrophilia and neutrophil accumulation in the metastatic lung

First of all, we aimed to corroborate our and published data that breast cancer cells growing on a host are elevating neutrophil counts throughout the body and in the metastatic lung (Fig. 3.1) (Acharyya et al., 2012, Casbon et al., 2015, Coffelt et al., 2015, Granot et al., 2011). In fact, transplantation of the mouse 4T1 mammary cancer cell line on syngeneic BALB/c and the MDA-MB-231 human breast cancer cell line on immunocompromised Rag1-/- mice also induced robust CD11b+Ly6G+ blood and/or lung neutrophil accumulation (Fig. 3.8).

The mechanisms how tumour presence systemically mobilises neutrophil accumulation are as important as complex. A recent study demonstrated one mechanism involving tumour-derived IL-1β-mediated activation of IL-17-producing gamma-delta T cells that in turn cause a systemic elevation of G-CSF levels resulting in neutrophilia (Coffelt et al., 2015). The actual cellular source of G-CSF was not identified in this study and we excluded tumour cell-derived G-CSF to be involved in lung neutrophil accumulation in the MMTV-PyMT mammary cancer model (Fig. 3.5). We were intrigued by the enhanced neutrophil accumulation particularly in the lung of mammary tumour-bearing mice, the preferential site of metastasis. Breast cancer cells, including MMTV-PyMT cells, have been shown to attract pro-tumourigenic neutrophil-like cells to mammary tumours and the metastatic lung via secretion of CXCL1/2, the ligands for CXCR2 (Acharyya et al., 2012). CXCL1 and 2 are highly effective chemokines that attract neutrophils to peripheral tissues in numerous pathologic settings through engagement of the neutrophil-expressed CXCR2 receptor. CXCR2 deficiency is frequently employed in experimental studies to prevent neutrophil infiltration into tissues that contain cancer lesions (section 1.3). This CXCL1/2-CXCR2 axis would explain why neutrophilia of advanced cancer stage MMTV-PyMT+ mice is most pronounced in the lung where cancer cells are present compared to other sites (Fig. 3.1). Hence, we aimed to investigate the molecular mediators of neutrophil recruitment to the metastatic lung of mammary tumour-harbouring mice and, firstly, wanted to confirm the involvement of a CXCL1/2-CXCR2-dependent mechanism. To this end, we generated bone marrow chimeric mice either reconstituted with wildtype or CXCR2-

deficient (CXCR2-/-) bone marrow isolated from donor mice (Cacalano et al., 1994) and grafted them with a syngeneic 4T1 mammary tumour (Fig. 3.9 a). The CXCR2-/- mice were in a BALB/c genetic background. Hence, we used BALB/c recipient mice and the BALB/c syngeneic 4T1 mammary cancer cell line instead of the spontaneous MMTV-PyMT model. CXCR2-deficiency of used mice was restricted to the radiosensitive immune cell compartment to avoid effects of CXCR2 expressed by other cell types involved in the metastatic process or neutrophil recruitment, such as endothelial cells or tumour cells themselves (Saintigny et al., 2013). We ensured a CXCR2-/- bone marrow reconstitution of more than 90% by absence of CXCR2 expression on bone marrow (data not shown) and lung neutrophils by flow cytometry for all mice included in the analysis (Fig. 3.9 c-d). Surprisingly, CD11b+Ly6G+CXCR2-/- neutrophils were perfectly able to infiltrate the metastatic lungs and constituted more than 90% of lung neutrophils of 4T1 tumour-bearing CXCR2-/- bone marrow-reconstituted wildtype mice (Fig. 3.9 c-d). Consequently we did not observe an alteration of total CD11b+Ly6G+ lung neutrophil frequencies in 4T1 tumour-bearing CXCR2-/- or wildtype bone marrow-reconstituted mice (Fig. 3.9 b), despite a study reporting CXCL1/2-dependent neutrophil-like cell recruitment to the metastatic lung in a breast cancer model (Acharyya et al., 2012). Also, primary tumour burden or spontaneous lung metastasis incidence was unaltered in these mice (Fig. 3.9 e-f). This evidence suggests that breast cancer-induced neutrophil accumulation in the lung is independent of CXCR2 expression by neutrophils, at least in the 4T1 mammary cancer model (discussed in detail in section 6.3.1).

Next, we wanted to determine if disseminated tumour cells present in the metastatic lung directly induce neutrophil recruitment via other mechanisms than CXCL1/2-CXCR2-mediated chemoattraction. To test this hypothesis, we directly seeded MMTV-PyMT cancer cells into the lung of otherwise tumour-free mice. We injected primary GFP-labelled MMTV-PyMT cells via the tail vein that were isolated from spontaneously developed tumours of MMTV-PyMT actin-GFP mice which constitutively express GFP protein under the control of the actin promoter (Okabe et al., 1997). Labelling of cancer cells with GFP allows for their quantification and analysis at early metastatic stages, however GFP expression triggers an adaptive immune response in wildtype animals interfering with the experimental setup. Therefore, immunocompromised Rag1-/- mice lacking functional B and T cells were

used for all experiments involving labelled cancer cells. We analysed mice three days after intravenous cancer cell injection (Fig. 3.10 a) to allow both, sufficient time to induce neutrophil recruitment and to assess the ability of single disseminated cancer cells and very small colonies (or the consequences of their death) to attract neutrophils. Thereby, the establishment of larger metastases that would additionally cause tissue damage and activation of the microenvironment which likely influences neutrophil infiltration was minimised. Alive GFP+ MMTV-PyMT cells were readily detected in the lungs of intravenously injected mice by flow cytometry (Fig. 3.10 b), however there was no increase in CD11b+Ly6G+ neutrophil frequencies in these lungs compared to untreated animals (Fig. 3.10 c). Hence, we concluded that neutrophil accumulation particularly in the metastatic lung of mammary cancer-bearing mice is likely facilitated by mechanisms independent from direct neutrophil recruitment by metastatic cancer cells present in the lung. On the same line, we observed that the frequencies of intravenously injected MMTV-PyMT cancer cells in the lung decrease about ten-fold within the first 24 hours post-injection (Fig. 3.10 d), which is very likely due to their death and apoptosis. Thus, death of disseminated cancer cells and potential activation of DAMP-associated immune responses in the lung at early metastatic stages appears unlikely to cause enhanced neutrophil influx. However, we did not formally check for the presence of apoptotic or necrotic cancer cells in the lung in our experiments and we also did not address the consequences of tissue damage induced by larger lung metastases that might contribute to lung neutrophil infiltration.

Next, we tested if the ability of tumours to induce lung neutrophil accumulation is dependent on its metastatic nature and preferential colonisation of the lung. To do so, we analysed the frequencies of neutrophils in the lung of a mouse model of non-metastatic, benign skin tumours. Viral-Ha-Ras transgene (TG.AC)-expressing mice were biweekly treated for 3.5 months with the inflammatory agent TPA (12-O-Tetradecanoylphorbol 13-acetate) topical on the backskin to develop benign skin papillomas (Leder et al., 1990). Interestingly, these mice harbouring non-metastatic skin papillomas also showed higher levels of lung neutrophils compared to TPA-treated wildtype controls (Fig. 3.11). This observation precludes a specific association of tumour-induced systemic neutrophil mobilisation or lung accumulation with the metastatic potential of a primary tumour.

Lastly, we aimed to determine if a growing mammary tumour would induce systemic neutrophilia and lung neutrophil accumulation in a persistent or reversible manner where tumour-derived signals are constantly required to maintain neutrophilia. To this end, we grafted primary MMTV-PyMT cancer cells onto the mammary gland of wildtype animals to grow a primary tumour of approximately 1x1x0.5 cm in size followed by surgical resection. Cancer cells were transplanted onto the mammary gland on this occasion to synchronise tumour growth and facilitate complete tumour resection. Notably, elevated neutrophil numbers persisted in the lungs of tumour-resected mice for at least one week despite the absence of a primary tumour (Fig. 3.12). This evidence suggests the interesting notion of a systemic "conditioning" of the host by the mammary tumour that appears to be an enduring alteration rather than an acute, reversible response that requires to be constantly sustained.

In conclusion, systemic neutrophil mobilisation in mammary tumour-bearing hosts is primary tumour-induced and persistent after tumour resection at least at in the lung. The mechanisms of neutrophil accumulation particularly in the metastatic lung appear to be independent of neutrophil-expressed CXCR2 as well as cancer cell-derived G-CSF and also not connected to the metastatic nature of a primary tumour. Additionally, distantly present metastatic mammary cancer cells do not appear to directly recruit neutrophils to the lung. This evidence highlights the complexity of the regulation of lung neutrophil presence in mammary cancer-bearing hosts and, as Coffelt et al. 2015 and Benevides et al. 2015 demonstrated, a dedicated study will be necessary to evaluate the precise mechanisms of systemic neutrophilia and specific neutrophil accumulation in the lung.

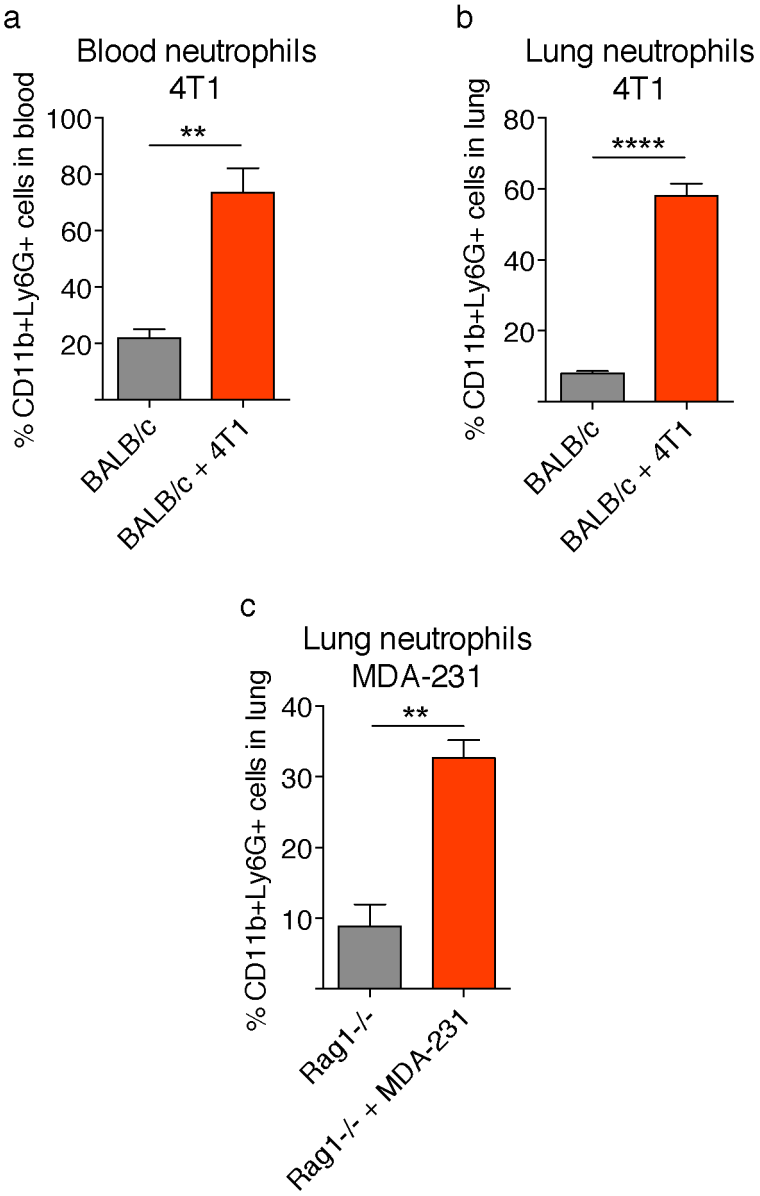


Figure 3-8 Lung metastatic mouse and human breast cancer cell lines grafted onto the mammary gland also elevate frequencies of lung neutrophils
(a-c) Flow cytometric quantification of CD11b+Ly6G+ neutrophils in the blood (a) and the metastatic lung (b-c) of wildtype mice harbouring late-stage mammary tumours after injection of syngeneic mouse 4T1 cells (a-b) or immunodeficient Rag1-/- mice grafted with human MDA-MB-231 cells at late carcinoma stages (n≥3 biological replicates (control or tumour cell-grafted mice) per group analysed in parallel).

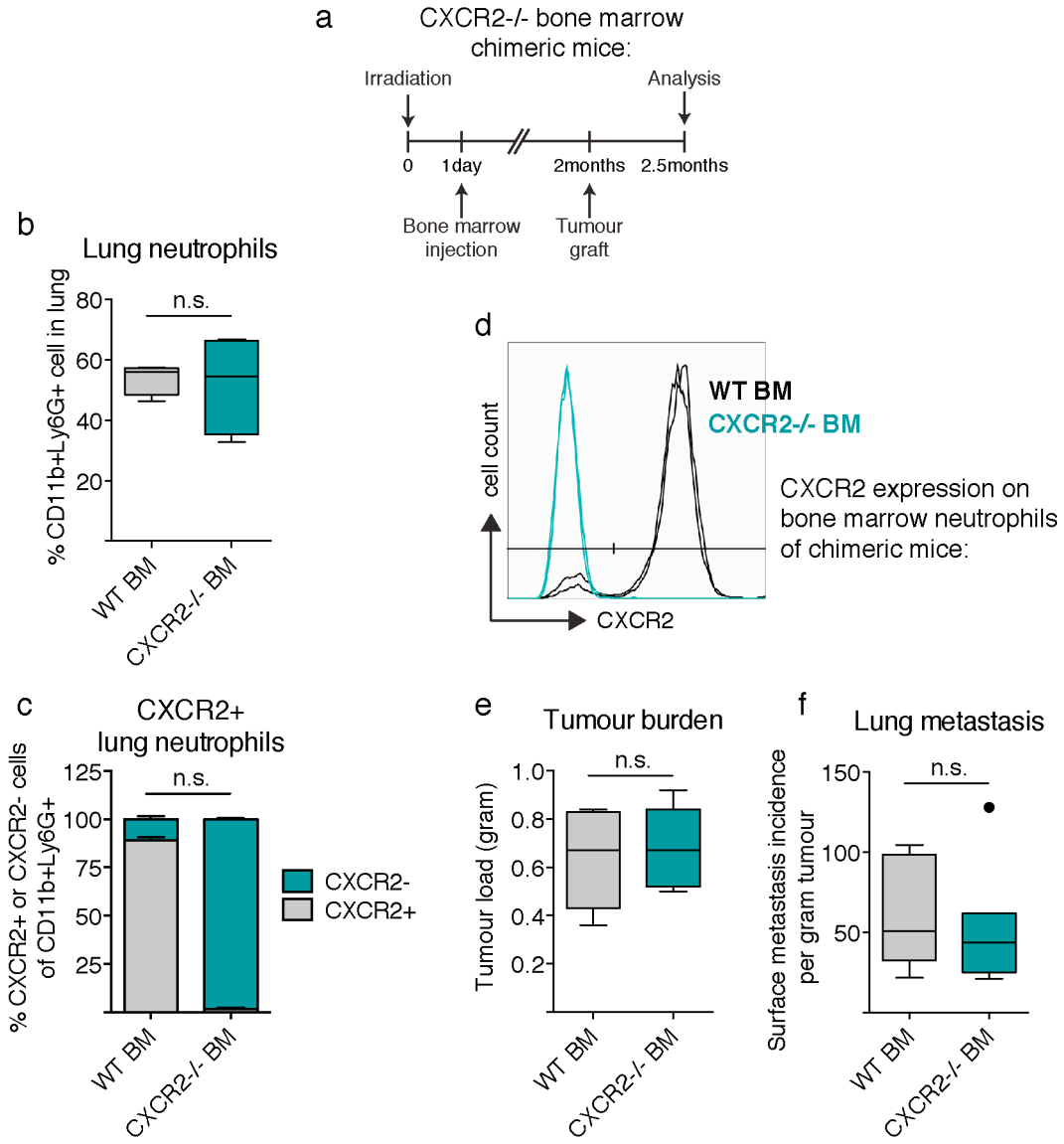


Figure 3-9 Neutrophil accumulation in the lung of tumour-bearing hosts is not depend on their CXCR2 expression

(a) Schematic representation of experimental setup and timeline for generation of wildtype and CXCR2-deficient (CXCR2-/-) bone marrow (BM)-reconstituted mice, grafting of 4T1 mammary tumours and analysis (n≥5 biological replicates (mice) per group analysed in parallel). (b-c) Quantification of total CD11b+Ly6G+ neutrophil frequencies in the lung (b) and CXCR2 expression on these CD11b+Ly6G+ lung neutrophils (c) of mammary tumour-bearing bone marrow-reconstituted mice by flow cytometry, gated on alive single cells (b) or CD11b+Ly6G+ cells (c). (d) Representative flow cytometric analysis of surface CXCR2 expression on lung CD11b+Ly6G+ neutrophils of quantification shown in (c). (e-f) Total primary mammary tumour burden in gram (e) and spontaneous metastasis incidence displayed as number of visible surface lung metastases relative to tumour load (f) of advanced stage 4T1 tumour-bearing bone marrow-reconstituted mice at time of lung neutrophil analysis.

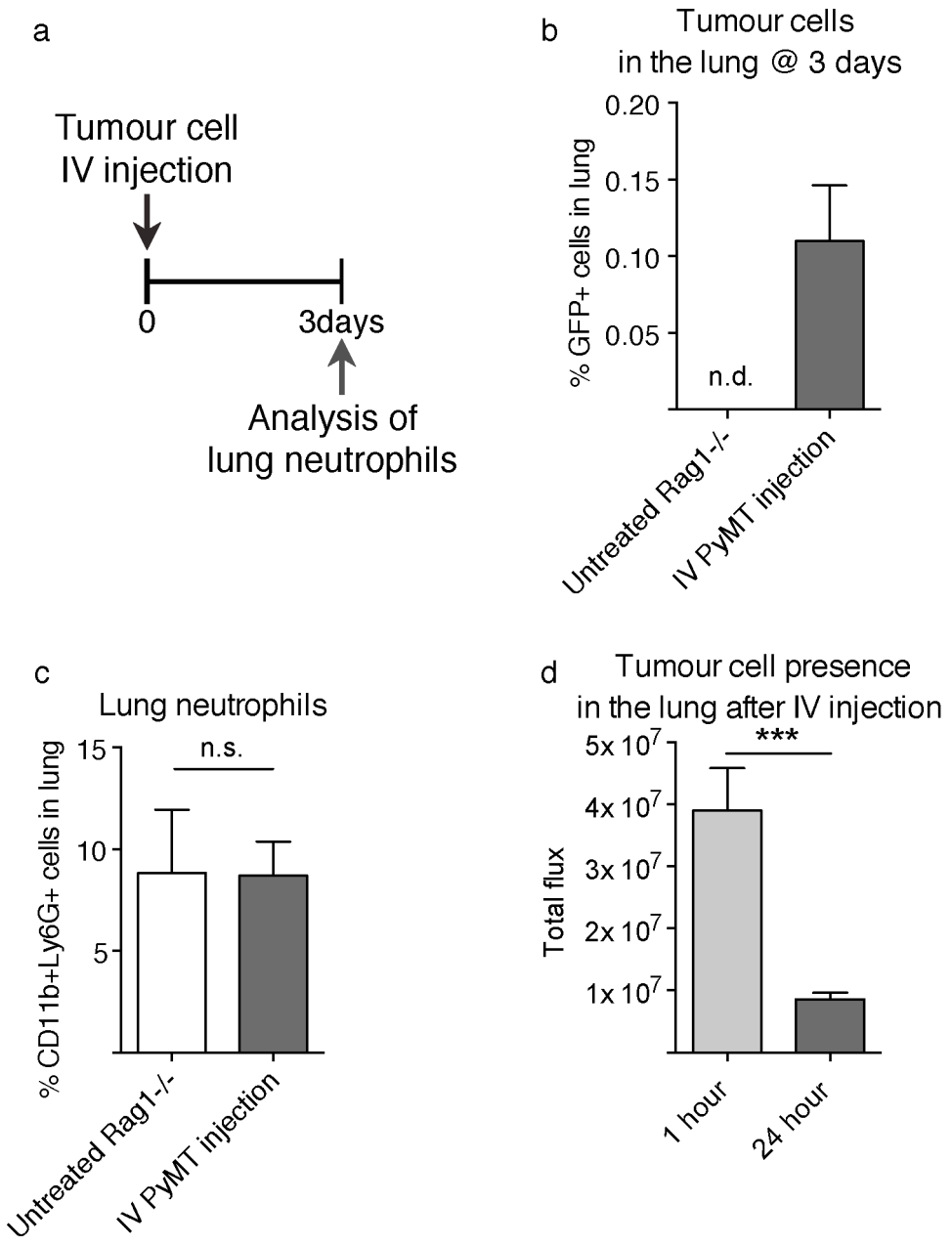


Figure 3-10 MMTV-PyMT cancer cells directly seeded into the lung of tumour-free mice do not induce lung neutrophil accumulation
(a-c) Schematic representation of experimental setup and timeline for intravenous (IV) GFP-labelled MMTV-PyMT cancer cell injection and lung analysis (one primary MMTV-PyMT cancer cell preparation injected into n≥3 mice per group (technical replicates) and analysed in parallel). Flow cytometric quantification of frequencies of total CD11b+Ly6G+ neutrophils (b) and GFP+ MMTV-PyMT cancer cells in the lung of cancer cell injected mice, gated on alive single cells. (d) Luciferase-labelled MMTV-PyMT cancer cells were intravenously injected into Rag1-/- mice and their presence in the lung analysed one hour or 24 hours after by determination of total flux bioluminescence imaging (n=7 mice per group pooled from 3 independent experiments using 3 different tumour cell preparations, section 2.2.16). N.d.: not detected.

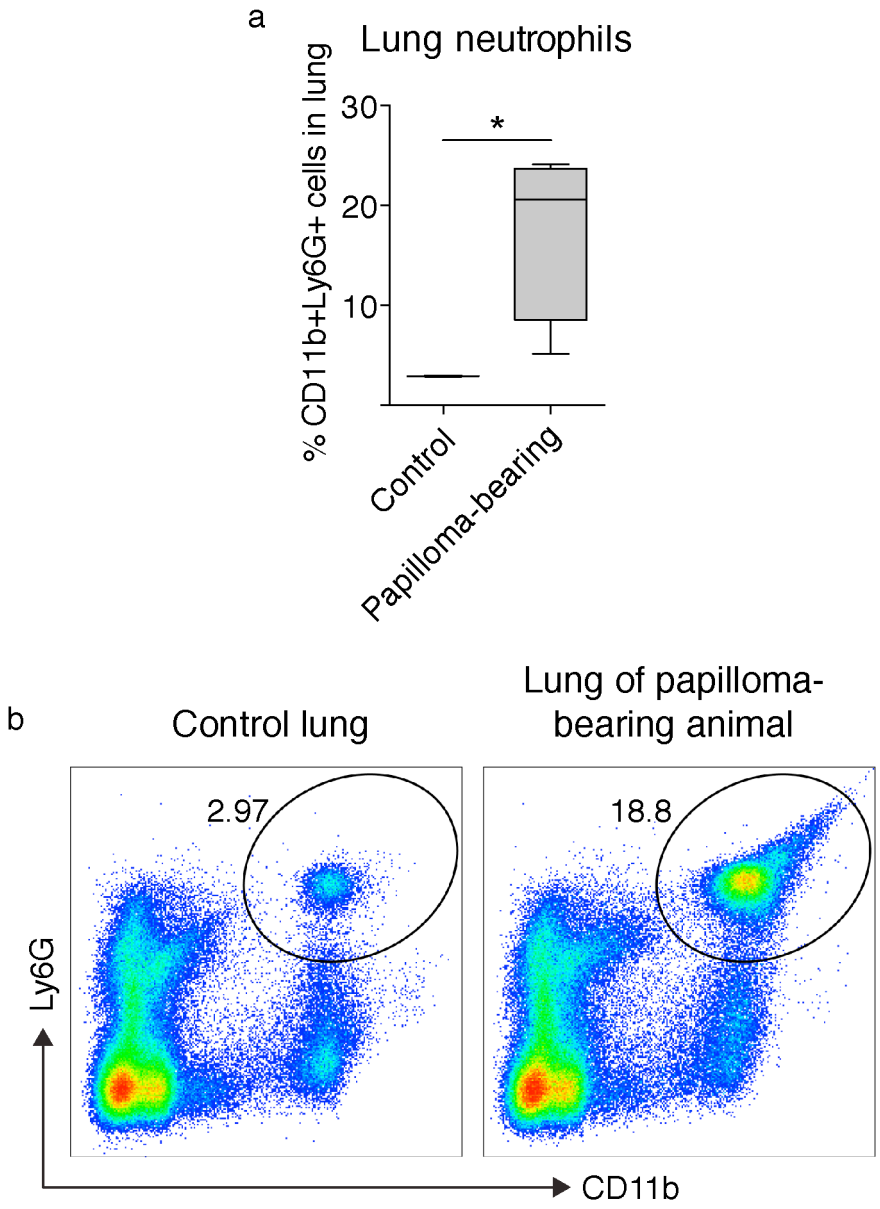


Figure 3-11 Tumour-mediated elevation of lung neutrophil levels does not depend on the metastatic potential of the tumour
(a-b) Flow cytometric quantification (a) and representative analysis (b) of CD11b+Ly6G+ neutrophils in the lung of TPA-treated tumour-free control and TPA-induced skin papilloma-bearing v-Ha-Ras transgenic mice (n≥3 biological replicates (control or papilloma-bearing mice) per group analysed in parallel), gated on alive single cells. Insert in (b) shows relative frequencies of double-positive cells contained in the displayed gate.

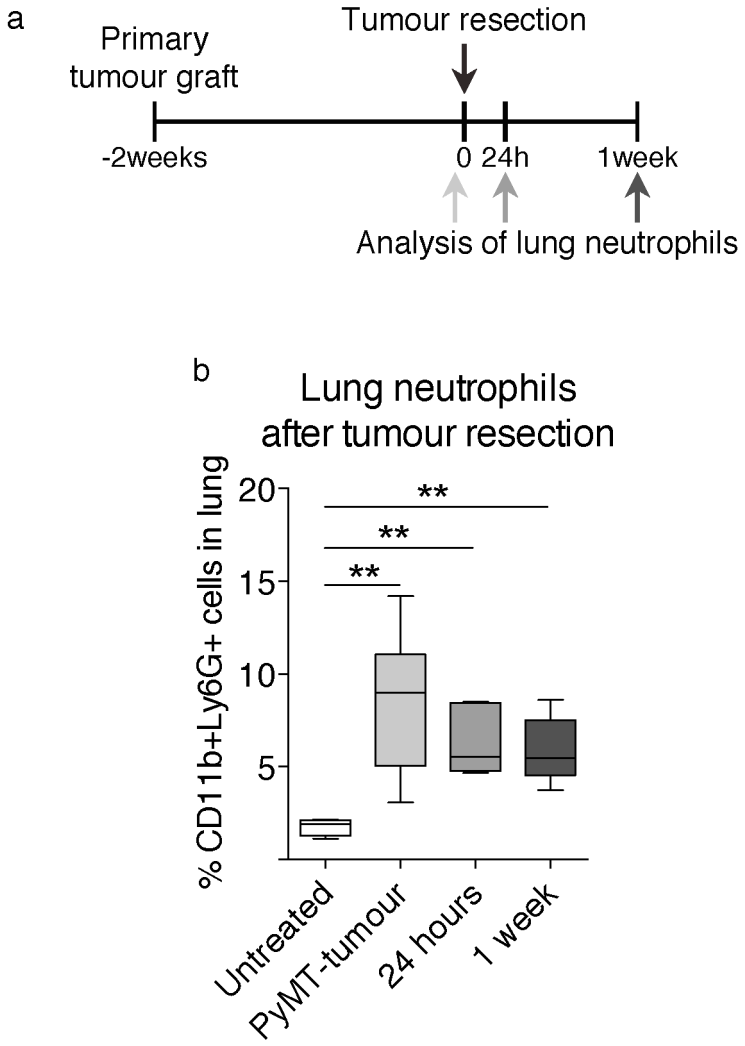


Figure 3-12 Lung neutrophil accumulation in MMTV-PyMT mammary tumour-bearing mice persists after surgical tumour resection
(a) Schematic representation and timeline of the experimental setup to investigate neutrophil presence in the lung after surgical removal of primary mammary tumours (n≥4 biological replicates (control or grafted mice using one MMTV-PyMT cell preparation) per group analysed in parallel). Wildtype mice were orthotopically transplanted with MMTV-PyMT mammary cancer cells and the established tumours resected two weeks thereafter. (b) Flow cytometric quantification of CD11b+Ly6G+ neutrophils in the lung of untreated control mice, MMTV-PyMT tumour-bearing mice at tumour resection, 24 hours and 1 week after tumour resection, gated on alive single cells.

3.2.3 Neutrophils infiltrate the pre-metastatic lung prior to tumour cells and are essential for early lung colonisation by mammary cancer cells

Our data from genetically neutropenic mammary cancer mouse models show a clearly overall pro-metastatic role of neutrophils and suggest a potential immunosuppressive activity at least at advanced metastatic stages (Fig. 3.3, 3.4 and 3.6). Neutrophils are highly present in the metastatic lung of mammary cancer bearing mice at late stages (Fig. 3.1 and Fig. 3.8), but have also been observed to infiltrate the lung earlier during cancer development (Acharyya et al., 2012, Casbon et al., 2015, Coffelt et al., 2015, Granot et al., 2011). Overall, the literature on the activities of neutrophils during the metastatic process appears controversial, which might be dependent on the precise stage of tumourigenesis (section 1.3.5). Hence, we focussed on characterising the functional contribution of lung neutrophils to metastasis and aimed to carefully determine at which steps of the metastatic process neutrophils are involved.

Firstly, we excluded previously described functions of neutrophils to promote angiogenesis or anti-tumour immunosuppression at the primary site (section 1.3.5) due to neutrophil-independent primary tumour growth in MMTV-PyMT+ mice (Fig. 3.3 a and 3.6 b). Neutrophils are also known to support dissemination and migration of cancer cells from the primary tumour site (section 1.3.5). To specifically address these neutrophil functions in the MMTV-PyMT mammary tumour model, we employed an experimental approach of metastasis induction that is independent of cancer cell dissemination or intravasation and intravenously injected primary GFP-labelled MMTV-PyMT cells isolated form spontaneously developed tumours. To test if the neutrophil pro-metastatic activity is independent of cancer cell dissemination or intravasation, Rag1-/- mice were grafted with primary unlabelled MMTV-PyMT tumour cells to induce neutrophil accumulation in the lung. Four weeks after tumour engraftment, mice were intraperitoneally injected with either control IgG- or anti-Ly6G antibody every 24 hours to systemically deplete neutrophils (Fig. 3.13 a). Daily intraperitoneal injections of the anti-Ly6G (clone 1A8) antibody have previously been used also in cancer settings to specifically ablate neutrophils in mice in a temporally controlled fashion (Daley et al., 2008, Granot et al., 2011). One day after the first antibody administration, GFP-

labelled MMTV-PyMT cancer cells were intravenously injected into these neutrophil-depleted mice and their presence in the lung tissue determined 20 hours thereafter. At this time point, cancer cells typically already extravasated into the tissue and metastatic lungs were perfused with PBS before analysis to remove remaining non-attached cancer cells in the vasculature. Therefore, this assay allows to determine if the neutrophil pro-metastatic activity involves both, facilitation of cancer cell dissemination from the primary tumour and intravasation as well as cancer cell trapping and extravasation at the distant metastatic site as previously suggested (section 1.3.5). Anti-Ly6G-treatment starting 24 hours before cancer cell intravenous injection efficiently depleted CD11b+Ly6G+ neutrophils from the lung of MMTV-PyMT tumour-grafted Rag1-/- mice (Fig. 3.13 b). Importantly, infiltration of cancer cells into the lung determined by flow cytometry for GFP+ MMTV-PyMT cancer cells was not affected by neutrophil absence/presence (Fig. 3.13 c). This observation precludes cancer cell dissemination, intravasation, trapping or extravasation to be the main metastatic stages supported by neutrophils during metastatic progression of the MMTV-PyMT mammary cancer mouse model. Nevertheless, a more detailed analysis would be necessary to assess the precise involvement of neutrophils in all individual aspects of these processes.

This evidence indicates that neutrophils appear to promote metastasis through mechanisms affecting cancer cells after arrival at the distant metastatic tissue. Previous reports showed neutrophil accumulation in the lung at very early tumourigenic stages (Acharyya et al., 2012, Casbon et al., 2015, Coffelt et al., 2015, Granot et al., 2011) and neutrophils strongly accumulate at the metastatic site at late stages (Fig. 3.1). Hence, we specifically assessed the dynamics of neutrophil presence in the lung of MMTV-PyMT+ mice that spontaneously-developed mammary tumours at pre-metastatic and metastatic stages. Importantly, neutrophils already accumulated in the lung before cancer cells started infiltrating the tissue (pre-metastatic lung) and their number progressively increased during metastatic outgrowth. We established these findings by assessing neutrophil presence in the lung by two independent strategies, quantification of CD11b+Ly6G+ cells by flow cytometry (Fig. 3.14 a) and S100A9+ cells by immunohistochemistry on lung sections (Fig. 3.14 c). S100A9 is a cytosolic protein that can also be released and is predominantly expressed in neutrophils (Kohler et al., 2011, Stroncek et al., 2005). We confirmed the pre-metastatic stage of

analysed lungs of spontaneously developed MMTV-PyMT tumour-bearing mice by the absence of detectable MMTV-PyMT cells by staining for the Polyoma middle T antigen (antibody clone sc-53481) in the consecutive histological sections that were stained for S100A9+ neutrophils (Fig. 3.14 b-c). This observation strengthens that neutrophils in the lung of MMTV-PyMT tumour-bearing mice do not appear to be recruited by metastatic cancer cells already present in the lung (Fig. 3.10) and suggests a potential role of neutrophils during very early metastatic lung colonisation.

To explore the functional contribution of pre-metastatic lung neutrophils to metastatic colonisation, we performed time-controlled neutrophil depletion experiments in immunocompromised Rag1-/- grafted with primary unlabelled MMTV-PyMT cells to establish tumours and trigger lung accumulation of neutrophils. This experimental setting allows for precise synchronisation of primary mammary tumour growth, lung neutrophil elevation and spontaneous metastasis, which is non-feasible in spontaneously tumour-developing MMTV-PyMT+ mice due to the naturally occurring temporal differences in primary tumour onset and burden. We administered anti-Ly6G blocking antibody daily from the fourth week post-tumour engraftment onwards to deplete neutrophils systemically around the time of onset of spontaneous metastatic dissemination and lung colonisation (Fig. 3.15 a). MMTV-PyMT tumour-bearing mice were sacrificed after two weeks of metastatic progression during anti-Ly6G antibody treatment and the lung analysed for presence of neutrophils and spontaneous metastasis. Efficacy of anti-Ly6G-mediated neutrophil depletion was confirmed by absence of CD11b+Ly6G+ neutrophils in the lung by flow cytometry (Fig. 3.15 b). Notably, this short-term neutrophil absence for only two weeks also caused a significant decrease of spontaneous lung metastasis determined by counting of visible, unlabelled surface lung metastases (Fig. 3.15 d and f, GFP- blue metastatic nodules). Neutrophil depletion did not affect grafted mammary tumour growth in this setting (Fig. 3.15 c). These results go hand in hand with our previous findings on the effects of genetic neutrophil-deficiency throughout the metastatic process (Fig. 3.3 and 3.6). Moreover, this data stresses the importance of the pro-metastatic activity of neutrophils in mammary cancer-bearing mice because their absence for only a limited time period already significantly impaired lung metastasis.

Next, we wanted to test the relevance of lung neutrophils precisely during arrival of disseminated cancer cells in the lung and early initiation of metastatic colonisation. To this end, lungs of the same unlabelled MMTV-PyMT cancer cell-grafted Rag1-/- mice used above (Fig. 3.15 a-d) were synchronously seeded with primary GFP-labelled MMTV-PyMT cancer cells by intravenous injection to induce experimental metastasis. GFP+ cells were injected one day after anti-Ly6G treatment start directly into neutrophil-depleted lungs. This setting allows the assessment of neutrophil activities exactly at the time of cancer cell influx and the very early beginning of metastatic growth of cancer cells in the lung. Two weeks after labelled cancer cell intravenous injection, their frequencies in the lung of neutrophil-sufficient or deficient MMTV-PyMT tumour-bearing mice were assessed concomitantly with spontaneous metastasis. Labelled MMTV-PyMT cells and thereby experimental metastasis could easily be distinguished from spontaneously metastasising unlabelled MMTV-PyMT cancer cells by flow cytometric quantification of GFP+ cells present in the lung or immunohistochemical staining for GFP in lung sections. Remarkably, synchronously seeded GFP+ cancer cells directly into neutrophil-depleted lungs were significantly impaired in their ability to colonise the lung compared to GFP+ cancer cells injected into neutrophil-containing lungs (Fig. 3.15 e-f). This result indicates the requirement of pre-metastatic lung neutrophils for efficient initiation of metastatic colonisation by disseminated MMTV-PyMT cancer cells when arriving in the lung of mammary tumour-bearing mice.

Collectively, this evidence indicated that primary mammary tumours might alter the microenvironment at the target site for metastatic spread in a promoting manner by inducing neutrophil accumulation in the lung prior to cancer cell infiltration. Hence, neutrophils present in elevated numbers in the pre-metastatic lung might provide a more hospitable environment for arriving cancer cells and aid their growth or survival reminiscent of a pre-metastatic niche (section 1.2.5). Hence, we focused on characterising these pre-metastatic CD11b+Ly6G+ cells accumulating in the pre-metastatic lung due to their notable metastasis-supporting nature during the very initial stages of lung colonisation. To phenotypically examine pre-metastatic lung neutrophils, MMTV-PyMT tumours were established by orthotopic injection of MMTV-PyMT cells into the mammary gland of wildtype mice to synchronise tumour growth and distant neutrophil accumulation. Lung pre-metastatic CD11b+Ly6G+

cells were analysed three weeks after primary tumour graft on wildtype mice, which still represents the pre-metastatic phase of spontaneous mammary tumour progression before growth of metastatic cancer cells in the lung (Fig. 3.16 a). The majority of isolated Ly6G+ cells from pre-metastatic lungs of MMTV-PyMT tumour-bearing mice appear morphologically very similar to mature neutrophils showing segmented nuclei (Fig. 3.16 b) (Dumitru et al., 2012, Gabrilovich et al., 2012, Pillay et al., 2012, Youn et al., 2012, Youn and Gabrilovich, 2010). Next, we compared tumour-induced lung CD11b+Ly6G+ cells three weeks after primary tumour graft with CD11b+Ly6G+ neutrophils from healthy lungs. In general, tumour-mobilised lung CD11b+Ly6G cells appear bigger and more granular than healthy wildtype neutrophils when assessed for flow cytometric parameters of forward and side scatter that determine size and granularity, respectively (Fig. 3.16 c). These features indicate their increased activation and maturity (section 1.3). We also tested the expression of factors previously associated with neutrophils in the tumour context in pre-metastatic lung Ly6G+ cells (Fridlender et al., 2009, Joyce and Pollard, 2009). Analysis of messenger RNA (mRNA) expression levels of secreted factors in isolated Ly6G+ cells and expression of surface markers on CD11b+Ly6G+ cells by flow cytometry revealed only some small variations (Fig. 3.16 c). Two of seven tested neutrophil-secreted factors showed differences on mRNA level and two of six analysed surface markers were altered. The upregulation of CD31 in the tumour context suggests increased lung infiltration activity of tumour-induced neutrophils (Kolaczkowska and Kubes, 2013, Luu et al., 2003, Malanchi, 2013) and reduction of their surface MHC-II expression a potential decline of their antigen presentation to impact T helper cell activation (Abi Abdallah et al., 2011). Together, these data indicate that, at this early time point, the tumour-induced CD11b+Ly6G+ cells in the lung appear to be mature neutrophils similar to the ones found in healthy lungs that likely display an activated state (section 1.3).

