INTERACTIONS BETWEEN MCF-7 AND MDA-MB-231 BREAST CANCER CELL LINES AND NEUTROPHILS

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Sherina Holland

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ABSTRACT

The tumour micro-environment (TME) has an essential role in tumour development and progression. Immune cells recruited to the site of the tumour secrete soluble factors such as proteinases, growth factors, survival factors and angiogenic factors into the TME. The secretion of these factors is up-regulated via inflammatory mediators secreted by tumour cells, resulting in a pro-malignant cycle between the cancer and immune cells. A greater understanding of the molecular mechanisms underpinning these interactions is required, as this will assist towards identifying potential new drug targets for cancer and ultimately, will aid in the long-term development of targeted and effective treatments for breast cancer and MBC.

The activities of certain immune cells, such as tumour associated macrophages, have been reasonably well characterised in cancer, however, until recently, less was known regarding the role of neutrophils in tumour progression. The goal of the research described in this thesis was to determine whether soluble factors secreted by breast cancer cells might alter the phenotype or lifespan of neutrophils. The latter may allow neutrophils sufficient time to participate in activities within the TME that may either help or hinder tumour progression, while soluble factors released by the neutrophils might influence the invasiveness of breast cancer cells.

To investigate whether soluble factors released by breast cancer cells could delay neutrophil apoptosis, neutrophils were cultured in conditioned medium (CM) prepared from highly metastatic MDA-MB-231 or poorly metastatic MCF-7 cells. Flow cytometry experiments showed a delay in apoptosis for neutrophils cultured in MDA-MB-231 CM, but not MCF-7 CM. Quantitative RT-PCR was used to measure neutrophil mRNA expression of pro- versus anti-apoptosis peptides; neutrophils incubated in MDA-MB-231 CM, but not MCF-7 CM, demonstrated a significantly higher expression of the anti-apoptosis peptide BCL2 (A1) and significantly lower expression of the pro-apoptosis peptide BAK compared to control. Western blots showed extensive caspase-8 activation for neutrophils cultured in MCF-7 CM, consistent with apoptosis, whilst neutrophils cultured in MDA-MB-231 CM showed little activation of caspase-8, indicating low levels

of apoptosis. The soluble factor contained within the MDA-MB-231 CM, responsible for the delay in neutrophil apoptosis was found to be heat stable and have a molecular weight of between 10-100kDA. Prostaglandin E_2 (PGE_2) was identified as a potential candidate molecule, as it is a heat stable lipid, and when bound to plasma proteins, fits the molecular weight criteria. In addition, neutrophils cultured with 10μM native or heat treated PGE_2 demonstrated a delay in apoptosis, however, this was to a lesser extent compared to neutrophils cultured in MDA-MB-231 CM. Cycoloxygenase-2 (COX-2), the enzyme responsible for PGE_2 synthesis, was shown to be expressed in MDA-MB-231 cells but not MCF-7 cells, which is in agreement with the results demonstrating a delay in apoptosis for neutrophils cultured in MDA-MB-231 CM but not MCF-7 CM.

Freshly isolated human neutrophils, obtained from the peripheral blood of healthy volunteers, cultured in MDA-MB-231 or MCF-7 CM for 7hrs were not polarised toward a pro or anti-tumour phenotype, as determined via the expression of ICAM-1 and MMP-9. Finally, to investigate whether neutrophils could influence the process of EMT and alter the migration of breast cancer cells, neutrophils were indirectly cultured, via transwell plates, with MDA-MB-231 or MCF-7 cells. Neutrophils were not found to enhance the migration of the cancer cells, as determined via a wound scratch assay. Likewise, neutrophils were not shown to influence the process of EMT in the cancer cells, as determined by changes to cell morphology or the expression of EMT Markers.

DEDICATION

This PhD is dedicated to the memory of my grandmothers, Angela Laity and Ruth James, and my aunt, Ruth Shorney.

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5-FU: 5-Fluorouracil

11-deoxyPGE$_{1:}$ 11-deoxy prostaglandin E$_1$

a2V-ATPase: a2 isoform V-ATPase

AA: Arachidonic acid

ACAMP: Apoptotic cell associated molecular patterns

ADCC: Antibody dependant cell-mediated cytotoxicity

APAF1: Apoptotic protease activating actor 1

APRIL: A proliferating-inducing ligand

ARG1: Arginase 1

ATP: Adenosine triphosphate

BAD: Bcl-2-associated death promoter

BAFF: B cell activating factor

BAK: Bcl-2 homologous antagonist killer

BASO: Basophil

BAX: Bcl-2-associated X protein

BCG: Bacillus Calmette-Guérin

BCL-2: B cell lymphoma

BCL-2 (A1): Bcl-2-related protein A1

BCL-XL: B-cell lymphoma-extra-large

BID: BH3 interacting-domain death agonist

BRCA1: Breast cancer 1 gene

BRCA2: Breast cancer 2 gene

BSA: Bovine serum albumin

BV8: Prokinecitin-2

CCL2: Chemokine ligand 2

CCL3: Chemokine ligand 3

CCL4: Chemokine ligand 4

CD15: 3-fucosyl-N-acetyl-lactosamine

CDK: Cyclin dependant kinase

cDNA: Complementary deoxyribonucleic acid

CM: Conditioned medium

COX-2: Cylooxygenase-2

CXCL1: Chemokine (C-X-C motif) ligand 1

CXCL2: Chemokine (C-X-C motif) ligand 2

CXCL5: Chemokine (C-X-C motif) ligand 5

CXCR2: Interleukin 8 receptor, beta

DAMP: Damage associated molecular pattern

DC: Dendritic cell

DCIS: Ductal carcinoma in situ

DISC: Death-inducing complex

DMEM: Delbucco's Modified Eagle Medium

DMSO: Dimethyl sulphoxide

DNA: Deoxyribonucleic acid

DNAse: Deoxyribonuclease

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EGFR: Epidermal growth factor receptor

ELISA: Enzyme-linked immunosorbent assay

EMT: Epithelial-mesenchymal transition

EnC: Endothelial cell

EO: Eosinophil

EpC: Epithelial cell

ER: Estrogen receptor

ERK: Extracellular signal-regulated kinases

FAS: FAS receptor

FBS: Foetal bovine serum

FGF: Fibroblast growth factor

FITC: Fluorescein isothiocyanate

FSC-A: Forward scatter area

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

G-CSF: Granulocyte-colony stimulating factor

GC/MS: Gas chromatography – Mass spectroscopy

GM-CSF: Granulocyte-macrophage colony-stimulating factor

G-MDSC: Granulocytic-myeloid derived suppressor cell

HA: Hyaluronic acid

HCL: Hydrochloric acid

HDN: High density neutrophil

HER-2: Human epidermal growth factor receptor 2

HGF: Hepatocyte growth factor

HIF-1: Hypoxia inducible factor 1

HIV: Human immunodeficiency viruses

HLA: Human leucocyte antigen

HMGB1: High mobility group box one protein

HNSCC: Head and neck squamous cell carcinoma

HPLC: High performance liquid chromatography

HRP: Horse radish peroxidase

HSC: Hematopoietic stem cell

HUVEC: Human umbilical vein endothelial cell

IAP: Inhibitor of apoptosis protein

ICAM-1: Intracellular adhesion molecule 1

IDC: Invasive ductal carcinoma

IgG: Immunoglobin G

IL-1β: Interleukin-1 beta

IL-4: Interleukin-4

IL-6: Interleukin-6

IL-8: Interleukin-8

IL-12: Interleukin-12

IL-17: Interleukin-17

ILC: Invasive lobular cancer

IFN- α: Interferon alpha

IFN- β: Interferon beta

IFN- γ: Interferon gamma

INOS: Inducible nitric oxide synthase

IRS-1: Insulin receptor substrate-1

kDa: Kilodalton

KRPG: Krebs ringer phosphate glucose

LC/MS: Liquid chromatography – Mass spectroscopy

LDN: Low density neutrophil

LHX2: LIM-homeobox gene 2

LPS: Lipopolysaccharide

MACS: Magnetic activated cell sorting

MBC: Metastatic breast cancer

MC: Mast cell

MCL-1: Induced myeloid leukaemia cell differentiation protein

MDSC: Myeloid derived suppressor cell

MET: Mesenchymal-epithelial transition

MF: Macrophage

MHC: Major histocompatibility complex

MICA: MHC class 1 chain-related protein A

MICB: MHC class 1 chain-related protein B

MMP-8: Matrix metalloproteinase-8

MMP-9: Matrix metalloproteinase-9

MOMP: Mitochondrial membrane permeabilization

MPO: Myeloperoxidase

mRNA: Messenger ribonucleic acid

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

MW: Molecular weight

MWCO: Molecular weight cut off

NE: Neutrophil elastase

NET: Neutrophil extracellular trap

NK: Natural killer cell

NLR: Neutrophil to lymphocyte ratio

NO: Nitric oxide

NOXA: Phorbol-12-myristate-13-acetate-induced protein 1

NSCLC: Non-small cell lung carcinoma

NST: No special type

OSM: Oncostatin M

PAMP: Pathogen associated molecular pattern

PBS: Phosphate buffered saline

PCNA: Proliferating cell nuclear antigen

PCR: Polymerase chain reaction

PDGFR: Platelet derived growth factor receptor

PGE_2: Prostaglandin E_2

PGE_2: Prostaglandin F_2

PGH_2: Prostaglandin H_2

PI: Propidium iodide

PICD: Phagocytosis-induced cell death

PMA: Phorbol 12-myristate 13-acetate

PMN: Polymorph nuclear cell or neutrophil

PR: Progesterone receptor

PVDF: Polyvinylidene fluoride

qRT-PCR: Quantitative reverse transcription polymerase chain reaction

RBC: Red blood cell

RCF: Relative centrifugal force

RNA: Ribonucleic acid

RNAse: Ribonuclease

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

RPM: Revolutions per minute

RPMI: Roswell park memorial institute medium

RT: Room temperature

RT-PCR: Reverse transcription polymerase chain reaction

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM: Standard error of the mean

SLUG: Snail family transcriptional repressor 2

SNAIL: Snail family transcriptional repressor 1

SSC-A: Side scatter area

TAM: Tumour associated macrophage

TAN: Tumour associated neutrophil

TEMED: Tetramethylethylenediamine

TGF-β: Transforming growth factor - beta

Th1: T helper cell 1

Th2: T helper cell 2

Th17: T helper cell 17

TIL: Tumour infiltrating lymphocyte

TIN: Tumour infiltrating neutrophil

TME: Tumour microenvironment

TNFα: Tumour necrosis factor-α

TRAIL: TNF-related apoptosis-inducing ligand

TRAIL-R1: TNF-related apoptosis-inducing ligand (TRAIL) receptor 1

TRAIL-R2: TNF-related apoptosis-inducing ligand (TRAIL) receptor 2

VEGF: Vascular endothelial growth factor

VEGF-A: Vascular endothelial growth factor alpha

XIAP: X-linked inhibitor of apoptosis protein

ZEB1: Zinc Finger E-Box Binding Homeobox 1

ZEB2: Zinc Finger E-Box Binding Homeobox 2

CHAPTER 1

INTRODUCTION & LITERATURE REVIEW

1.1 Introduction

The average human being is comprised of at least 37.2 trillion cells (1), each working together in an orderly manner, participating in processes essential to life. In most adult tissues, cells are continuously turned over; the cells growing, dividing and dying in response to signals from the environment in which they are located (2). This is a tightly controlled process; when cells become old they should die (3), and if cells become infected or damaged they should be destroyed, either by cell-autonomous mechanisms or by the immune system (4). Nonetheless, if a cell incurs mutations or epigenetic changes to tumour suppressor or proto-oncogenes, it may begin to proliferate uncontrollably and form a tumour (5). Over time the tumour cells may acquire certain biological capabilities; these include evasion of growth suppressors, resistance to cell death, replicative immortality and avoidance of immune destruction (6). Consequently, whilst the immune system can detect and destroy abnormal cells during the early stages of tumour formation (reviewed in 7,8), eventually the tumour cells may evade the immune response, prompting unrestrained growth and the development of cancer (9).

Whilst great progress has been made towards the prevention and treatment of cancer, it remains one of the leading causes of morbidity and mortality worldwide (10). An estimated 18.1 million new cases and 9.6 million cancer associated deaths occurred globally during 2018 (11). These numbers are projected to rise by approximately 63% over the next two decades, with 29.5 million new cases and 16.4 million deaths expected per year, by 2040 (12). This is partly due to the increased size and lifespan of the global population (13) and partly due to adverse environmental and lifestyle factors such as cigarette smoking, poor diet and air pollution that increase the risk of genetic mutations, and consequently, cancer development (14). Certain cancers are more common than others, especially those due to lifestyle factors; for example lung cancer is the most common cancer in men, with tobacco use accounting for 87% of (male) lung cancer associated deaths (15). Whilst other cancers (such as colon or breast cancer in women) are more common because the cells contained within the tissue undergo more frequent cell divisions, thus increasing the chance of mutations (16).

A substantial body of research (reviewed in 17,18) from the last 2-3 decades suggests that the micro-environment of the tumour may have a pivotal role in tumour development and progression. Large numbers of immune cells are recruited to the site of tumours; the inflammatory infiltrate secretes proteinases, growth factors, survival factors and angiogenic factors into the microenvironment (reviewed in 6,19,20). Unfortunately, rather than helping the host, these substances can also promote cancer growth, angiogenesis, tissue invasion, and ultimately, metastatic dissemination (reviewed in 6,19,20). The activities of certain innate immune cells, such as tumour associated macrophages or "TAM's", have been reasonably well characterised in this process (21–23) and are now considered to be an important treatment target for oncology, in conjunction with chemotherapy and immunotherapy (24). However, until recently, less was known regarding the role of neutrophils in cancer.