Neutrophils in the tumour and metastasis context were also reported to act immunosuppressive in a similar fashion as immature granulocytic myeloid-derived suppressor cells (G-MDSCs) preventing the activation of especially NK and cytotoxic T cell anti-tumour responses (section 1.2.3.2 and 1.3.5). In fact, immature G-MDSCs with pronounced immunosuppressive properties have been described to infiltrate metastatic sites in the literature (Quail and Joyce, 2013, Sceneay et al., 2013). Moreover, mature neutrophils and G-MDSCs share similar surface markers

and hence have to be distinguished based on their morphology, phenotype and function (Dumitru et al., 2012, Gabrilovich et al., 2012, Pillay et al., 2013). The morphology and expression of mRNA levels as well as surface markers of CD11b+Ly6G+ cells accumulating in the pre-metastatic lung of MMTV-PyMT tumour-bearing mice suggests them to rather represent mature neutrophils than immature G-MDSCs (Fig. 16 and discussed in detail in section 6.2.3.2). Nevertheless, we specifically assessed eventual immunosuppressive properties of pre-metastatic CD11b+Ly6G+ neutrophils that might account for their pro-metastatic activity. To this end, we functionally investigated the presence of an anti-cancer immune environment within the pre-metastatic lung of immunocompetent wildtype mice grafted with MMTV-PyMT mammary tumours. We continuously depleted neutrophils during the pre-metastatic stage by anti-Ly6G antibody treatment starting one day before tumour engraftment and analysed lungs three weeks thereafter (Fig. 3.17 a). As expected, levels of CD11b+Ly6G+ neutrophils significantly increased in the pre-metastatic lung, which is also reflected in increased total CD45+ immune cell frequencies in these temporally controlled experiments, and was prevented by Ly6G administration (Fig. 3.17 b-c and f). No significant differences in frequencies and/or activation status of the following immune cell types were found in pre-metastatic lungs, neutrophil-depleted pre-metastatic lungs and lungs of healthy wildtype mice: CD11b+SiglecF+ eosinophils, alveolar macrophages, interstitial macrophages, dendritic cells and, most importantly, NK cells and cytotoxic T cells including the ratio of immunosuppressive regulatory T cells vs. cytotoxic T cells (Fig. 3.17 d-e+g, Fig. 3.18 a-b+e and Fig. 3.19 a-d). B cell activation seemed to be reduced in the pre-metastatic lung compared to tumour-free controls, however in a neutrophil-independent manner (Fig. 3.18 c-d+f). Interestingly, while neutrophil absence throughout cancer development correlated with rescued NK cell presence in the metastatic lung of mammary MMTV-PyMT tumour-bearing mice (Fig. 3.4), we did not observe a similar effect of neutrophil-depletion in the pre-metastatic lung. This observation highlights the difference in composition of the microenvironment in a pre-metastatic compared to a metastatic setting and suggests that neutrophils might gain NK cell inhibitory features at later metastatic stages. Alternatively, MMTV-PyMT+ neutrophil-deficient mice displayed a decreased metastatic burden compared to MTV-PyMT+ controls, which might also contribute to reduced NK cell recruitment to

the lung. Nevertheless, the absence of a neutrophil-controlled inhibition of NK and cytotoxic T cell presence or activation in the pre-metastatic lung together with a paralleled significant lung neutrophil accumulation suggests other mechanisms contributing to neutrophil pro-metastatic activity at initial stages of cancer cell lung colonisation rather than immunosuppression.

Additionally, we functionally excluded a dependence of the metastasis-promoting features of pre-metastatic lung neutrophils on T or B cell-mediated mechanisms, because neutrophil depletion in MMTV-PyMT tumour-bearing Rag1-/- mice efficiently impaired metastatic initiation and lung colonisation of cancer cells (Fig. 3.15).

Together, our results indicate that primary mammary tumours trigger accumulation of neutrophils in the lung that precedes cancer cell arrival and metastatic spread to the lung. Pre-metastatic lung neutrophils appear phenotypically mature and likely create a favourable pre-metastatic niche in the tissue targeted for metastatic dissemination independent of a potential immunosuppressive activity.

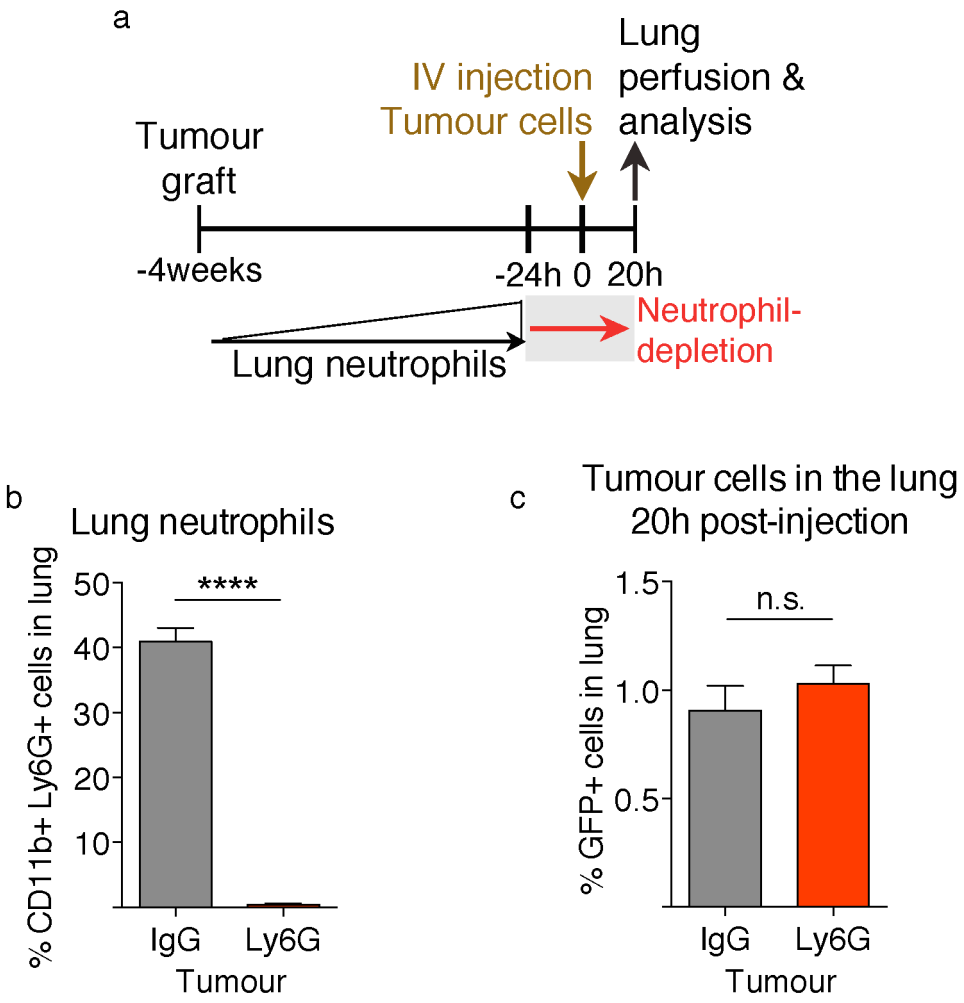


Figure 3-13 Neutrophils do not appear to significantly influence MMTV-PyMT cancer cell extravasation and arrival at the distant lung
(a) Schematic representation and timeline of the experimental setup to test the involvement of tumour-induced neutrophils to MMTV-PyMT cancer cell trapping and extravasation in the lung. Rag1-/- mice were orthotopically transplanted with MMTV-PyMT mammary cancer cells, treated with neutrophil blocking anti-Ly6G antibody followed by intravenous injection of labelled MMTV-PyMT cancer cells. 20 hours later lungs were perfused to remove cells present in the circulation and analysed (one primary MMTV-PyMT cancer cell preparation injected into n≥4 mice per group (technical replicates) and analysed in parallel). (b-c) Flow cytometric quantification of frequencies of total CD11b+Ly6G+ neutrophils (b) and GFP+ MMTV-PyMT cancer cells (c) in the lung, gated on alive single cells.

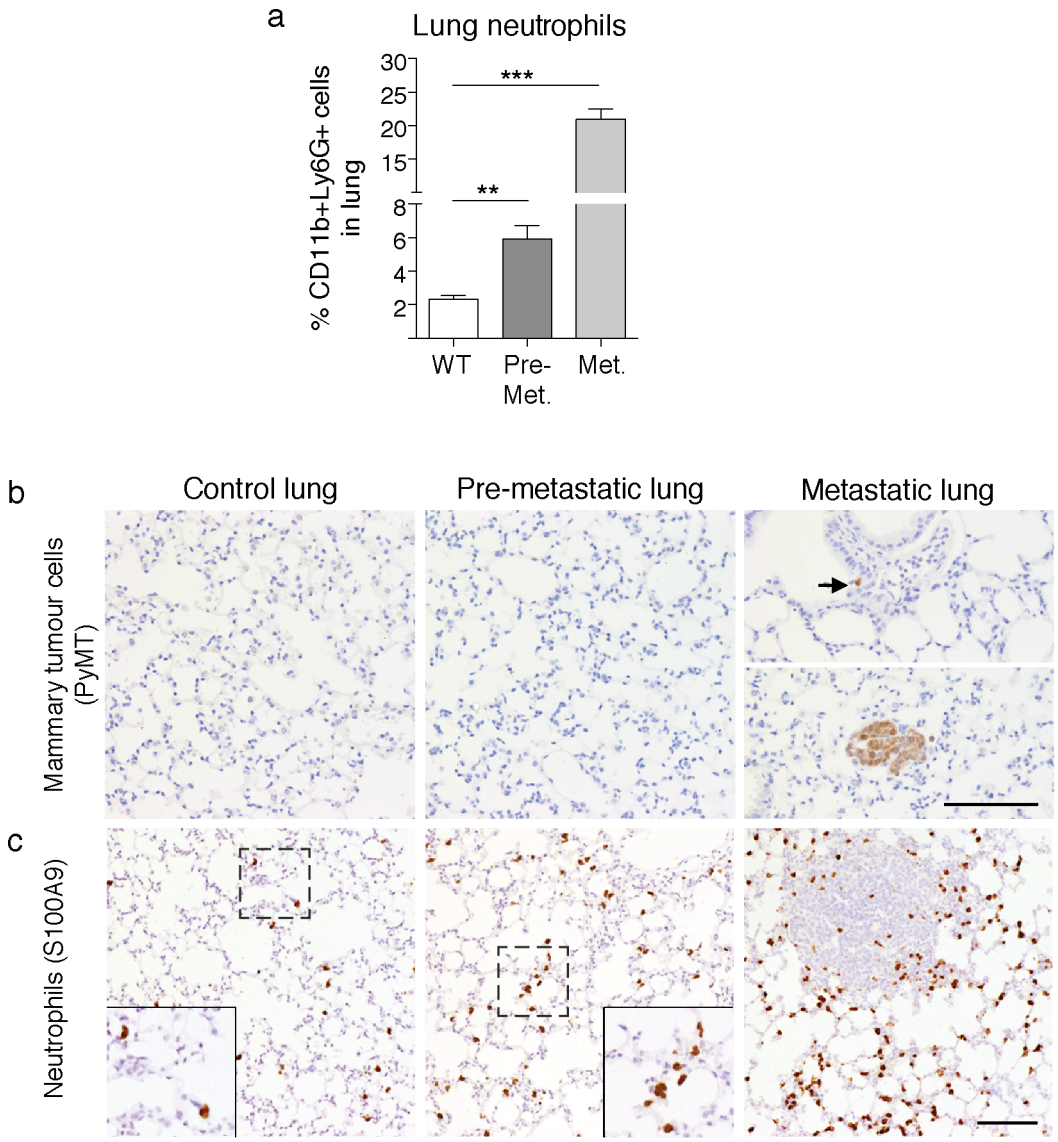


Figure 3-14 Neutrophils infiltrate the pre-metastatic lung of mammary MMTV-PyMT cancer-bearing mice preceding cancer cell infiltration
(a) CD11b+ Ly6G+ neutrophils present in wildtype control, pre-metastatic and metastatic lungs of MMTV-PyMT+ mice were quantified by flow cytometry (n≥3 per group pooled from ≥3 independent litters). (b-c) Histological lung section of a wildtype control, pre-metastatic and metastatic MMTV-PyMT+ mouse stained for either the neutrophil-marker S100A9 (c, brown) or the exclusively cancer cell-expressed Polyoma middle T antigen (PyMT, b, brown) and haematoxylin (blue) to visualise nuclei, scale bars are 100µm. Representative of ≥3 analysed lung sections is shown. Pre-metastatic status of the lung was confirmed by examination of six sections per lung for PyMT staining, 100µm apart. Arrow in (b) indicates a single PyMT+ cancer cell. Close-ups on inserts in lung sections in (c) highlight presence of S100A9+ neutrophils in the lung. WT: wildtype littermate control. Pre-Met. and Met.: MMTV-PyMT+ mice without detectable cancer cells in the lung or with established stage lung metastases, respectively.

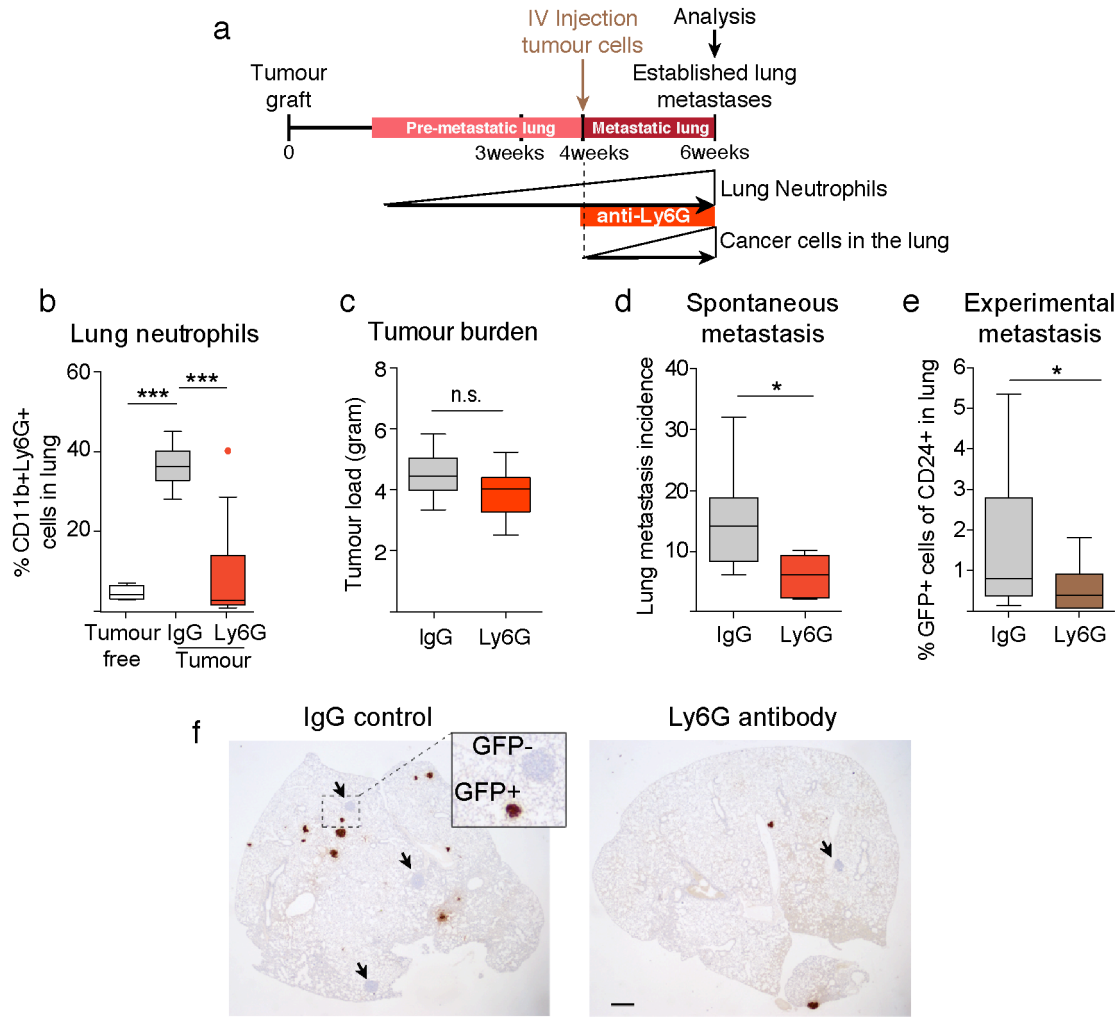
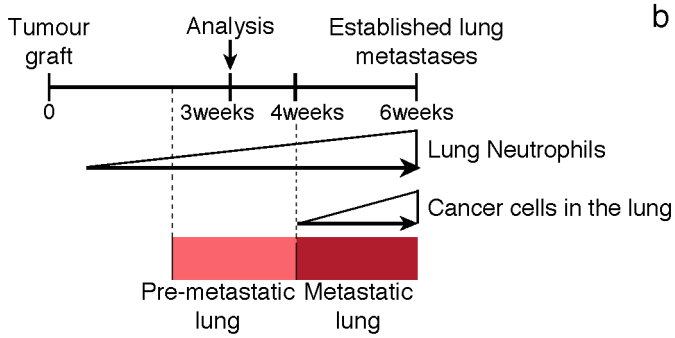


Figure 3-15 Lung neutrophil presence in mammary cancer-bearing mice is critical for efficient initiation of early metastatic lung colonisation by disseminated cancer cells

(a) Schematic representation and timeline of the experimental setup for neutrophil-depletion during lung metastasis initiation. Unlabelled MMTV-PyMT cells were grafted onto the mammary gland of Rag1-/- mice and GFP-labelled MMTV-PyMT cells injected via the tail vein (IV) 4 weeks thereafter. Daily treatment with control IgG- or anti-Ly6G antibody by intraperitoneal injection started one day prior to GFP-labelled cell injection and continued for two weeks until analysis. (b) CD11b+Ly6G+ neutrophil presence in the lung was determined by flow cytometric quantification at the end of the experiment, gated on alive single cells (n≥4 per group pooled from 3 independent experiments using different MMTV-PyMT cancer cell preparations). (c-f) Tumour burden was assessed by weighing of dissected tumours (c), spontaneous metastatic progression by quantification of visible, non-GFP surface lung metastases (d) and experimental metastasis initiation by flow cytometric quantification of GFP+CD24+ MMTV-PyMT cells in the lung (n≥12 per group pooled from 3 independent experiments using different MMTV-PyMT cancer cell preparations). (f) Representative histological lung sections were stained with GFP in brown to visualise tail-vein injected GFP-labelled MMTV-PyMT cells and with haematoxylin in blue to stain nuclei. Arrows indicate spontaneous metastases originating from the primary tumour; scale bar is 100μm. Close-ups on inserts highlight spontaneous and experimental, labelled metastases.

a



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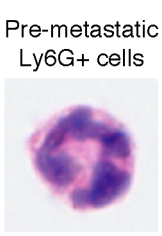

c

Analysis of neutrophils in the lung				
	Wildtype	**Pre-metastatic**		
Cytometric analysis:	Mean intensity of CD11b+ Ly6G+		Significance	
Cell size (FSC)	84295 ± 199.0	87223 ± 628.1	N=4, P=0.0044	**
Cell granularity (SSC)	54932 ± 1467	62774 ± 2020	N=4, P=0.0200	*
Surface expression:	Percent positive of CD11b+ Ly6G+			
CXCR2+	97.90 ± 0.524	98.45 ± 0.150	N=4, P=0.3522	
CD31+	31.58 ± 3.728	49.45 ± 1.900	N=4, P=0.0053	**
MHC-I+	30.60 ± 1.696	28.77 ± 2.381	N=4, P=0.5533	
MHC-II+	28.18 ± 0.820	22.82 ± 1.415	N=4, P=0.0170	*
ICAM1+	23.90 ± 2.515	24.20 ± 1.119	N≥3, P=0.9008	
Fas+	99.35 ± 0.050	99.50 ± 0.058	N≥2, P=0.1697	
mRNA expression:	Change relative to wildtype		Neutrophil purity ≥ 90%	
TNFα	1	0.9585 ± 0.1735	P=0.8334	
Arginase 1	1	0.7667 ± 0.1924	P=0.3492	
VEGF-A	1	0.6814 ± 0.1494	P=0.1666	
CCL2	1	0.4157 ± 0.0932	P=0.0245	*
CCL3	1	0.7391 ± 0.1584	P=0.2414	
iNOS	1	1.0360 ± 0.4653	P=0.9506	
CCL5	1	0.0517 ± 0.0042	P<0.0001	***

Figure 3-16 Comparison of wildtype lung neutrophils with tumour-induced, pre-metastatic lung neutrophils

(a) Representation of timing and dynamics of neutrophil and cancer cell infiltration into the lung of mice grafted with mammary MMTV-PyMT tumours by orthotopic injection of MMTV-PyMT tumour cells. (b) Haematoxylin & eosin (H&E) stained Ly6G+ cell isolated from a pre-metastatic lung. (c) Flow cytometric analysis for cell size (forward scatter, FSC) and granularity (side scatter, SSC), gated on alive single cells, as well as expression of surface markers CXCR2, CD31, MHC-I, MHC-II, ICAM1 and Fas, gated on CD11b+Ly6G+ cells, of CD11b+Ly6G+ wildtype or pre-metastatic lung neutrophils three weeks after primary tumour graft (one primary MMTV-PyMT cancer cell preparation injected into n≥3 mice per group (technical replicates) and analysed in parallel). Data represent mean intensity ± standard error of the mean (SEM) for flow cytometric data. mRNA expression analysis by quantitative PCR of isolated Ly6G+ cells from the lung of wildtype or tumour-bearing mice for TNFα, Arginase1, VEGF-A, CCL2, CCL3, iNOS and CCL5 (biological triplicates (isolated neutrophils) of the pre-metastatic lung compared to a representative control (wild-type lung neutrophil) value). mRNA expression levels are displayed normalised to internal GAPDH control and represent fold-changes ± SEM.

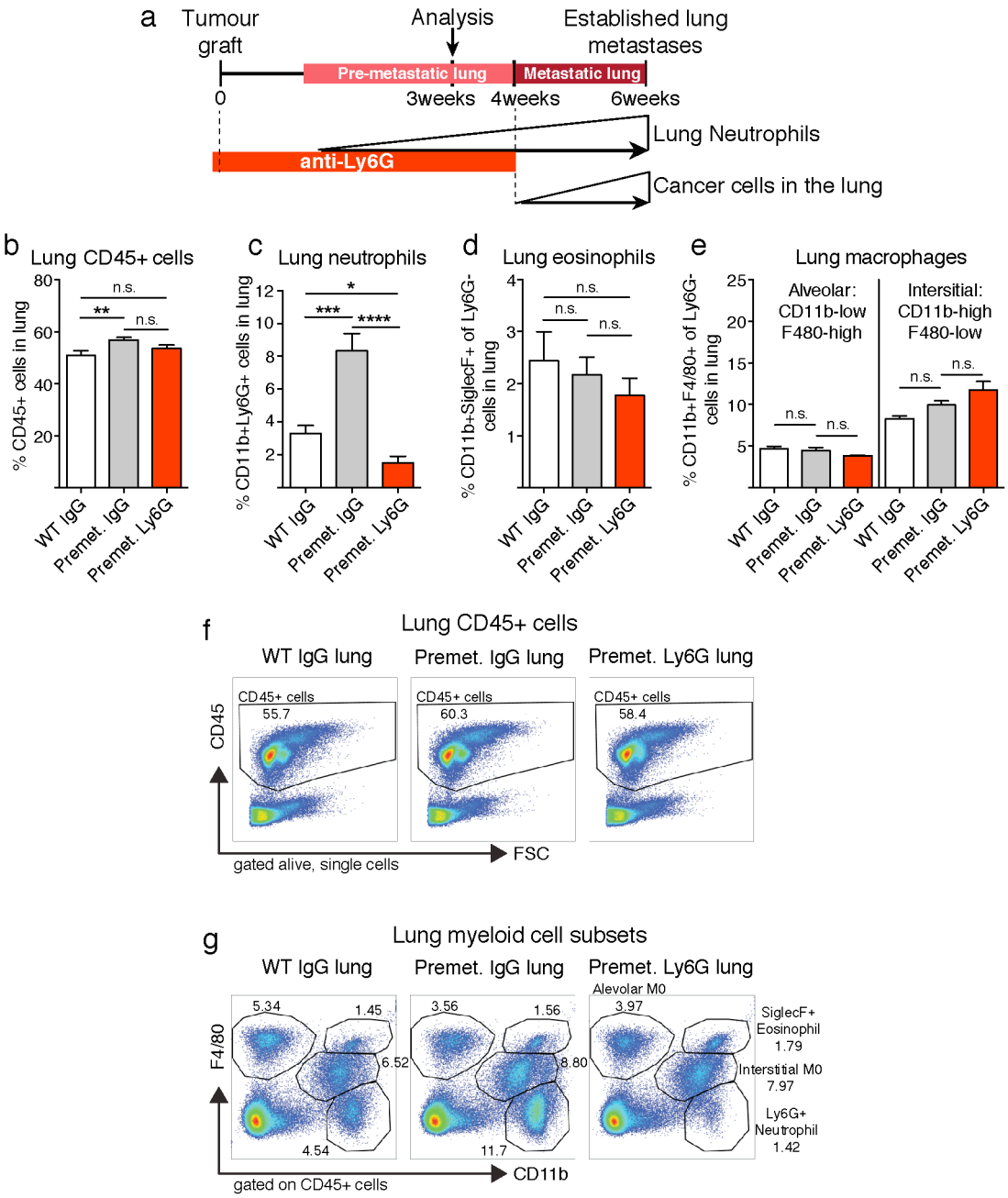

Figure 3-17 Immune cell frequencies and activation in the pre-metastatic lung of MMTV-PyMT tumour-bearing mice is independent of neutrophil presence (part 1)
(a) Representation of timing and dynamics of neutrophil and cancer cell infiltration into the lung of mice grafted with mammary MMTV-PyMT tumours. Mice were analysed three weeks after primary tumour graft (n≥4 biological replicates per group pooled from 1-2 independent experiments). (b-g) Flow cytometric quantification and representative analysis of the following immune cell types in wildtype (WT) or pre-metastatic lungs treated daily with either control IgG or anti-Ly6G antibody from tumour onset onwards: (b+f) total CD45+ immune cells, (c) CD11b+Ly6G+ neutrophils, (d+g) CD11b+SiglecF+ eosinophils, (e+g) CD11b-low F4/80-high alveolar macrophages and CD11b-high F4/80-low interstitial macrophages, gated on alive single cells (b-c+f), Ly6G-negative cells (d-e) or CD45+ cells (g). Inserts in (f-g) show relative frequencies of positive cells contained in the displayed gate.

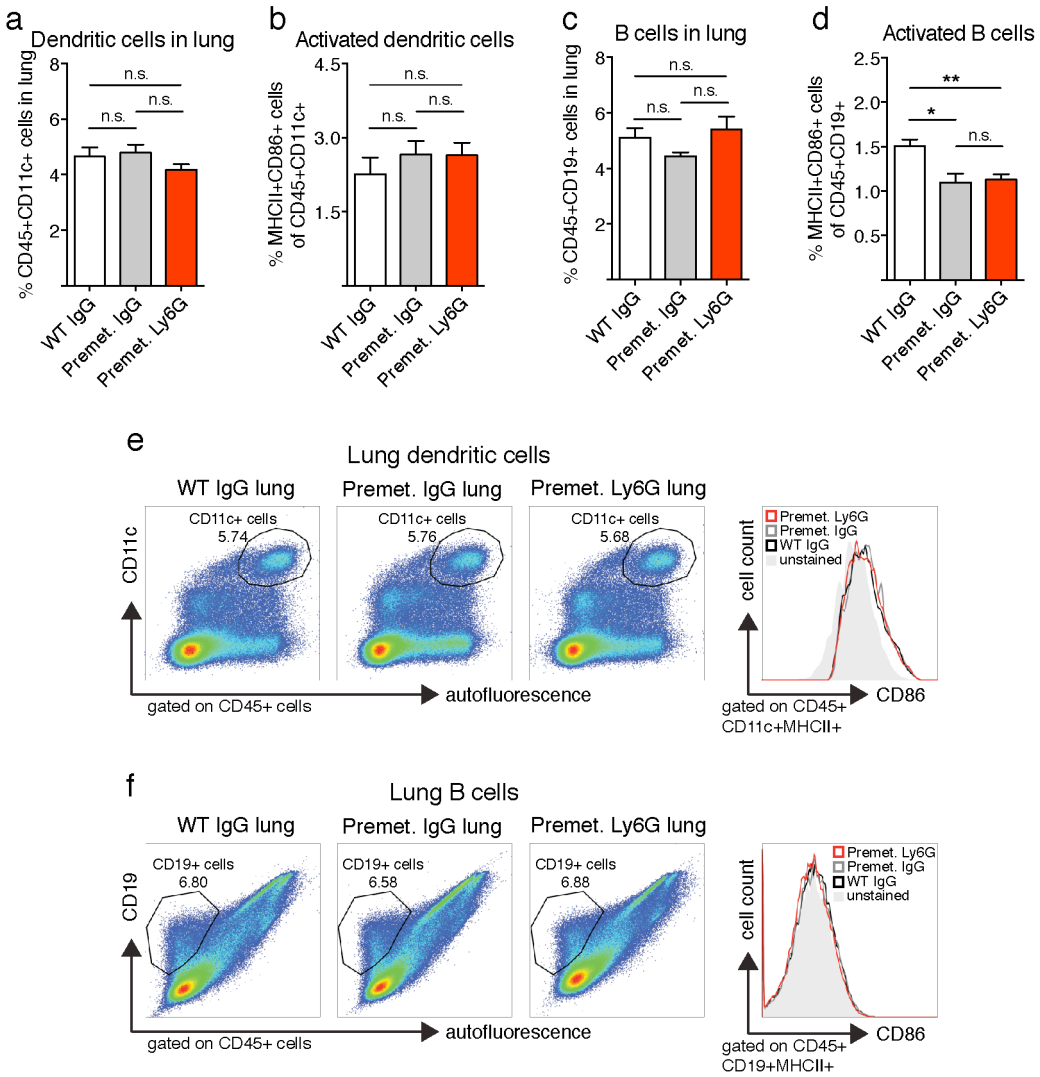

Figure 3-18 Immune cell frequencies and activation in the pre-metastatic lung of MMTV-PyMT tumour-bearing mice is independent of neutrophil presence (part 2)
(a-f) Flow cytometric quantification and representative analysis of the following immune cell types in wildtype (WT) or pre-metastatic lungs treated daily with either control IgG or anti-Ly6G antibody from tumour onset onwards (n≥4 biological replicates per group pooled from 1-2 independent experiments). (a+e) CD45+CD11c+ dendritic cells, (b+e) MHCII+CD86+ activated dendritic cells, (c+f) CD45+CD19+ B cells and (d+f) MHCII+CD86+ activated B cells, gated on either: alive single cells (a+c), CD45+ cells (e-f), CD45+CD11c+ cells (b+e) or CD45+CD19+ cells (d+f), as indicated. Inserts in (e-f) show relative frequencies of positive cells contained in the displayed gate.

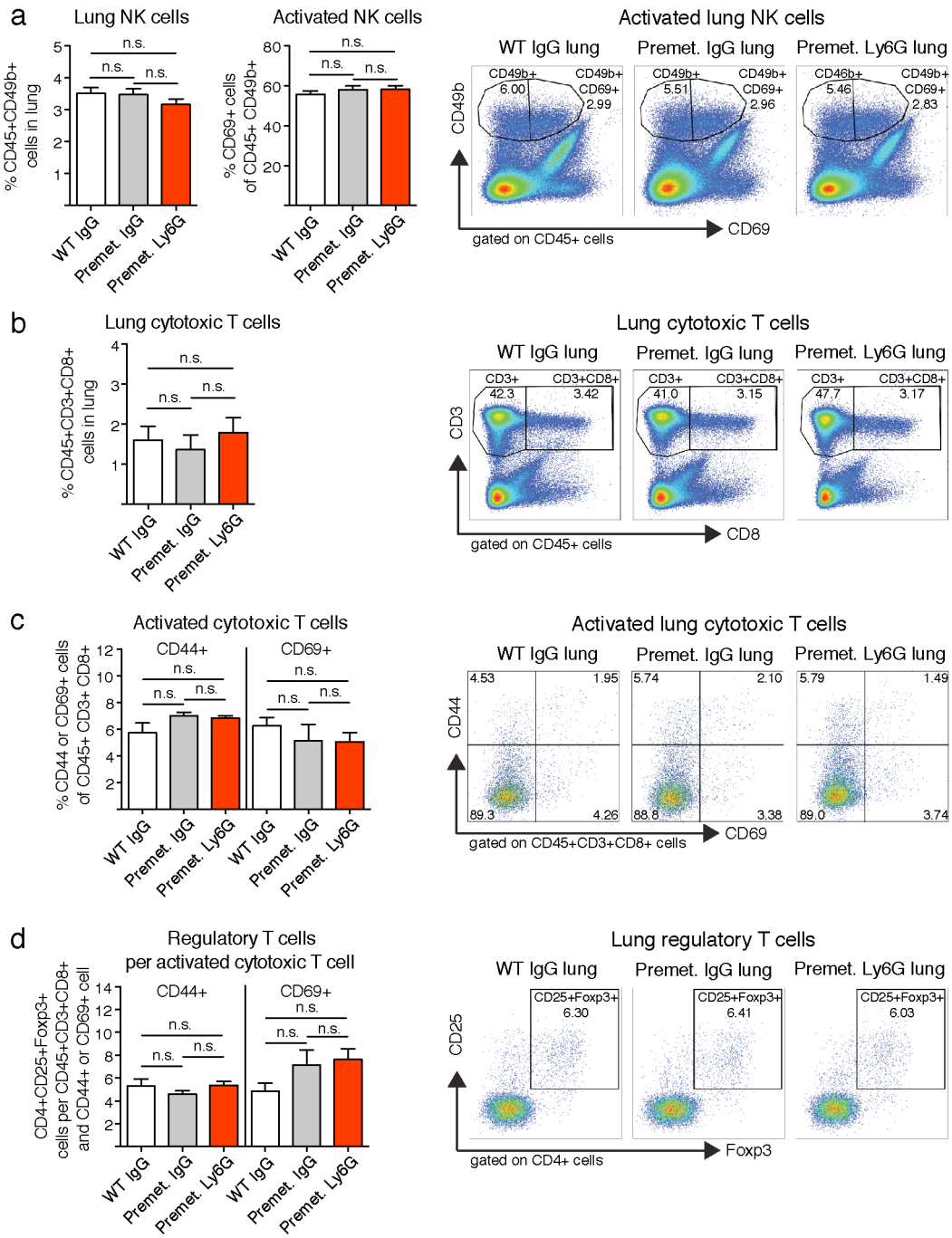

Figure 3-19 Immune cell frequencies and activation in the pre-metastatic lung of MMTV-PyMT tumour-bearing mice is independent of neutrophil presence (part 3)
(a-d) Flow cytometric quantification and representative analysis of the following immune cell types in wildtype (WT) or pre-metastatic lungs treated daily with either control IgG or anti-Ly6G antibody from tumour onset onwards (n≥4 biological replicates per group pooled from 1-2 independent experiments). (a) CD45+CD49b+ natural killer (NK) cells, CD69+ activated NK cells, (b) CD45+CD3+CD8+ cytotoxic T cells, (c) CD44+ or CD69+ activated T cells and (d) CD45+CD3+CD4+CD25+Foxp3+ regulatory T cells and the ratio of regulatory T cells per activated T cell, gated on either: alive single cells, CD45+ cells, CD45+CD49b+ cells, CD45+CD3+CD8+ cells or CD4+ cells, as indicated. Inserts show relative frequencies of positive cells in the displayed gate.

3.2.4 Neutrophils boost lung colonisation potential of mammary cancer cells by directly supporting metastasis-initiating cells

In light of our previous observations, we hypothesised that neutrophils might directly influence arriving metastatic cancer cells in the lung and investigated a potential direct effect of neutrophil-secreted factors on tumour cells *in vivo* and *in vitro*. Cancer cells are very heterogeneous and they might respond differently to environmental stimulations (section 1.1.4). Disseminated cancer cells in patients also show a high level of heterogeneity (Yu et al., 2013). We therefore probed whether neutrophils alter cancer cell heterogeneity in favour of highly metastatic cells by monitoring the previously described metastasis-initiating cancer cell (MIC) population (CD24+CD90+) of MMTV-PyMT cells (Malanchi et al., 2012). To this end, we isolated Ly6G+ neutrophils from the bone marrow and pre-metastatic lung of MMTV-PyMT tumour-grafted wildtype mice about three weeks after primary mammary tumour graft by magnetic-activated cell sorting (MACS). We confirmed viability and purity of bone marrow and pre-metastatic lung neutrophils by flow cytometry for percentage of Ly6G+ cells and staining with PI at time of isolation and only preparations of ≥85% alive and pure neutrophils were used (Fig. 3.20). Neutrophils were not viable for prolonged periods of time *in vitro* after isolation and showed a viability of only about 10-20% after 24-hour culture. Hence, we used either freshly isolated bone marrow or lung neutrophils immediately or used them to condition cell culture medium for 14 hours, bone marrow neutrophil-conditioned (BMN) or lung-neutrophil-conditioned (LuN) medium. To treat cancer cells with lung neutrophils or lung neutrophil-derived factors (LuN medium) *in vivo*, we seeded freshly isolated total GFP+ MMTV-PyMT cancer cells into the lung of tumour-free Rag1-/- mice by intravenous injection followed 12 hours later by intravenous injection of either LuN medium (three injections, every 12 hours) or freshly isolated pre-metastatic lung neutrophils (one injection). Lungs of treated mice were harvested three days after intravenous cancer cell injection and GFP+ MMTV-PyMT cancer cells analysed for their expression of CD24 and CD90 by flow cytometry (Fig. 3.21 a). Strikingly, both, neutrophil-secreted factors and isolated lung neutrophils induced a doubling of MIC frequencies among the total cancer cell population (Fig. 3.21b+d-e). Interestingly, the overall presence of MMTV-PyMT

cancer cells in the lung of recipient mice did not significantly change after transfer of freshly isolated lung neutrophil or LuN medium within three days (Fig. 3.21 c+f). In order to show that this relative increase of the MIC subpopulation upon neutrophil/LuN medium treatment is functionally relevant, we continued treatment of intravenously GFP+ cancer cell-injected mice to assess early metastatic colonisation (Fig. 3.22 a). Indeed, five intravenous injections of LuN media every twelve hours after Luciferase-expressing MMTV-PyMT cancer cell injection led to enhanced metastatic outgrowth in Rag1-/- mice after three weeks as determined by bioluminescence imaging for Luciferase activity (Fig. 3.22 b). Luciferase-expressing MMTV-PyMT cancer cells were isolated from spontaneously developed tumours in MMTV-PyMT+ Actin-Luciferase mice that constitutively express Firefly Luciferase under the control of the actin promoter (Lassailly et al., 2013). In an alternative setting, freshly purified neutrophils were co-injected with GFP+ MMTV-PyMT cancer cells followed by two additional neutrophil injections 48 hours and 96 hours later (Fig. 3.22 a) triggered a modest increase of experimental metastatic burden within one week (Fig. 3.22 c). Overall, these results suggest that neutrophil-derived factors expand the MIC pool among the total cancer cell population to favour metastatic lung colonisation. Please note that the observed boosting effects by lung neutrophil/LuN medium transfer on metastatic incidence appear partial in these experimental settings, which is likely due to the only sporadic injection of either neutrophil-conditioned media or purified neutrophils. Our *in vivo* gain-of-function strategy has the limitation of lacking the constant simulation within the metastatic microenvironment, where neutrophils are persistently present in a physiologic situation. Taking this into consideration, the ability of lung neutrophils to expand the MIC population and promote early metastatic seeding of MMTV-PyMT cancer cells in the lung seems very potent.