For many years, neutrophils were thought to have a negligible role in cancer, primarily because they were classically viewed to be short lived, terminally differentiated cells. Whilst there was initial interest during the late 80's and early 90's, after this point the number of published papers regarding neutrophils and cancer gradually declined (25). However, somewhat unexpectedly, research from the last decade has shown that neutrophils demonstrate a heterogenous phenotype (26) and, within the context of certain inflammatory diseases, demonstrate an enhanced lifespan (27). This, taken in conjunction with the knowledge that neutrophils represent a significant proportion of the immune cell infiltrate within many tumours (25,28,29), has prompted fresh investigation into their potential role in cancer.

To date there is a some evidence that neutrophil lifespan might be enhanced in certain cancers, and that cross talk between neutrophils may enhance cancer cell motility and invasiveness (30–35). However, overall, there is conflicting data regarding the role of neutrophils in cancer, with both "pro" and "anti-tumour" effects being reported (Section 1.2.5.5.1 – 1.2.5.5.2). Research (36,37) suggests that neutrophils may demonstrate an altered phenotype in cancer which could explain their dual, opposing functions. A great deal of work is required to characterise the role of neutrophils in cancer, partly because their influence appears to depend on the type or stage of tumour development.

The purpose of this research was to determine how interactions between cancer cells (specifically breast cancer cell lines) and neutrophils might help or hinder tumour progression. Knowledge gained during this process may contribute in the long term, towards the development of a targeted treatment for breast cancer.

This project had the following main objectives:

1. To determine if soluble factors released by breast cancer cell lines could delay neutrophil apoptosis, thus allowing the neutrophils more time to participate in activities within the tumour microenvironment that could either help or hinder cancer progression or metastatic dissemination.
2. To investigate possible molecular pathways responsible for the delay in neutrophil apoptosis and to partially characterise the molecule(s) responsible for this effect.
3. To determine if soluble factors released by breast cancer cell lines could polarise the neutrophils towards a pro or anti-tumour phenotype.
4. To determine if soluble factors released by neutrophils could alter the phenotype of breast cancer cell lines by a) activating or inhibiting the epithelial mesenchymal transition or "EMT", b) altering the expression of growth factors and cytokines associated with tumour growth and progression and c) enhancing or inhibiting tumour cell migration.

1.2 Literature review

1.2.1 Breast cancer background

Breast cancer is the most common cancer in women worldwide, accounting for more than a quarter of all female cancer cases, more than double the occurrence of any other cancer in women. Globally, breast cancer was associated with an estimated 2,093,876 new cases and 626,679 deaths during 2018 (11).

Risk factors for breast cancer include intrinsic, non-modifiable factors such as having a family history of breast cancer or inherited genetic mutations, increased age; with most breast cancers being diagnosed after the age of 50, and gender; being female (reviewed in 38,39). To date more than 200 hereditary cancer syndromes (genetic disorders in which inherited mutations predispose the affected individual to cancer) have been identified, these are believed to account for approximately 5-10% of all cancers worldwide (40). Most of these are associated with tumour suppressor genes, such as p53 which codes for a protein responsible for regulating the cell cycle and apoptosis (41). Although germline mutations in p53 are associated with early onset breast cancer in women (lifetime risk of disease being 49% by age 60 (42)), this condition (referred to as Li Fraumini syndrome) is very rare and has only been identified within 500 families globally (43). Hereditary breast-ovarian cancer syndrome is far more common, as germline mutations in the genes breast cancer 1 (BRCA1) and breast cancer 2 (BRCA2) have a population incidence of between 1/500 – 1/1000 (40). BRCA1 and BRCA2 are tumour suppressors that encode for proteins involved in repairing damaged DNA or destroying cells if the damage cannot be repaired. Mutations within these genes are associated with a 70-85% risk of developing breast cancer by age 70 in females (40), along with a 44-63% (BRCA1) or 27-31% (BRCA2) lifetime risk of ovarian cancer (44,45). However reproductive factors are also critically important, since gonadal hormones such as progesterone and oestrogen may act as tumour promoters by increasing the rate of cellular proliferation, thereby driving tumour growth (46,47). Thus, factors that increase exposure to these hormones, such as early menarche, late menopause, hormone replacement therapies and oral contraceptives, are associated with a moderate increase in breast cancer risk, whilst factors that decrease

exposure to these hormones such as parity and breast feeding are associated with a moderate decrease in breast cancer risk (38,39,47). Furthermore, lifestyle factors, such as obesity and alcohol consumption, may increase breast cancer risk by raising circulating oestrogen levels (38,48,49), whilst exercise may decrease risk by altering oestrogen metabolism (50).

1.2.1.1 The anatomy of the female breast and histology of breast cancer

The adult female breast sits above the pectoralis major muscle, held in place by the suspensory Ligaments of Cooper which run from the fascia beneath the breast to the dermis of the skin. The breast extends across the rib cage from the sternum (breast bone) into the axilla (arm pit) via the Tail of Spence. The glandular tissue of the breast is comprised of 15-20 lobes which radiate around the nipple in a petal like fashion. Each lobe contains smaller lobules and ducts which produce and transport the milk toward the nipple (Figure 1.1). More than 95% (51,52) of diagnosed breast cancers are termed "adenocarcinomas"; these arise from the cells that line the smaller lobules and ducts.

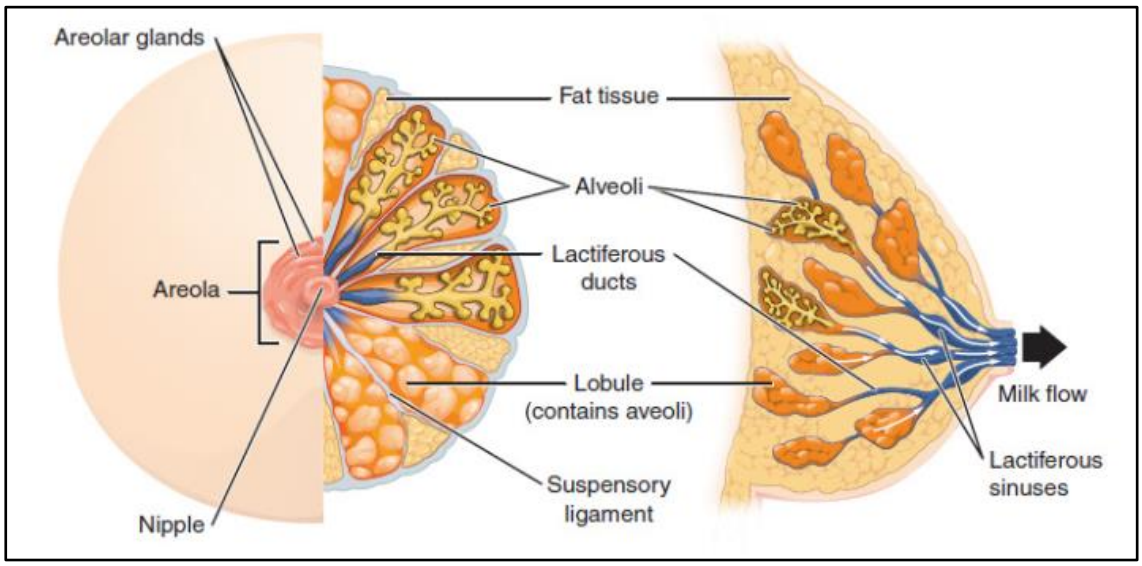


Figure 1.1: Anatomy of the female breast

Reprinted from OpenStax, 27.2 Anatomy and Physiology of the Female Reproductive System. OpenStax CNX. 3 May 2019 http://cnx.org/contents/9cccba49-6490-4e5b-a366-9991b7dbc56c@9; (Modified) (53). Gratis Reuse.