Intrigued by this possibility, we set out to corroborate the effect of neutrophil-derived factors to increase “stemness” and metastatic potential of the total cancer cell population directly *in vitro*. We isolated MMTV-PyMT cancer cells from spontaneous mammary tumours and cultured them in control, bone marrow neutrophil (BMN) or lung neutrophil-conditioned medium (LuN) in either non-adherent sphere formation or adherent conditions on collagen-coated dishes. Notably, LuN medium displayed cell toxicity and reduced viable cancer cell numbers exclusively *in* vitro in adherent conditions, likely as a consequence of

potentially toxic factors in the medium, including granzymes, reactive oxygen species and lactoferrin, following isolation-induced neutrophil degranulation *in vitro* (Fig. 3.23 a-b). LuN medium triggered apoptosis of cancer cells as detected by TUNEL positivity leading to cell death by day five of adherent culture (Fig. 3.23 c-d). However, LuN medium showed to be less toxic for MMTV-PyMT cancer cells in non-adherent culture conditions and they thrived seven days post-plating (Fig. 24 a-b). Importantly, *in vitro* LuN medium toxicity was not restricted to tumour cells in adherent conditions, but also affected cultured primary lung stromal cells and reduced their remaining cell numbers in a similar fashion (Fig. 23 b-c). Hence, we suspected a potentially non-physiological effect of lung neutrophil-derived factors in *in vitro* culture, which does not appear to be relevant but actually notably different *in vivo* in the lung. In fact, the lung microenvironment appears to counteract neutrophil-derived harmful factors because, strikingly, the same LuN medium caused no obvious lung damage or toxicity towards cancer cells *in vivo* when injected intravenously into Rag1-/- mice (Fig. 3.21 c+f and Fig. 3.22 b-c). Nevertheless, we also isolated Ly6G+ neutrophils from the bone marrow and produced conditioned cell culture medium (BMN medium) to potentially avoid *in vitro* toxicity. BMN media resulted non-toxic *in* vitro and remaining cancer cell numbers in adherent culture were unaltered compared to untreated culture media (Fig. 3.23 a), however in expense of neutrophil immaturity that might not entirely reflect the functions of lung neutrophils. Strikingly, freshly isolated MMTV-PyMT cancer cells cultured in LuN medium in non-adherent conditions showed enhanced sphere formation potential already after seven days of culture (Fig. 3.24 a-b). This observation strongly suggests a support of neutrophil-derived factors to specifically the cancer cells that intrinsically retain higher anoikis resistance and self-renewal activity. BMN medium appeared to be less potent compared to LuN medium, however a significant increase in sphere formation was also detected after longer cancer cell exposure time (Fig. 3.24 b). Hence, neutrophils appear to have a promoting activity that enhances the potential of the total cancer cell population *in vitro*, potentially by directly expanding the subpool of more potent cancer stem cell-like cells. This pro-tumourigenic feature of lung neutrophils of mammary tumour-bearing hosts seems to be independent from the side effect of neutrophil-derived factor-induced cell toxicity in adherent culture (discussed in detail in section 6.2.3.1). More immature neutrophils from the bone marrow might not cause *in vitro*

toxicity, but appear to be less potent in supporting cancer stem cell-like cells than tumour-induced neutrophils in the pre-metastatic lung. Hence, we focussed on lung neutrophils and their derived factors that comprise part of the pre-metastatic lung microenvironment *in vivo*.

Next, we aimed to substantiate our hypothesis that neutrophil-derived factors might modify the composition of the total cancer cell population favouring more tumourigenic or metastatic subpools. To this end, we tested the potential of total cancer cells after short-term treatment with neutrophil-derived factors, rather that continuous exposure. We pre-treated primary MMTV-PyMT cancer cells with LuN medium for three days in adherent culture (Fig. 3.25 a) followed by plating them in fresh, unmodified culture medium in non-adherent conditions *in vitro* to form spheres. Alternatively, we injected three-day LuN-treated cancer cells orthotopically into the mammary gland to assess tumour initiation potential or intravenously into the lung of Rag1-/- mice to determine their ability to initiate metastases *in vivo*. For some experiments, we used primary Luciferase-expressing MMTV-PyMT cancer cells to quantify metastatic growth in the lung. Short-term LuN medium pre-treatment boosted the tumourigenic potential of MMTV-PyMT cancer cells *in vitro* as well as *in vivo* and enhanced both, their sphere formation as well as their tumour formation potential in 4 independent experiments (Fig. 3.25 b-d). Importantly, LuN exposure also significantly enhanced the experimental metastatic initiation and lung colonisation competence of the total cancer cell population assessed by quantification of visible surface nodules or bioluminescence imaging for Luciferase activity (Fig. 3.25 e-f). Unfortunately, we were unable to monitor the expression of MIC markers CD24+CD90+ on MMTV-PyMT cells cultured in LuN medium to prove our hypothesis that neutrophil-derived factors mediate an expansion of the MIC pool *in vitro*, likely due to the side effect of cell toxicity in adherent culture. Nevertheless, we confirmed direct cancer cell-promoting functions of neutrophil-derived factors that significantly enhance the tumourigenic and metastatic potential of MMTV-PyMT cancer cells.

Collectively, we observe that neutrophil-derived factors alter the heterogeneity of cancer cells favouring CD24+CD90+ metastasis-initiating cells (MICs) and expand the MIC subpool among the total cancer cell population *in vivo* (Fig. 3.21). This expansion might be caused by induction of MIC proliferation or, eventually, a

reversion of nonMICs to a MIC state due to high plasticity among cancer cells (Plaks et al., 2015). Thereby, neutrophils directly enhance the tumourigenic and metastatic competence of mammary cancer cells *in vitro* and *in vivo* that results in increased sphere formation, initiation of primary tumour formation as well as metastatic colonisation ability of total cancer cells (Fig. 3.22, Fig. 3.24 and Fig. 3.25).

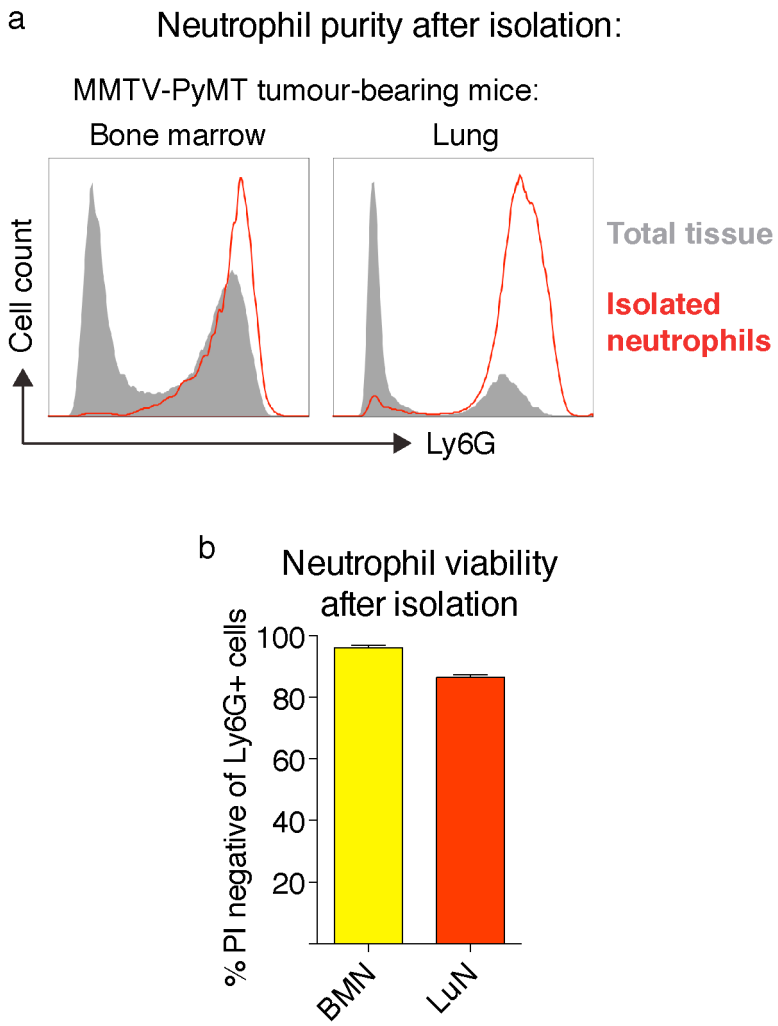

Figure 3-20 Neutrophil Purity and viability of neutrophils isolated from the bone marrow and the pre-metastatic lung of MMTV-PyMT tumour-bearing mice
(a) Representative flow cytometric analysis of neutrophil purity after isolation from the bone marrow or lung compared to total lung tissue, gated on alive single cells. Only neutrophil purity of ≥90% was used for further experiments. (b) Neutrophil viability was assessed by flow cytometry for propidium iodide (PI) after isolation (n=8 biological replicates per group pooled from 8 independent experiments).

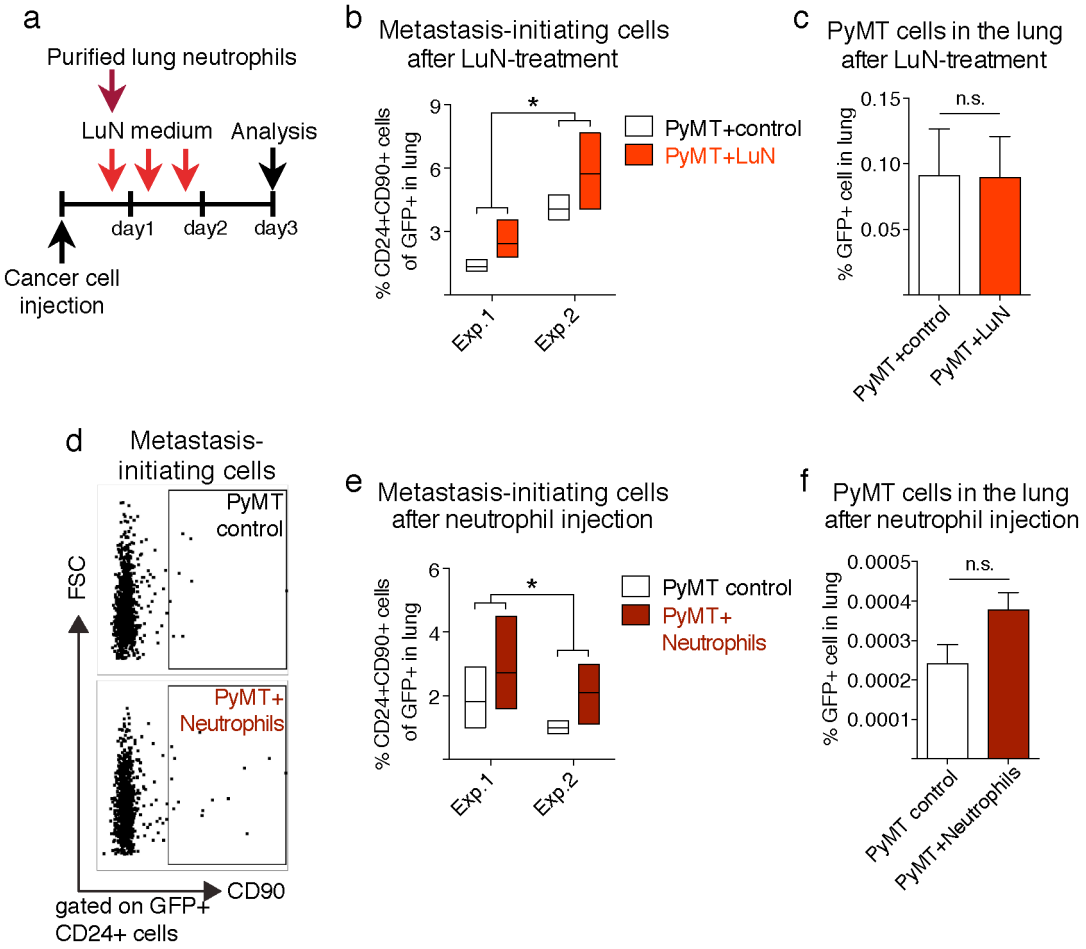

Figure 3-21 Lung neutrophils of MMTV-PyMT cancer-bearing mice enrich for the subpool of cancer cells driving metastatic initiation
(a) Schematic representation and timeline of the experimental setup to assess neutrophil-mediated effects on the heterogeneous cancer stem cell population. Rag1-/- mice were intravenously (IV) injected with GFP-labelled MMTV-PyMT cells and subsequently three times with control or LuN medium or once with neutrophils freshly isolated from the pre-metastatic lung (n≥3 per group per experiment, two independent experiments are shown individually (b+e) or merged into the same graph (c+f)). (b-f) Flow cytometric quantification of frequencies of total present GFP-labelled MMTV-PyMT cells, gated on alive single cells (c+f) or frequencies of CD24+CD90+ MICs among total GFP-labelled MMTV-PyMT cells, gated on GFP+ cells (b+e) in the lung three days after cancer cell injection. (d) Representative flow cytometric analysis of surface CD90+ MMTV-PyMT cancer cells among GFP+CD24+ cells in the lung of quantification shown in (d).

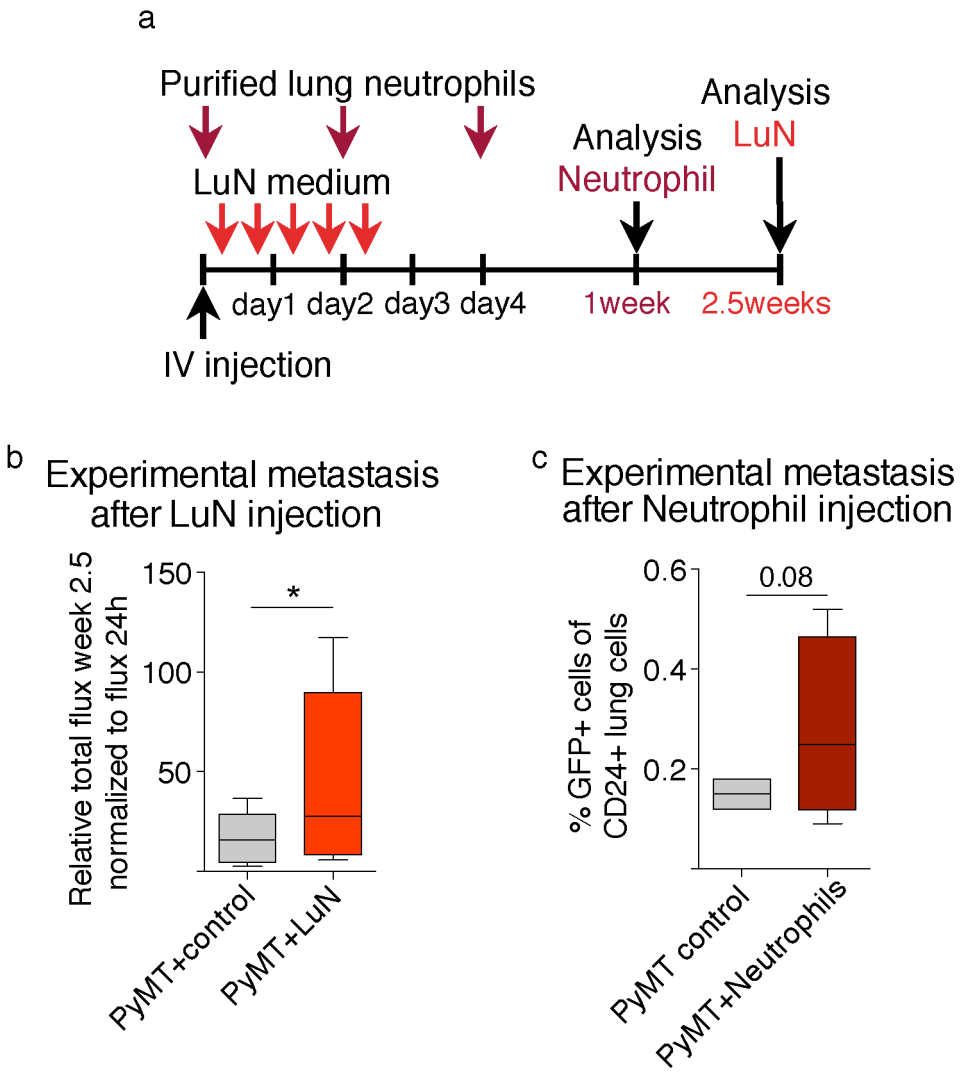

Figure 3-22 Transfer of mammary tumour-induced lung neutrophils or their secreted factors enhance lung colonisation competence of MMTV-PyMT cancer cells *in vivo*

(a) Schematic representation and timeline of the experimental setup to determine if neutrophil-mediated enrichment of MICs within the total MMTV-PyMT cancer cell population leads to enhanced metastatic burden. (b-c) Rag1-/- mice were intravenously (IV) injected with total Luciferase-expressing (b) or GFP-labelled MMTV-PyMT cells (c) followed by either three to five times intravenous injection with control or LuN medium (b) or by three times intravenous injection with neutrophils freshly isolated from a pre-metastatic lung (c). Quantification of experimental metastatic incidence by determination of bioluminescence intensity at 2.5 weeks relative to 24 hours post-cancer cell injection (b, n≥11 per group pooled from 3 independent experiments using different MMTV-PyMT cancer cell preparations) or flow cytometric analysis of GFP+ cancer cells in the lung, gated on CD24+ cells (c, one primary MMTV-PyMT cancer cell preparation injected into n≥4 mice per group (technical replicates) and analysed in parallel) is shown.

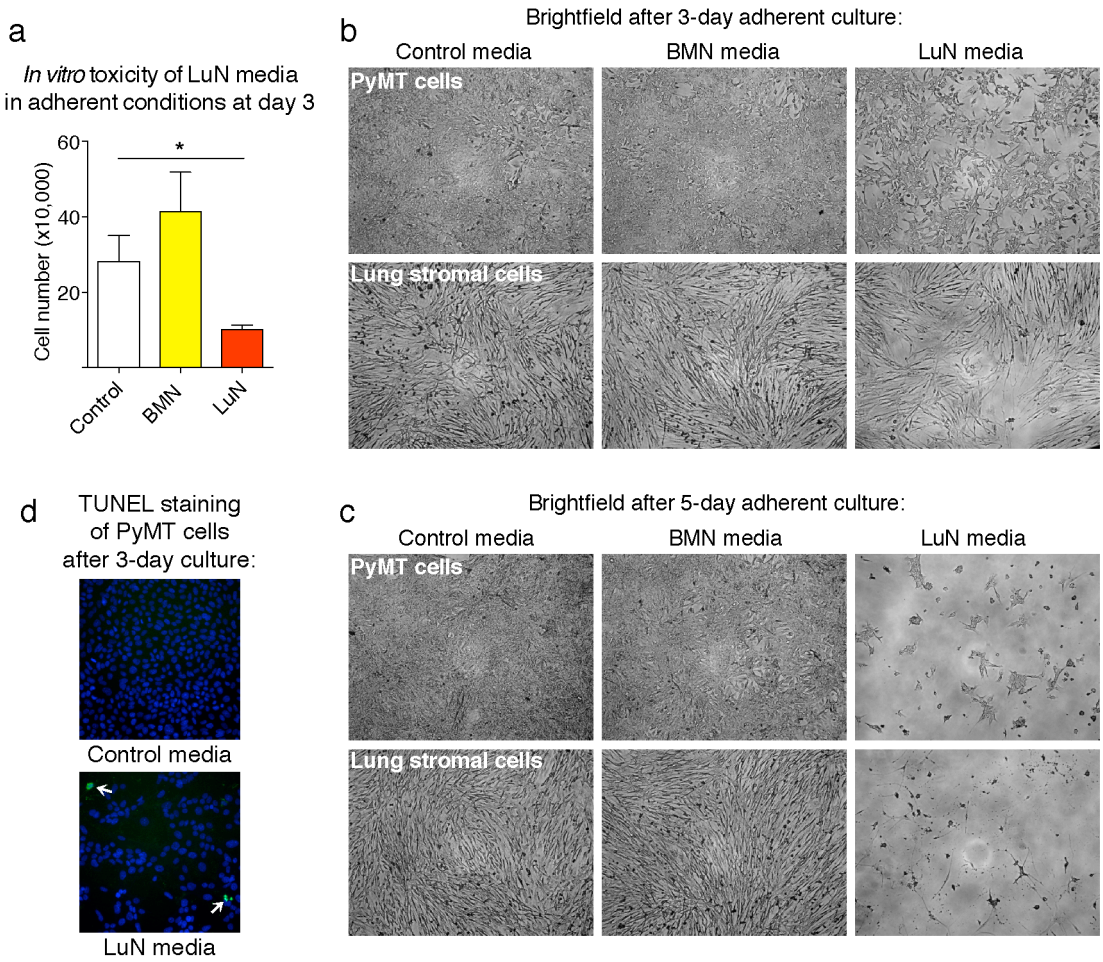

Figure 3-23 Pre-metastatic lung neutrophil-conditioned medium is toxic towards different cell types in adherent conditions *in vitro* that is likely a side effect of culture

(a) Quantification of remaining, alive primary MMTV-PyMT cancer cells cultured in control, BMN or LuN medium in adherent conditions for three days by detaching, trypan blue-staining and cell counting (n≥4 biological replicates per group pooled from ≥4 independent experiments). (b-c) Bright field microscopic images of primary MMTV-PyMT cancer cells or freshly isolated lung stromal cells grown in control, BMN or LuN medium for three (b) or five (c) days in adherent conditions, magnification 10x. (d) Immunofluorescence microscopic images of primary MMTV-PyMT cancer cells after three-day culture in control or LuN media in adherent condition stained for DAPI (blue) to visualise nuclei and TUNEL reagent (green) to detect apoptotic cells, magnification 20x. Arrows indicate TUNEL+ cells. (b-d) Representative experiment of at least two independent repetitions is shown.

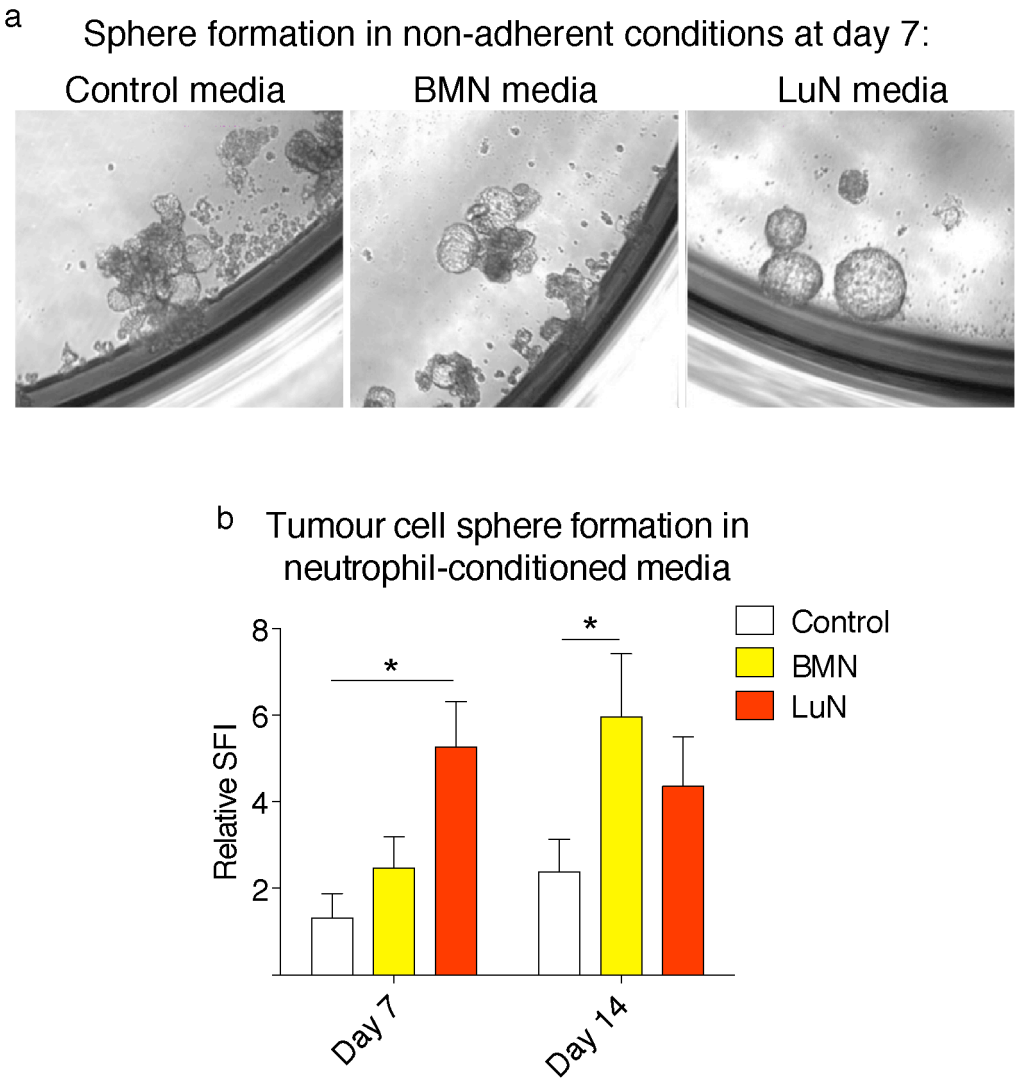

Figure 3-24 Lung neutrophil-derived factors promote self-renewal and anoikis resistance of the total MMTV-PyMT cancer cell population
(a-b) Bright field microscopic images (a) and quantification (b) of ≥7 independent experiments using different primary MMTV-PyMT cell preparations grown for seven days or two weeks in ultra low-attachment conditions that promote tumour cell sphere formation in control cell culture medium or medium conditioned by neutrophils isolated from the bone marrow (BMN) or the lung (LuN) of MMTV-PyMT mammary tumour-bearing mice, scale bar is 10µm. Sphere formation index (SFI) in (b) was calculated as the combination of area of all formed spheres per experiment to incorporate sphere number and size.

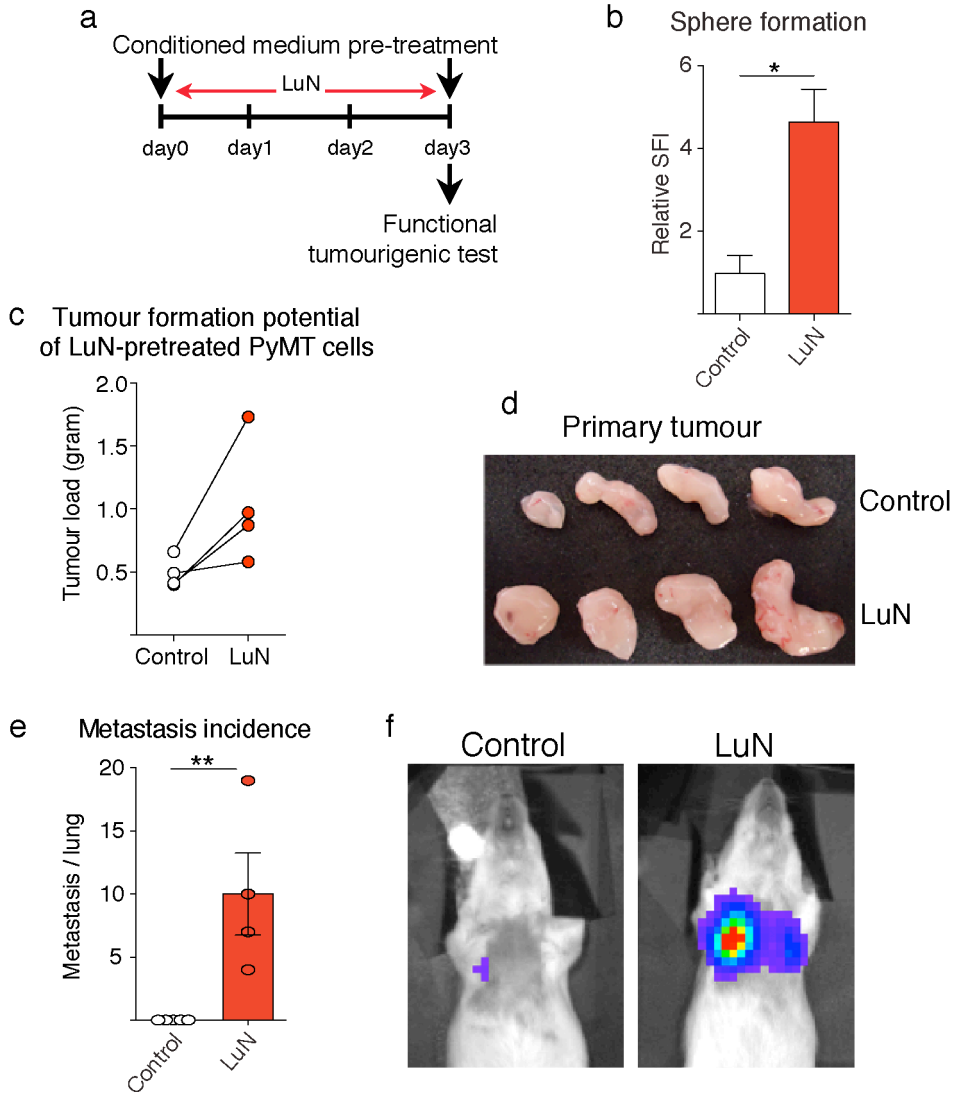

Figure 3-25 A pre-metastatic lung neutrophil-derived paracrine mechanism promotes *in vitro* self-renewal as well as *in vivo* tumour initiation and metastatic lung colonisation potential of mammary cancer cells

(a) Schematic representation of *in vitro* pre-treatment of MMTV-PyMT cancer cells with control or lung neutrophil-conditioned (LuN) medium in adherent culture for three days followed by a functional test of tumourigenic potential. (b-f) LuN-medium pre-treated unlabelled or Luciferase-expressing MMTV-PyMT cells were detached and plated in ultra-low attachment conditions followed by sphere-quantification at day 10 post-seeding for ≥3 independent experiments using different MMTV-PyMT tumour cell preparations (b), orthotopically injected into the mammary gland of Rag1-/- mice and tumour burden assessed by dissection and weighing (c-d; n≥2 per group of 4 independent experiments and average tumour weights per experiment are depicted) or intravenously injected into Rag1-/- mice and the resulting surface lung metastases counted 20 days after injection (e-f, n≥8 per group pooled from 2 independent experiments using different MMTV-PyMT cancer cell preparations). (d) Representative photograph of dissected mammary tumours quantified in (c). (f) Representative image of cancer cell-derived bioluminescence intensity in intravenously injected mice shown in (e). Sphere formation index (SFI) in (b) was calculated as the combination of area of all formed spheres per experiment to incorporate sphere number and size.

3.3 Chapter conclusion

Neutrophils appear to be systemically mobilised by growing (mammary gland) tumours and their increase is most pronounced in the lung, the predisposed site of distant metastasis. This lung neutrophil accumulation does not seem to be instigated by metastatic cancer cells in the lung or their secretion of G-CSF and is also independent of CXCR2 presence on neutrophils. Strikingly, tumour-induced lung neutrophils appear very similar to wildtype lung neutrophils and their frequencies increase prior to infiltration of metastatic cancer cells.

Importantly, neutrophils act in a pro-metastatic fashion, as their genetic or antibody-mediated depletion reduces metastatic burden, and directly supports early lung colonisation by arriving cancer cells. This pro-metastatic activity of neutrophils driving metastasis initiation is independent from effects on cancer cell dissemination from the primary tumour or extravasation in the lung. Neutrophils appear to be involved in recruitment of NK cells to the metastatic site at late metastatic stages, however they do not create an immunosuppressive environment during the onset of metastatic lung colonisation by cancer cells *in vivo*. In fact, pre-metastatic lung neutrophils provide a likely coincidental niche for arriving metastatic cancer cells in the organ targeted for metastasis. Neutrophil-derived paracrine signals appear to change the composition of the heterogeneous cancer cell population in favour of cells in the lung that are intrinsically more competent for metastatic initiation (Fig. 3.21 and Fig. 3.22) and thereby facilitate metastatic lung colonisation (Fig. 3.26).

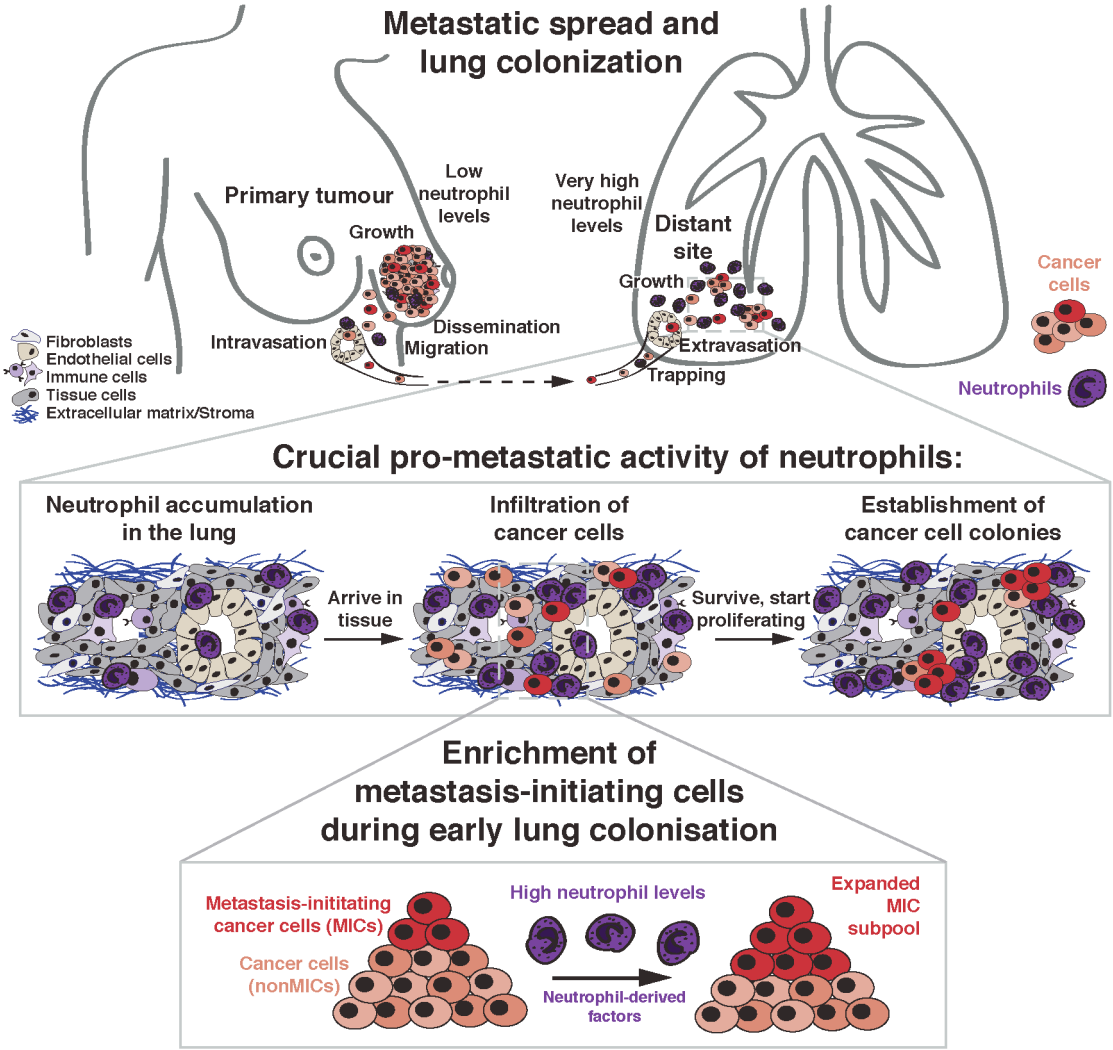

Figure 3-26 Lung neutrophils expand the metastasis-initiating subpopulation of mammary cancer cells to drive metastatic lung colonisation a direct fashion
In order to metastasise, mammary cancer cells disseminate from the primary tumour, migrate through the circulation to arrive at the distant metastatic site, the lung. There, neutrophils are present in elevated numbers and provide a direct pro-tumourigenic signal that promotes expansion of the subpool of cancer cells that intrinsically has a superior metastatic initiation potential (MICs) within the total cancer population. Thereby, neutrophils directly facilitate successful metastatic lung colonisation of mammary cancer cells.

Chapter 4. Alox5-derived metabolites mediate the metastasis supporting activity of neutrophils by induction of proliferation in metastasis-initiating cells during early lung colonisation

4.1 Chapter Introduction

We unravelled a previously unknown function of pre-metastatic lung neutrophils to support lung colonisation of mammary cancer cells by directly promoting the highly metastatic cancer cell subpopulation (section 3). In order to improve our understanding of the mechanisms behind this neutrophil activity, we set out to identify the responsible neutrophil-derived factor(s). In fact, neutrophils can secrete a plethora of factors, many of which with known pro-tumourigenic functions including proteins like CCL2, MMP9, HGF and IL-1 (section 1.3) and (Mitchem and DeNardo, 2012, Tamassia et al., 2012). Various cells in the tumour microenvironment can be an additional source of these mediators (sections 1.2) and might likely compensate for neutrophil depletion, therefore we concentrated on factors more characteristic of innate leukocytes.

The eicosanoid inflammatory mediators leukotrienes comprise a family of lipids that are mainly produced by innate leukocytes including neutrophils, macrophages, monocytes, eosinophils, basophils and mast cells via the lipoxygenase pathway (section 1.2.3.5). The enzyme Arachidonate 5-lipoxygenase (Alox5) is crucial for leukotriene synthesis as it converts arachidonic acid into an unstable intermediate Leukotriene A4 (LTA4). LTA4 is further metabolised by Leukotriene-A4 hydrolase into the dihydroxy-leukotriene B4 (LTB4) or by Leukotriene C4 synthase into the cysteinyl-leukotriene C4 (LTC4). LTB4 and LTC4 are secreted by innate leukocytes and extracellular LTC4 can additionally be modified to leukotriene D4 and E4 (LTD4 and LTE4). LTB4 acts on its two receptors LTB4 receptor 1 and 2 (BLT1 and 2) and cysteinyl leukotrienes LTC4, LTD4 and LTE4 (LTC-D-E4) signal via their receptors cysteinyl leukotriene receptor 1 and 2 (CysLT1 and 2), all G protein-coupled receptors that are expressed on a variety of cell types (section 1.2.3.5 and Fig. 4.1). Leukotrienes have previously been reported to be involved in

tumourigenic processes with rather pro-tumourigenic roles for LTB4 and more antagonistic functions of cysteinyl leukotrienes LTC-D-E4 (section 1.2.3.5.1).

We addressed the role of neutrophil-derived Alox5 metabolites/leukotrienes in metastatic lung colonisation of mammary cancer mouse models taking advantage of Alox5-deficient mice (Chen et al., 1994) and the Alox5 inhibitor Zileuton (section 1.2.3.5.1) as well as the leukotriene receptor inhibitors LY255283 (BLT2 inhibitor) (Herron et al., 1992) and BAY-u9773 (CysLT2 inhibitor) (Tudhope et al., 1994). Also, we stimulated cancer cells with the lipids LTB4 or a mixture of cysteinyl-leukotrienes LTC4, LTD4 and LTE4 (LTC-D-E4).

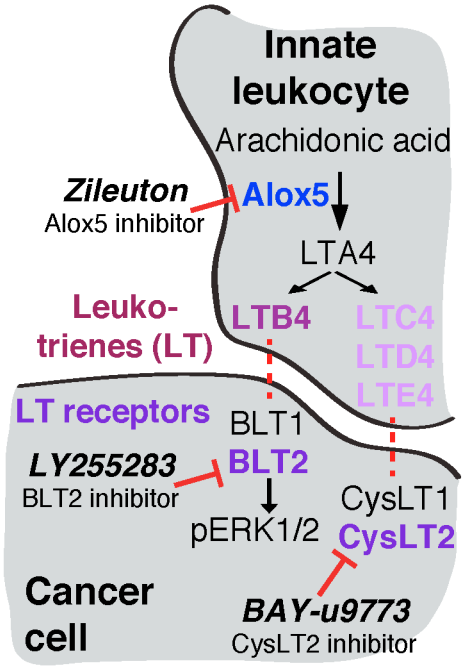

Figure 4-1 Overview on leukotriene synthesis and their signalling through cell surface receptors
Leukotrienes are lipid signalling mediators that are synthesised from arachidonic acid by the enzyme Alox5. Subsequently, they are secreted by leukocytes like neutrophils and eventually further modified. Leukotrienes signal via their cell surface receptors on target cells, for example BLT2 and CysLT2.

4.2 Results

4.2.1 Pre-metastatic lung neutrophils secrete the Alox5 products/leukotrienes which enhance the metastatic potential of mammary cancer cells comparably to the cocktail of neutrophil-derived factors

Lung neutrophil-conditioned medium (LuN) contains the factors facilitating the neutrophil-mediated pro-metastatic activity because we obtained similar results when treating MMTV-PyMT cancer cells with LuN medium or freshly isolated pre-metastatic lung neutrophils (section 3.2.4). Hence, we performed a candidate screening approach to identify present protein and lipid signalling mediators. Commercially available Protein Profiler Arrays (R&D systems) were used to assess presence of proteins and Enzyme Immunoassays to measure the content of lipids. Numerous secreted factors were detected in LuN medium and we selected proteins and lipids present in high levels as well as more innate immune cell-specific factors for further functional tests, in particular LTB4 as well as LTC4, LTD4 and LTE4, prostaglandin E2 (PGE2), CCL2, CCL6, CCL22 and MMP9 (Fig. 4.2 and Fig. 4.3). Importantly, direct leukotriene-treatment of MMTV-PyMT cancer cells in ten-day non-adherent culture recapitulated the features of LuN medium by boosting tumourigenic sphere formation potential, while all other tested factors failed to do so even in different concentrations (Fig. 4.4). Hence, we suspected the Alox5 products leukotrienes to be the potential paracrine mediators of lung-neutrophil pro-metastatic activity. To address this hypothesis, we firstly tested if leukotrienes enhance the tumourigenic and metastatic potential of primary mammary cancer cells. To this end, we stimulated total MMTV-PyMT cancer cells for three days in adherent culture with both types of leukotrienes simultaneously followed by detachment and the determination of their tumourigenic and metastatic potential (Fig. 4.5 a) in equal settings like for lung neutrophil-derived factors (Fig. 3.25). We decided to test the effects of co-stimulation of cancer cells with LTB4 and cysteinyl leukotrienes together because both leukotriene types are produced by the same enzyme and readily present in lung neutrophil-conditioned medium. Hence, leukotrienes are likely to be released by pre-metastatic lung neutrophils simultaneously *in vitro* and in the lung tissue (discussed in detail in section 6.2.2).

Three-day leukotriene pre-treatment of MMTV-PyMT cancer cells increased cancer cell *in vivo* competence for initiation of mammary tumour formation upon injection into the mammary gland of Rag1-/- mice compared to control Ethanol (EtOH)-treated cells as measured by increased tumour burden in 4 out of 5 independent experiments (Fig. 4.5 b-c). Moreover, leukotriene-stimulated GFP-labelled MMTV-PyMT cancer cells showed a significantly enhanced lung metastasis initiation potential *in vivo* when intravenously injected in Rag1-/- mice determined by flow cytometric analysis of GFP+ cells present in the lung (Fig. 4.5 d-e). Thus, the Alox5 products leukotrienes recapitulated the features of LuN medium to boost tumourigenicity of mammary cancer cells *in vitro*. Next, we tested the ability of leukotrienes to induce a similar alteration of the composition of the total MMTV-PyMT cancer cell population in favour of intrinsically highly metastatic cells (MICs) *in vitro* that we observed by lung neutrophil-conditioned medium treatment. In line with functionally superior metastatic ability of the total MMTV-PyMT cancer cell population after *in vitro* leukotriene-exposure, we observed that leukotrienes increased MIC frequency within MMTV-PyMT tumour cells as assessed by flow cytometric analysis for CD24 and CD90 (Fig. 4.6 a). Importantly, this data shows that the Alox5 products leukotrienes corroborate the effects of neutrophil-derived factors/LuN medium on mammary cancer cells seeded in the lung (Fig. 3.21) and appear to expand the MIC subpopulation *in vitro*. In concert, leukotriene-stimulation of mouse 4T1 mammary cancer cells enriched the CD49f-high cell subpool (Fig. 4.6 b), a surface marker previously described to correlate with highly tumourigenic cancer stem cell-like populations (Chou et al., 2013, Stingl et al., 2006, Yu et al., 2012).

In summary, the Alox5 metabolites leukotrienes appear to shift heterogeneous cancer cell populations in favour of intrinsically highly tumourigenic or metastatic cells and thereby increase metastatic competence in a similar fashion as pre-metastatic lung neutrophil-conditioned medium. This evidence indicates that leukotrienes might be the neutrophil-derived paracrine factors mediating their pro-metastatic activity during early lung colonisation of mammary cancer cells.

a

Protein name	detected in LuN, array position
Adiponectin	ARY013, B1-B2
CCL2	ARY013, D1-D2 and ARY015, D11-D12 and ARY006, D9-D10 and ARY020, C13-C14
CCL3	ARY006, D15-D16
CCL4	ARY006, D17-D18
CCL5	ARY013, D15-D16 and ARY006, D21-D22
CCL6	ARY020, B7-B8
CCL9/10	ARY020, D13-D14
CCL12	ARY006, D11-D12 and ARY020, D5-D6
CCL22	ARY020, D7-D8
CXCL1	ARY015, D7-D8 and ARY006, D5-D6
CXCL2	ARY006, D19-D20
CXCL4	ARY015, E13-E14
CXCL9	ARY006, D13-D14
CXCL10	ARY015, D5-D6 and ARY006, D1-D2
DPPIV	ARY013, B9-B10 and ARY015, B7-B8
Endoglin	ARY015, B11-B12
Fetuin A	ARY013, B13-B14
G-CSF	ARY006, B5-B6
GM-CSF	ARY006, B7-B8
HGF	ARY013, B19-B20
ICAM-1	ARY013, B21-B22 and ARY006, B13-B14
IGFBP-5	ARY013, C9-C10
IL-1 alpha	ARY006, B17-B18
IL-1 beta	ARY006, B19-B20
IL-1 receptor antagonist	ARY006, B21-B22
IL-6	ARY013, C13-C14
IL-16	ARY006, C7-C8 and ARY020, C7-C8
LIF	ARY013, C21-C22
M-CSF	ARY013, D3-D4 and ARY006, D7-D8
MMP3	ARY015, D15-D16
MMP8	ARY015, D17-D18
MMP9	ARY015, D19-D20
Osteopontin	ARY015, E3-E4
RAGE	ARY013, D13-D14
RBP4	ARY013, D17-D18
Serpin E1	ARY013, D21-D22 and ARY015, F5-F6
TIMP-1	ARY013, D23-D24 and ARY006, E3-E4
TNF alpha	ARY006, E5-E6
TREM-1	ARY006, E7-E8
VEGF-A	ARY013, E3-E4
Positive control: FGF basic	ARY015, B19-B20

b

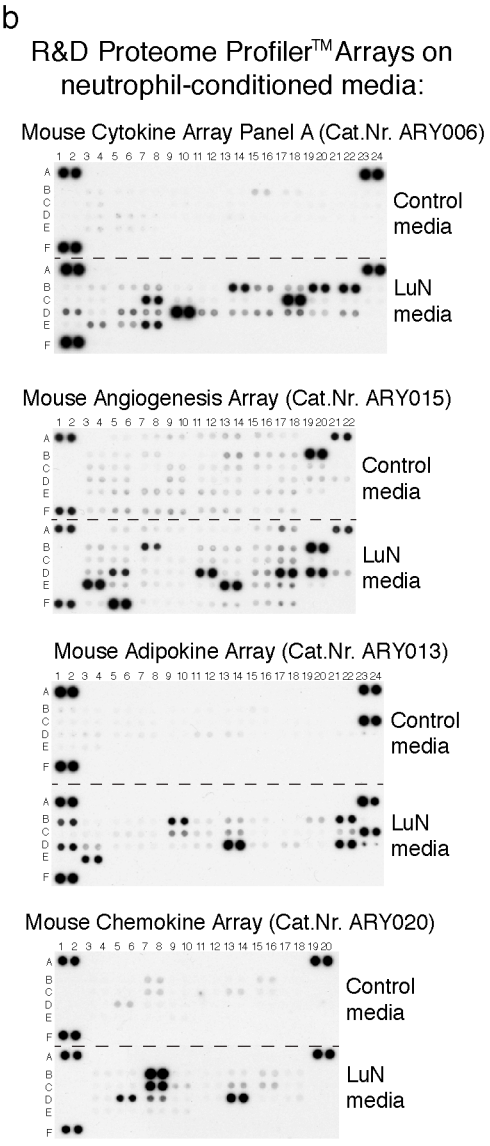

Figure 4-2 Analysis of secreted proteins present in conditioned media from pre-metastatic lung neutrophils of MMTV-PyMT+ mice

(a-b) R&D Proteome Profiler™ Array dot blot membranes were incubated with either untreated control or LuN medium from lung neutrophils of MMTV-PyMT+ mice to identify present proteins (one membrane array was used per group). A list of all detected proteins in LuN medium including their array position (a) as well as the films showing chemiluminescence signal for all 4 used arrays (b) are shown. FGF basic served as a positive control as the recombinant protein was added to the medium. Cat.Nr. Catalogue number of R&D.

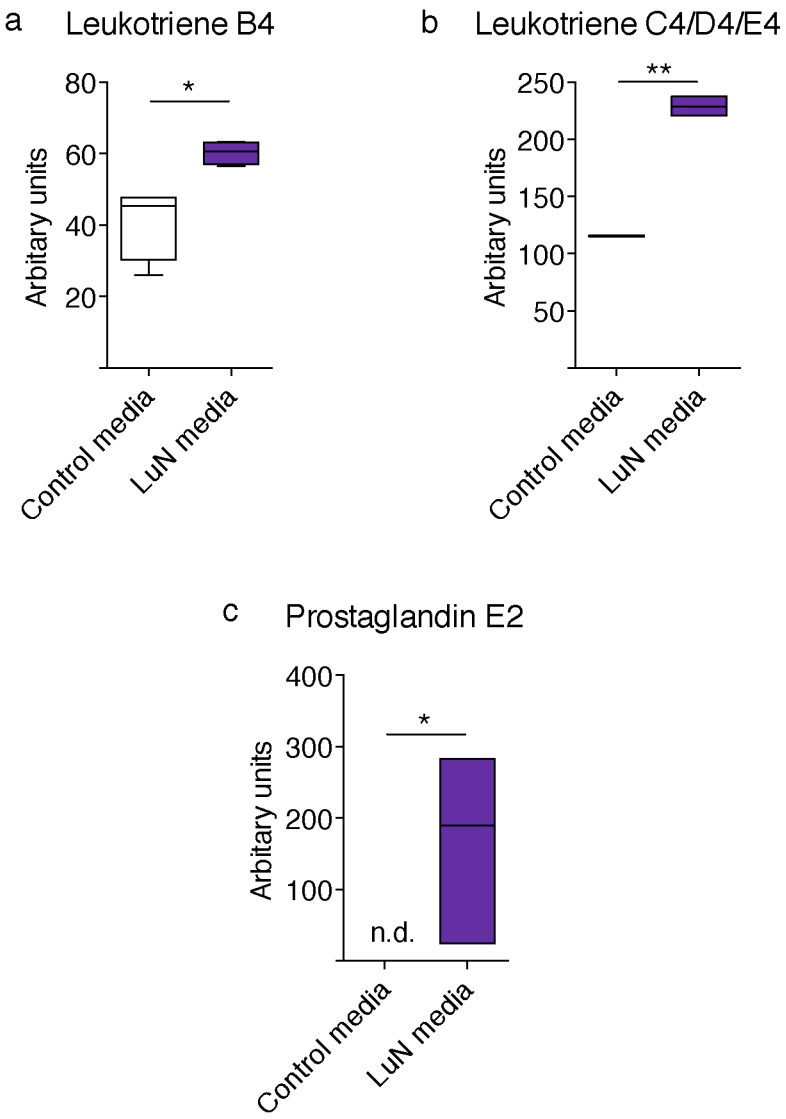

Figure 4-3 Determination of lipid factors present in conditioned media from pre-metastatic lung neutrophils of MMTV-PyMT+ mice
(a-c) Enzyme immunoassay (EIA) analysis was used to identify lipid components in control and LuN medium. The detailed results of the EIA analysis for LTB4 (a), cysteinyl leukotrienes (b) and PGE2 (c) are depicted (n≥4 different media preparations per group analysed in 2 independent experiments). Results are displayed in arbitrary units relative to background signal detected in negative controls without added medium. Of note, the EIA for cysteinyl leukotrienes equally detected leukotriene C4, D4 and E4. N.d.: not detected.

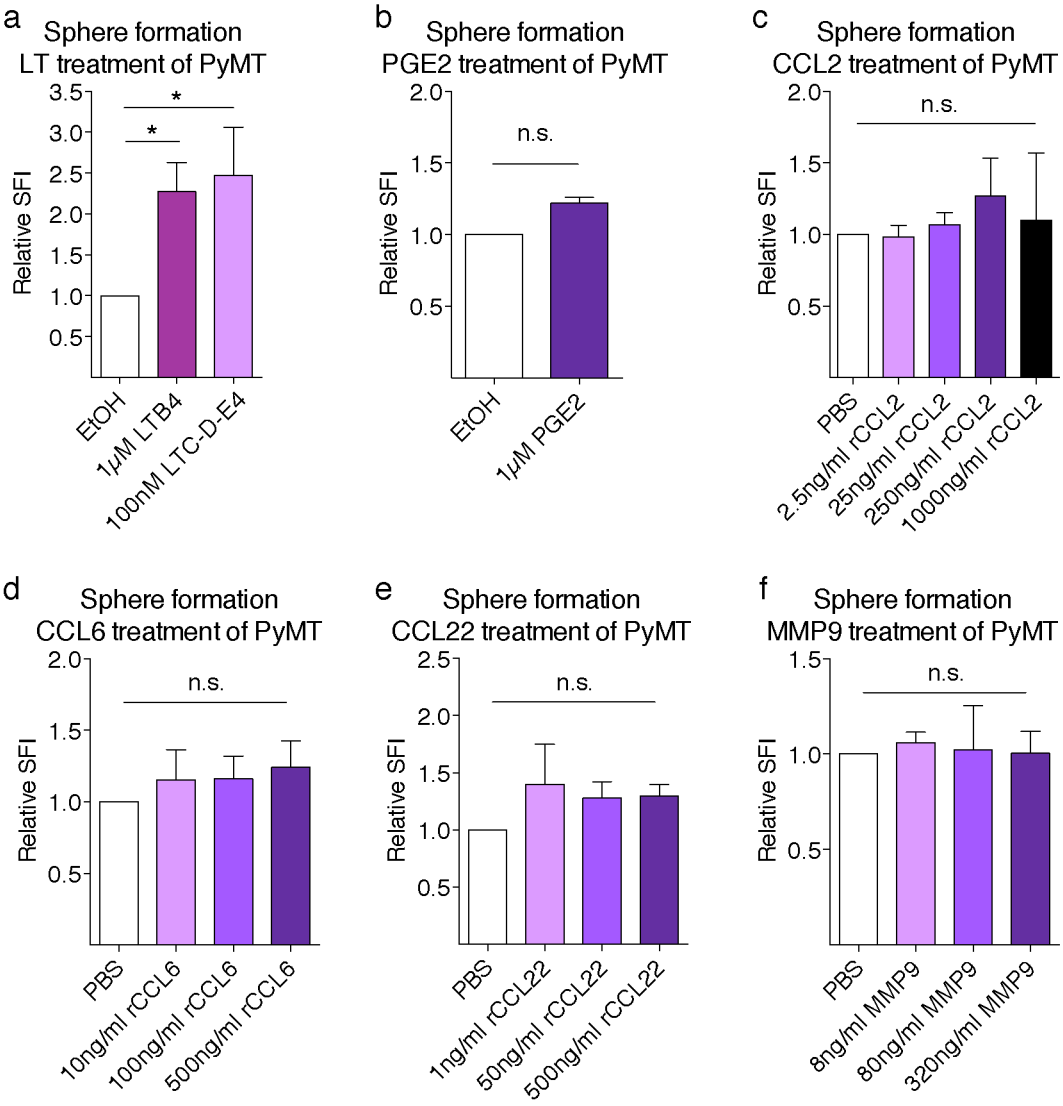

Figure 4-4 Effect of selected neutrophil-derived factors on self-renewal ability of MMTV-PyMT cancer cells in sphere formation assays
MMTV-PyMT cells were seeded in single cell suspensions in ultra-low attachment plates and treated with the indicated concentrations of lipid mediators or recombinant (r) proteins for ten days followed by quantification of size and number of formed spheres. Quantification was made of ≥3 independent experiments using different MMTV-PyMT tumour cell preparations for every condition. Sphere formation index (SFI) was calculated as the combination of area of all formed spheres per experiment to incorporate sphere number and size and is shown relative to Ethanol (EtOH) or PBS (Phosphate-buffered saline)-treated control. LT: Leukotriene

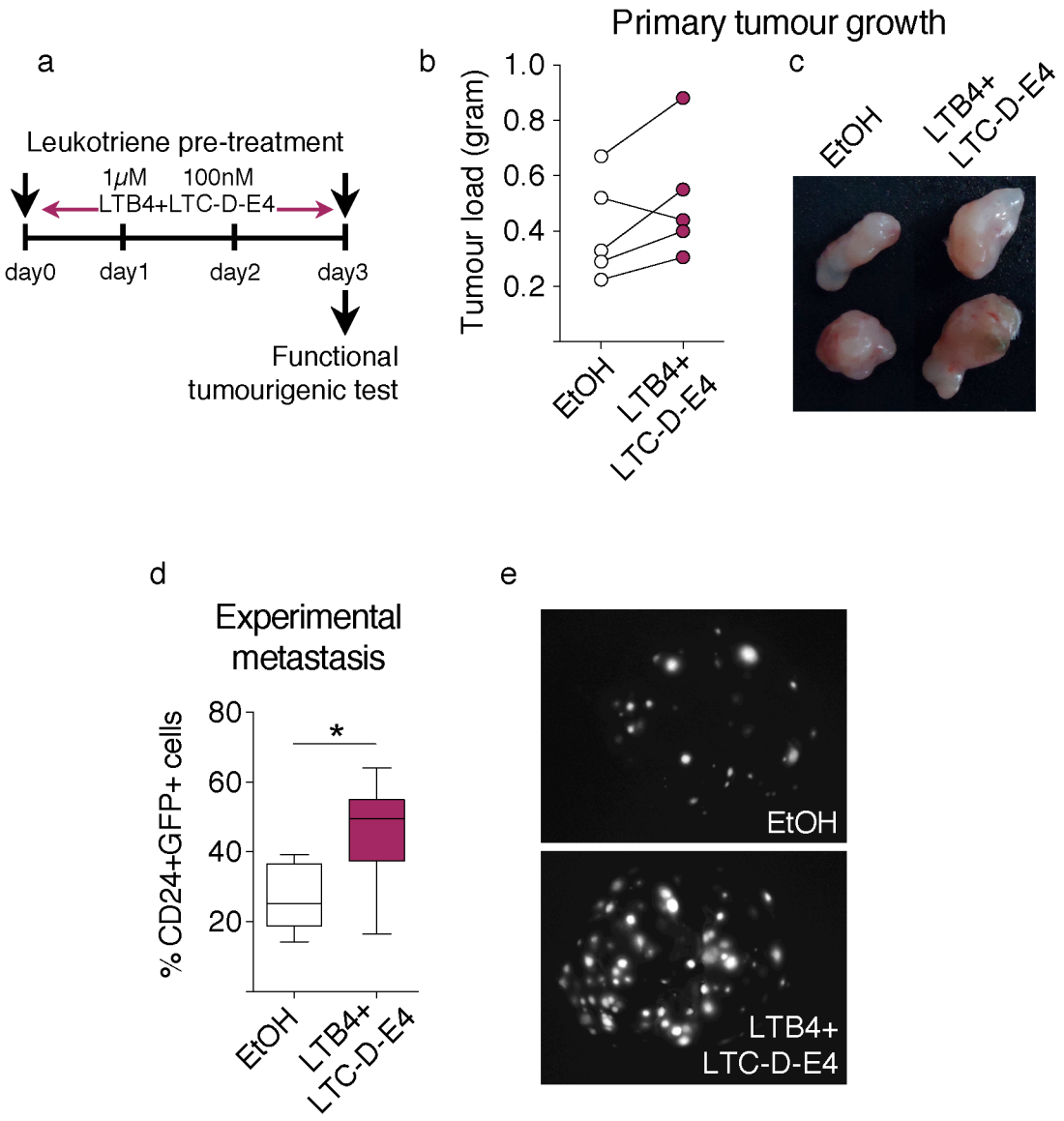

Figure 4-5 Leukotrienes promote primary tumour initiation and metastatic lung colonisation potential of the total MMTV-PyMT cancer cell population
(a-c) Three-day LTB4- and LTC-D-E4-treated MMTV-PyMT cells in adherent culture (a) were detached and analysed for primary tumour initiation potential by orthotopic transplantation in Rag1-/- mice, tumour resection and weighing (b; n≥2 per group of 5 independent experiments and average tumour weights per experiment are depicted). Representative image of resected tumours (c) is shown. (d-e) Experimental metastasis initiation competence of leukotriene pre-treated GFP-labelled MMTV-PyMT after IV injection was determined by quantification of CD24+GFP+ cells in the lung by flow cytometry, gated on alive single cells (d, n=6 per group pooled from 2 independent experiments with different MMTV-PyMT cell preparations), and representative stereomicroscopic images of GFP+ cancer cell colonies in the lung is shown (e).

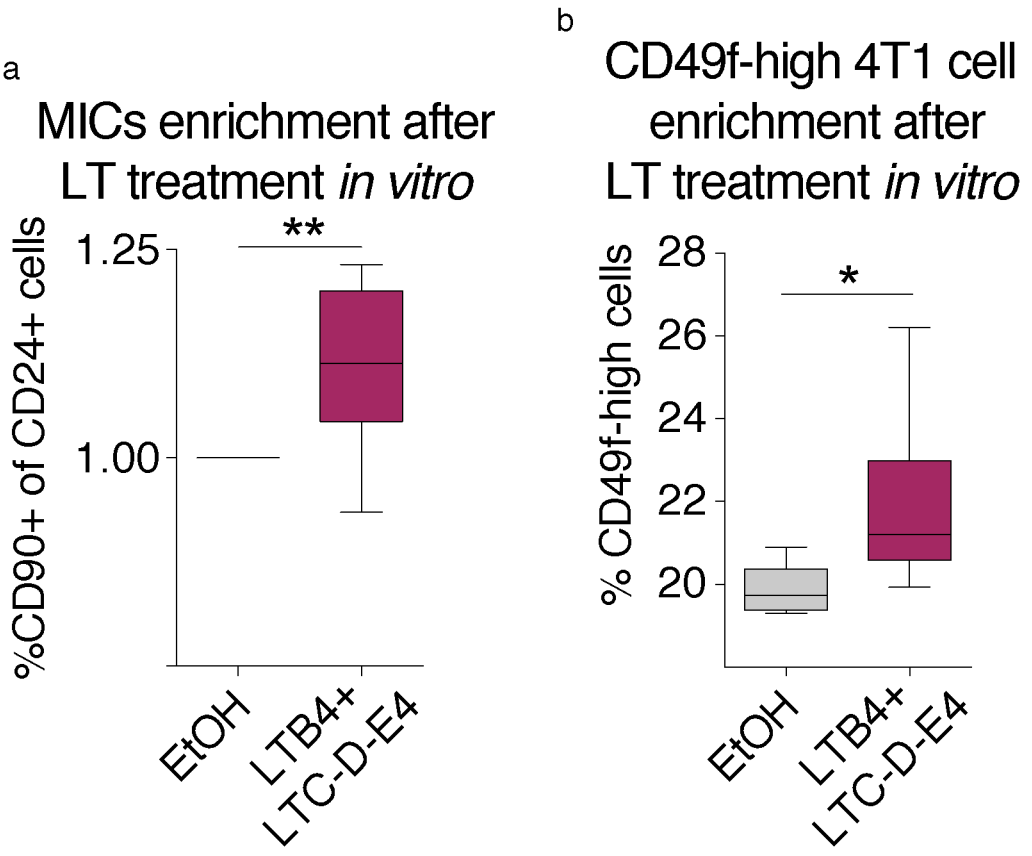

Figure 4-6 Leukotrienes specifically enrich for cancer cell subpopulations with superior tumourigenic or metastatic competence
Three-day LTB4- and LTC-D-E4-treated MMTV-PyMT (a, n=8 per group pooled from 4 independent experiments with different MMTV-PyMT preparations, relative to EtOH control) or 4T1 cells (b, n=6 per group pooled from 6 independent experiments) in adherent culture were analysed for the MIC marker CD90, gated on CD24+ cells (a), or the CSC marker CD49f, gated on alive single cells (b), by flow cytometry. LT: Leukotriene.

4.2.2 Leukotrienes induce proliferation of specifically the metastasis-initiating cancer cell subset because it is enriched for expression of leukotriene receptors

Our results demonstrate that leukotriene-treatment increases the tumourigenic and metastatic potential of cancer cells likely by expanding the intrinsically more potent subpopulations. Hence, we set out to unravel the molecular mechanisms behind the actions of leukotrienes on the target cancer cells. First of all, we focussed on examining the presence of leukotriene receptors (LTRs) on mammary cancer cells. The Alox5 products leukotrienes signal through their cell surface receptors, LTB4 through BLT1 and BLT2 and cysteinyl-leukotrienes LTC-D-E4 through CysLT1 and CysLT2 and, in fact, leukotriene receptor expression has been previously observed in cancer cells (section 1.2.3.5). We analysed expression of all 4 known leukotriene receptors on the surface of primary MMTV-PyMT, 4T1 and MDA-MB-231 cells by flow cytometry and found BLT2 and CysLT2 to be present on a small proportion. However, we did not detect surface expression of BLT1 or CysLT1 (Fig. 4.7 a-b). The fact that BLT2 and CysLT2 are expressed on small subsets of mammary cancer cells (about 2.5% or 5% respectively) is a very interesting observation in light of metastasis-initiating or cancer stem cell like cells also being small subpopulations of total cancer cells that are expanded upon leukotriene treatment (Fig. 4.6). Hence, we hypothesised that leukotriene receptors might be preferentially present on these cancer cell subpools which intrinsically have a higher tumourigenic or metastatic potential. In fact, we observed a noteworthy expression pattern of both, BLT2 and CysLT2, within the heterogeneous mammary cancer cell population. Leukotriene receptors appear to be highly enriched on the MIC subpopulation of primary MMTV-PyMT cancer cells compared to nonMICs (Fig. 4.7 c-e) as well as on other well-known higher tumourigenic subsets of breast cancer cell lines. These include Aldefluor-active (Ginestier et al., 2007, Hiraga et al., 2011) or CD44-high (Al-Hajj et al., 2003, Sheridan et al., 2006) human MDA-MB-231 cells and CD49f-high mouse 4T1 cells (Stingl et al., 2006, Yu et al., 2012) (Fig. 4.7 f-h). The enrichment of leukotriene receptor-expressing cells in mammary cancer cell subsets with higher tumour initiation potential suggests that leukotriene receptors themselves might identify a cancer cell population with enhanced

tumourigenic competence. In order to test this hypothesis, freshly isolated MMTV-PyMT cancer cells were flow-sorted based on the presence (LTR+) or absence (LTR-) of BLT2 and/or CysLT2 followed by two functional tests for tumourigenic potential. Firstly, LTR+ and LTR- MMTV-PyMT cancer cells were cultured in non-adherent conditions to assess their self-renewal ability by quantification of sphere formation. Secondly, these cancer cell subpopulations were orthotopically injected into Rag1-/- mice to compare their tumour formation competence. Strikingly, LTR+ MMTV-PyMT cells showed enhanced sphere formation and mammary tumour initiation ability compared to LTR- cancer cells (Fig. 4.8). Here, we analysed MMTV-PyMT cancer cell populations that contained single BLT2 or CysLT2 as well as double-expressing cells with the reason that both of their ligand types, LTB4 and cysteinyl leukotrienes, enhance tumourigenic and metastatic potential of cancer cells and cause metastasis-initiating and cancer stem cell-like cell expansion (Fig. 4.5 and 4.6). However, the individual potential of BLT2 and CysLT2 single-positive or double-positive mammary cancer cell populations would have to be analysed in dedicated experiments (discussed in detail in section 6.3.2). Nevertheless, in agreement with the enrichment of leukotriene receptor-expressing cells in cancer stem cell-like cell subsets, leukotriene receptor expression identifies a novel subpopulation of MMTV-PyMT cancer cells with enhanced intrinsic tumourigenic potential independent from ligand stimulation.

Next, we aimed to elucidate the signalling downstream of BLT2 and CysLT2 induced by leukotriene stimulation in mammary cancer cells. In line with previous reports on LTB4-mediated signalling (Choi et al., 2010, Kim et al., 2010a, Woo et al., 2002), the analysis of the intracellular response in cancer cells revealed that both, LTB4 and LTC-D-E4, increased levels of intracellular reactive-oxygen-species (ROS) immediately upon stimulation (Fig. 4.9 a). Further, we were intrigued by reports in the literature that demonstrate the LTB4-BLT2 axis-dependent activation of the mitogen-activated protein kinases ERK1/2 in cancer cells, which is directly triggering cell proliferation (Ihara et al., 2007, Park et al., 2012, Tong et al., 2002, Tong et al., 2005, Woo et al., 2002, Zhai et al., 2010). In general, activation of the MAPK/ERK pathway is usually associated with increased cell proliferation and a well-studied target in cancer therapy (section 1.1.3). Hence, the induction of proliferation via ERK1/2 activation in leukotriene receptor-expressing cells would be a likely explanation for the observed leukotriene-mediated enrichment of MICs and

cancer stem cell-like cells (Fig. 4.6) as these subpopulations are enriched for leukotriene receptor expression (Fig. 4.7). In fact, we observed enhanced activation of ERK1/2 in primary MMTV-PyMT cells starting 30 minutes after stimulation with LTB4 or LTC-D-E4 in adherent culture as determined by Western blotting for phosphorylated ERK1/2 (pERK1/2) on protein lysates (Fig. 4.9 b-d). Moreover, we confirmed that LTB4 treatment leads to activation of ERK1/2 also in the human breast cancer cell line MDA-MB-231 (Fig. 4.9 e). In order to validate that LTR-expressing cells are required within the total cancer cell population to detect a leukotriene-mediated pERK1/2 increase, we largely depleted mammary 4T1 cancer cells from LTR+ cells by flow sorting. Next, we stimulated cancer cells with leukotrienes three days after sorting, at which time the frequency of LTR+ cells was significantly reduced (Fig. 4.10 a). This LTR+ cell-reduced cancer cell population failed to activate ERK1/2 upon LTB4 treatment, while unsorted total 4T1 cancer cells showed a clear increase in ERK1/2 phosphorylation (Fig. 4.10 b-c). In an alternative approach to test the functional relevance of leukotriene receptors for leukotriene-mediated ERK1/2 stimulation, we took advantage of specific leukotriene receptor inhibitors. Both, the BLT2 inhibitor LY255283 and the CysLT2 inhibitor BAY-u9773 interfered with LTB4 or LTC-D-E4-mediated ERK1/2 activation in 4T1 cancer cells, respectively (Fig. 4.11). Of note, leukotrienes and leukotriene receptor inhibitors are supplied in 100% Ethanol, therefore cancer cells are exposed to elevated Ethanol levels in culture when adding both. This high Ethanol concentration (about 2%) led to unexpected reactions of primary MMTV-PyMT cancer cells and made this experiment unfeasible. The 4T1 cancer cell line appeared to be more resistant, however addition of elevated amounts of Ethanol to the culture medium caused an initial decrease of ERK1/2 phosphorylation for 5-15 minutes. Hence, we displayed pERK1/2 levels in 4T1 cancer cells starting at five minutes after leukotriene-stimulation under presence of leukotriene receptor inhibitors. Nevertheless, these data demonstrate that LTB4 and LTC-D-E4 trigger an intracellular increase of ROS levels in concert with ERK1/2 activation in a BLT2 or CysLT2-dependent manner, respectively.

Next, we aimed to determine if this leukotriene-induced ERK1/2 phosphorylation leads to enhanced proliferation of leukotriene receptor-expressing cells, which are enriched among intrinsically highly metastatic or tumourigenic cell subsets. Indeed, three-day leukotriene-treatment of total human MDA-MB-231 cancer cells resulted

in an increased frequency of LTR+ cells, suggesting that ERK1/2 activation functionally boosts their proliferation (Fig. 4.12 a). Also, leukotriene-stimulation specifically increased the proliferation of MICs, but not nonMICs, in accordance with the MIC subpopulation being enriched for leukotriene receptor-expressing cells (Fig. 4.12 b-d). Cell proliferation was assayed by pulse-chase incorporation of BrdU followed by BrdU detection by flow cytometry. Inhibition of MEK1/2, the upstream kinases of ERK1/2, using the specific inhibitor PD0325901 prevented the leukotriene-mediated elevation in MIC proliferation, stressing its functional dependency on ERK1/2 activation (Fig. 4.12 c).

In summary, the Alox5 products leukotrienes appear to alter the composition of the total mammary cancer cell population by providing a selective proliferative signal and ERK1/2 activation to cancer cell subsets that retain intrinsically higher tumourigenic and metastatic competence due to their enrichment for leukotriene receptor-expressing cells.

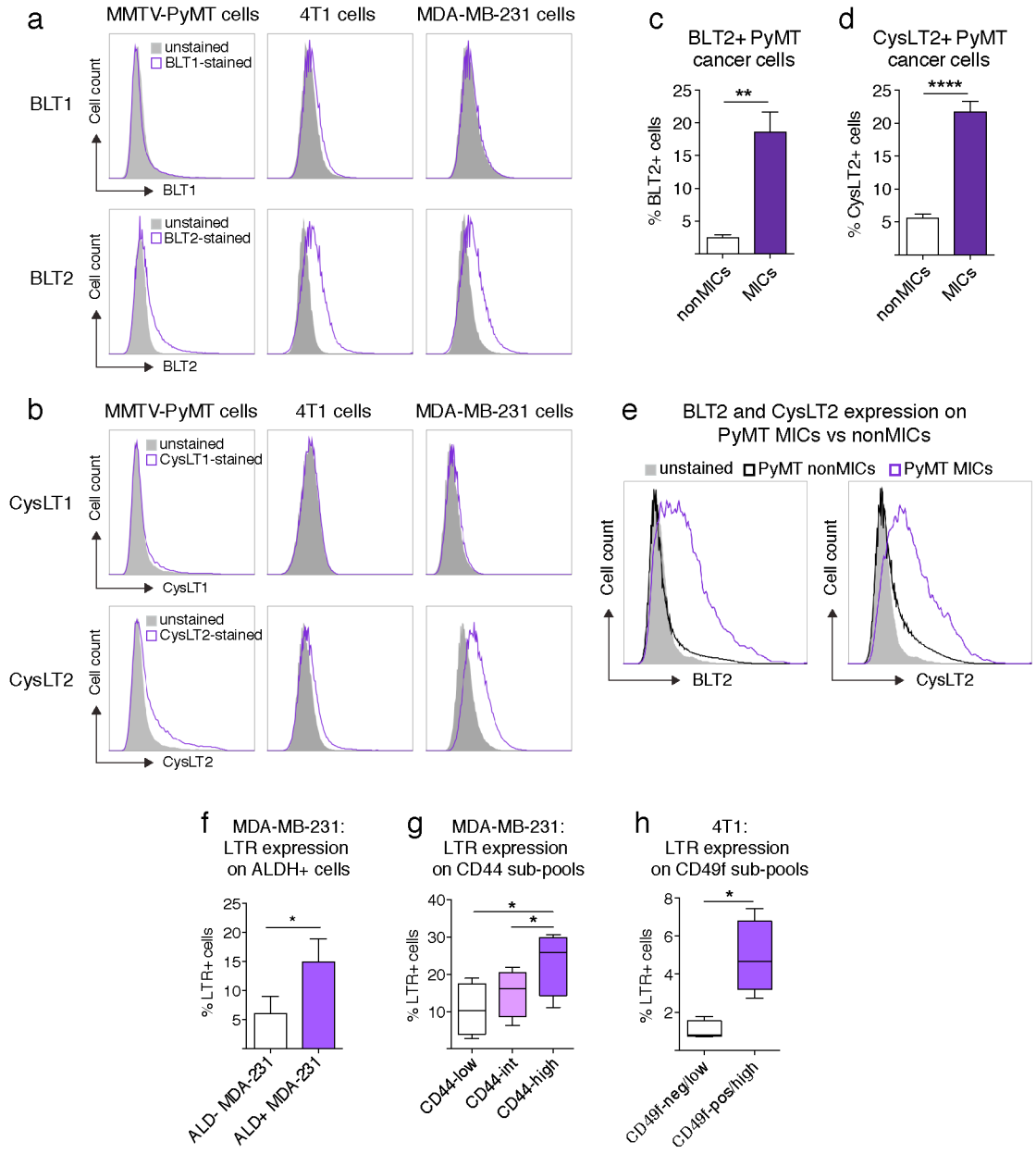

Figure 4-7 Leukotriene receptors BLT2 and CysLT2 are expressed on mouse and human breast cancer cells and enriched in the metastasis-initiating or cancer stem cell-like cell subpopulations

(a-b) Representative flow cytometric analyses of primary MMTV-PyMT cancer cells, the mouse mammary cancer cell line 4T1 and the human breast cancer cell line MDA-MB-231 for expression of the leukotriene B4 receptors 1 (BLT1) and 2 (BLT2) (a) as well as the cysteinyl leukotriene receptors 1 (CysLT1) and 2 (CysLT2) (b). (c-e) Quantification (c-d) and representative flow cytometric analysis (e) of BLT2+ (c) and CysLT2+ cells (d) among MMTV-PyMT non-MICs and MICs (n≥4 per group, 2 independent experiments). (f-h) Flow cytometric quantification of frequencies of leukotriene receptor BLT2+ and CysLT2+ single or double-positive cells (LTR+) among cancer stem cell-like Aldefluor (ALDH)+ or CD44-high subpopulations of human MDA-MB-231 cells (f-g) or CD49f+ mouse 4T1 cells (h), (n≥4 per group, 4 independent experiments). All flow cytometric analyses were gated on alive single cells.