Breast tissue is highly vascularised and is supplied by branches of the internal thoracic, axillary and intercostal arteries; its venous return occurring primarily via the axillary and internal thoracic veins. A large number of lymphatic vessels traverse alongside these blood vessels, draining their lymph into the axillary, parasternal and posterior intercostal nodes. Whilst both the lymphatic and venous blood vessels provide a pathway for metastatic dissemination, breast tumours preferentially utilise the lymphatic system (54,55) and typically invade the nodes in sequence, starting with the nearest (the sentinel node) (56,57) then distal (non-sentinel) nodes followed by the axillary node (55).

Cancers localised to the inside of ducts, referred to as "Ductal Carcinoma in Situ" or DCIS" are non-invasive, whereas those that have begun to invade the fatty tissue outside of the duct, referred to "Invasive Ductal carcinoma" or "IDC" are considered malignant. IDC is the most commonly diagnosed invasive breast cancer (~80% of cases) (58), with the majority of IDC cases being classified as "No Special Type" (NST) (59). Rarer IDC subtypes include "tubular carcinoma of the breast", in which the tumours are comprised of tube-like structures (~2-4.4% of cases); "medullary carcinoma of the breast", in which the tumours are soft and fleshy (~1-7% of cases) and "papillary carcinoma of the breast", in which the tumours display finger-like projections (~0.4.-2% of cases) (reviewed in 59). Invasive adenocarcinomas that commence inside the lobes, referred to as "Invasive Lobular Cancer" (ILC), usually affect women later in life than IDC and represent the second most common type of invasive breast cancer (~5-15% of cases) (52). Although rare, breast cancer can occur outside of the ducts and lobes, for example in cells that line the blood and lymphatic vessels or within connective tissue (e.g. "Phyllodes tumours", ~0.3-0.5% female breast tumours) (60).

1.2.1.2 Breast cancer symptoms, detection and survival rates

The earlier a breast cancer is detected, the better the prognosis. Most cancers are described according to their stage (severity); Stage 0 describes cancer in situ, stage I a small cancer or tumour that has not invaded deeply into surrounding tissue, Stages II-III refer to larger cancers that have spread regionally and may have entered the blood stream and lymph glands and stage IV refers to a cancer that has spread to distal sites throughout the body.

In the USA, the five-year relative - survival rate is ~100% for women diagnosed with stage 0 or 1 breast cancer compared to ~22% for those diagnosed with metastatic or stage IV breast cancer (61). Although breast cancer begins in non-essential tissue, it preferentially metastasises to the bone and vital organs such as the brain, liver and lungs (62), disrupting essential biological functions. Whilst more research is required to determine the proportion of women with non-metastatic breast cancer that go on to develop metastatic breast cancer (MBC), a recent study from the USA found that three in four patients living with MBC were initially diagnosed with stage I-III breast cancer (63).

A significant proportion of breast cancers are detected via either self-examination or accidently by a partner or spouse (64). Early breast cancer symptoms are variable and can include a lump in the breast or armpit; thickening, swelling of the breast or dimpling, redness and flakiness of the skin; discharge or pain within the nipple area; general breast pain and changes in the shape and size of the breast (65). However, many women do not display symptoms, consequently mammograms are used as a screening tool for women aged over 40 as these can identify very small tumours (~2mm in size (66)), prior to a lump being felt. Approximately 3000 women and 20 men are diagnosed with breast cancer each year in New Zealand, with Maori women (on average) having a 1.4 times higher incidence rate than non-Maori women (67); the average ten year survival rate is approximately ~80%, however this increases to ~95% if the breast cancer is identified on an early screening mammogram (66).

1.2.2 The human immune system and cancer

It has long been recognised that the immune system plays a pivotal role in the control and development of cancer, indeed the first indications occurred as early as the 1700's when it was observed that febrile infections in cancer patients were occasionally associated with spontaneous tumour remission (68). However, it was not until 1909 that Paul Ehrlich specifically suggested that the immune system might prevent abnormal cells from developing into tumours (as cited by D.Ribatti, 69), with Sir Frank Macfarlane Burnet adding to this hypothesis in 1957, suggesting that tumour cells may possess antigenic "potentialities" capable of provoking an immune response (70). This concept eventually

evolved into the theory of “immunosurveillance”; with Burnet proposing that immune cells continuously patrol bodily tissues in order to eliminate neoplastic cells, made recognisable by their tumour associated antigens (71). The immune system is now recognised as performing three distinct roles in tumour prevention: firstly, it protects the body against virus induced tumours by destroying and supressing viral infections (Section 1.2.2.1); secondly, it eliminates pathogens and resolves inflammation in a timely manner, thereby preventing the formation of an inflammatory micro-environment conducive to tumour formation (Section 1.2.2.2); and thirdly, it can identify and eliminate aberrantly transformed cells before they can form a malignant tumour (reviewed in 7,8).

1.2.2.1 An overview of the human immune system

The human immune system is a network of interacting cells, tissues and organs that functions to protect the body against pathogens and infectious disease. The immune system is categorised into two subsystems, innate and adaptive immunity, both of which participate in cancer cell recognition and control (72). Each system contains white blood cells (leucocytes) that patrol freely through body fluids and tissues in order to seek out and remove cellular debris, damaged or infected cells, invading microorganisms and viruses. Leucocytes (both innate and adaptive) are derived from hematopoietic stem cells (HSCs) contained in bone marrow and are categorised according to their structure (granulocytes or agranulocytes) or their progenitor cell lineage (myeloid or lymphoid) (Figure 1.2).

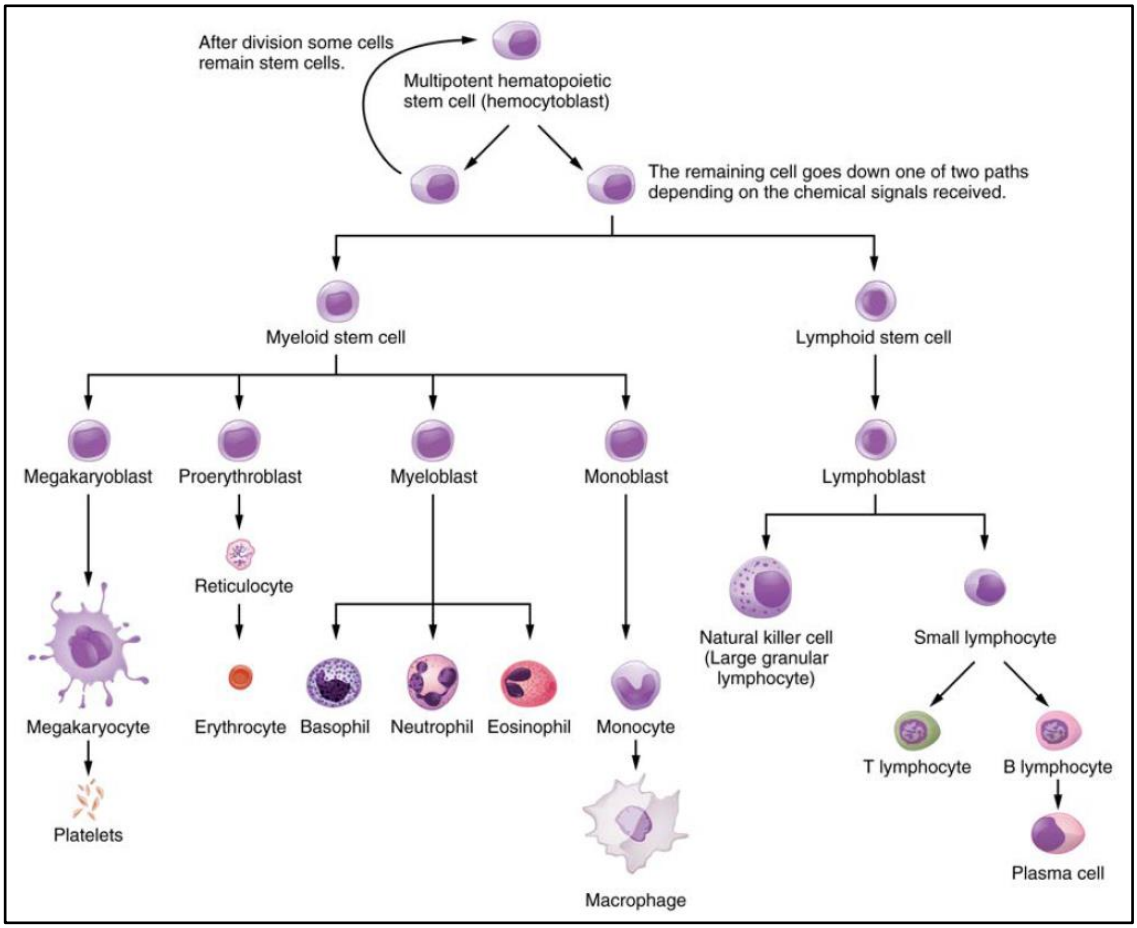


Figure 1.2: Haematopoiesis of myeloid and lymphoid cells

Multipotent hematopoietic stem cells differentiate into myeloid and lymphoid stem cells in response to environmental signals, which in turn differentiate into mature blood cells. Reprinted from OpenStax, 3.6 Cellular Differentiation. OpenStax CNX. 3 May 2019 http://cnx.org/contents/966c32cc-3d6f-4f4e-af4f-ea0c975e825c@8. (Modified), (73). Gratis Reuse.

1.2.2.2 Innate immunity

Innate immunity is a host defence mechanism in which cells respond to a pathogen immediately, in a generic non-specialised manner (74). It is comprised of physical barriers (e.g. epithelium of the skin), inflammation, soluble factors (referred to as complement) and a cellular component; specifically, neutrophils, macrophages, mast cells and dendritic cells (collectively known as phagocytes) along with basophils, eosinophils and natural killer (NK) cells.

Phagocytes primarily remove pathogens and dead/dying cells by engulfing them within their plasma membrane, destroying them with toxic reactive oxygen compounds (superoxide and hypochlorite) and degradative enzymes such as acid hydrolases and proteases (75,76). In contrast, basophils and eosinophils destroy pathogens via the release of cationic proteins contained within their granules (77), whereas natural killer cells are cytotoxic, releasing perforin and granzymes (serene proteases) to trigger apoptosis within damaged or infected cells (78). Importantly, natural killer cells (NK cells) are believed to have a pivotal role in immunosurveillance (Section 1.2.2.4) during the early stages of cancer formation (79)). Leucocytes distinguish between "self" and "non-self" via the expression of MHC molecules, yet MHC class 1 molecules are frequently down regulated by tumour cells, enabling them to avoid destruction by cytotoxic T Cells (80). However, NK cells recognise and destroy cells that lack the expression of these molecules (referred to as "missing-self") on their surface (79). The apoptotic tumour cells are subsequently phagocytosed by macrophages and dendritic cells prompting the secretion of inflammatory cytokines and the presentation of ingested tumour antigens to adaptive immune cells (Section 1.2.2.3) (81,82).

Tissue inflammation is primarily stimulated by the release of inflammatory cytokines, eicosanoids and chemokines by macrophages and mast cells that have been activated in response to pathogen derived molecular products (e.g. Lipopolysaccharide or "LPS") or endogenous stress signals (such as extracellular ATP) at the site of infection or injury (reviewed in 83). These inflammatory mediators trigger a cascade of effects, including the activation of pain receptors (84), vasodilation of blood vessels, and critically, the recruitment and activation of additional leucocytes (83). This inflammatory response is tightly regulated, as too little will not resolve the infection or injury, whereas too much will cause damage to nearby host cells; an inappropriate response (either to a non-pathological threat such as pollen or gluten or to the host cells themselves) can lead to allergies (85), or tissue destruction and the development of autoimmune diseases (86,87). In the context of cancer, acute inflammation frequently heralds the development of protective, adaptive immune responses against the early stages of tumour development (Sections 1.2.2.3-4) (88). Chronic inflammation, either from infection, disease or lifestyle factors such as obesity may contribute to tumour initiation (by generating genotoxic stress – damage to genetic information that results in mutations), tumour

promotion (by inducing cellular proliferation) and disease progression (by augmenting angiogenesis and tissue invasion) (9,88–91).

1.2.2.3 Adaptive immunity

The adaptive immune system is highly specific in its response towards pathogens or pathogen infected cells. It is comprised of two types of cells, B cells and T cells, which (along with NK cells) are collectively referred to as "lymphocytes". Newly created B cells and T cells circulate the body as immature "naïve" cells until they become activated by antigens, B cells directly and T cells indirectly via antigen presenting cells (85). The naïve B & T cells subsequently differentiate into mature "effector cells" or "memory cells"; the effector cells directly destroy pathogens, whereas the memory cells provide long lasting protection to the host, by mounting a rapid, enhanced response towards any subsequent encounters with the same pathogen (85).

Effector B cells (plasma cells) secrete antibodies *specific* to the original activating antigen into blood and lymph, which bind to target antigens and destroy pathogens by neutralising them, activating complement and/or marking infected cells for destruction by phagocytes (92). In the context of cancer, B cells may exert an anti-tumour response by secreting antibodies specific to tumour antigens (93), or by presenting tumour antigens directly to naïve T cells (94). Effector T cells include Killer T cells or CD8+ T cells, Helper or CD4+ cells, regulatory or CD4+T reg cells. CD8+ T cells destroy damaged and infected cells by releasing cytotoxins and proteases to trigger apoptosis (95). Whereas CD4+ T cells release cytokines such as IFN-γ and IL-4 that help mediate the immune response (96), for example by activating CD8+ T cells or stimulating an increase in antibody production by B cells (97). Finally, CD4+ T reg cells function to supress the immune response by a) downregulating the activation and proliferation of CD8+ T cells once a pathogen has been dealt with and b) triggering the apoptosis of auto-reactive CD8+ T cells thereby preventing autoimmune disease (98). Both CD8+ and CD4+ T cells may play a critical role in anti-tumour immunity, as they can recognise tumour cell antigens and subsequently eliminate the tumour cells (99,100).