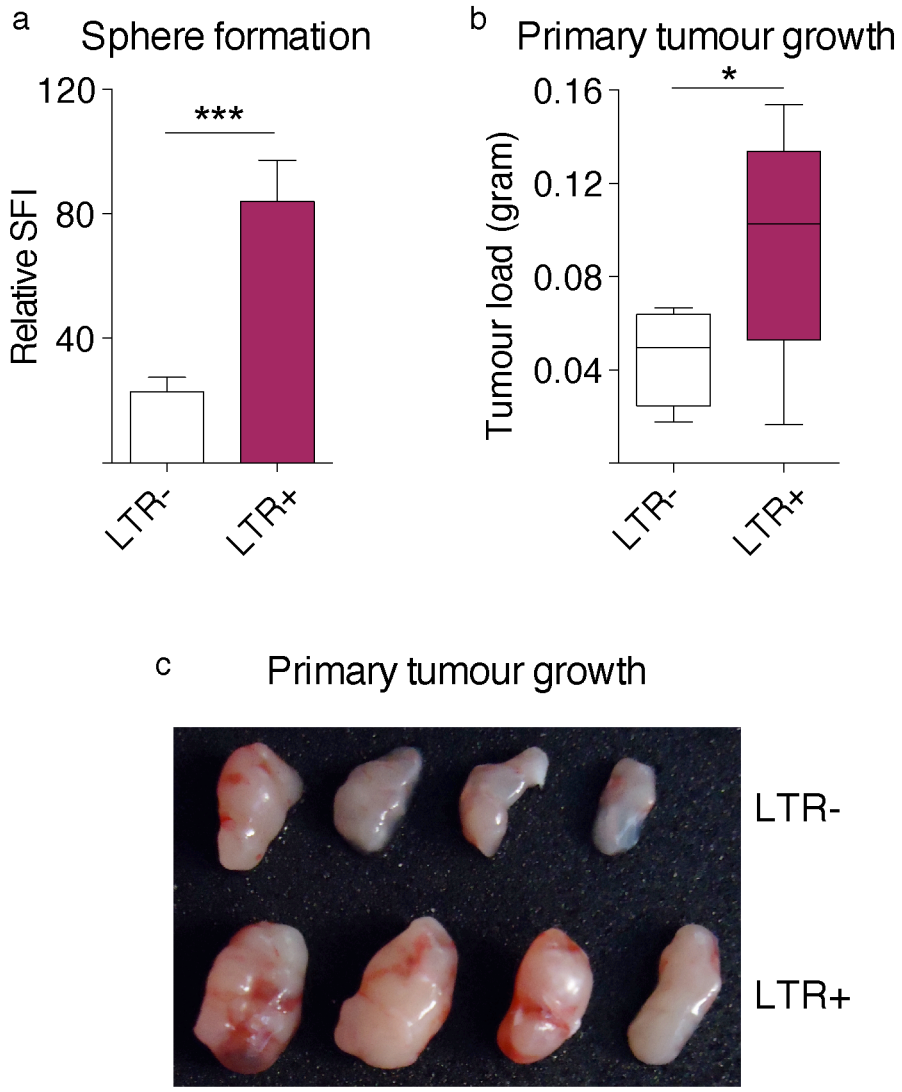

Figure 4-8 BLT2 and CysLT2 leukotriene receptor expression identifies a novel MMTV-PyMT cancer cell population with enhanced tumourigenic potential
(a-b) Sorted leukotriene receptor (LTR)+ or LTR- MMTV-PyMT tumour cells were plated in non-attachment conditions followed by sphere-quantification at day ten post-seeding for 3 independent experiments (a) or grafted onto the mammary gland of Rag1-/- mice for analysis of tumour formation potential (b). Tumour burden was determined by weighing (n=8 per group pooled from 2 independent experiments isolating cells from different spontaneous primary tumours) after three weeks and (c) representative image of tumours is shown. Sphere formation index (SFI) was calculated as the combination of area of all formed spheres per experiment to incorporate sphere number and size.

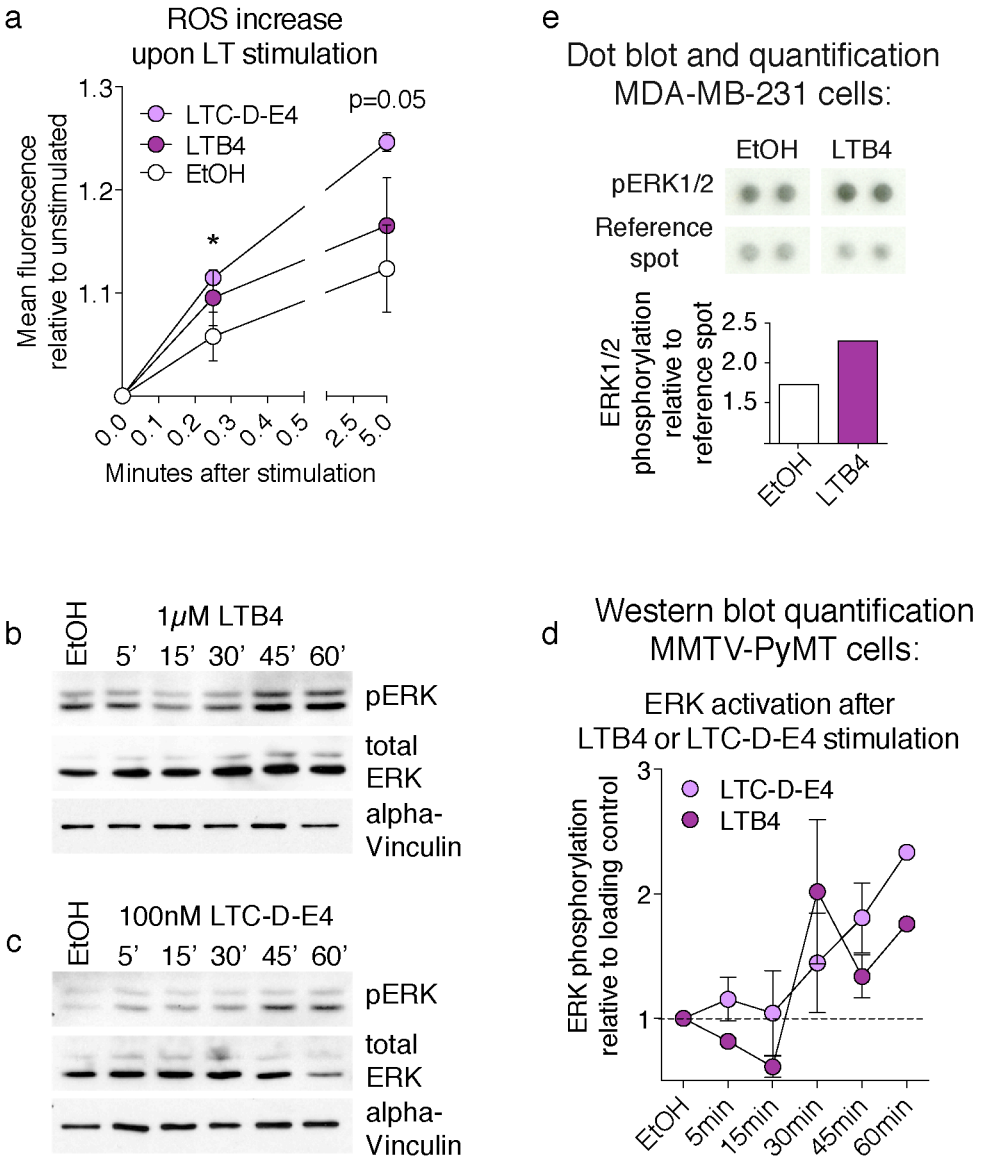

Figure 4-9 LTB4 and cysteinyl leukotriene-stimulation induces ERK1/2 activation in primary MMTV-PyMT cancer cells and human MDA-MB-231 breast cancer cells
(a) Primary MMTV-PyMT cancer cells were stimulated with Ethanol (EtOH) control, LTB4 or LTC-D-E4 in suspension under presence of the DCF-DA dye that becomes fluorescent upon oxidation by reactive oxygen species (ROS) and immediately analysed by flow cytometry. Mean fluorescence intensity relative to unstimulated control is shown over time (4 independent experiments). (b-d) Western blots of ERK1/2 phosphorylation and total ERK1/2 levels (b-c) and quantification (d) of LTB4- or LTC-D-E4-treated MMTV-PyMT cells for the indicated period of time (≥2 independent experiments). Quantification of ERK1/2 phosphorylation in (d) is shown relative to the internal loading control alpha-Vinculin. (e) Dot blot and quantification of ERK1/2 phosphorylation in MDA-MB-231 cells after three-hour stimulation with LTB4 measured by R&D Proteome Profiler™ Human Phospho-Kinase Array (ARY003B). Quantification of ERK1/2 phosphorylation is relative to reference spot on the array membrane (one membrane array was used).

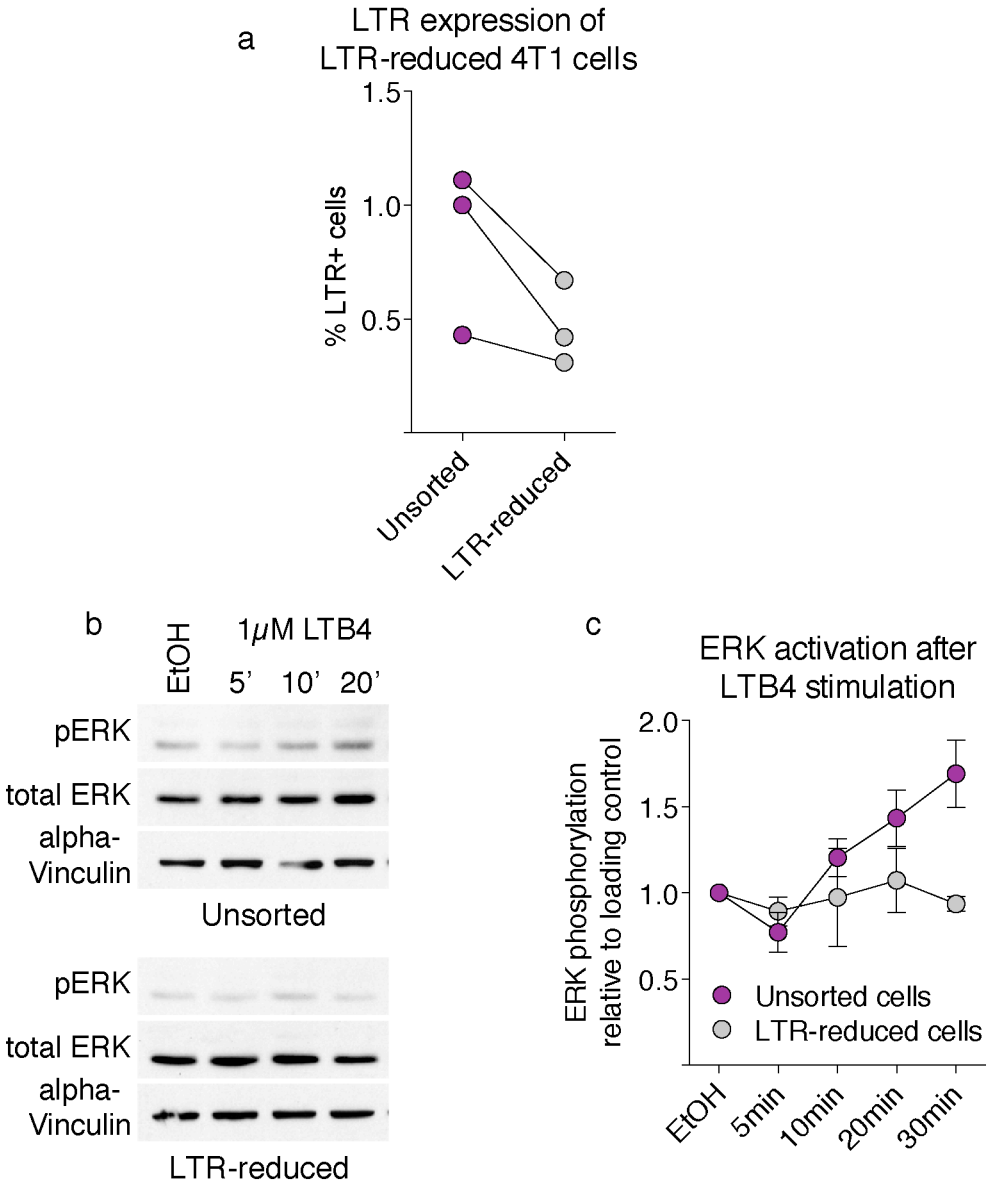

Figure 4-10 ERK1/2 phosphorylation upon treatment with LTB4 is dependent on the presence of leukotriene receptor-expressing cells within total mammary 4T1 cancer cells

(a) Flow cytometric quantification of leukotriene receptor expression (BLT2 and CysLT2) of sorted LTR-reduced 4T1 cells, gated on alive single cells (n=3). (b-c) Representative analysis (b) and quantification (c) of 3 independent experiments of Western blots for total ERK1/2 and ERK1/2 phosphorylation relative to internal alpha-Vinculin of unsorted 4T1 cells or 4T1 cells sorted for LTR negativity shown in (a). EtOH: Ethanol.

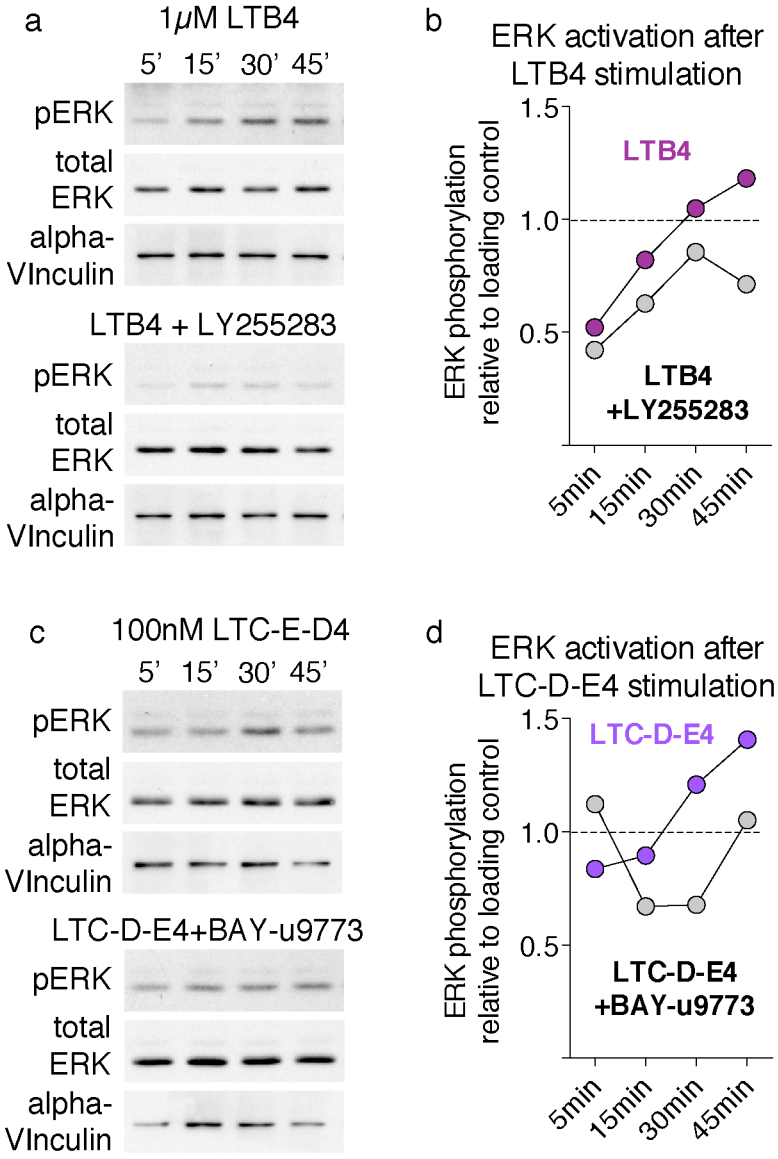

Figure 4-11 BLT2 and CysLT2 inhibitors prevent LTB4 and cysteinyl leukotriene-induced ERK1/2 activation in mammary 4T1 cancer cells
(a-d) Analysis and quantification of Western blot for total ERK1/2 and ERK1/2 phosphorylation relative to internal loading control alpha-Vinculin of 4T1 cells following (a-b) LTB4 or (c-d) LTC-D-E4-stimulation for the indicated period of time in presence of BLT2 inhibitor LY255283 or CysLT2 inhibitor BAY-u9773, respectively (n=1). Dotted lines in (b+d) indicate the Ethanol only control level of ERK1/2 phosphorylation. Note the decrease of ERK1/2 phosphorylation observed after 5-15 minutes when adding both, leukotrienes and their receptor inhibitors, is due to the increase in Ethanol concentration. Data are shown as ERK1/2 phosphorylation recovery and increase from 5 to 45 minutes after stimulation. These experiments were performed once.

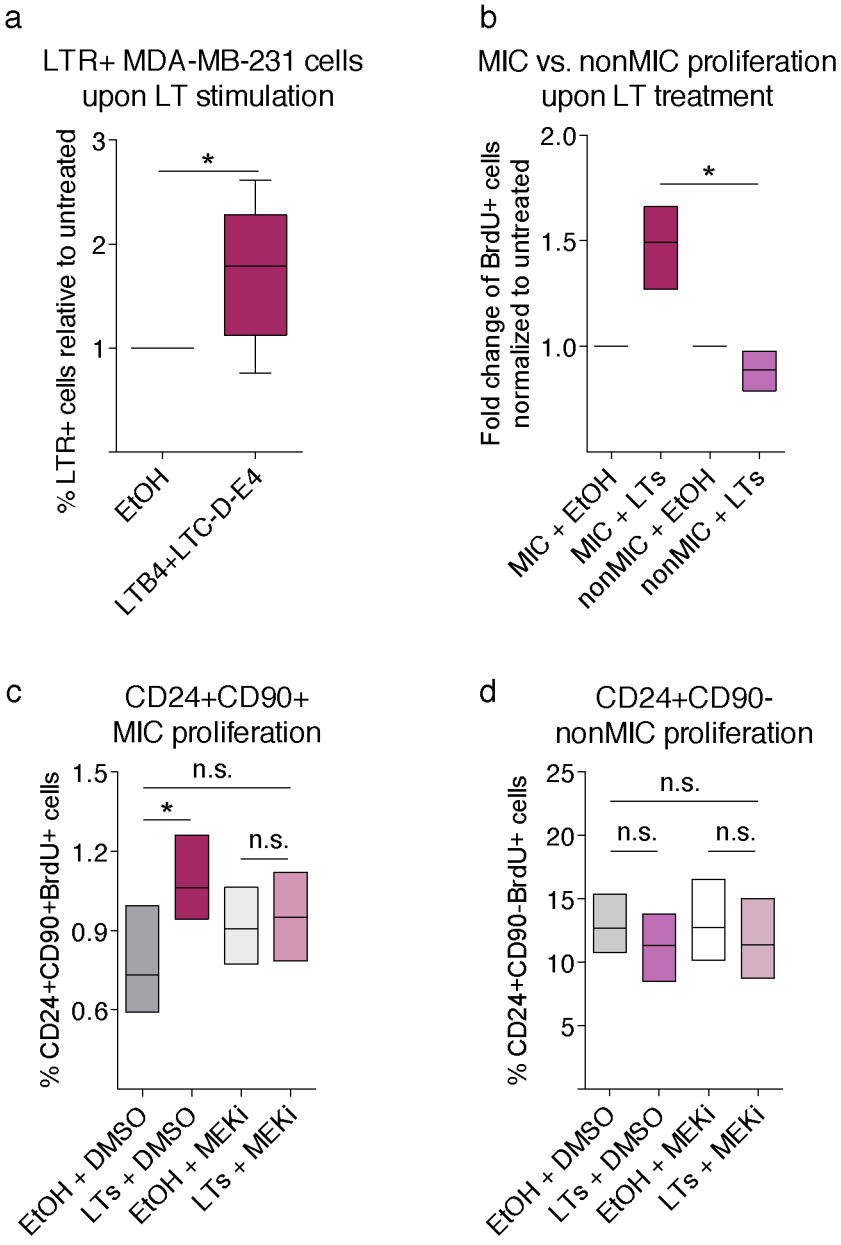

Figure 4-12 Leukotriene directly expand the metastasis-initiating MMTV-PyMT cancer cell subpopulation by specifically inducing their proliferation
(a) Flow cytometric quantification of 5 independent experiments of three-day LTB4 and LTC-D-E4-treated MDA-MB-231 cells for frequency of leukotriene receptor (BLT2 and CysLT2)-expressing cells displayed relative to Ethanol (EtOH)-treated control, gated on alive single cells. (b-d) Three-day leukotriene-treated MMTV-PyMT cells in adherent culture were analysed for BrdU incorporation of CD24+CD90+ MICs and CD24+CD90- nonMICs three hours after BrdU addition by flow cytometry, gated on alive single cells. Fold change of percentage of BrdU+ cells among CD24+CD90+ or CD24+CD90- cells is displayed relative to Ethanol (EtOH)-treated control in (b, 3 independent experiments). (c-d) BrdU incorporation upon leukotriene-stimulation in CD24+CD90+ MICs (c) or CD24+CD90- nonMIC MMTV-PyMT cells in additional presence of PD0325901 MEK inhibitor (MEKi, 3 independent experiments). DMSO=Dimethyl sulfoxide-treated control.

4.2.3 Neutrophil-derived Alox5 metabolites/leukotrienes are required to promote lung metastasis *in vivo*

So far, we established that the Alox5 products leukotrienes induce the proliferation of specifically the highly metastatic subset of mammary cancer cells leading to their relative expansion (Fig. 4.6 and 4.12). Consequently, the total cancer population showed increased tumourigenicity and metastatic competence upon leukotriene treatment *in vitro* (Fig. 4.4 and Fig. 4.4) thereby mimicking the effects of neutrophil-derived factors *in vitro* and *in vivo* (Fig. 3.21, 3.24 and 3.25). Next, we intended to test if Alox5-derived leukotrienes are functionally relevant in mediating the pro-metastatic activity of neutrophils supporting early lung colonisation *in vivo*. We took advantage of a knock-out mouse model for the gene encoding Alox5, Alox5-/- mice (Chen et al., 1994). Firstly, to test the immune cell-specific relevance of Alox5 during metastatic progression *in vivo*, we generated bone marrow chimeric mice where genetic Alox5 deficiency is restricted to the radiosensitive immune cell compartment. Wildtype recipient mice were lethally irradiated and 24 hours later intravenously injected with either wildtype or Alox5-/- bone marrow isolated from donor mice (Fig. 4.13 b). Percentage of bone marrow reconstitution was determined by semi-quantitative PCR for the Alox5 null allele of DNA isolated from the bone marrow at the end of the experiment (Fig. 4.13 a). Alox5-/- bone marrow-reconstituted mice were grafted with MMTV-PyMT cells onto the mammary gland (Fig. 4.13 b) and displayed elevated levels of neutrophils in the lung compared to tumour-free controls. Importantly, there was no difference in lung neutrophil accumulation between MMTV-PyMT tumour-grafted wildtype and Alox5-/- bone marrow-recipient mice (Fig. 4.13 c-d). Also, Alox5-/- bone marrow chimeric mice showed unaltered primary tumour growth compared to controls (Fig. 4.13 e). Significantly, the efficiency of spontaneous lung metastasis was impaired in the absence of leukotriene production from bone marrow-derived cells in these mice (Fig. 4.13 f), thus being similar to the effects of neutropenia (Fig. 3.3, 3.6 and 3.15). This observation highlights the importance of Alox5-derived products from bone marrow-derived cells, likely neutrophils that accumulate in the lung, for spontaneous lung metastasis of mammary MMTV-PyMT tumours. To address the potentially pro-metastatic function of exclusively neutrophil-derived leukotrienes, we

isolated pre-metastatic lung Alox5-/- neutrophils from Alox5-/- mice grafted with MMTV-PyMT mammary tumours. These neutrophils were used to generate leukotriene-deficient neutrophil-conditioned medium (LuN-Alox5ko), which showed the same extend of cell toxicity as wildtype LuN medium (Fig. 4.14 b). Three-day *in vitro* pre-treatment of Luciferase-expressing MMTV-PyMT cancer cells with LuN-Alox5ko medium in adherent conditions (Fig. 4.14 a) showed mildly reduced potency to increase sphere formation compared to wildtype LuN medium. Strikingly, leukotriene-deficient lung neutrophil-conditioned medium failed to boost metastatic competence of pre-treated MMTV-PyMT cancer cells following intravenous injection into Rag1-/- mice (Fig. 4.14 d-e). This loss-of-function approach validates the importance of specifically neutrophil-derived leukotrienes to promote efficiency of lung metastasis initiation of mammary cancer cells.

Taken together, these data confirm the Alox5 products leukotrienes as crucial mediators of lung neutrophil pro-metastatic *in vivo* activity in altering the composition of heterogeneous cancer cell populations favouring highly metastatic cells and thereby enhancing metastatic potential.

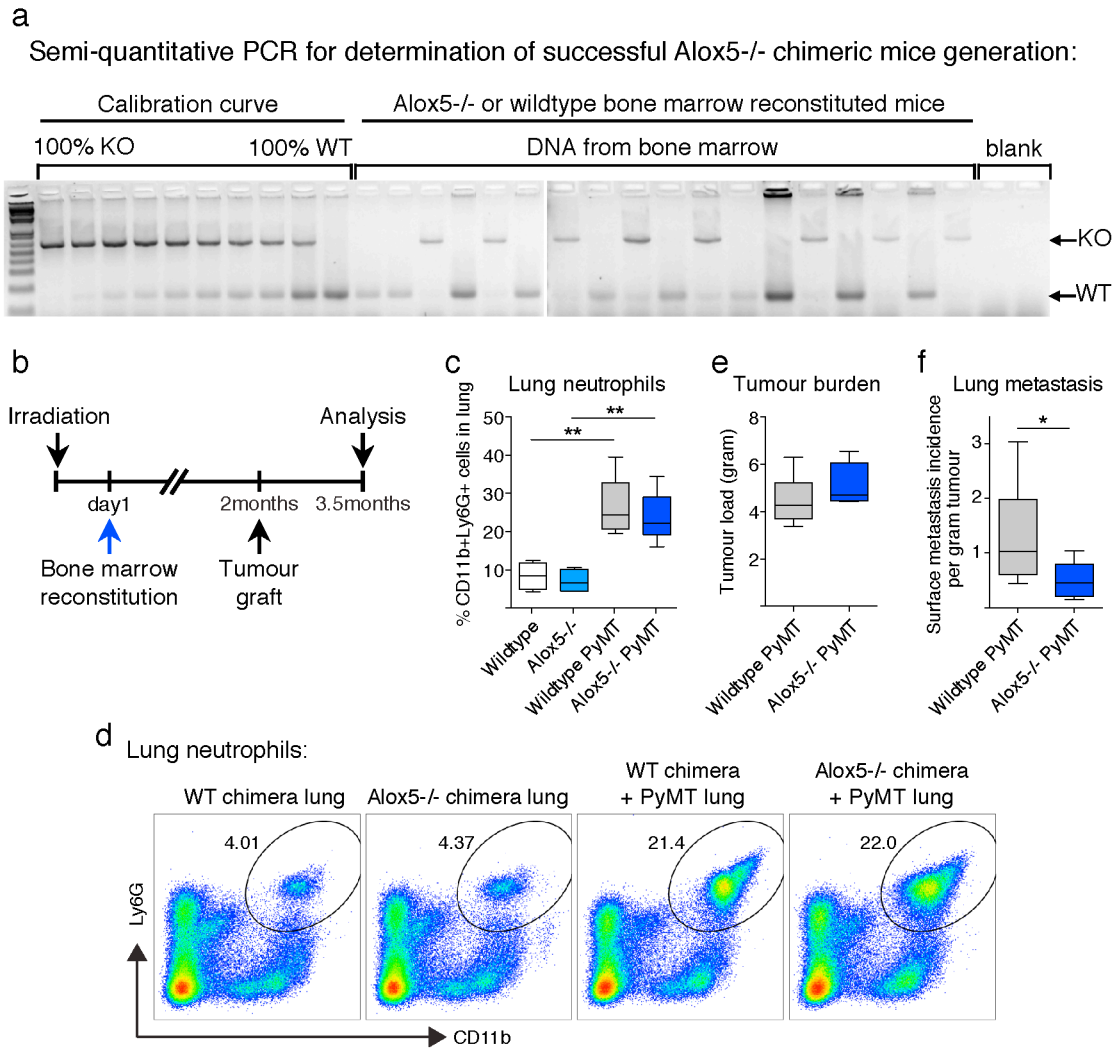

Figure 4-13 Alox5-/- bone marrow-reconstituted mice display significantly ameliorated lung metastatic burden when grafted with a primary mammary MMTV-PyMT tumour

(a-b) Wildtype mice were lethally irradiated and one day later intravenously injected with either wildtype (WT) or Alox5-/- (KO) bone marrow. Two months post-irradiation primary MMTV-PyMT cells were engraftment onto the mammary gland (b; n≥6 per group pooled from 2 independent experiments). Bone marrow reconstitution efficiency was determined by semi-quantitative PCR analysis of DNA isolated from the bone marrow (a). A calibration curve of the ratio between the PCR-band amplified from the wildtype and Alox5 null allele was used to calculate the percentage of reconstitution. Tests of eight representative mice are shown. Only mice with >80% Alox5 null bone marrow reconstitution where used for experiments. (c-f) Bone marrow-reconstituted mice 1.5 months post-tumour-graft and/or tumour-free controls were analysed. Quantification (c) and representative flow cytometric analysis (d) of CD11b+Ly6G+ neutrophil present in the lung, gated on alive single cells. Primary tumour load was determined by weighing (e) and spontaneous metastasis to the lung by quantification of surface lung metastases shown relative to tumour load (f).

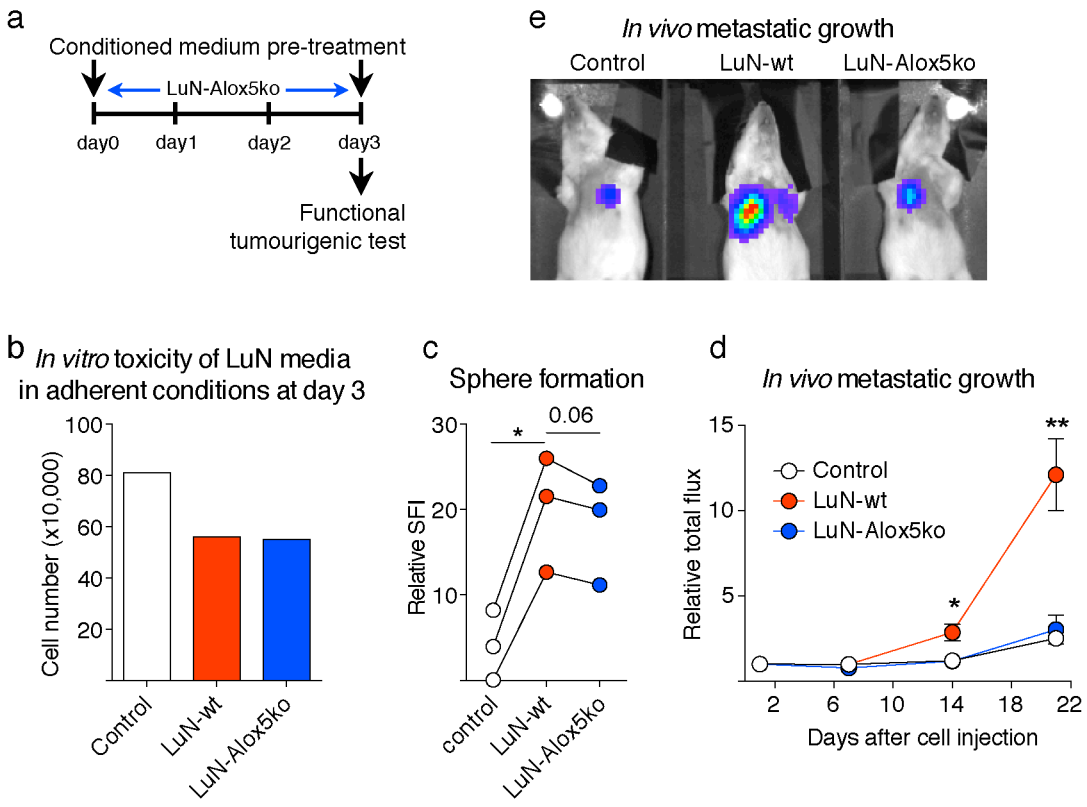

Figure 4-14 The lung neutrophil-derived Alox5 products leukotrienes mediate the activity of neutrophils to boost metastatic competence of mammary cancer cells
(a) Luciferase-expressing MMTV-PyMT cells were treated with control, wildtype LuN (LuN-wt) or Alox5-deficient neutrophils-derived LuN (LuN-Alox5ko) medium for three days in adherent culture. (b) Level of toxicity after three-day LuN treatment displayed as number of remaining alive MMTV-PyMT cells (one representative experiment of 3 repetitions is shown. (c-e) Neutrophil-conditioned medium pre-treated MMTV-PyMT cells were plated in non-adherent conditions and their sphere formation assessed (c; 3 independent experiments) or intravenously injected into Rag1-/- mice for determination of experimental metastasis initiation (d-e). Quantification of cancer cell-derived bioluminescence intensity in the lung over time is depicted relative to 24 hours (d; n≥8 per group pooled from 2 independent experiments) and representative image is shown (e). Sphere formation index (SFI) in (c) was calculated as the combination of area of all formed spheres per experiment to incorporate sphere number and size.

4.3 Chapter conclusion

The Alox5 products leukotriene B4 and cysteinyl-leukotrienes C4, D4 and E4 are secreted by pre-metastatic lung neutrophils of mammary cancer bearing mice and increase the tumourigenic and metastatic potential of mammary cancer cells comparably to the cocktail of neutrophil-derived factors. Leukotrienes specifically promote proliferation of the metastasis-initiating subset of cancer cells in an ERK1/2 activation-dependent manner. This function is mediated by the enrichment of leukotriene receptor BLT2 and CysLT2-expressing cells among metastasis-initiating cancer cells. In fact, BLT2 and/or CysLT2-expressing MMTV-PyMT cells represent a distinct cancer cell population, which is significantly enriched among mammary cancer cells that retain an intrinsically higher tumourigenic potential. Hence, leukotriene receptors BLT2 and CysLT2 might serve as novel, functional markers for cancer stem cell-like cells at least in the MMTV-PyMT mammary cancer mouse model. Overall, LTB4 and cysteinyl leukotriene stimulation appears to lead to an alteration of the composition of the total cancer cell population in support of leukotriene receptor-expressing, intrinsically highly metastatic cancer cells. This observation reflects the novel neutrophil-mediated pro-metastatic function identified earlier (chapter 3). Moreover, depleting neutrophils from leukotriene synthesis by genetically targeting the Alox5 enzyme deprives them from their ability to facilitate the metastatic competence of total cancer cell populations.
Hence, we are providing a mechanism for the metastasis initiation/lung colonisation-promoting activity of neutrophils *in vitro* and *in vivo* via a neutrophil-mediated leukotriene-leukotriene receptor-ERK1/2 axis that drives proliferation specifically of intrinsically highly metastatic cancer cells among the total population (Fig. 4.15).