1.2.2.4 Immunosurveillance and immunoediting

Whilst the theory of immunosurveillance is supported by studies that demonstrate a significant increased risk of cancer in immunocompromised individuals (101–103), this does not explain the incidence of cancer in healthy, immunocompetent individuals. A series of animal studies (reviewed in 104) suggested that tumour development might be shaped in response to the immunological environment in which they form. For example, Shankaran *et al.* (105), demonstrated that whilst lymphocytes and IFN-γ could protect against the development of carcinogen-induced sarcomas or spontaneous carcinomas in immunodeficient mice, this process might also lead to the positive selection of cancer cells capable of evading that particular immune response (105). It was subsequently recognised, that the immune system might play a dual role in cancer, with both host protective and tumour promoting actions. As a consequence, the theory of "immunosurveillance" was refined and extended into the concept of "immunoediting", the interplay between tumour cells and the host immune system being made up of three stages, elimination, equilibrium and escape (Figure 1.3) (104).

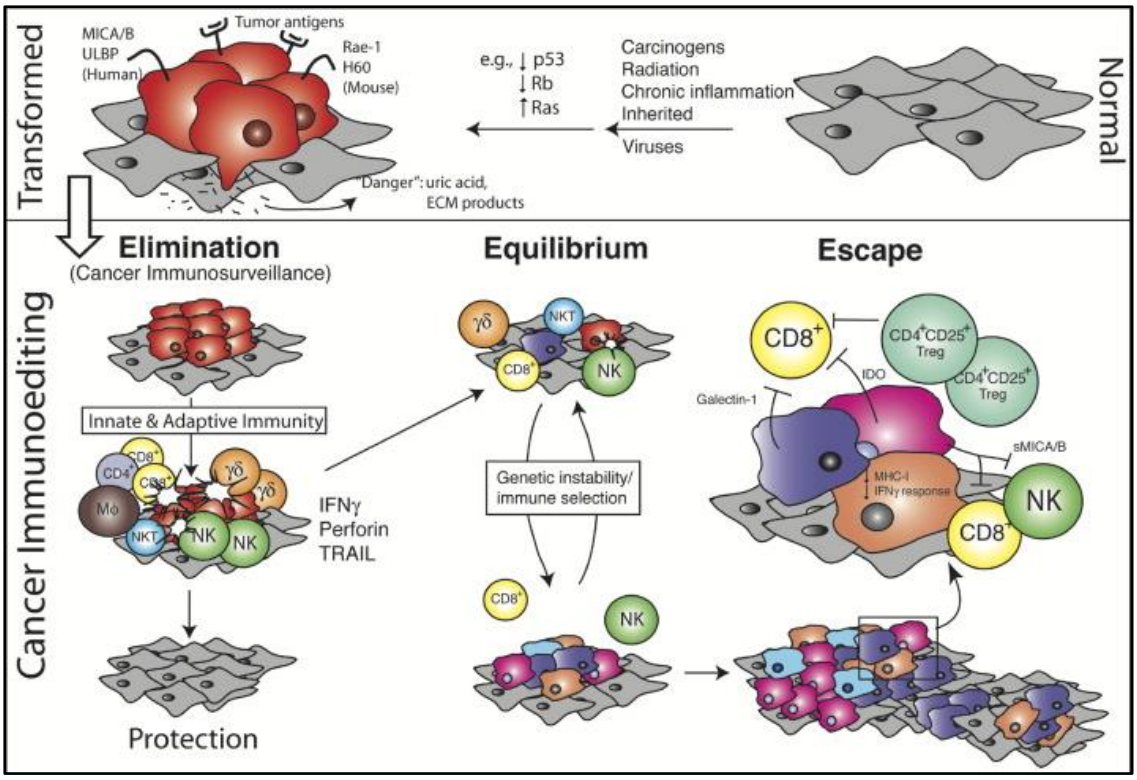


Figure 1.3: The process of cellular transformation and immunoediting

Healthy cells exposed to carcinogens, chronic inflammation or viruses incur genetic mutations and undergo neoplastic transformation. The transformed cells are initially detected and destroyed by the immune system, via the recognition of tumour antigens, DNA damage ligands or danger signals. Any surviving cells enter into equilibrium. Overt growth is initially inhibited by NK and CD8+T cells, however this results in the positive selection of traits that enable tumour cell escape. Reprinted from Immunity, 21 (2), Dunn G, Old LJ, Schreiber RD, The immunobiology of cancer immunosurveillance and immunoediting, Page 138, with permission from Elsevier; (Modified), (106).

The elimination phase of immunoediting is similar to the theory of immunosurveillance; the innate and adaptive immune system work together to recognise and destroy tumours before they become clinically detectable (for mechanisms of action see Sections 1.2.2.2 and 1.2.2.3) (8,9). An infiltrate of immune cells can be observed within the stroma of most solid tumours (Section 1.2.3); however, the precise mechanisms by which they are initially recruited are not yet completely understood. It has been suggested that NK cells may be activated by MHC class 1 chain-related protein A and B (MICA & MICB) stress ligands expressed on tumour cells in response to DNA damage, (107), or IFN-γ secreted as a "danger signal" by healthy cells in close proximity to the tumour cells (108).

Whereas dendritic cells may be activated by high mobility group box one protein (HMGB1), released by apoptotic tumour cells (109), thereby prompting the release of inflammatory cytokines, recruitment of immune cells and activation of the adaptive immune system. Consequently, the elimination phase of immunoediting is associated with the induction of an acute inflammatory response.

The end result of the elimination phase, should it go to completion, is the clinical absence of a tumour. Cancer cells that survive the elimination phase enter into the equilibrium phase, in which primarily adaptive immune cells continuously destroy tumour cells, preventing overt tumour growth (8,9,104). Support for this theory has been found in mouse tumour models (reviewed in 8). For example, research by Koebel *et al.* (110) demonstrated that CD4+ and CD8+ T cells could restrain carcinogen-induced sarcoma growths. Unfortunately, this process exerts a selective pressure on the genetically unstable tumour cells (reviewed in 111), and may lead to the emergence of tumour cell variants that can a) escape immune cell recognition, for example by down regulating the expression of MHC class 1 molecules (112) and/or b) avoid immune cell effector mechanisms by increasing the expression of anti-apoptosis peptides such as "B-cell lymphoma-2" or "BCL-2" (113). As a consequence, the tumour cells become "invisible" to the host immune system and thus acquire the capacity for unrestrained growth (9).

However, tumour escape may also result from the creation of an immunosuppressant microenvironment. For example, tumour cells can recruit T reg cells and myeloid-derived suppressor cells (MDSCs) to the site of the tumour, both of which can inhibit the function of anti-tumour CD8+T cells (9). Furthermore, tumour cells can secrete immunosuppressant factors such as VEGF and TGF-β, which act to inhibit dendritic cell stimulation of naive CD8+T cells (114), as well as the activation, proliferation and differentiation of T cells (115). Lastly, tumour escape may also be aided by the recruitment of inflammatory immune cells to the tumour microenvironment, as these engage in cross talk with the cancer cells, prompting the secretion of factors that work to promote tumour growth and tissue invasion (Section 1.2.4).