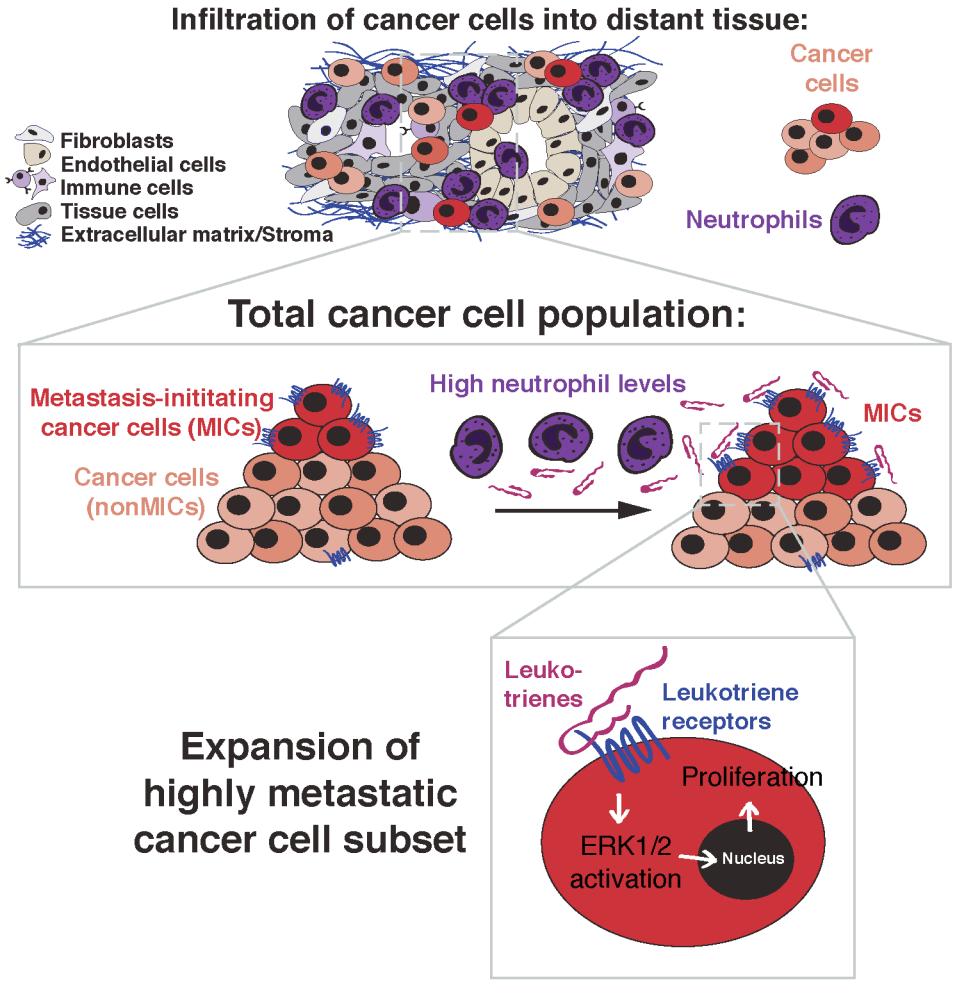

Figure 4-15 Pre-metastatic lung neutrophils
Neutrophils accumulate in the pre-metastatic lung of mammary cancer bearing mice prior to arrival of disseminated cancer cells. These neutrophils secrete the eicosanoid mediators leukotrienes LTB4, LTC4, LTD4 and LTE4 that are synthesised by the enzyme Alox5. Leukotrienes differentially affect arriving mammary cancer cell populations. Subsets of cancer cells with intrinsically minimal lung colonisation ability do express very limited amounts of leukotriene receptors BLT2 and CysLT2, while their potent metastasis-initiating counterparts are strongly enriched for leukotriene receptor expressing cells. Hence, specifically the metastasis-initiating cancer cell subpool is susceptible to leukotriene-induced activation of ERK1/2 kinases that stimulate cell proliferation leading to the expansion of the population. Thereby, neutrophils directly provide a selective growth-promoting signal for intrinsically highly potent mammary cancer cells and facilitate initiation of metastatic lung colonisation.

Chapter 5. Pharmacologic inhibition of leukotrienes-producing enzyme Alox5 limits mammary cancer metastatic progression to the lung

5.1 Chapter Introduction

Metastatic spread of primary tumours remains the main cause of cancer-related deaths (section 1.1), hence the discovery of a novel function of neutrophils to promote metastatic progression of breast cancer to the lung provides a promising opportunity for therapeutic intervention. This approach would allow the combination of drugs targeting disseminated cancer cells directly with tackling the pro-metastatic microenvironment and might increase efficacy. However, directly targeting neutrophils is therapeutically unfeasible, as neutropenia is associated with severe side effects especially during chemotherapy of cancer patients, mainly because of an increased susceptibility to lethal infections (section 1.3).

The identification of the mechanism behind the neutrophil pro-metastatic activity offers the unique opportunity to specifically deprive neutrophils from their ability to promote metastasis while maintaining other crucial functions to manage infections. The specific Alox5 inhibitor Zileuton is regularly used to treat inflammatory asthma because of the important role of leukotrienes in this disease (Lazarus et al., 1998, Watkins et al., 2007, Wenzel and Kamada, 1996) and the therapeutic and preventive effect of Zileuton was/is tested in two clinical trials for lung cancer (Edelman et al., 2008, Szabo et al., 2013). Given the efficacy of genetic Alox5 depletion in mouse models, we wondered if pharmacologic inhibition of Alox5 using Zileuton would prove effective to prevent early stages of lung colonisation and metastasis of mammary cancer. To this end, we explored Zileuton-mediated inhibition of Alox5/leukotriene synthesis and thereby the neutrophil-mediated leukotriene-leukotriene receptor-ERK1/2 axis in a therapeutic approach to treat metastatic breast cancer in mouse models.

5.2 Results

5.2.1 Zileuton inhibits Alox5 activity in neutrophils when systemically administered to mammary tumour-bearing mice and Zileuton-treated neutrophils lose metastasis-supporting activity

In order to test the efficacy of Zileuton to block leukotriene synthesis in lung neutrophils in mammary tumour-bearing mice, we treated wildtype mice daily with Zileuton via the oral route and grafted MMTV-PyMT cells onto the mammary gland one day after treatment start (Fig. 5.1 a). Neutrophils readily accumulated in the pre-metastatic lung of Zileuton-treated mice (data not shown) and mammary tumour growth was not affected (Fig. 5.1 b). We isolated pre-metastatic lung neutrophils four weeks after tumour graft and used to them to condition *in vivo* Zileuton-treated neutrophil-conditioned medium (LuN-Zil). Enzyme Immunoassay analysis of LuN-Zil medium showed a significant reduction of LTB4 levels, confirming the activity of the drug (Fig. 5.1 c). Adherent culture of MMTV-PyMT cells for three days in LuN-Zil medium caused an equal toxicity like wildtype LuN medium (Fig. 5.1 d-e). In accordance with previous results using leukotriene-deficient LuN-Alox5ko medium, LuN-Zil medium was slightly less potent in boosting *in vitro* sphere formation after pre-treatment (Fig. 5.1 f). Strikingly, primary MMTV-PyMT cancer cells cultured for three days in LuN-Zil medium displayed dramatically decreased *in vivo* metastatic initiation competence compared to wildtype LuN medium (Fig. 5.1 g). This evidence indicates that pharmacologic inhibition of Alox5 by Zileuton is effective in mammary tumour mouse models and limits the metastasis-boosting ability of neutrophils *in vitro*.

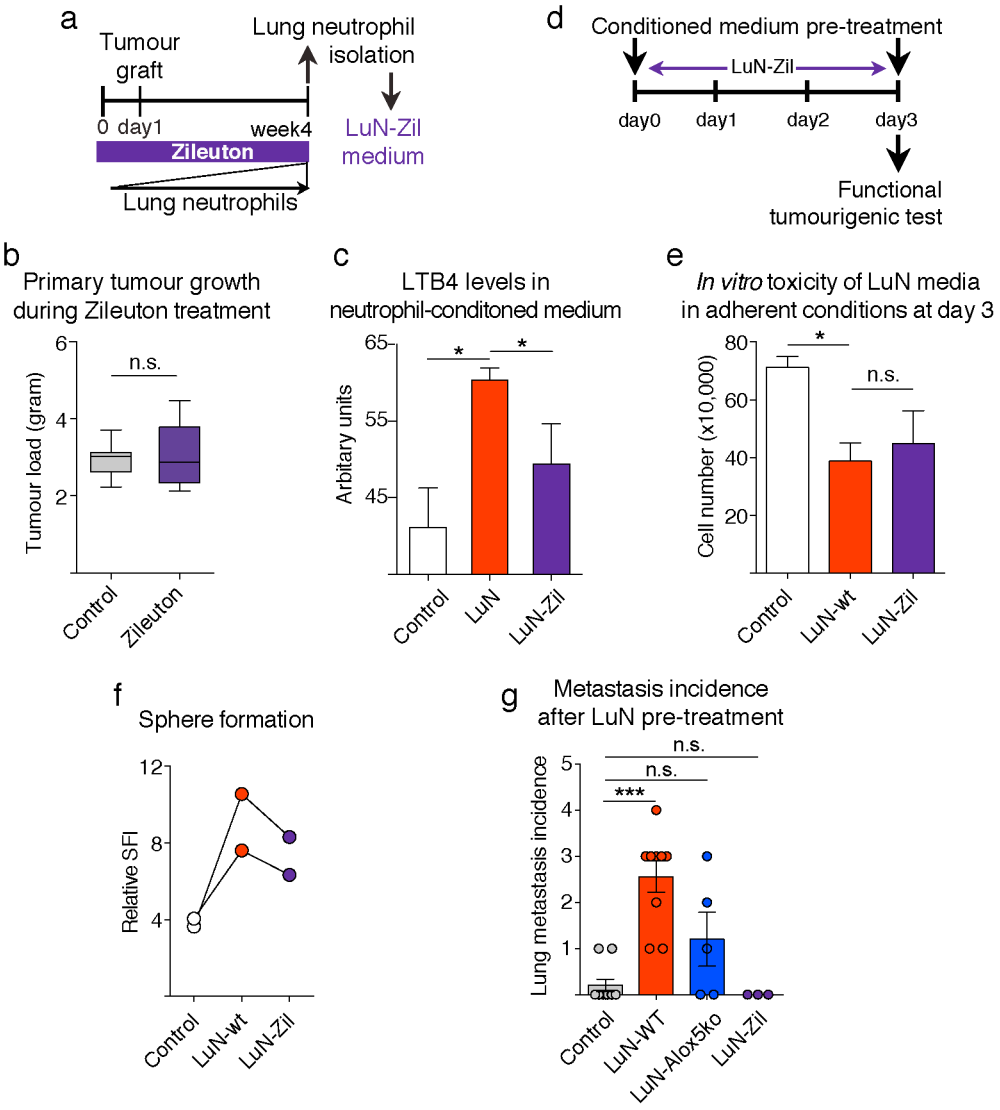

Figure 5-1 *In vivo* pharmacological inhibition of leukotriene synthesis in lung neutrophils limits their potential to enhance metastatic competence of mammary cancer cells

(a) Neutrophils were isolated from the lungs of MMTV-PyMT mammary tumour-grafted wildtype mice treated daily with Zileuton for four weeks and used to condition culture media (LuN-Zil). (b) Quantification of mammary tumour growth of control DMSO- or Zileuton-treated wildtype mice by dissection and weighing (n≥8 per group, pooled from 2 independent experiments). (c) Enzyme immunoassay analysis of leukotriene B4 levels in control, wildtype LuN or LuN-Zil medium (b, n≥3 per group pooled from 3 independent experiments). (d) Primary MMTV-PyMT cells were treated with control, LuN-wt, LuN-Alox5ko or LuN-Zil medium for three days in adherent culture. (e) Level of toxicity after three-day LuN treatment displayed as number of remaining MMTV-PyMT cells (3 independent experiments). (f-g) Neutrophil-conditioned medium pre-treated MMTV-PyMT cells were plated in non-adherent conditions and their sphere formation assessed (f; 2 independent experiments) or intravenously injected into Rag1-/- mice for determination of experimental metastasis initiation (g; n≥8 per group pooled from 2 independent experiments). Lung metastasis was quantified by counting of visible surface nodules. Sphere formation index (SFI) in (f) was calculated as the combination of area of all formed spheres per experiment to incorporate sphere number and size.

5.2.2 Therapeutic Zileuton treatment phenocopies neutrophil depletion and inhibits metastatic initiation ability of mammary cancer cells

Next, we aimed to test if pharmacological Alox5 inhibition limits metastatic progression of mammary cancer to the lung in a therapeutic setting. We grafted Rag1-/- mice with MMTV-PyMT cells onto the mammary gland followed by daily Zileuton treatment starting four weeks later for two weeks during stages of spontaneous metastasis initiation in the lung followed by analysis (Fig. 5.2 a). Strikingly, Zileuton-treated MMTV-PyMT tumour-harbouring mice showed a significant reduction of spontaneous metastasis without consistent alterations in primary tumour growth or lung neutrophil recruitment (Fig. 5.2 b-d+f). Additionally, we used the same mice to investigate the effects of Zileuton treatment on initiation of metastatic lung colonisation, similar to previous experiments when blocking neutrophils (Fig. 3.15). To this end, we seeded GFP-labelled MMTV-PyMT cancer cells directly into the lung by intravenous injection 24 hours after Zileuton treatment start (Fig. 5.2 a). Significantly, experimental metastasis initiation was diminished when cancer cells were injected during pharmacologic inhibition of Alox5 (Fig. 5.2 e-f) corroborating the promoting effect of leukotrienes on early lung colonisation. We also confirmed that metastatic MMTV-PyMT cancer cells were impaired in proliferation very early after infiltrating lungs of Zileuton-treated, mammary tumour-bearing mice by determination of *in vivo* BrdU incorporation 24 hours after intravenous injection of cancer cells (Fig. 5.2 g). Importantly, these data validate our findings of the pro-metastatic activity of lung neutrophil-derived leukotrienes and shows the efficacy of pharmacological targeting of the Alox5 enzyme to limit mammary cancer metastasis to the lung.

Next, we aimed to determine if Zileuton treatment during later stages of growth of established metastases in the lung would also result effective. To test this hypothesis, we employed a comparable setting used previously (Fig. 5.2 a) and grafted Rag1-/- mice with MMTV-PyMT mammary tumours followed by intravenous injection of GFP-labelled cancer cells two weeks after tumour graft. We started daily Zileuton treatment another two weeks post-cancer cell intravenous injection and analysed mice two weeks thereafter (Fig. 5.3 a). This setting allows formation of established metastases in the lung prior to Alox5 inhibition and the assessment

of effects during progression of metastatic growth. To our surprise, primary tumour burden was ameliorated in Zileuton-receiving mice (Fig. 5.3 c), which is probably due to the induction of an altered, Alox5-sensitive growth dynamic of MMTV-PyMT cancer cells in the mammary gland because of the injection of large amounts of circulating tumour cells very early during cancer development. This trend is not consistent among our experimental approaches (Fig. 5.1 b and Fig. 5.2 c) and would, if at all, even improve the anti-tumourigenic effects of Zileuton therapy. Importantly, neutrophil accumulation in the lung of these MMTV-PyMT tumour-bearing Zileuton-receiving mice was unaltered compared to controls (Fig. 5.3 b). However, Zileuton treatment of established lung metastases showed only a non-significant trend of reducing experimentally induced metastatic burden (Fig. 5.3 d-e). This observation highlights the crucial role of neutrophil-derived Alox5 products to induce proliferation and formation of cancer cell colonies very early during the process of metastatic lung colonisation. Nevertheless, the effects of Zileuton in limiting initiation of metastatic lung colonisation are vital for the overall efficiency of lung metastasis development, as spontaneous metastasis originating from the primary mammary tumour showed a significant reduction upon Alox5 blockade (Fig. 5.2 d+f).

Importantly, the efficacy of Zileuton treatment to limit initiation of metastatic lung colonisation was also confirmed in two metastatic breast cancer cell lines, mouse 4T1 cells and human MDA-MB-231 cells in comparable settings. Rag1-/- mice were grafted with unlabelled 4T1 or MDA-MB-231 cells onto the mammary gland and Zileuton administered daily starting four weeks thereafter. GFP-labelled 4T1 or MDA-MB-231 cancer cells were intravenously injected into these mice one day after start of Zileuton treatment and experimental metastasis quantified 1.5 weeks (4T1 cells) or two weeks later (MDA-MB-231 cells) (Fig. 5.4 a). This experimental setting reflects our approach for the analysis of Zileuton treatment on the potential of MMTV-PyMT cells to initiate metastatic lung colonisation (Fig. 5.2). As expected, Zileuton administration did not alter 4T1 or MDA-MB-231 tumour-induced lung neutrophil infiltration or growth of grafted tumours (Fig. 5.4 b-c+f-g). Importantly, experimental metastasis initiation originating from both, GFP-labelled intravenously injected 4T1 and MDA-MB-231 cancer cells, was significantly reduced upon Zileuton treatment (Fig. 5.4 d-e+h-i). These results confirm the efficacy of Zileuton therapy to impede lung colonisation of mammary cancer cells.

In summary, our observations suggest pharmacological inhibition of Alox5 by the inhibitor Zileuton as promising therapeutic approach to limit breast cancer metastasis to the lung. Alox5 products appear to play their most crucial pro-metastatic role during initiation of lung metastases rather than their continuous growth, consistent with the identified novel activity of leukotrienes towards intrinsically highly potent metastasis-initiating cells. The effects of pharmacological Alox5 inhibition complement our findings of genetic Alox5 deficiency and strengthen leukotrienes as mediators of the neutrophil-derived pro-metastatic activity. Taken together, these data suggest a potential therapeutic approach to target the Leukotriene/Alox5-dependet function of neutrophils supporting early metastatic colonisation.

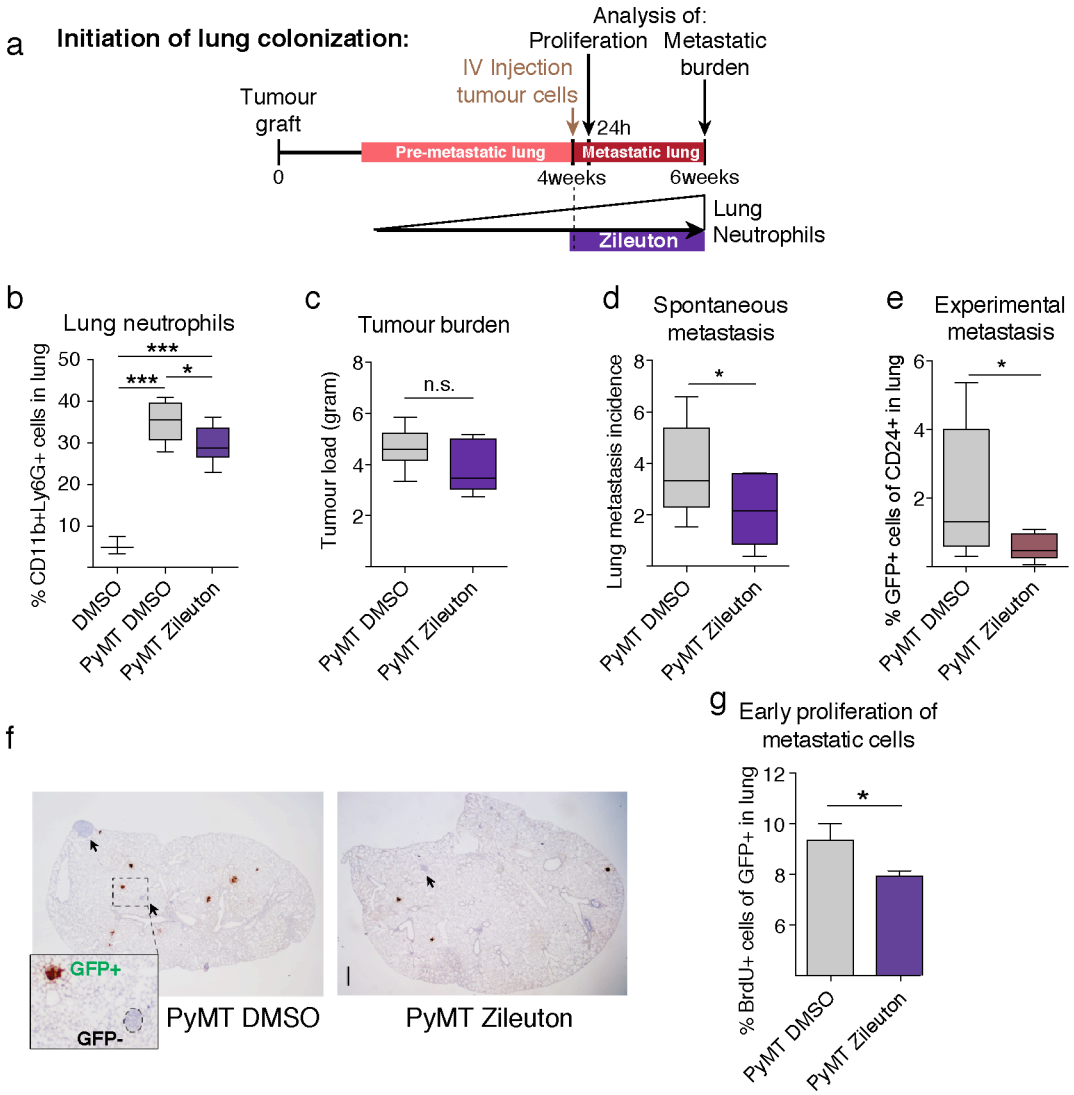

Figure 5-2 Pharmacological inhibition of leukotriene synthesis significantly decreases initiation of metastatic lung colonisation by MMTV-PyMT cancer cells
(a) Unlabelled MMTV-PyMT cells were grafted onto the mammary gland of Rag1-/- mice and GFP-labelled MMTV-PyMT cells intravenously (IV) injected. Daily Zileuton or DMSO treatment started one day prior to GFP-labelled cell injection and continued for two weeks (n≥7 per group pooled from 2 independent experiments). (b-f) Flow cytometric quantification of CD11b+LyG6+ neutrophils in the lung (b), primary tumour burden (c) and spontaneous metastatic progression determined by quantification of visible, non-GFP surface lung metastases (d). (e) Experimental metastasis initiation in Zileuton-treated or control mice assessed by flow cytometric quantification of GFP+ MMTV-PyMT cells in the lung, gated on CD24+ cells. (f) Representative histological lung sections stained with GFP in brown to visualise experimental metastasis and haematoxylin in blue to stain nuclei. Arrows indicate spontaneous metastases originating from the transplanted primary tumour, scale bar is 100µm. Close-ups on inserts highlight spontaneous and experimental, labelled metastases in the lung. (g) Determination of *in vivo* cancer cell proliferation 24 hours after intravenous injection of GFP-labelled MMTV-PyMT cancer cells into MMTV-PyMT tumour-bearing, Zileuton-treated mice by six hours BrdU chase and flow cytometric quantification of BrdU+ cancer cells in the lung, gated on GFP+ cells (n≥3 per group, one experiment).

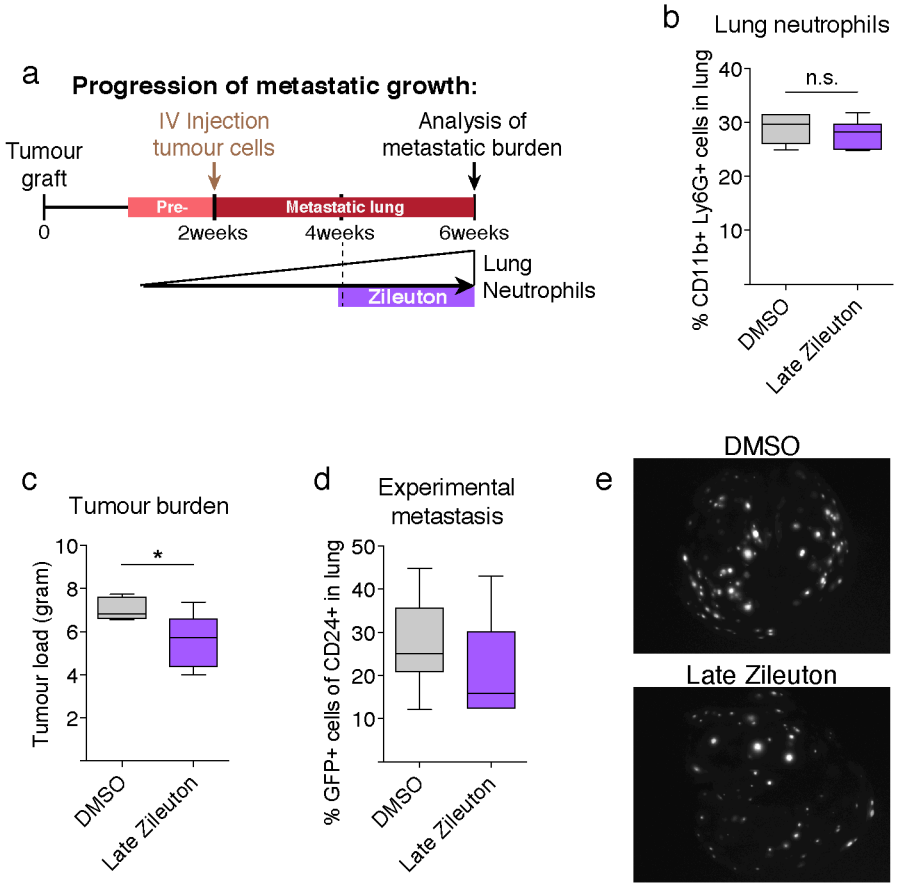

Figure 5-3 Zileuton administration does not significantly affect growth of established metastases in the lung

(a-e) Unlabelled MMTV-PyMT cells were grafted onto the mammary gland of Rag1-/- mice and GFP-labelled MMTV-PyMT cells intravenously (IV) injected. Daily Zileuton or control DMSO treatment started two weeks after GFP-labelled cell injection and continued for two weeks prior to analysis (a, n=5 per group, one experiment). Mammary gland tumour burden was assessed by resection and weighing at the end of the experiment (c). Presence of CD11b+Ly6G+ neutrophils (b) and experimental metastasis as frequency of GFP+ MMTV-PyMT cancer cells in the lung (d) was determined by flow cytometry, gated on alive single cells in (b) and CD24+ cells in (d). Representative stereomicroscopic images of GFP signal in the lung (e) are shown.

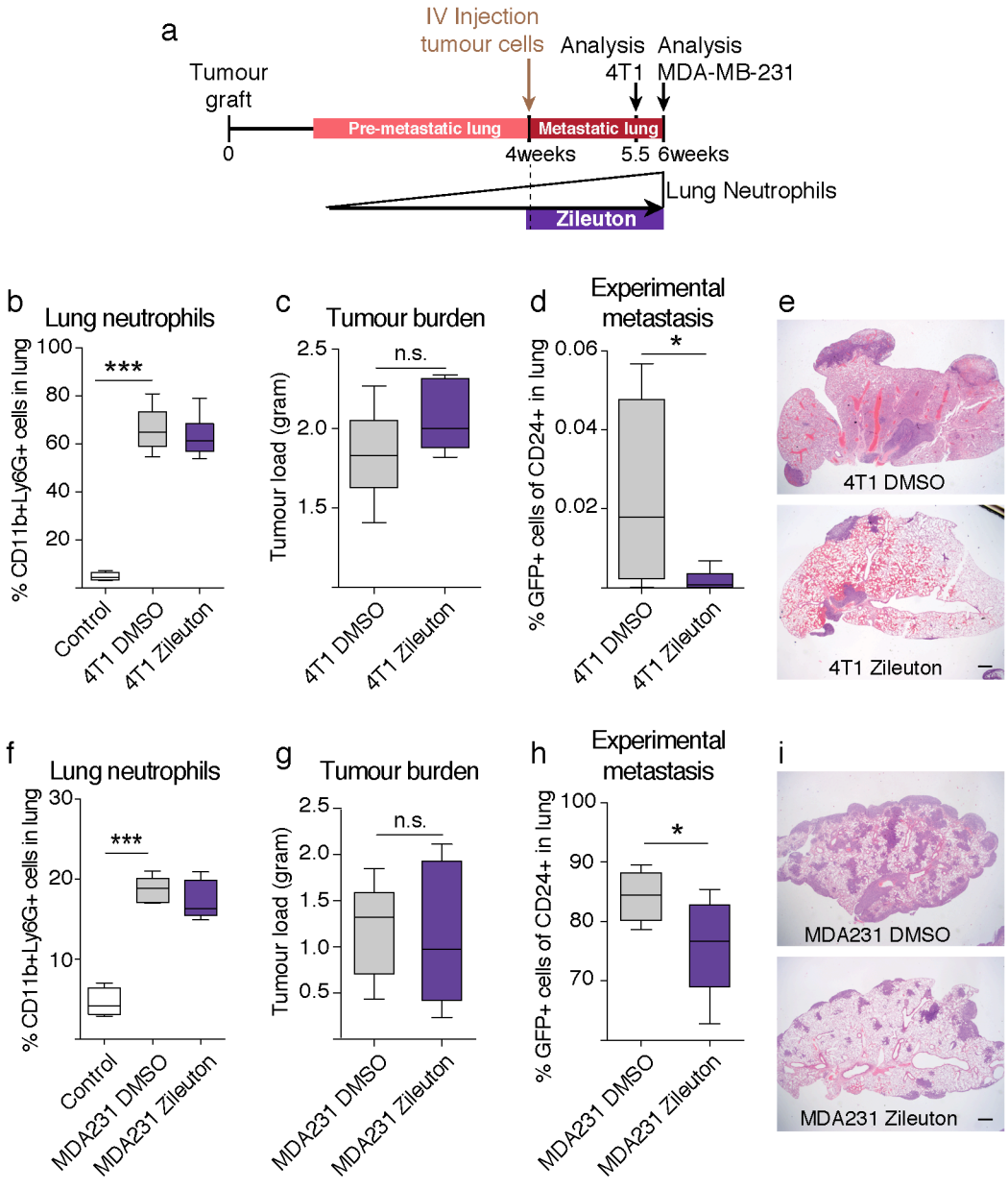

Figure 5-4 Pharmacological blockade of Alox5 activity decreases lung metastasis initiation in several advanced breast cancer models
(a-i) Unlabelled mouse 4T1 mammary (b-e) or human MDA-MB-231 breast cancer cells (f-i) were grafted onto the mammary gland of Rag1-/- mice, GFP-labelled cancer cells intravenously (IV) injected followed by daily Zileuton or control DMSO administration (a, n≥5 per group, one experiment). Flow cytometric quantification of CD11b+LyG6+ neutrophils in the lung, gated on single alive cells (b+f), primary tumour burden at time of analysis (c+g) and experimental metastasis initiation analysed by flow cytometric quantification of GFP+ cancer cells in the lung, gated on CD24+ cells, (d+h) of mice intravenously injected with the GFP-labelled mouse cancer cell line 4T1 (b-e) or GFP-labelled human cancer cell line MDA-MB-231 (f-i) and treated with Zileuton. Representative haematoxylin and eosin-stained lung sections are shown of mice that were grafted and intravenously injected with 4T1 (e) or MDA-MB-231 cancer cells (i).

5.2.3 Zileuton treatment does not directly affect mammary cancer cells, but the tumour microenvironment

We propose a novel strategy to target the tumour microenvironment to limit metastatic progression of breast cancer using the Alox5 inhibitor Zileuton. In order to elucidate eventual effects of Zileuton directly on mammary cancer cells, we did the following series of experiments. Firstly, we already established that Zileuton treatment had no effect on long-term primary tumour growth in wildtype mice (Fig. 5.1 b). Further, we treated primary MMTV-PyMT or 4T1 cancer cells in adherent culture with Zileuton for 24 hours followed by determination of cell proliferation by BrdU incorporation after a three-hour pulse (Fig. 5.5 a). Also, we monitored the frequencies of metastasis-initiating MMTV-PyMT cancer cells (CD90+ MICs) among the total cancer cell population after three day Zileuton exposure *in vitro* (Fig. 5.5 b). Lastly, MMTV-PyMT cells treated with Zileuton in non-adherent culture for three days were plated in non-attachment conditions and their self-renewal ability determined by assessment of sphere formation potential (Fig. 5.5 c). In short, Zileuton treatment did not influence cancer cell behaviour *in vitro* in all these assays and *in vivo* mammary tumour growth, indicating that Alox5 products are not part of an autocrine loop of cancer cells but derived from the lung microenvironment *in vivo* (Fig. 5.1 b and 5.5).

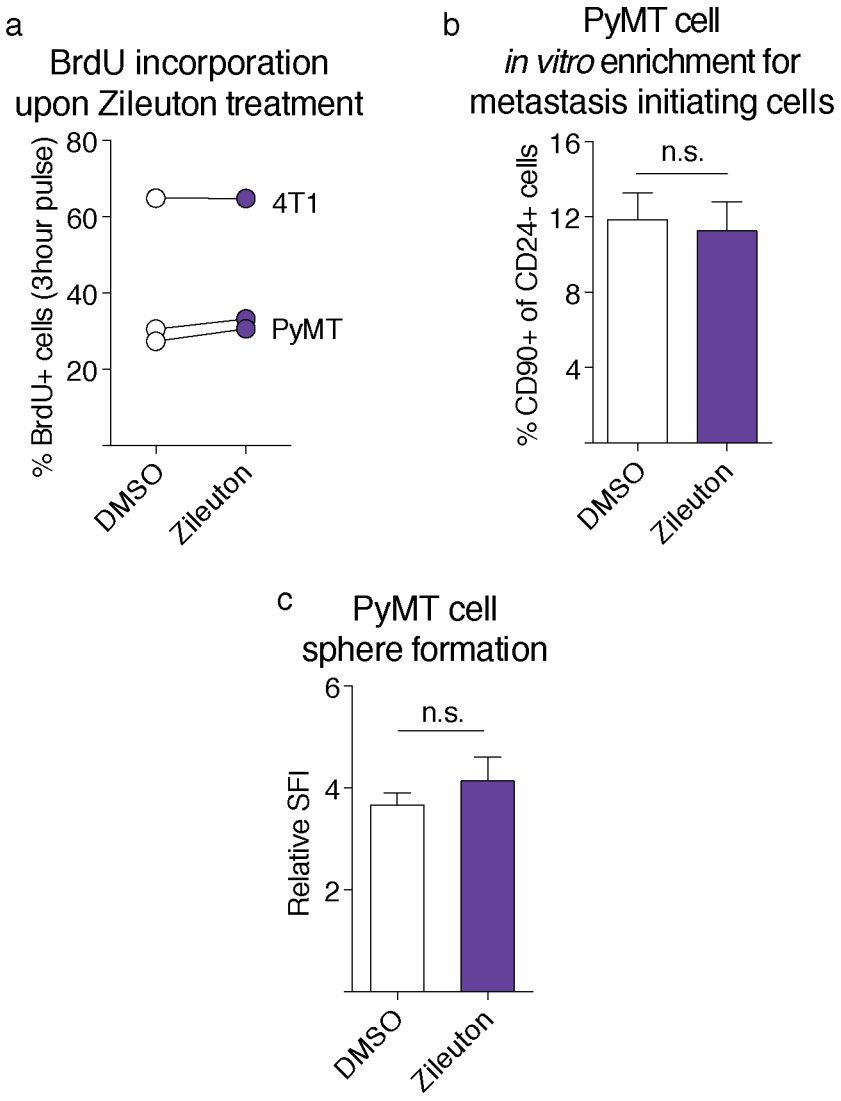

Figure 5-5 Breast cancer cell proliferation and self-renewal are not directly affected by treatment with the Alox5 inhibitor Zileuton
(a) Flow cytometric quantification of BrdU incorporation after a 3 hour-pulse of primary MMTV-PyMT cells (2 independent experiments) and the mouse 4T1 mammary cancer cell line (one experiment) treated with 1μM Zileuton or control DMSO for 24 hours in adherent culture, gated on alive single cells. (b) Flow cytometric analysis of frequency of CD90+ MICs in total MMTV-PyMT cells after three-day treatment with 1μM Zileuton in adherent culture, gated on CD24+ cells (3 independent experiments). (c) Quantification of sphere formation of MMTV-PyMT cancer cells in presence of 1μM Zileuton (2 independent experiments). Sphere formation index (SFI) was calculated as the combination of area of all formed spheres per experiment to incorporate sphere number and size.

5.2.4 Leukotriene receptors are expressed in the majority of examined human breast cancers and maintained in lymph node metastases with varying intensities and frequencies

Finally, we aimed to investigate if the leukotriene receptors BLT2 and CysLT2 are expressed in breast cancer cells in patient tissue, which would be crucial for a potential clinical application of blocking the neutrophil-mediated leukotriene-leukotriene receptor-ERK1/2 axis by Zileuton. We obtained commercially available sections of paraffin-embedded breast cancer tissue biopsies with paired lymph node metastases (n≥30) and immunohistologically stained them with antibodies detecting either human BLT2 or CysLT2. The human breast cancer samples comprised metastatic ductal as well as lobular carcinoma of stages II to IV. Frequency of stained cancer cells, not stromal cells, and staining intensity was determined and combined to a final score value for every sample (Fig. 5.6, details on analysis are discussed in section 2.3.2). BLT2 is expressed in about 50% of analysed human breast cancers and CysLT2 in about 75% of cases with increased intensity compared to BLT2. Of note, the antibody detecting CysLT2 is very efficient, while the BLT2 antibody shows an in general weaker signal. Importantly, expression of leukotriene receptors is maintained in metastases, suggesting susceptibility to the promoting signal of neutrophil-derived leukotrienes (Fig. 4.15). No significant correlation was found between staining score and cancer stage, probably due to the quite small number of samples.

This evidence further suggests that a comparable neutrophil-mediated pro-metastatic mechanism via a leukotriene-leukotriene receptor-ERK1/2 axis, as observed in mammary cancer mouse models, might boost human breast cancer progression to the lung.

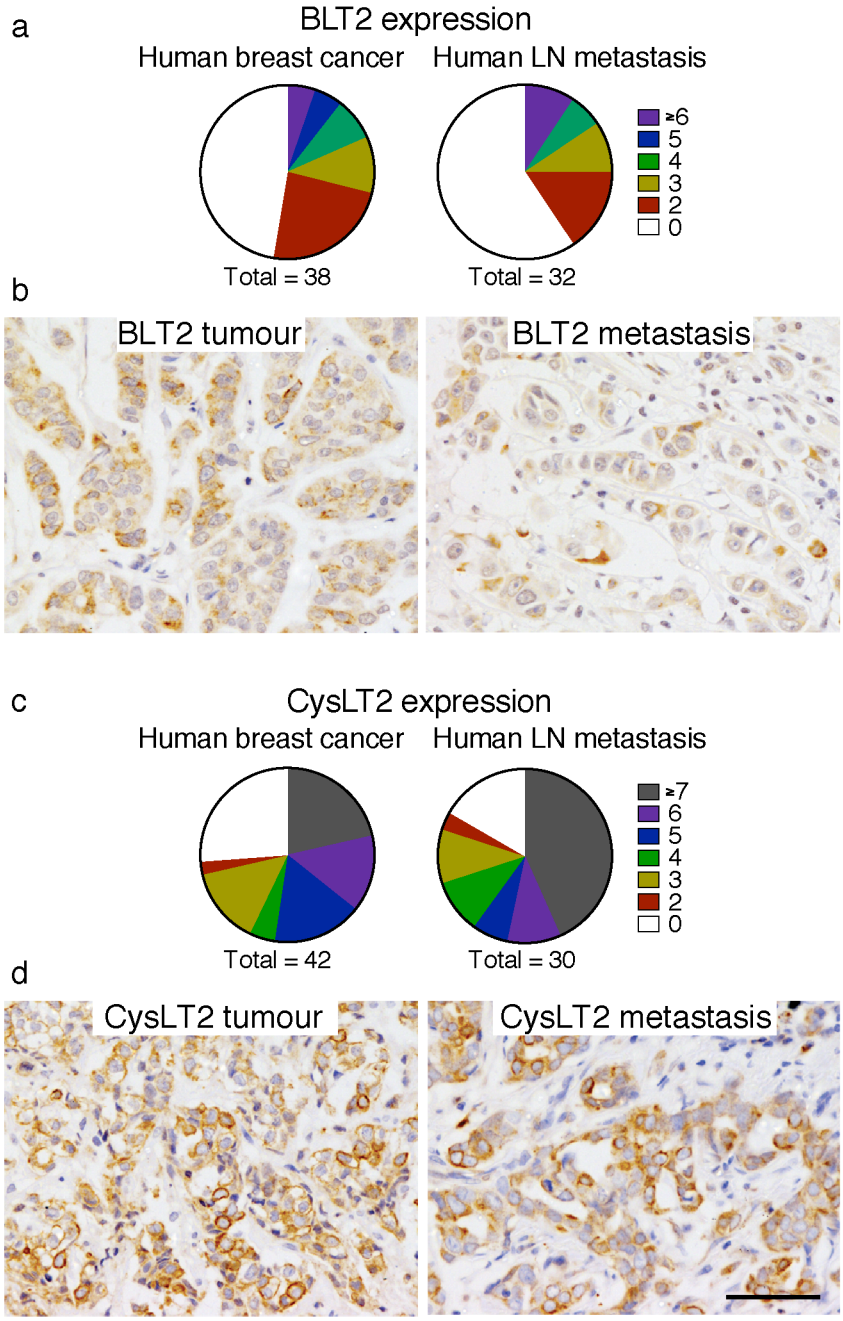

Figure 5-6 The leukotriene receptors BLT2 and CysLT2 are expressed in human breast cancer tissue
(a-d) Primary human breast cancer samples and matched lymph node (LN) metastases were stained by immunohistochemistry for leukotriene receptors BLT2 (a-b) and CysLT2 (c-d; brown) and haematoxylin (blue). Representative microscopic images of human breast cancer tissue (b+d) and scoring (score 0 to 7) of BLT2 and CysLT2 presence determined by incorporation of staining intensity and frequency on breast cancer cells (a+c; section 2.3.2) is shown, scale bar is 50µm. *n* numbers are depicted below the respective quantifications.