1.2.3 The tumour stroma and mechanisms of tumour invasion

Aside from certain hematopoietic cancers, most cancers develop as a solid mass of tissue comprised of two interconnected compartments. The tumour parenchyma contains the cancer cells, whereas the tumour stroma is comprised of connective tissue, blood vessels, extracellular matrix constituents (as well as their cellular components such as endothelial cells, pericytes and fibroblasts respectively) and innate and adaptive immune cells (116). For most tumours, including epithelial cancers such as breast cancer, the tumour parenchyma is separated from the stroma by a layer of extracellular matrix referred to as the basal lamina (116). In normal (healthy) tissue, the stroma functions to maintain epithelial tissue integrity and homeostasis, whereas in cancer the stroma forms a progressively reactive micro-environment (Figure 1.4).

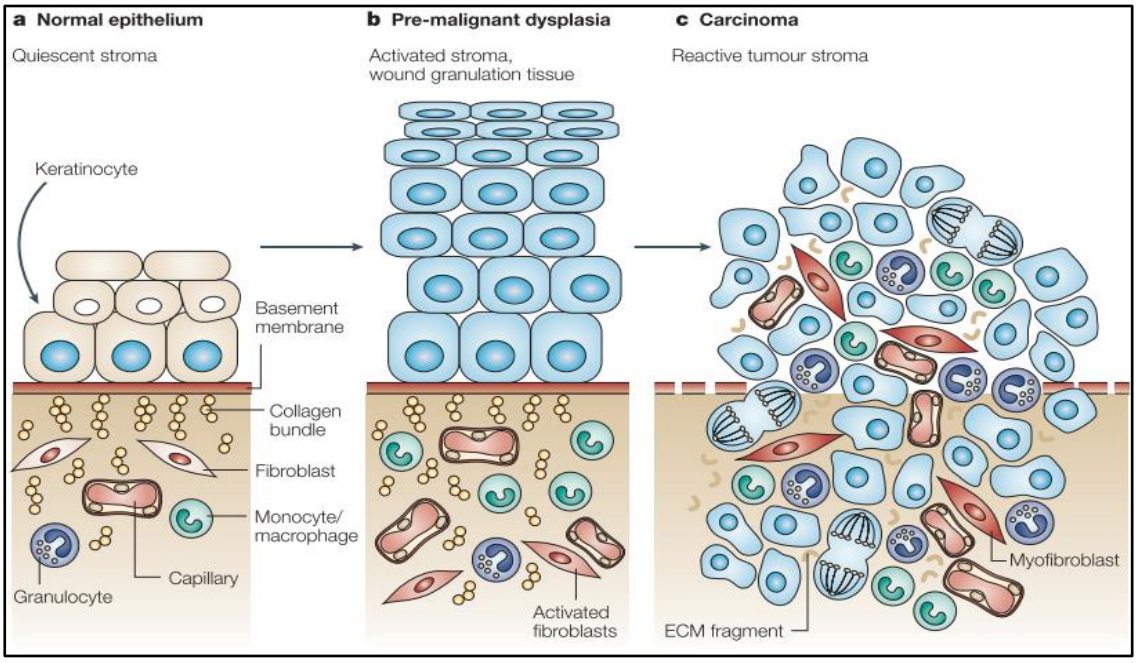


Figure 1.4: Tumour stroma during disease progression

Left: Normal stroma, contains collagen bundles, resting fibroblasts & tissue resident leucocytes separated from the epithelium by the basal lamina; Middle: Pre-malignant dysplasia, stroma contains increased number of leucocytes, capillaries & activated fibroblasts. Right: Carcinoma, reactive stroma contains increased leucocytes, tumour cells invade the basal lamina. Reprinted by permission from Springer Nature: Nature/Springer/Palgrave, Nature Reviews Cancer, Friends or foes – bipolar effects of the tumour stroma in cancer, Mueller MM, Fusenig NE. Copyright 2004; (Modified), (117).

Tumour cells invade their surrounding tissue via passive cell movement or active cell migration. In terms of passive movement, as the tumour develops it gains an increased number of structural components (both parenchymal and stromal) so that the tumour begins to stiffen (118). This generates a mechanical force that pushes against the surrounding tissue and enables the tumour to grow outwards, displacing normal cells (119,120).

In terms of active movement, the tumour cells either migrate collectively, as a multi-cellular group, or individually as single cells (118,121–124). The key difference between these two processes being that collectively migrating cells retain cell to cell contacts (including adherence, tight and gap junctions and desmosomes (122), thus allowing whole groups of cells to penetrate the tissue in the form of strands, irregular sheets or clusters. Whereas, in single cell invasion the individual cancer cells invade the surrounding tissue independently of each other via either amoeboid or mesenchymal migration (possibly shifting between the two) (121–123).

Amoeboid cancer cells are small, approximately 10-30μm (122), with a round-elliptical shape that can readily deform to fit through pre-existing gaps and spaces within the extracellular matrix (121–123). This type of movement is most frequently seen in lymphomas and certain lung cancers (122,125). In contrast, mesenchymal cancer cells are larger, approximately 50-200μm (122), with an elongated spindle shaped; mesenchymal cancer cells release proteolytic enzymes, such as metalloproteinases and caspases, that degrade tissue structures in order to create a path for their migration (121–123). This type of movement is primarily seen in connective tissue cancers, such as gliomas, or dedifferentiated epithelial cancers (122), as described below.

As previously noted, collective cell migration is characterised by the movement of whole cell groups. Normally seen within epithelial cancers (including breast cancers (126)), these structures have two zones, a leading edge in which mesenchymal-like “leader” cells secrete proteolytic enzymes that degrade the extracellular matrix to create a microtrack within the tissue, and a trailing edge of “following” cells which are pulled along by traction movement (127), thereby widening the microtrack into a larger macrotrack

(118,123). As the tumour progresses, the leader cells located at the invasive front may transition from a collective cell migration pattern towards a detached, single cell migration pattern (122). This process, during which the epithelial cells dedifferentiate and gain a mesenchymal phenotype, is referred to the epithelial-mesenchymal-transition or "EMT", (128). Genes responsible for initiating EMT are classically expressed during embryogenesis, organ development and wound repair, however they can also be re-expressed by cancer cells, and this process is thought to be a critical step towards malignancy (128,129), as it provides the cancer cells with enhanced motility, invasiveness and increased resistance towards apoptosis (129). Research suggests soluble factors present within the tumour micro-environment, including inflammatory mediators secreted by immune cells, may play a key role in activating the EMT process (see Section 2.9.5.2) (as reviewed by Chockley *et al.* (130)). The reversal of EMT, termed the mesenchymal-epithelial-transition or "MET", is associated with decreased migration and the acquisition of an epithelial phenotype, as defined by the adoption of apical-basal polarity and formation of tight junctions (reviewed by Pei *et al.* (131)). Subsequently whilst EMT is believed to trigger the migration and dissemination of carcinoma cells, MET is believed to halt their migration and induce the proliferation of tumour cells at distal sites (132). It is important to note that tumour cells do not oscillate between a full EMT or MET state, the process is highly plastic with many intermediary stages in which cells display a hybrid phenotype (as reviewed by Nieto *et al.* (132)).