5.3 Chapter conclusion

We show here that systemic Zileuton treatment/Alox5 inhibition reduced metastatic progression to the lung, specifically the initiation of lung colonisation, of three different models for breast cancer with a varying degree of metastatic potential (mouse spontaneous, mouse and human cell lines). Pharmacological inhibition of leukotriene synthesis appears to impair the ability of neutrophils to directly increase the metastatic competence of the total cancer cell population in a similar fashion as genetic Alox5 deficiency and does not directly affect cancer cells *in vivo* or *in vitro*. These results stress the importance of a pro-metastatic neutrophil-mediated leukotriene-leukotriene receptor-ERK1/2 axis during early lung colonisation and provide a strategy for therapeutic intervention. Importantly, the majority of human breast cancers and metastases express the receptors for Alox5 metabolites/leukotrienes, BLT2 and CysLT2, and are, hence, theoretically responsive to leukotriene stimulation. Considering that generalised neutrophil-blocking approaches are unsuitable to treat cancer patients, Zileuton allows targeting of the novel pro-metastatic activity of neutrophils, without inducing the life-threatening consequences of neutropenia. Overall, Zileuton administration might provide a well-tolerated therapeutic approach to target the metastasis-supporting microenvironment (neutrophils) to complement conventional breast cancer treatments and reduce the risk of initiation of cancer cell growth at a distant site.

Chapter 6. Discussion

Please note that part of the data contained in this PhD thesis was first published by the Nature Publishing Group (Wculek and Malanchi, 2015).

6.1 Summary of findings

Collectively, we demonstrate a pro-tumourigenic role of neutrophils during early phases of metastatic colonisation of breast cancer cells to the lung. In detail, neutrophils are systemically mobilised by a primary mammary tumour and accumulate at the metastatic site prior to infiltration of disseminated cancer cells, and further increase during metastatic progression in three independent *in vivo* mouse models for spontaneous metastasis; one genetic mammary cancer model, one mouse and one human breast cancer cell line. Three different approaches for neutropenia, two genetic models and one antibody-mediated ablation, resulted in the reduction of metastatic burden without affecting primary tumour growth, indicating a supportive role of neutrophils during metastasis. We excluded potential effects of neutrophils on cancer cell invasion, intra- or extravasation by directly seeding cancer cells into the lung and detected no difference following neutrophil depletion. Hence, we hypothesised that neutrophils aid cancer cell colonisation of the metastatic organ and metastatic growth. In fact, as suggested by neutrophil accumulation in the pre-metastatic lung, we established an essential role of neutrophils during the very early stages of metastatic initiation at the distant site through temporal neutrophil-depletion experiments. We did not observe any changes of NK or T cell frequencies or their activation upon neutrophil deficiency at this early stage of metastatic colonisation and we corroborated our findings of the pro-metastatic activity of neutrophils in immunocompromised mice lacking functional B and T cells. Thus, we dismissed a functional contribution of suppression of anti-cancer immunity towards the observed neutrophil-mediated boost of metastatic potential in our system. In fact, in a series of *in vitro* and *in vivo* experiments using lung neutrophil-conditioned medium or adoptive transfer of lung neutrophils, we established that neutrophils enrich for the subpopulation of cancer cells that show an intrinsically superior competence for metastatic colonisation

(MICs) at the metastatic site that goes hand in hand with promoting overall metastatic competence. Mechanistically, we elucidated that the neutrophil-mediated increase of MIC frequencies in the lung is a result of a direct neutrophil-derived proliferative signal. Screening for neutrophil-secreted factors present in conditioned medium and receptor expression on cancer cells, we noticed an enrichment of leukotriene receptors BLT2 and CysLT2 on the MIC subpopulation as well as other CSC-like population in cell lines and confirmed the release of leukotrienes LTB4 and Cysteinyl leukotrienes LTC4, LTD4 and LTE4 by pre-metastatic lung neutrophils. *Ex vivo* leukotriene stimulation of the total cancer cell population mirrored neutrophil-conditioned medium treatment and enhanced cancer stemness, tumour initiation potential and metastatic competence. Additionally, LTB4 and Cysteinyl leukotrienes induced phosphorylation of ERK1/2 in cancer cells in a BLT2 and CysLT2-dependent manner, respectively. This ERK1/2 activation caused proliferation of the highly potent MIC subpool of cancer cells specifically leading to their expansion within the total cancer cell population. We also confirmed the increase of leukotriene receptor-expressing cancer cells upon leukotriene treatment in a human breast cancer cell line. Strikingly, Alox5 deficiency in the haematopoietic system and treatment with a pharmacologic Alox5 inhibitor *in vivo* also mirrored the lack of neutrophils and significantly decreased overall metastatic burden by, specifically, limiting initial metastatic lung colonisation with moderate effects on progression of established metastases. This observation went hand in hand with a reduction of cancer cell proliferation in the lung at very early stages of metastatic initiation upon Alox5 inhibition. Importantly, we confirmed that neutrophil-derived leukotriene mediate these observed effects as *ex vivo* neutrophil-mediated boost of stemness and metastatic competence of the total cancer cell population dependent on leukotriene presence. In fact, the ability of Alox5-deficient or *in vivo* Alox5 inhibitor-treated neutrophils to enhance cancer cell tumourigenicity or metastasis initiation was largely diminished compared to wildtype lung neutrophils. These results confirm that neutrophils promote metastasis by directly supporting the proliferation of intrinsically highly metastatic cells within the total cancer cell population at the metastatic site via a neutrophil-derived leukotriene–leukotriene receptor–ERK1/2 axis. We corroborated the metastasis-decreasing effect of Alox5 inhibition in three different pre-clinical models for spontaneous lung metastasis of mammary cancer cells including one human

breast cancer cell line and detected expression of the leukotriene receptors BLT2 and CysLT2 on the majority of human breast cancer tissue and lymph node metastases. These data suggest that a neutrophil-leukotriene-ERK1/2 axis might also be relevant for supporting human breast cancer metastasis.

6.2 Novelty and context of findings

We report a novel activity of neutrophils to support metastatic lung colonisation of breast cancer cells by secretion of Alox5 products/leukotrienes. In detail, we show that pre-metastatic lung neutrophil-derived leukotriene boost the most recognised hallmark of cancer, the ability to proliferate indefinitely, and directly induce one of the best-described mechanisms to facilitate that, the MAPK kinase ERK1/2. Thereby, neutrophil-derived leukotrienes promote specifically mammary MIC proliferation by engagement of leukotriene receptors that are preferentially expressed on MICs. This promotion of MIC proliferation likely overcomes cancer cell dormancy at the target site and allows successful establishment of metastases. Metastasis constitutes the highest cause of cancer-related mortality and neutrophils largely correlate with poor survival in the clinic, which includes breast cancer patients. Despite that, our knowledge about the precise roles of neutrophils in the metastatic process are mainly confined to their potential to induce cancer cell invasiveness and tumour angiogenesis and only very few reports address their functions during distant organ colonisation (as discussed in section 1.3.5.2). Hence, our identification of a previously unknown ability of neutrophils to promote metastatic colonisation and outgrowth has high relevance for experimental science, but also for a potential clinical application.

6.2.1 A previously unknown function of neutrophils and leukotrienes in metastasis

Metastatic colonisation is a very inefficient process with only 0.02% cells that arrive at the target site actually being able to successfully initiate metastases as the vast majority of cells remain dormant (Luzzi et al., 1998). This observation might not be surprising in light of metastatic cancer cells arriving in a rather inhospitable environment at the metastatic site without established survival or proliferative support. In fact, dormancy of disseminated cancer cells in distant organs has been observed in the clinic (sections 1.1.2 and 1.2.5), most notably in breast cancer (Braun et al., 2000, Pierga et al., 2003), and suggests that cancer cells survive in a non-proliferative state for up to several years. Cancer cell dormancy is mainly

caused by nutrient limitation/autophagy, cell cycle arrest or inhibitory signals from immune cells and has to be overcome for successful establishment of metastases (Aguirre-Ghiso, 2007, Barkan et al., 2010, Giancotti, 2013, Kenific et al., 2010, Naumov et al., 2006, Teng et al., 2008).

Firstly, we propose that neutrophils that accumulate in the pre-metastatic lung of breast-cancer bearing hosts provide a niche for arriving cancer cells and help them to exit dormancy and grow by providing the proliferation-inducing signal leukotrienes. Most likely, neutrophils specifically aid overcoming the cellular type of dormancy caused by an arrest in the cell cycle as we characterise a direct stimulation of proliferation of cancer cell subsets by neutrophils/leukotrienes and thereby cell cycle progression. A lack of available nutrients is likely an issue at slightly later metastatic stages that require neo-vessel formation (section 1.1.2 and 1.2.5) and we did not observe effects of neutrophils/leukotrienes on anti-cancer immunosuppression during initiation of metastatic colonisation (Fig. 3.17, Fig. 3.18 and Fig. 3.19). Hence, we cannot conclude neutrophils to be involved in modulation of cancer cell exit from nutrient limitation- or immunity-induced dormancy at least at very early stages of metastatic colonisation. In general, sustained proliferation is one of the hallmark features of cancer (Hanahan and Weinberg, 2011) and activation of ERK1/2 in cancer cells was shown to be crucial for induction of cancer cell proliferation as well as overcoming cellular dormancy (section 1.2.5). EGFR-ERK signalling triggered *in vivo* growth as well as proliferation of carcinoma cells (Liu et al., 2002) and limitation of ERK1/2 activation caused cancer cell dormancy (Ranganathan et al., 2006). Also, leukotrienes themselves have been shown to promote tumourigenesis and their receptors are often upregulated in many types of human cancers (section 1.2.3.5.1). For example, the LTB4-BLT2 cascade was functionally implicated in tumourigenesis as Ras-mediated transformation of a rat fibroblast cell line enhanced their LTB4 production and depended on BLT2 expression by these cells while BLT2 suppression reduced tumour formation (Yoo et al., 2004). In breast cancer cells, increased BLT2 expression was reported and BLT2 inhibition caused apoptosis via loss of a ROS-dependent survival signalling (Choi et al., 2010). In particular, leukotrienes induced proliferation of cancer cells via induction of ERK1/2 phosphorylation in several studies (section 1.2.3.5.1). LTB4 stimulation directly enhanced proliferation of fibroblasts, neutroblastoma, colon and pancreatic cancer cell lines via, among others, activation of ERK1/2 and

that was prevented by inhibition of LTB4 receptors BLT1 or BLT2 (Bortuzzo et al., 1996, Ihara et al., 2007, Sveinbjornsson et al., 2008, Tong et al., 2002, Tong et al., 2005, Woo et al., 2002). LTD4 induced proliferation of neutroblastoma cells (Sveinbjornsson et al., 2008) and caused ERK1/2 activation mainly via CysLT1 in intestinal epithelial cells enhancing proliferation (Mezhybovska et al., 2006, Ohd et al., 2000, Paruchuri et al., 2005, Paruchuri et al., 2002, Paruchuri et al., 2006). Importantly, our study is the first one to directly link cancer cell proliferation by leukotriene receptor-mediated ERK1/2 activation at the (pre-) metastatic site with locally accumulating neutrophils and their direct stimulation of metastatic cancer cells with Alox5 products/leukotrienes. Moreover, we show that this neutrophil-leukotriene-receptor-ERK1/2 axis is essential for efficient establishment of metastasis as depleting neutrophils or blocking the Alox5 enzyme limits metastatic burden (Fig. 3.3, Fig. 3.6, Fig. 3.15, Fig. 4.14, Fig. 5.2 and Fig. 5.4).

Secondly, neutrophil-derived leukotrienes selectively support proliferation of specific cancer cell subsets at the metastatic site and not the entire population, likely due to the differential expression of leukotriene receptors BLT2 and CysLT2. The cancer cell subpool that was experimentally proven to have the highest ability to form metastases, and is therefore referred to as metastasis-initiating cells (Malanchi et al., 2012), is strongly enriched for leukotriene receptor-expressing cells (Fig. 4.7). In fact, leukotrienes and their receptors have previously been involved in the modulation of homeostasis and induction of proliferation of very potent cells, tissue stem cells (section 1.2.3.5.1). For example, LTB4 activated proliferation and expansion of human haematopoietic stem cells via BLT2 and mouse neural stem cells via BLT1 (Chung et al., 2005, Wada et al., 2006). LTD4 stimulated proliferation of human haematopoietic progenitor cells by activation of ERK1/2 through CysLT1 and of mouse embryonic stem cells via CysLT1 and CysLT2 (Boehmler et al., 2009, Kim et al., 2010b). We provide the previously unknown finding that neutrophils/neutrophil-derived leukotrienes can directly support the highly potent metastasis-initiating cells (MICs), enrich for them within the total cancer cell population and thereby promote the metastatic process. We show that MIC enrichment in the lung during metastatic colonisation is directly proportional to increased metastasis formation (Fig. 3.21 and 3.22) and clinical studies correlate the frequency of MICs or cancer stem cells (CSCs) with poor clinical/metastatic outcome (Baccelli et al., 2013, Charafe-Jauffret et al., 2010,

Terwijn et al., 2014, Zeppernick et al., 2008). Our observations further suggest that at least part of the superior potency of these mammary MICs to colonise the lung is based on their responsiveness to the neutrophil-derived proliferative signal leukotrienes. This microenvironmentally-induced stimulation of proliferation might provide cancer cells with a way to circumvent cellular dormancy and, thereby, could contribute to their enhanced metastasis-initiating potential (Malanchi, 2013).

During initiation of metastatic colonisation we observed that neutrophils/leukotrienes induce a pronounced enrichment of MICs over nonMICs (Fig. 3.21 and Fig. 4.6) that is likely a combination of nonMIC death or continued dormancy and induction of MIC proliferation. However, this effect of neutrophils on MIC expansion appears transient and affects only the very initial stage of single cancer cells/small clusters arriving at the metastatic site that are then challenged to grow. Upon successful establishment of metastases, MIC frequencies normalise and they subsequently reconstitute a small percentage within the total cancer cell population similar to the primary tumour (Malanchi et al., 2012). The fact that neutrophils continue to accumulate in the lung throughout the metastatic process raises the question why MICs are not longer enriched. The most straightforward explanation would be that neutrophils decrease their leukotriene secretion and shift to other functions due to tumour cell- or metastatic environment-derived signals at late metastatic stages, such as the suppression of NK (Fig. 3.2 f) and other anti-tumour immune responses (Coffelt et al., 2015). The leukotriene-mediated induction of MIC proliferation could also reach a threshold level where the ratio of available leukotriene versus MICs in the lung is not longer sufficient. Alternatively, MICs could simply be not susceptible to the neutrophil-derived proliferative signal anymore. This feature could be achieved by downregulation of expression of leukotriene receptors by MICs induced by the established metastasis microenvironment at later stages or, alternatively, the withdrawal of a complementary signal that is required in concert with leukotrienes and their receptors to induce MIC proliferation. In fact, it appears likely that the nonMICs or the metastasis microenvironment influence the proliferative ability of MICs. Our preliminary experiments indicate that isolated MICs alone do not appear to react to leukotriene stimulation in the same fashion as they do within the total cancer cell population *in vitro*. Hence, MICs might require an additional signal from the

nonMICs or the pre-metastatic microenvironment together with neutrophil-derived leukotriene stimulation to activate ERK1/2 and/or induce their proliferation. Additionally, MICs have been shown to directly modulate and depend on their microenvironment, precisely involving the ECM components periostin or tenascin C, at the metastatic site (Malanchi et al., 2012, Oskarsson et al., 2011). Another plausible mechanism preventing MIC proliferation in response to leukotrienes in established metastases could be a direct inhibitory signal specifically for MICs from the nonMICs or the now established metastasis microenvironment. In a way it sounds tempting that MICs might be uniquely responsive to growth inhibitory factors as they are to proliferative factors like leukotrienes, depending on the specific context. Rather non-proliferative MICs arriving in the lung are acutely challenged to divide and establish successful metastases, reminiscent of a wound response of tissue stem cells. However, MIC proliferation has to be tightly controlled to avoid induction of apoptotic or senescence-inducing pathways and prevent susceptibility to pool exhaustion or – in the case of cancer – therapy-induced death. In concert with the rather quiescent nature of highly tumourigenic cells or cancer stem cells, a growing metastatic nodule that is not longer entirely dependent on MIC proliferation might ensure the quiescent status of its most potent and precious cells for their protection. Importantly, similar mechanisms might explain the very low frequencies of leukotriene receptor-expressing cells in the primary tumour, which is probably further explained by the largely limited presence of neutrophils and hence their proliferative signal (Fig. 3.1 e). Overall, the ability of highly potent cancer cells to respond to a proliferation-promoting signal from neutrophils might represent an acquired and eventually risky backup mechanism to react to exceptional challenge. This notion would make neutrophil-derived signals ideal candidates to be hijacked by cancer cells, as neutrophil presence in peripheral tissues is usually very low at steady state but they naturally respond very quickly and potently to numerous insults a tissue can be faced with.

6.2.2 Proposal of a novel, potential therapeutic strategy for breast cancer metastasis

Numerous approaches to target key cancer cell-intrinsic pathways causing tumour growth and progression are routinely used in the clinic, but often result in heterogeneous responses of cancer cells and subsequently ineffective due to therapy resistance (section 1.1). Hand in hand with our increasing knowledge on the positive interactions of a tumour with its tumour microenvironment (TME) grows the notion to target supportive functions of stromal cells together with therapies directed to cancer cells in order to improve current anti-cancer treatments (Quail and Joyce, 2013). Additionally, metastasis is not only the most detrimental feature of cancer progression, but has also been shown to be strongly dependent on a supportive microenvironment. However, successful therapies for metastatic cancer are currently rare (Malanchi, 2013, Nguyen et al., 2009, Steeg, 2006).

In specific regard to inflammatory and immune cells in the TME, a few successful approaches reached the clinic, such as immune checkpoint inhibitor blockade therapies that sustain anti-cancer immunity like inhibition of CTLA-4, PD-L1 or PD-1 (section 1.2.3.2.1). These treatments show outstanding success in the clinic (Postow et al., 2015, Allison, 2015, Pardoll, 2012, Topalian et al., 2015), however our understanding of the exact mechanisms behind and their controlled modulation have to be improved as immune checkpoint blockade can be accompanied by drug-related adverse side effects (Camacho, 2015, Gelao et al., 2014, Postow, 2015). Additionally, mimicking an acute rather than a chronic smouldering inflammation at cancer sites by treatment with bacterial products based on Coley's toxin or bacillus Calmette-Guerin-related vaccination showed promising results in animal models and the clinic (section 1.2.3 and 1.3.5). However, these approaches or the use of bacterial strains for cancer therapy could not yet be sufficiently improved to be routinely used in patient therapy – except in a few cases of late-stage ovarian cancer where little other options are currently available (Wei et al., 2008) – despite their discoveries many decades ago followed by long-term research efforts. Another proposed approach to target the inflammatory TME is changing the polarisation of microenvironmental cells from pro-tumourigenic to anti-tumourigenic features or limit their recruitment to tumour sites, for example for macrophages (section 1.2.3.3). This idea is based on a potential re-education of

myeloid cells towards acute inflammatory functions, which might be difficult to control on an individual cancer patient-basis and can come with the high risks of lacking specificity due to potential damaging of tumour-adjacent tissue by activated inflammatory cells (section 1.2.3 and 1.3). Nevertheless, blockade of CSF-1R, CCR2 and CXCR2 are currently being investigated in clinical trials based on the success in pre-clinical models (Quail and Joyce, 2013).

Neutrophils largely represent a pro-tumourigenic problem in the clinic due to the correlation of their increased presence with worse prognosis (section 1.3.5); however, they cannot be entirely depleted due to the detrimental effects of neutropenia (section 1.3.3). A mechanism of re-polarising neutrophils towards cancer toxicity rather than pro-tumourigenic activities via TGF-beta has been described (Fridlender et al., 2009) and might have clinical relevance. However, the potential of modulating neutrophil functions has to be carefully controlled because over-activated cytotoxic neutrophils represent an enormous danger for uncontrolled tissue damage (section 1.3.3), similar to potential issues with inflammation induced by Coley's toxin and the Calmette-Guerin-related vaccination. Hence, the most straightforward approach to tackle tumour-supportive activities of neutrophils by identifying and specifically blocking the molecular mediators of these functions might be the most promising and controllable therapeutic strategy and should at least be considered. Additionally, this approach theoretically does not interfere with anti-tumourigenic functions of neutrophils as their depletion or blocking their recruitment would.

We propose the novel complementary therapeutic strategy of inhibiting Alox5 function to limit metastasis of breast cancer to the lung based on our findings in pre-clinical models. We show that the Alox5 inhibitor Zileuton successfully reduced initiation of metastatic colonisation and overall metastatic burden in three lung metastatic mammary cancer mouse models, including one of spontaneous mammary tumour formation and one human breast cancer cell line (Fig. 5.2 and Fig. 5.4). This approach might hold promise to treat breast cancer patients with the risk of metastasis due to the expression of leukotriene receptors BLT2 and CysLT2 in a subset of cancer cells in the majority of examined breast cancer tissue samples (Fig. 5.6). Moreover, were observed that human cancer cells functionally responded to leukotriene stimulation *in vitro* with activation of ERK1/2 and an enrichment of LTR-expressing cells over time (Fig. 4.9 e and Fig. 4.12 a).

Our data suggest that Alox5 products facilitate metastasis establishment by stimulating the exit of disseminated cancer cells from cellular dormancy and reactivating the cell cycle. In fact, dormancy of disseminated breast cancer cells has been described to occur in patients (Braun et al., 2000, Pierga et al., 2003). Zileuton treatment might reduce the risk of cancer cells overcoming cellular dormancy by depleting a proliferation-inducing neutrophil-derived signal that might likely come along during an inflammatory response. Additionally, we show the persistence of elevated neutrophil numbers in the lung of mice even after resection of the primary mammary tumour (Fig. 3.12). This observation suggests a pro-metastatic "conditioning" of the host by the tumour and the creation of a cancer cell-supportive pre-metastatic niche in the lung that appears to be long lasting. This concept would suggest the administration of Zileuton to breast cancer patients with inoperable tumours as well as patients post-surgical tumour resection, since both groups might carry a sufficient risk for relapse due to disseminated, dormant cancer cells. However, Zileuton treatment would have to be continued long-term, if not for life. Importantly, the usage of Zileuton in the clinic poses one advantage in contrast to many other emerging cancer therapies; the safety of long-term administration of Zileuton in the clinic was already assessed and no severe side effects have been reported. In fact, it has been routinely used in the treatment of asthma since 1996 (section 1.2.3.5.1).

Eicosanoids, their producing enzymes and receptors attracted previous attention in the cancer field, however the focus lied on the prostaglandin-producing enzymes COX1 and COX2 (section 1.2.3.5.1). In regard to the use of the Alox5 inhibitor Zileuton in cancer, two clinical trials were conducted for treatment of lung cancer based on successful pre-clinical data and correlations in human patients. Zileuton administration did not show beneficial effects for advanced lung cancer patients (Edelman et al., 2008) and the assessment of Zileuton efficacy in preventing development of lung cancer in high risk patients with bronchial dysplasia is on-going (Szabo et al., 2013). It is in general difficult to comment on the outcome of studies conducted in different cancer types. However, some general concepts may be considered due to the common affected organ of the clinically assessed tumour type and our pre-clinical metastases (the lung). Our data in mouse lung metastatic breast cancer shows efficacy of Zileuton to limit initial outgrowth of metastatic cancer cells in the lung and is less effective at later stages of growth of established

metastases. These data could eventually be related to the unaltered outcome of Zileuton treatment in the clinical trial for advanced lung cancer. Following this notion, the result of the clinical trial determining the risk of actual cancer development in the lung might be more related to our findings. Nevertheless, primary lung cancer and lung metastases of breast cancer cells are clearly two different types of disease. No data for the expression of leukotriene receptors on cancer tissue is available for the aforementioned clinical trials testing the efficacy of Zileuton treatment in lung cancer. Furthermore, different cancer cell types could respond differently to the same stimulus even if it would be transmitted through the same receptor. Therefore, the effect of Zileuton on metastatic progression of human breast cancer has to be assessed in a dedicated, independent clinical trial to allow conclusions on Zileuton efficacy in the same clinical setting and cancer type where our pre-clinical observations were made in.

Our data do not suggest blocking of individual leukotriene receptors in the clinic as an alternative to Alox5 inhibition and we did not attempt this strategy in our pre-clinical models. We observe a functional overlap and the induction of the same signalling effector ERK1/2 in cancer cells being mediated by two distinct receptors for Alox5 products. Moreover, these different Alox5 products are secreted by the same cell and, hence are simultaneously available in the environment. This observation indicates a potential compensation mechanism when inhibiting only one leukotriene receptor rather than the production of the entire group of lipid signalling mediators.

In summary, we demonstrate strong evidence for the use of Zileuton as a novel treatment strategy for the clinically highly relevant metastatic progression of breast cancer. This therapeutic approach might represent a novel way to directly tackle cancer stem cell-like cells as its functionality is based on depleting the metastasis-supportive microenvironment from a factor that directly induces proliferation of cancer cells with enhanced metastatic potential. However, the exact nature and potential of human leukotriene receptor-expressing breast cancer cells has to be assessed before Zileuton can be considered to prevent leukotriene-mediated proliferation of human cancer stem cell-like cells during metastasis initiation.

6.2.3 The novel pro-metastatic neutrophil function driving lung colonisation in the context of current knowledge

6.2.3.1 Controversial role of neutrophils during metastasis – cytotoxicity and killing of metastatic cells

Mouse neutrophils have been frequently reported to have direct cytotoxic activity against cancer cells and can distinguish between target cells *in vitro*. Isolated human neutrophils showed similar potential in culture and a few clinical observations suggest anti-tumourigenic activities of neutrophils. However, neutrophils mostly have to be strongly activated either *ex vivo* or by tumour-associated factors *in vivo* to mediate cancer cell lysis. All this evidence together with contradicting pro-tumourigenic functions of neutrophils is described in section 1.3.5.

In the context of metastasis, three studies in lung-metastatic breast cancer reports pro-tumourigenic activities of neutrophils (Acharyya et al., 2012, Benevides et al., 2015, Coffelt et al., 2015), while another work characterises direct killing of lung metastatic mouse mammary cancer cells by neutrophils (Granot et al., 2011). This evidence appears to be in concert or clear contrast to our data, highlighting the controversial roles ascribed to neutrophils in cancer. Granot et al. 2011 show that isolated circulating mouse or human neutrophils are cytotoxic towards mammary cancer cells *in vitro* and direct *in vivo* evidence in the non-manipulated situation was not shown. Moreover, these neutrophils have been isolated from the blood and needed to be activated by direct exposure to cancer cells *in vitro*. It appears that this strategy might not be physiologic as cancer cells on a plate are different from those in the lung because other TME factors as well as the changes happening in cancer cells upon seeding in the lung might significantly contribute to cancer cell behaviour. Additionally, there was no difference in primary cancer burden of 4T1 cell line-derived tumours upon neutrophil ablation that is rather reminiscent of cultured 4T1 cancer cells than metastases and, unfortunately, no explanation was provided. Hence, it would probably have been the more ideal approach to isolate naturally *in vivo* activated neutrophils from the lung were they are in normal contact with cancer cells. Also, it would have been desirable to confirm the direct cytotoxic activity of neutrophils towards cancer cells *in vivo* by, for example, histological

stainings of lung sections for apoptosis or necrosis markers. In a gain-of-function approach, the authors transferred *in vitro* stimulated neutrophils into mice and suggest that they limit metastasis, however this experiment did not actually reach significance. Nevertheless, the authors clearly show that neutrophil depletion increased spontaneous metastatic burden of two mammary cancer mouse models, including the 4T1 mammary cancer cell line that we also employed for leukotriene inhibition experiments (Granot et al., 2011). Moreover, another study in several mouse cancer models, including the MMTV-PyMT mammary cancer mouse model, reported that HGF-stimulation of Met receptor expressed on neutrophils induced a cytotoxic phenotype of neutrophils that rendered them anti-tumourigenic with cancer cell killing properties (Finisguerra et al., 2015). In contrast, Benevides et al. 2015 utilised the same 4T1 mammary cancer mouse model like Granot et al. 2011 and observed a pro-tumourigenic role of neutrophils likely by secretion of CXCL1, MMP9, VEGF and TNF-alpha, despite not directly addressing metastasis (Benevides et al., 2015, Granot et al., 2011).

4T1 cells mediate a pronounced neutrophilia were the amount of neutrophils in the lung appears to largely exceed physiologic levels comparable to human patients. Granot et al. 2011 show an approximately 100-fold increase of neutrophils in the lung of tumour bearing mice, however do not give a precise number. Their immunohistochemistry data indicates that clearly more than 50% of cells present in the lung are neutrophils at late metastatic stages. Additionally, our own observations indicate that up to 60% of cells in the lung are neutrophils at late stages in this mammary cancer model (Fig. 3.8 b). Interestingly, several studies show the dose-dependency of neutrophil functions, where moderate neutrophil levels are pro-tumourigenic and excessive neutrophil frequencies show anti-tumourigenic properties (section 1.3.5). For instance, a melanoma study, where melanoma cells modulated neutrophil recruitment into the TME by different levels of IL-8 expression, clearly demonstrated this notion (Schaider et al., 2003). Hence, the presence of extraordinarily high neutrophil numbers in the lung is a likely explanation for the controversial effects of neutrophil depletion on metastatic outcome in different mouse mammary cancer models. However, Granot et al. 2011 also used a probably more physiological mouse cancer model in regard to neutrophil accumulation, the MMTV-PyMT/MMTV-cMyc model. There, they saw an increased primary tumour burden upon neutrophil deletion that they did not explain

in the context of their studies, which as the authors indicate precludes conclusions of spontaneous metastasis (Granot et al., 2011).

Additionally, this difference in neutrophil functions in individual contexts could be explained by a modulation of neutrophil activity by their microenvironment and tumour cells itself. A polarisation of neutrophil phenotypes by TGF-beta presence in the TME was elegantly demonstrated (Fridlender et al., 2009). For example, engagement of the kinase receptor Met by its ligand HGF provided by the microenvironment or the tumour was shown to be required for cytotoxic functions of neutrophils and, additionally, Met expression was dependent on tumour cell-derived TNF-alpha (Finisguerra et al., 2015). Also, Granot et al. 2011 report a dependency of cytotoxic ability of neutrophils on tumour cell-derived CCL2. Nevertheless, in light of tumour cells themselves inducing an anti-tumourigenic phenotype of neutrophils, it appears tempting to think that these cancer cells might easily downregulate or lose the expression of anti-cancer neutrophil-stimulating factors over the course of the long-term disease in humans compared to mouse models and thereby escape neutrophil cytotoxicity. Additionally, neutrophil cytotoxicity was usually assessed *in vitro* in the present literature and *in vivo* data are largely based on correlations or neutrophil injections after initial isolation (section 1.3.5). Hence, direct evidence in the non-manipulated state *in vivo* is rare. In light of several reports describing a direct lysis of non-malignant cells by neutrophils *in vitro* (Becker, 1988) and over-activated neutrophils posing a risk for tissue damage during inflammatory reactions (section 1.3.3), a proof for the specific killing activity of neutrophils towards cancer cells and not normal host cells *in vitro* and *in vivo* during metastasis would have been desirable in the studies of Granot et al. 2011 and Finisguerra et al. 2015.

Our data show how isolation and use of neutrophils *ex vivo* can lead to potential miss-interpretations of neutrophil activities in the context of cancer *in vivo*. In fact, conditioned medium from neutrophils isolated from the pre-metastatic lung showed toxicity against cancer cells and stromal lung cells and reduced the amount of viable cells approximately by half upon three-day exposure (Fig. 3.23). This *in vitro* observation stood in clear contrast to our *in vivo* data, where continuous genetic neutrophil absence using two strategies and short-term antibody-mediated neutrophil depletion reduced metastatic burden while not affecting primary mammary cancer growth (Fig. 3.3, Fig. 3.6 and Fig. 3.15). Additionally, there was

no obvious damage or toxicity of neutrophils towards lung cells in metastatic lungs of control tumour-bearing mice when examining histological sections. Hence, we hypothesised an artificial activation of neutrophils during the isolation process that led to degranulation and toxicity of the conditioned medium. However, when injecting the very same neutrophil-conditioned medium that was toxic towards cancer cells *in vitro* intravenously into the lungs of mice, no adverse effects on metastatic cancer cell presence or normal lung cell viability were observed (Fig. 3.21). In fact, neutrophil-conditioned media injection and lung neutrophil transfer experiments displayed the opposite effect and boosted lung metastasis burden with one of our two approaches reaching statistical significance (Fig. 3.22). These observations have important implications for the use of anti-tumourigenic and cytotoxic neutrophils in the clinic, as their potency appears to be sufficiently prevented *in vivo*. Our *in vivo* evidence suggests that the *in vitro* toxicity of neutrophil-conditioned medium could be regarded as a side effect of culture and, also, appears to be entirely independent from the pro-metastatic function of neutrophils that we report. Importantly, lung neutrophil conditioned medium from Alox5-deficient or *in vivo* Zileuton-treated neutrophils exhibited the same level of *in vitro* toxicity compared to wildtype lung neutrophil-conditioned medium, but the promoting effect on cancer cell sphere formation potential and *in vivo* metastatic growth competence was significantly reduced (Fig. 4.14 and Fig. 5.1). Moreover, we confirmed our findings using 4T1 mammary cancer cells, the same cancer cell line as employed in the study of Granot et al. 2011 that characterises cytotoxic effects of metastatic lung neutrophils. We did not graft 4T1 cells onto syngeneic wildtype BALB/c mice due to our observations of exceedingly high neutrophil levels in this particular mouse strain and the fact that we used GFP-labelled 4T1 cells that were rejected by wildtype mice. In FVB/N Rag1-/- mice with grafted 4T1 tumours, we still observed pronounced neutrophil levels in the lung (about 50% of lung cells), however inhibition of Alox5/leukotriene production in this model by administration of Zileuton significantly decreased metastatic burden (Fig. 5.4). Moreover, leukotriene stimulation of 4T1 cells caused ERK1/2 activation and enrichment of a the CD49f+ cancer cell subpool previously reported to have higher tumourigenic potential (Stingl et al., 2006, Yu et al., 2012) (Fig. 4.6 b, Fig. 4.10 and Fig. 4.11). These observations went hand in hand with the increased frequency of leukotriene receptor-expressing cells among CD49f+ 4T1 cells within the total population (Fig.

4.7 h). These data suggest that the novel mechanism of the proliferative support of neutrophil-derived leukotrienes towards highly potent metastasis-initiating cells seems to be preserved in the 4T1 mammary cancer model, despite the reported anti-cancer cytotoxicity of at least some neutrophil subpopulations (Granot et al., 2011). Additionally, as mentioned above, a cytotoxic role of 4T1 tumour-induced neutrophils was recently challenged (Benevides et al., 2015). In fact, Benevides at al. 2015 demonstrated that neutrophil depletion in 4T1 tumour-bearing BALB/c mice significantly reduced primary tumour burden and growth when using the same anti-Ly6G neutrophil blocking antibody like Granot et al. 2011. We only used the 4T1 cancer cell line complementary to the spontaneous MMTV-PyMT and the human MDA-231 mammary cancer models due to the issues of extremely high neutrophil mobilisation and the controversies around neutrophil function in this mouse model.

In conclusion, neutrophils might release toxic factors that kill cultured cells in a direct artificial exposure *in vitro*, however – at least in our system – cytotoxicity of these identical factors *in vivo* is somehow balanced or counteracted by the microenvironment and not sufficient *in vivo*. Hence, the observed killing effect towards cancer and stromal cell types of prolonged exposure to *in vitro* produced neutrophil-conditioned medium appears irrelevant for the *in vivo* establishment of lung metastases. However, there is convincing evidence in the literature for context-dependent anti-tumourigenic functions of neutrophils (section 1.3.5), highlighting the need for further research efforts in order to understand the complex role of neutrophils in cancer.

6.2.3.2 Pro-metastatic effects of neutrophils at the target site: immunosuppression and promoting cancer cell survival

In cancer, neutrophils have been ascribed to promote metastasis by aiding cancer cell dissemination, invasion and extravasation (section 1.3.5). We assessed if neutrophils facilitate cancer cell extravasation by intravenous injection of mammary cancer cells into the circulation *in vivo*. Mammary tumour-induced neutrophil presence or absence did not alter the ability of circulating cancer cells to infiltrate the lung (Fig. 3.13). Moreover, adoptive transfer of neutrophils or neutrophil-derived

factors also did not affect the presence of intravenously injected cancer cells in the lung shortly after cancer cell extravasation is usually accomplished (Fig. 3.21 c+f). Hence, we excluded a significant contribution of the ability of neutrophils to aid cancer cell dissemination or extravasation to the identified novel pro-metastatic function of neutrophils.

Additionally, neutrophils were shown to possess immunosuppressive properties that prevent proper anti-cancer T and NK cell responses and therefore facilitate cancer progression and metastasis (section 1.3.5). Casbon et al. 2015 reported the systemic increase of neutrophil-like or G-MDSC-like cells in the MMTV-PyMT breast cancer mouse model. These Ly6G+ neutrophils were isolated from the spleen and did suppress CD4+ and CD8+ T cell proliferation likely via ROS release *in vitro*, however these findings were not corroborated *in vivo*. Also, the authors prevented systemic neutrophilia in the MMTV-PyMT mammary cancer model by administration of a G-CSF blocking antibody or genetic deficiency for G-CSFR in the bone marrow, however they do not comment on the effects of these manipulations on cancer progression (Casbon et al., 2015). Coffelt et al. 2015 describe a pro-metastatic function of neutrophils in suppressing CD8+ T cell responses that prevents T-cell mediated killing of lung-metastatic breast cancer cells in the K14cre, $Cdh1^{flox/flox}$, $Trp53^{flox/flox}$ mammary cancer mouse model. Puzzlingly, they report a reduced metastatic burden hand in hand with reduced lung neutrophil presence in the lung when crossing their mammary tumour model with B and T cell-deficient Rag1-/- mice (Coffelt et al., 2015). These data strongly suggest other pro-metastatic functions of neutrophils that are independent from T cells. Nevertheless, our observations partially corroborate the ability of neutrophils to inhibit immune cells that can mount potent anti-cancer responses at advanced metastatic stages. Interestingly, NK cell frequencies were decreased in late stage metastatic lungs of spontaneous MMTV-PyMT cancer-bearing mice and normalised in neutrophil-deficient mice (Fig. 3.4 e). Hence, neutrophils might be involved in recruitment or survival of NK cells that have a potent ability to directly eliminate cancer cells (section 1.2.3.1) at the metastatic site and thereby positively contribute to tumourigenesis. In contrast, activation CD8+ T cells in the bone marrow, which also show powerful cancer cell killing functions (section 1.2.3.1), appeared partially enhanced in late stage mammary cancer and is prevented upon neutropenia (Fig. 3.7 b). This observation might suggest an opposing effect of

neutrophils on anti-cancer immunity. However, these data could simply be a reflection of the difference in late stage lung metastasis burden between tumour-bearing wildtype and neutrophil-deficient mice or, in regard to the bone marrow, alterations in the aberrant myelopoiesis in cancer-bearing hosts upon neutrophil loss.

In general, analysis of neutrophils during long lasting processes like cancer is hindered by limitation of available genetic markers or surface marker overlap in mice. Inhibition of the receptor involved in neutrophil recruitment, CXCR2, was frequently used to block neutrophil infiltration into peripheral tissues. This strategy might not always be effective (Fig. 3.9) and CXCR2 is expressed on many other cells than neutrophils such as endothelial cells and tumour cells (discussed in detail in section 6.3.1). Also, G-CSF deficiency or inhibition might not be sufficient to deplete neutrophils in every setting, as it causes neutropenia but does not entirely ablate neutrophils (Fig. 3.3 d). Similarly, neutrophil elastase and myeloperoxidase are suggested genetic markers for neutrophils, but are not very effective, probably due to their limited expression during neutrophil maturation (section 1.3). Hence, neutrophil studies mostly employed the Gr.1 antibody that recognises both, the Ly6C molecule on monocytes/macrophages and the Ly6G molecule on neutrophils, and might therefore be suboptimal to specifically study the role of neutrophils in cancer. Despite the proposed specificity of the anti-Ly6G (clone 1A8) antibody for neutrophils (Daley et al., 2008), it does also sufficiently deplete CD11b+Ly6G+/Ly6C-low G-MDSCs. Moreover, neutrophil-like cells with MDSC properties have been reported to accumulate in MMTV-PyMT cancer-bearing mice in a G-CSF-dependent manner (Abrams and Waight, 2012, Casbon et al., 2015). Hence, in the context of suppression of anti-cancer immunity by neutrophils and when using the anti-Ly6G antibody or G-CSF deficiency, the population of immature neutrophil-like myeloid cells that shows pronounced capacity to inhibit NK an T cell activity has to be addressed. These immature bone marrow-derived cell populations have been termed myeloid-derived suppressor cells (MDSCs) according to their predominant activity to suppress anti-cancer NK and T cell responses. MDSCs accumulate in cancer-bearing hosts and comprise a heterogeneous population containing a granulocytic, neutrophil-like (G-MDSCs, CD11b+Ly6G+/Ly6C-low) and a monocytic or macrophage-like (M-MDSCs,

CD11b+Ly6G-/Ly-6C+) component, which are present in the spleen upon tumour development. Their pro-tumourigenic actions due to potent immunosuppressive activity have been reported numerous times to be a driving force of cancer progression (section 1.2.3.2). In metastasis, the accumulation of G-MDSCs at (pre-) metastatic sites and their pro-metastatic effect mainly by protection of disseminated cancer cells from T and NK-cell mediated killing is widely accepted (Condamine et al., 2015). Interestingly, Alox5 products released by mast cells during intestinal polyposis were shown to attract MDSCs (Cheon et al., 2011) in a similar fashion like neutrophils. In light of our findings, neutrophils and neutrophil-derived Alox5 products might further enhance breast cancer metastasis to the lung by recruitment of MDSCs and thereby immunosuppression, which might also explain the reinstated NK cell frequencies upon neutropenia at late metastatic stages (Fig. 3.4 e). Nevertheless, transcriptomic analysis of cancer-associated neutrophils that are found in the proximity of tumours or neutrophils from healthy mice compared with G-MDSCs stress their differences (Fridlender et al., 2012, Youn et al., 2012, Youn and Gabrilovich, 2010). Moreover, circulating immature G-MDSCs were also clearly differentiated from more mature neutrophils in cancer settings (Sagiv et al., 2015). Hence, G-MDSCs and neutrophils are considered to be morphologically, phenotypically and functionally distinct cell populations, despite common origins, related mechanisms of their generation and shared surface markers (Dumitru et al., 2012, Gabrilovich et al., 2012) while some reports consider G-MDSC as novel, distinct phenotype of neutrophils (Pillay et al., 2013).

Our data indicate that CD11b+Ly6G+ myeloid cells, which are mobilised by G-CSF and express neutrophil elastase, play an important promoting role during the initiation phase of metastatic lung colonisation before the presence of established lung metastases. We draw this conclusion from the decreased metastasis incidence in G-CSF-deficient or neutrophil elastase-expressing cell-depleted mammary cancer mouse models (Fig. 3.3 and Fig. 3.6) together with the same observation in mammary cancer bearing mice where Ly6G+ cells were only depleted during the initiation phase of lung metastases (Fig. 3.15). Moreover, co-injection of lung neutrophils or neutrophil-derived products with cancer cells intravenously into healthy mice followed by only sporadic additional treatments enhanced metastatic burden (Fig. 3.22). These very limited time frames (one or 2.5 weeks) may already insinuate limited involvement of activated CD8+ T cells that

specifically recognise tumour-associated antigens, since antigen-specific T cell responses in mice usually need about seven days to fully develop in, for example, acute and chronic viral infections (Althaus et al., 2007). Nevertheless, we specifically assessed the relevance of a potential immunosuppressive function of neutrophils to our observed pro-metastatic neutrophil activity at very early metastatic initiation stages. Additionally, these approaches were also evaluating the potential that our anti-Ly6G antibody neutrophil-depleting strategy might target G-MDSCs by assessing their characteristic functions of immunosuppression and their mediators. Firstly, we investigated surface marker and mRNA expression of wildtype lung and PyMT-tumour-induced pre-metastatic lung Ly6G+ cells. Pre-metastatic lung cells appeared bigger and more granular than wildtype lung Ly6G+ cells, indicating their maturity (section 1.3). Also, they expressed similarly high CXCR2, increased CD31 and decreased MHC-II surface levels compared to wildtype Ly6G+ lung cells and their mRNA levels of TNF-alpha, Arg1, iNOS and VEGF were unaltered while some CCL chemokines were downregulated (Fig. 3.16). This phenotypic and transcriptional comparison of healthy lung and tumour-induced pre-metastatic lung Ly6G+ cells revealed only minor changes and in general suggests the similarity of wildtype lung neutrophils with tumour-induced Ly6G+ cells. Importantly, Arg1 and iNOS are the classic molecules to be present at increased levels and CXCR2 is usually downregulated in immature G-MDSCs compared to mature neutrophils (Dumitru et al., 2012, Gabrilovich et al., 2012, Pillay et al., 2013, Youn et al., 2012). Overall, pre-metastatic Ly6G+ cells appeared mature with segmented nuclei, pronounced expression of CXCR2 and increased CD31 levels, which are characteristics of neutrophils (Borregaard, 2010, Joyce and Pollard, 2009, Luu et al., 2003). Secondly, we performed an immune cell profile of the pre-metastatic lung of immunosufficient MMTV-PyMT cancer-bearing control IgG-treated or Ly6G+ cell-depleted mice and observed no differences in NK or cytotoxic T cell frequencies or activation status (Fig. 3.19). This observation suggests the absence of a Ly6G+ cell-dependent anti-cancer immune response in the pre-metastatic lung during the early stages of metastasis initiation. Lastly, the experiment demonstrating that Ly6G+ cell-ablation during only initial stages of metastatic lung colonisation by mammary cancer cells significantly reduced metastatic burden was performed in Rag1-/- mice (Fig. 3.15) that lack functional B and T cells. This observation functionally excludes the involvement of T cells in the

Ly6G+ cell-dependent promotion of early metastatic initiation. Of note, T cells represent the main cell type mediating anti-cancer immune responses that was reported to be suppressed by neutrophils in lung metastatic mouse mammary cancer models (Casbon et al., 2015, Coffelt et al., 2015). Overall, these series of experiments show that neutrophils play a clearly pro-metastatic role in the pre-metastatic lung of mammary cancer-bearing mice during the onset of metastatic initiation, which is independent from the suppression of anti-tumourigenic NK or T cells. Together with the very neutrophil-like morphological and transcriptional characterisation of pre-metastatic lung Ly6G+ cells, we functionally and phenotypically exclude the contribution of G-MDSCs at this early metastatic stage. Hence, we propose that pre-metastatic lung Ly6G+ cells in the MMTV-PyMT mammary cancer model are mature neutrophils, which display a novel, pro-metastatic and non-immunosuppressive function.

Additionally, neutrophil-like cells that are recruited to the mammary tumour and the metastatic lung were directly shown to have non-immunosuppressive, pro-metastatic effects. Acharyya et al. 2012 demonstrated that neutrophil-like cell-derived S100A8/A9 is protecting mammary cancer cells from chemotherapeutic agent-induced death, at least partially via p38 kinase, and promotes mammary cancer cell survival during metastasis (Acharyya et al., 2012). Importantly, this action of S100A8/A9 was entirely independent from ERK1/2 activity and, hence, constitutes a pro-metastatic activity of neutrophil-like cells that is distinct from the novel leukotriene-leukotriene receptor-ERK1/2 axis we report. Benevides et al. 2015 describe 4T1 tumour-induced neutrophils to possess properties promoting mammary tumour and metastatic growth likely via the secretion of CXCL1, MMP9, VEGF and TNF-alpha. However, the authors did not address the mechanisms behind this pro-tumourigenic activity of neutrophils due to another focus of this study (Benevides et al., 2015). We excluded the potential of MMP9 to enhance cancer stemness in sphere formation assays with mammary MMTV-PyMT cells (Fig. 4.4 f), suggesting that MMP9 is not involved in enriching or promoting mammary cancer stem cell-like cells.

Neutrophils or neutrophil-like cells such as G-MDSCs that share common surface markers certainly have the potential to prevent NK and T cell activation during the process of metastasis. The mechanisms for some immunosuppressive features of

neutrophils in lung-metastatic mammary cancer mouse models were previously reported in the literature (Casbon et al., 2015, Coffelt et al., 2015). In fact, neutrophil-mediated inhibition of NK cells or recruitment of G-MDSCs is likely to contribute to the pro-tumourigenic features of neutrophils that we observe upon genetic neutropenia throughout the metastatic process in mammary cancer models. This notion is illustrated by a neutrophil-dependent limitation of NK cell presence at advanced metastatic stages (Fig. 3.2 f). However, our data demonstrate that neutrophils have another, previously unknown pro-metastatic function that is independent from cancer cell dissemination or extravasation, immunosuppression, metastatic cancer cell survival and MMP9 (Fig. 3.13, Fig. 3.17, Fig. 3.18, Fig. 3.19 and Fig. 4.4 f). We report for the very first time that neutrophils directly aid initiation of metastatic lung colonisation of breast cancer cells by selectively inducing ERK1/2-mediated proliferation of the highly potent metastasis-initiating cancer cell subset via the secretion of Alox5 products/leukotrienes (Fig. 4.15).

6.2.3.3 Integration of neutrophil and macrophage functions aiding metastatic lung colonisation of mammary cancer cells in mice

The establishment of successful secondary tumours or metastases at distant organs is a process consisting of several steps. In general, disseminated cancer cells have to arrest within blood or lymphatic vessels at the target site, extravasate to infiltrate the tissue, survive in the foreign microenvironment and start to proliferate while preventing apoptosis to form micro- and at later stages macro-metastases (Nguyen et al., 2009, Valastyan and Weinberg, 2011) and (Fig. 1.1). Our work identified a distinct activity of neutrophils to promote metastatic lung colonisation of mammary cancer cells during the stages of initiation of cell proliferation and micro-metastasis establishment (section 6.2.3.2.).

Other myeloid cells like macrophages are also known to aid multiple steps of distant lung tissue colonisation by breast cancer cells. First of all, in murine melanoma models, tumour cell-induced clot formation within the lung vasculature recruited CD11b+CD68+F4/80+ macrophages that enhanced tumour cell survival (Gil-Bernabe et al., 2012). CD45+F4/80+ macrophages in the pre-metastatic lung of mammary cancer-bearing mice express Integrin-alpha4-beta1 that serves as a

docking site and survival signal for arriving human breast cancer cells via VCAM-1 and downstream PI3K signalling (Chen et al., 2011). Moreover, in concert with known functions of neutrophils in promoting metastatic cancer cell transendothelial migration and extravasation (Reymond et al., 2013, Wu et al., 2001) and (section 1.3.5.2.); a distinct, recruited population of macrophages that likely differentiated from inflammatory monocytes (metastasis-associated macrophages, MAMs) engaged with arriving mammary cancer cells and also aided their extravasation from blood vessels in the metastatic lung at least partially via VEGF-A (Qian et al., 2009, Qian et al., 2011). MAMs were also suggested to support growth of established metastases, since their depletion or prevention of their recruitment after seeding of mammary cancer cells into the lung reduced metastatic burden at later stages (Qian et al., 2009, Qian et al., 2011), this effect appears to be partially mediated by a Flt1/MCSF cascade in macrophages (Qian et al., 2015). Potential macrophage-mediated mechanisms supporting metastatic growth in the lung include promotion of angiogenesis, inhibitory activity towards anti-cancer immunity and activation of an inflammatory programme in macrophages (Mazzieri et al., 2011, Qian and Pollard, 2010, Qian et al., 2015) and (section1.2.3.3.). Interestingly, these CD11b+F4/80+ MAMs were shown to be recruited to the metastatic lung by metastatic mammary cancer cell-derived CCL2, in contrast to CD11b+Ly6G+ neutrophils that we and others detected in pre-metastatic lungs before cancer cell arrival (Fig. 3.14 and section 6.3.1). In fact, CCR2+ inflammatory monocytes migrated to the metastatic lungs instead of the primary mammary tumour that expresses low levels of CCL2 and these monocytes further differentiated to cancer cell extravasation- and survival-promoting MAMs (Qian et al., 2011). Further, CCL2-CCR2 signalling-induced chemokine secretion (CCL3) by these macrophages mediated the retention of MAMs in the lung and deficiency of CCL3 or its receptor CCR1 decreased MAM numbers in the lung of mammary cancer-bearing mice likely by limiting their interactions with metastatic cancer cells (Kitamura et al., 2015). Importantly, recruited CD11b+ MAMs appear to be distinct from resident CD11c+ (alveolar) lung macrophages, because only ablation of CD11b+, but not CD11+ macrophages reduced extravasation and metastatic growth of breast cancer cells in the lung (Qian et al., 2009, Qian and Pollard, 2010). Additionally, it has to be mentioned that the currently available tools for *in vivo* macrophage depletion might also target CD11b+Ly6G+ neutrophils. The mainly

used strategies to interfere with macrophages in the mentioned studies consist of CD11b-DTR and clodronate-containing liposome-mediated ablation, Mac1 and MCSF-deficient mice or administration of CCL2-blocking antibodies. Importantly, Qian et al. 2009 also observed the increase of CD11b+Gr.1+ neutrophils in the metastatic lung of mammary cancer-bearing mice, however the effect of macrophage-depletion on lung neutrophil levels was not assessed in these studies (Gil-Bernabe et al., 2012, Qian et al., 2011, Qian et al., 2009).

Here, we report a novel function of neutrophils to promote lung metastasis of mammary cancer cells by leukotriene-mediated promotion of ERK1/2-dependent proliferation during very early stages of lung colonisation. Despite known functions of macrophages at the primary tumour site to promote cancer cell proliferation via secretion of growth factors (Lewis and Pollard, 2006), direct evidence for MAM-mediated support of metastatic mammary cancer cell proliferation during initial stages of metastatic establishment are missing. This notion together with the timing of neutrophil increase in the pre-metastatic lung prior to the described macrophage recruitment suggests likely functionally and temporally distinct activities of neutrophils and macrophages to foster metastasis. Nevertheless, these myeloid cell types might secrete the same or similarly acting factors (such as leukotrienes) that would likely mediate similar cancer cell responses and could act in concert or during different stages (Fig.6.1). Further research will be necessary to shed light on potentially distinct or complementary functions of neutrophils and macrophages during lung metastasis establishment in mammary cancer-bearing hosts.

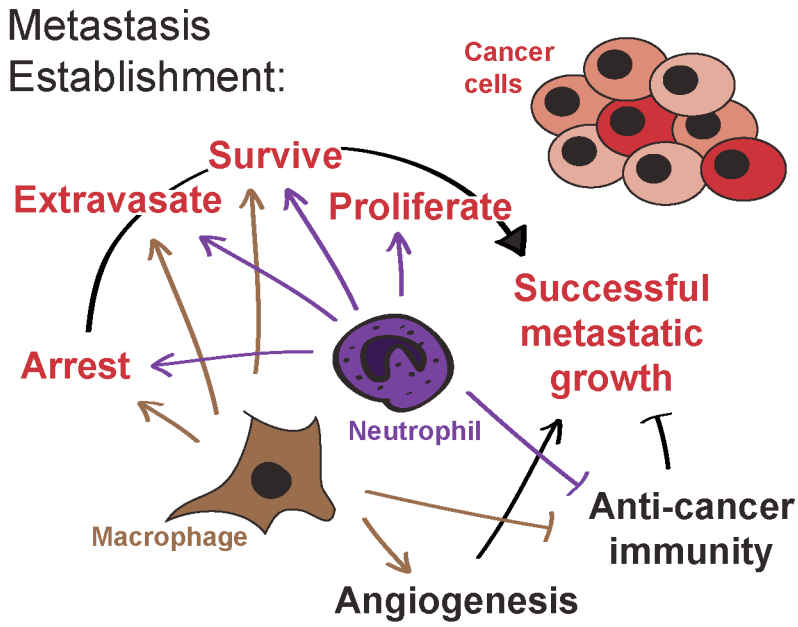

Figure 6-1 Overview on main pro-metastatic activities of macrophages and neutrophils during early metastatic colonisation
Schematic representation of the stages cancer cells likely undergo to establish metastatic nodules (red) and their support by macrophages (brown) or neutrophils (purple).

6.2.3.4 Restoring normal neutrophil levels during chemotherapy in the clinic

Neutrophilia represents an independent risk factor in multivariate analyses for poor clinical outcome in many cancer types including breast cancer. Neutropenia is a frequent issue arising during the treatment of cancer patients with chemotherapeutic agents. Normal neutrophil counts are routinely restored by the administration of recombinant G-CSF and reduce the risk of non-cancer related complications like the susceptibility to infections and, hence, appear to extend the life of cancer patients (sections 1.3.3 and 1.3.5). This notion should not necessarily be influenced by our and other data demonstrating a pro-tumourigenic function of neutrophils, despite several reports that patients developing neutropenia during chemotherapy have a better cancer-related prognosis (Cameron et al., 2003, Di Maio et al., 2005, Shitara et al., 2009, Eskander and Tewari, 2012, Tewari et al., 2014). In our pre-clinical MMTV-PyMT mammary cancer mouse model, complete neutrophil depletion strongly reduced the incidence of lung metastasis (Fig. 3.3, Fig.

3.6 and Fig. 3.15). Also, in the clinic, G-CSF administration was correlated with cancer progression (Aliper et al., 2014, Voloshin et al., 2011). However, the likelihood of a life-threatening infection in mouse experiments in isolated facilities that last several weeks or months is far lower than that for cancer patients who are in continuous contact with potential pathogens for years. In the clinic, enhancing the risk of cancer progression by increasing neutrophil levels has to be counterbalanced with additional, possibly life-threatening side effects like the inability to fight infections. Additionally, restoring normal neutrophil counts in cancer patients does not necessarily mean causing drug-induced neutrophilia. In fact, G-CSF is only administered to neutropenic cancer patients in controlled doses and usually normalises neutrophil counts but does not continuously enhance their presence above the physiological baseline (Morstyn et al., 1989). Therefore, the neutrophil levels of cancer patients receiving G-CSF with chemotherapy are unlikely to represent the ones of a patient with tumour-induced neutrophilia that constitutes a worse prognosis.

In fact, this issue of balancing neutrophil counts to an acceptable but not cancer-promoting level highlights the potential power of our suggested novel therapeutic strategy for the limitation of breast cancer metastasis. Zileuton specifically blocks a pro-tumourigenic function of neutrophils likely without interfering with neutrophil presence or their additional essential functions. Moreover, leukotriene blockade in long-term use in the clinic was proven to be safe for asthmatic patients (section 6.2.2).

6.3 Outlook and arising questions

6.3.1 Neutrophil recruitment to the pre-metastatic site

Our study confirmed systemic mobilisation and lung accumulation of neutrophils in additional pre-clinical mouse models of lung-metastatic breast cancer complementing previous reports (Acharyya et al., 2012, Casbon et al., 2015, Coffelt et al., 2015, Granot et al., 2011). Coffelt et al. 2015 conduced an entire study dedicated to uncover the mechanism for neutrophil accumulation in the lung of K14cre, $Cdh1^{flox/flox}$, $Trp53^{flox/flox}$ breast cancer-bearing hosts. They describe that mammary cancer cell-derived IL-1-beta activates circulating gamma-delta T cells to increase IL-17 production, which in turn upregulates systemic G-CSF levels and causes neutrophil expansion. In fact, tumour-bearing K14cre, $Cdh1^{flox/flox}$, $Trp53^{flox/flox}$ crossed with T and B cell-deficient Rag1-/- mice showed reduced systemic IL-17 and G-CSF levels in concert with decreased presence of neutrophils in the lung (Coffelt et al., 2015). Moreover, Benevides et al. 2015 corroborated the importance of IL-17 in supporting mammary tumour progression by promoting neutrophil influx to the metastatic site. The authors describe that tumour cell derived-IL-6 and CCL20 induce the recruitment of T lymphocytes to the primary mammary tumour and their differentiation towards an IL-17-secreting phenotype (Benevides et al., 2015). The IL-17/G-CSF axis is central to regulate neutrophil mobilisation during infection and other inflammatory processes, where tissue-resident macrophages and mast cells were shown to release IL-23 which stimulates IL-17 production by T cells (Borregaard, 2010). However, we observe that neutrophils readily infiltrate the pre-metastatic and metastatic lung of T and B cell-deficient Rag1-/- mice when grafted with MMTV-PyMT, 4T1 and MDA-MB-231 mammary tumours (Fig. 3.17 c, Fig. 5.2 b and Fig. 5.4 b+f), in contrast to K14cre, $Cdh1^{flox/flox}$, $Trp53^{flox/flox}$ Rag1-/- mice (Coffelt et al., 2015). This evidence indicates the existence of T cell-independent mechanisms that lead to neutrophil accumulation in the lung of mammary tumour-bearing hosts, at least for the three mouse models that we employed. However, systemic expansion of neutrophils by elevated levels of G-CSF does not entirely clarify the preferential accumulation of neutrophils in the pre-metastatic lung of mammary tumour-bearing mice before the arrival of disseminated cancer cells. A likely explanation would be the fact that the

lung is one of the very few organs where neutrophils are present in significant amounts at steady state (Kolaczkowska and Kubes, 2013, Kreisel et al., 2010, Kruger et al., 2015) and, accordingly, their presence would increase predominantly at this sites upon neutrophilia independent of the lung becoming the site of metastasis. Additionally, tumour-induced G-CSF increase could directly mediate the expansion and proliferation of this lung-resident neutrophil pools. However, lung-resident neutrophil populations would have to be replenished by radiosensitive bone marrow-derived cells based on our observation of the pronounced presence of CXCR2-/- neutrophils in the lung of mammary tumour-bearing mice that were reconstituted with CXCR2-/- bone marrow (Fig. 3.9).

Interestingly, another study is partially addressing elevated neutrophil presence in the metastatic (but not the pre-metastatic) lung of mammary cancer-bearing mice and implicated CXCR2 ligands-mediated neutrophil recruitment (Acharyya et al., 2012). The authors report cancer cell-derived CXCR2 ligands CXCL1/2-induced attraction of neutrophils to primary mammary tumours as well as metastatic lungs. They clearly demonstrate that CXCL1/2-depleted cancer cells seeded into the mammary gland or the lung show diminished growth correlating with reduced neutrophil infiltration in two different lung metastatic mammary cancer models. Additionally, they show that systemic CXCR2 inhibition enhances the effect of chemotherapy in pre-clinical mouse models while CXCR2 blockade itself did not inhibit mammary tumour growth. CXCL1 and CXCL2, the murine homologs of IL-8, are strong chemoattractants for neutrophils and CXCR2 deletion is commonly associated with limiting neutrophil infiltration into peripheral tissues even in cancer settings (Gong et al., 2013, Jamieson et al., 2012, Keane et al., 2004). Based on these observations, Acharyya et al. 2012 hypothesise a direct cancer cell-derived CXCL1/2-mediated recruitment of pro-tumourigenic neutrophils via engagement of their CXCR2 receptor. Interestingly, our data demonstrate that the neutrophil accumulation in the metastatic lung is independent from neutrophil-expressed CXCR2. We detected unaltered levels of CXCR2-deficient neutrophils in the metastatic lungs of mammary tumour-bearing mice compared to wildtype controls, however using a different lung metastatic mammary cancer model (Fig. 3.9). Moreover, cancer cells in the mammary tumour itself express CXCL1/2 that is attracting neutrophils (Acharyya et al., 2012), but we found only minimal neutrophil presence at the primary site (Fig. 3.1 e). This evidence raises the interesting

possibility of the existence of another (unknown) CXCL1/2 receptor on neutrophils in a cancer setting, eventually a result of the aberrant myelopoiesis in cancer-bearing hosts. Alternatively, there might be an additional cellular player that is responsive to cancer cell-derived CXCL1/2 and facilitates neutrophil recruitment to the lung, but less to the mammary gland. In fact, Acharyya et al. 2012 did not directly investigate if cancer cell-derived CXCL1/2-mediated neutrophil recruitment was dependent on CXCR2 expression by neutrophils in mammary cancer mouse models. Also, the authors did not confirm reduced neutrophil frequencies in mammary tumours or the lung when systemically inhibiting CXCR2. CXCR2 is expressed on numerous other, non-haematopoietic cells and correlates with poor prognosis when present on tumour cells that could additionally account for the observed effects on neutrophil infiltration or cancer response to chemotherapy when systemically blocking CXCR2 (Acharyya et al., 2012, Ijichi et al., 2011, Mestas et al., 2005, Saintigny et al., 2013, Warner et al., 2008). The CXCL1/2-mediated chemoattraction via neutrophil-expressed CXCR2 might be crucial for neutrophil recruitment by cancer cells present in several different organs, however does not seem to directly mediate neutrophil influx in the metastatic lung of 4T1 tumour-bearing mice.

Our findings that pro-metastatic neutrophils accumulate in the pre-metastatic lung prior to disseminated cancer cell infiltration and promote metastasis initiation might indicate that neutrophils represent an initial part of a pre-metastatic niche. Elevation of neutrophil levels that induce cancer cell proliferation could be a means of the primary tumour to create a more hospitable environment at the pre-metastatic organ, hence paving the way for disseminated cancer cells. However, enhanced neutrophil presence in the distant metastatic organ might be a coincidental event, since we also observed lung neutrophil accumulation in non-metastatic tumour-bearing mice (Fig. 3.11). This observation was not corroborated when grafting the non-metastatic mammary cancer cell line 67NR (Benevides et al., 2015), however that is likely a reflection of the slow *in vivo* growth of this cell line and the analysis of neutrophil mobilisation in this study before the grafted mammary tumour reached a sufficient size. Nevertheless, changes in the pre-metastatic environment through primary cancer cell or stromal cell-derived factors might be the cause for neutrophil infiltration. Known niche-inducing factors include soluble mediators like cytokines and chemokines as well as exosomes that contain a plethora of factors (Oskarsson

et al., 2014, Plaks et al., 2015, Psaila and Lyden, 2009, Quail and Joyce, 2013). For example, VEGF, TNF-alpha and TGF-beta are known to be secreted by mammary tumours and caused production of S100A8 and S100A9 by pre-metastatic lung endothelial and myeloid cells (Hiratsuka et al., 2006). S100A8/A9, in turn, are potent inducers of neutrophil chemotaxis (Ryckman et al., 2003) and could mediate neutrophil recruitment to the lung, apart from their described role in promoting mammary cancer cell survival when secreted from neutrophil-like cells in the primary tumour (Acharyya et al., 2012). Moreover, ECM remodelling by upregulation of MMP9 in the pre-metastatic microenvironment (Hiratsuka et al., 2002) might cause a subtle tissue damage response by resident mast cells and macrophages that facilitate neutrophil recruitment (Kim and Luster, 2015, Kolaczkowska and Kubes, 2013). In fact, lung macrophages have been shown to be activated in mammary-cancer bearing hosts prior to cancer cell arrival (Chen et al., 2011). Metastatic colonisation-preceding activation of tissue resident fibroblasts by soluble or exosome-transported factors might also promote lung neutrophil attraction. Activated fibroblasts are known to attract bone marrow-derived VEGFR1+ haematopoietic progenitor cells to the metastatic site (Kaplan et al., 2005) and fibroblasts can mediate neutrophil recruitment to the lung via LTB4, IL-8 or G-CSF release upon inflammatory insults like bleomycin or cigarette smoke (Sato et al., 1999, Takamizawa et al., 1999). Granot et al. 2011 did not directly address this question of neutrophil recruitment to the pre-metastatic lung in mammary cancer-bearing hosts, although they did provide some insights. They excluded 4T1 cancer cell-derived CCL2 as a neutrophil chemoattractant to the metastatic lung in mice carrying 4T1 mammary tumours since cancer cell-specific knock down of CCL2 did not alter neutrophil accumulation in the lungs. Also, the involvement of stromal cell-derived CCL2 in the recruitment of neutrophils appears unlikely, since the authors shown that cancer cell-derived CCL2 is required for functional properties of neutrophils and that these are not induced by the stroma (Granot et al., 2011). However, CCL2 was also reported to facilitate recruitment of monocytes and neutrophil-like myeloid-derived suppressor cells (G-MDSCs) to tumour or metastatic sites (Qian et al., 2011, Talmadge and Gabrilovich, 2013, Wesolowski et al., 2013), stressing the difference between these cell types and neutrophils.

In summary, the accumulation of neutrophils in the pre-metastatic lung in breast cancer mouse models emerges as a very complex process. It appears connected to a systemic IL-17/G-CSF axis-induced neutrophil mobilisation (Benevides et al., 2015, Coffelt et al., 2015), but is likely also mediated by a combination of additional mechanisms including CXCL1/2 at least at later metastatic stages (Acharyya et al., 2012).

6.3.2 Leukotriene receptors as functional cancer stem cell markers?

A common definition of stem cells is their ability to self-renew and differentiate into multiple lineages that are distinct from their current state. This notion led to the proposal of the existence of stem cells within tumours that showed the unique potential to re-initiate the entity of a tumour even from a single cell level onwards. However, “stemness” in cancer appears to be a dynamic cell state and not comprised within rigid cell populations. Also, monitoring the key feature of stemness, the ability to self-renew, appears very challenging within tumour tissues. Hence, the term “cancer stem cell” has to be used with caution (section 1.1.4). In fact, the most accepted assay to determine stem cell potential of cancer cells is their competence to initiate an entire tumour upon xenografting them into immunocompromised mice (Kreso and Dick, 2014). We show that mouse BLT2 and/or CysLT2-expressing spontaneous MMTV-PyMT tumour cells have enhanced competence to grow a mammary tumour upon injection of 10^3 cells into immunocompromised Rag1-/- mice compared to leukotriene receptor-deficient cells (Fig. 4.8 b+c). Also, leukotriene receptor-expressing MMTV-PyMT cells show enhanced sphere-forming ability (Fig. 4.8 a). Moreover, leukotriene treatment of the total cancer cell population is increasing the frequency of cancer cell subpools that are enriched for leukotriene receptor-expressing cells as well as the overall competence of cancer cells for sphere formation, tumour and metastasis initiation (Fig. 4.6, Fig. 4.7 and Fig. 4.12 a). These results indicate that leukotriene receptor-expressing mouse cancer cells have a functional growth/proliferation advantage over leukotriene receptor-deficient cells and at least contain a population of cancer cells with increased tumour-formation potential. However, additional studies are necessary to confirm leukotriene receptor-expressing cells as tumour-initiating cells.

We attempted xenografting experiments of BLT2 and/orCysLT2-expressing 4T1 mammary cancer cells, however this cancer cell line was exceedingly aggressive and even as little as 10 transplanted cells always formed a mammary tumour upon transplantation independent from their leukotriene receptor expression. Additionally, the tumour formation potential of individually grafted leukotriene receptor-expressing cancer cells compared to leukotriene receptor-deficient cells has to be tested according to current definitions of tumour-initiating cells (Kreso and Dick, 2014). Given the difficulty of single cells derived from solid tumours to grow after injection into mice, this approach would likely require more immunocompromised hosts (Rongvaux et al., 2013). However, this strategy might actually be counterproductive and diminish the authentic potential of these cancer cells, as we propose the leukotriene receptor-mediated growth advantage to be dependent on the interaction with the microenvironment/neutrophils. This notion might also tackle the general view of intrinsic potency of cancer stem cells or tumour-initiating cells (Kreso and Dick, 2014) that mostly ignores the importance of the microenvironment regulating the potential of a cell, especially in cancer (Malanchi, 2013, Plaks et al., 2015, Quail and Joyce, 2013).

Another related question is if leukotriene receptor expression would identify a population of metastasis-initiating cells. This idea is very attractive, due to BLT2 and CysLT2 being enriched but not restricted to previously functionally defined CD90+ MICs (Fig. 4.7 c-e) and both leukotriene receptors providing increased responsiveness to neutrophil-derived proliferation-promoting signals (Fig. 4.9, Fig. 4.10 and Fig. 4.11). Unfortunately, proof of this hypothesis is missing. The fact that leukotriene receptor-expressing MMTV-PyMT cells are a very small subpopulation (Fig. 4.7 a-b) represents a key technical caveat and all trials to isolate and intravenously inject a sufficient cell number ended up failing. We also tried the intravenous injection of up to $4x10^4$ isolated leukotriene receptor-expressing 4T1 cells, which are more aggressive and metastatic. In this experiment, we found that 0/6 leukotriene receptor-deficient and 1/6 leukotriene receptor-expressing cells established metastases, which is again not conclusive enough. However, the intravenous injection of cancer cells into healthy mice represents a test for their intrinsic metastasis initiation potential without taking the influence of a pre-metastatic niche into account (Malanchi, 2013), which we demonstrated to be the

crucial mechanism how leukotriene receptors confer a growth advantage to metastasis-initiating cells.

The tumour or metastasis initiation potential of spontaneous human LTR+ breast cancer cells was to our knowledge never reported to be tested. Expression of leukotriene receptors including BLT2 and CysLT2 was demonstrated on subsets of many different types of human cancer tissues and cancer cell lines including breast cancer, frequently even in increased levels. Interestingly, expression of CysLT1 correlated with worse prognosis while cancer cell CysLT2 expression indicated better outcome (section 1.2.3.5.1). However, it largely remains to be seen if leukotriene receptor expression in different combinations correlates with metastatic prognosis for breast cancer patients. We confirmed that both leukotriene receptors, BLT2 and CysLT2, are expressed on cancer cells of more than 50% of tested primary breast cancer and lymph node metastases samples with largely varying intensity and frequency of positive cells (Fig. 5.6). This observation confirms phenotypic differences between human cancer cell subpopulations according to leukotriene receptor expression, however the functional consequence is largely unknown. Of note, BLT2- and CysLT2-expressing human cancer cells of the human breast cancer cell line MDA-MB-231 are enriched within the CD44-high cell and Aldefluor+ cell subset (Fig. 4.7 f-g) that was previously reported to exhibit cancer stem cell-like properties (Al-Hajj et al., 2003, Sheridan et al., 2006). *In vitro*, leukotriene treatment of the human MDA-MB-231 cell line caused ERK1/2 phosphorylation and enrichment of LTR+ cells among the total cancer cell population (Fig. 4.9 e and Fig. 4.12 a), confirming the responsiveness of human cells to a leukotriene-mediated proliferation signal. In xenograft assays, MDA-MB-231 cells displayed a generally very low tumour initiation capacity. 0/6 LTR- cells and 1/6 LTR+ cells formed tumours after five months upon injection of 10^3 cells into the mammary gland, suggesting a tendency that is not yet convincing.

To eventually refine a cancer cell subset with enhanced cancer stem cell/tumour-initiating ability by leukotriene receptor expression, the individual potential of BLT2+ and CysLT2+ cancer cells should to be assessed. *In vitro*, engagement of either receptor on mammary cancer cells by its respective ligand is sufficient to cause ERK1/2 phosphorylation (Fig. 4.9, Fig. 4.10 and Fig. 4.11), suggesting a functional commonality that made us assess BLT2 and CysLT2 receptors and their stimulation jointly in our metastasis study. Our *in vivo* experiments so far assessed

the tumour initiation potential of a cancer cell population expressing at least one leukotriene receptor and, therefore, contained single BLT2+, single CysLT2+ and double leukotriene receptor-expressing cells (Fig. 4.8). Preliminary experiments xenografting BLT2+ or CysLT2+ MMTV-PyMT cancer cells suggested a clear tumour formation advantage of BLT2+ over BLT2- cells, which was not as pronounced for CysLT2. Additionally, testing the tumour initiation potential of double BLT2+ and CysLT2+ MMTV-PyMT cancer cells versus leukotriene receptor-deficient cells might provide an additional approach to refine markers for highly potent mammary cancer cells.

At this point, we can conclude that leukotriene receptor-expressing cancer cells have a higher potency for tumour formation compared to cells lacking leukotriene receptors at least in the MMTV-PyMT mammary cancer mouse model. However, it remains to be established if leukotriene receptor-expressing cancer cells have an intrinsically higher tumour formation potential or, more likely, if this is at least partially mediated by the crosstalk with the microenvironment/neutrophils, which is the crucial functional relevance of leukotriene receptor expression during metastasis. In any case, normal and some types of tumour or metastasis-initiating cancer cells are known to depend on a microenvironmental niche that regulates their maintenance and differentiation (Li and Neaves, 2006, Oskarsson et al., 2014, Plaks et al., 2015). A main hallmark of cancer cells is their ability to proliferate (Hanahan and Weinberg, 2011), however cancer stem cells or tumour-initiating cells are frequently in a rather quiescent state (Plaks et al., 2015). Leukotriene receptors appear to functionally provide highly potent cancer stem cell-like cells with the ability to respond to microenvironmental, neutrophil-derived signals inducing proliferation in challenging situations, such as during cancer cell dormancy, metastatic colonisation or relapse after therapy. Hence, leukotriene receptor-expressing cells likely define a more potent population of mammary cancer cells, despite the need of further experimental proof.

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