1.2.4 The tumour microenvironment

The tumour microenvironment (TME) is the cellular environment in which a growing tumour exists, and is comprised of proliferating tumour cells, blood vessels, the tumour stroma, immune cells, other non-malignant tissue cells and signalling molecules, all of which play an important role in tumour development and progression (133,134). The TME is created by the tumour, since tumour cells are predisposed, much like normal cells to interact with the cellular and extracellular components of their surrounding tissue, hence its structure and composition evolves during tumour progression (133,135) A detailed evaluation of all the interactions that occur between the tumour and the TME is

beyond the scope of this review (for reference see (20,136,137) hence, only interactions between tumour cells and immune cells will be considered here.

Infiltrating immune cells are a key component of the TME. Whilst the composition varies from tumour to tumour, they typically include NK cells, macrophages, MDSCs neutrophils, eosinophils, dendritic and mast cells, as well as various subsets of T cells and B cells (138,139). These cells can be found throughout the tumour, specifically, in the tumour itself (B cells and T cells within this category are referred to as "tumour infiltrating lymphocytes or "TILs"), around the tumour including at the invasive edge or within the tumour stroma (139), in varying densities from minor infiltrations to gross inflammations (138). In the past, the presence of infiltrating immune cells was thought to reflect a failed attempt of tumour destruction (see Section 1.2.2.4) (6,133). Indeed, a number of studies suggest that an increased density of adaptive immune cells (particularly CD4+ Th1 helper cells or CD8+ T cells) might be associated with an improved prognosis for certain breast cancers (140–143). For example, recent research (142), in which 3371 breast biopsies were assessed, found that a 10% increase in TIL was associated with a significantly longer disease-free survival in both triple negative breast cancer, and HER-2 breast cancer. However, it should also be noted increased TILs were found to be an *adverse* prognostic factor within luminal HER-2 negative breast cancer, suggesting these cells have a different immunological phenotype to those within triple negative or non-luminal HER-2 breast cancer (142).

However, for the majority of cancers, the presence of innate immune cells is typically associated with tumour progression (22). As previously noted, (Section 1.2.2.2) chronic inflammation is a key driver of malignancy. However chronic, pro tumour - inflammation is present within the micro-environment of most established tumours, even in the absence of known infection or inflammatory disease (144,145). Research suggests that this may be caused by mutations within oncogenes such as RAS, MYC and RET, which subsequently lead to the constitutive expression of inflammatory mediators, such as cytokines, chemokines and prostaglandins by the tumour cells (reviewed in 144). The inflammatory mediators recruit and activate immune cells to the site of the tumour, whilst those that are already present within the tissue are co-opted by tumour cells via the same

mechanism (18). The immune cells subsequently engage in cross talk with the tumour cells, prompting the release of further inflammatory mediators that feedback into the disease cycle, as well as soluble factors that promote tumour growth, angiogenesis and tissue invasion (Figure 1.5) (6).

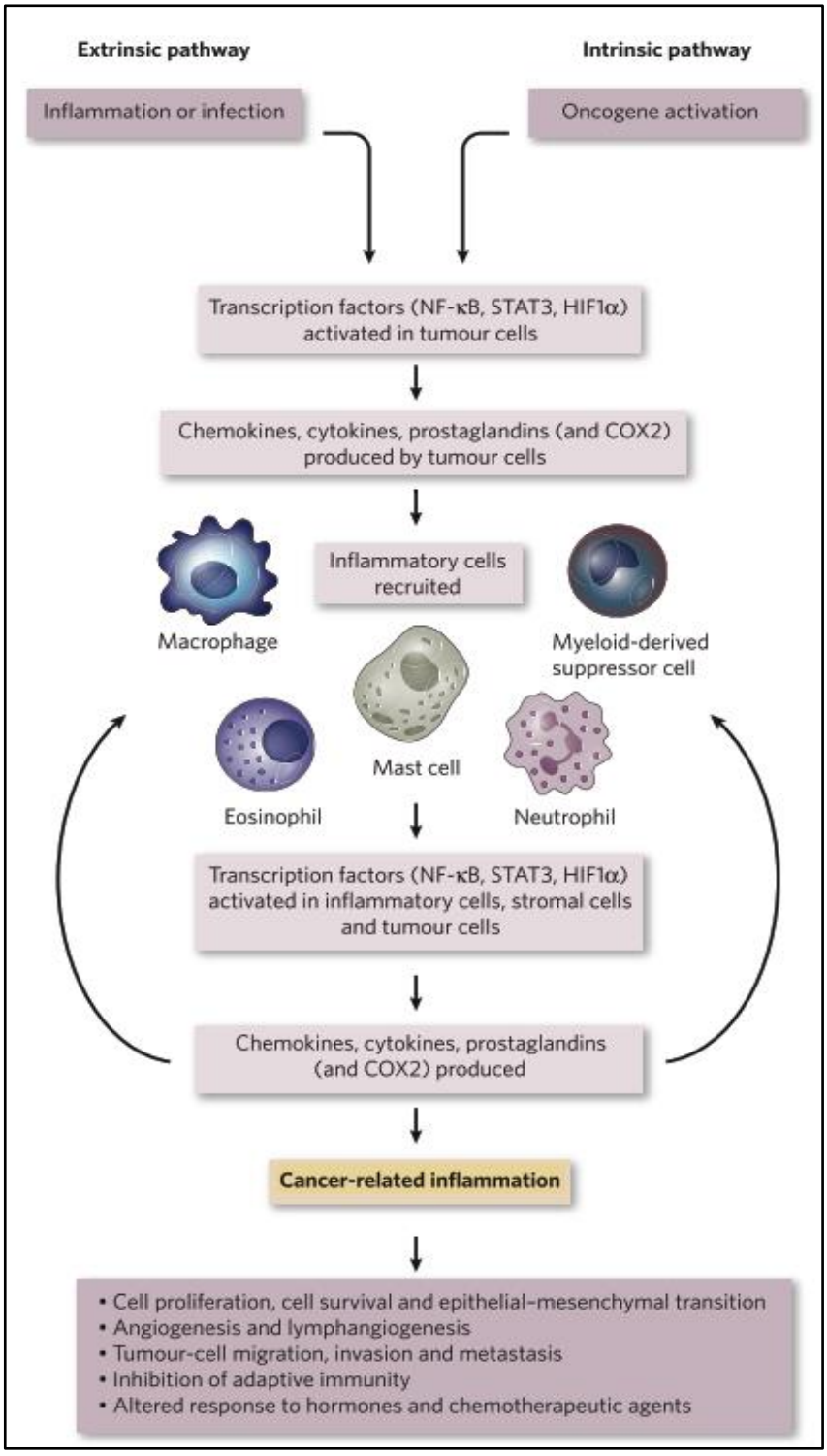


Figure 1.5: The two pathways of inflammation in cancer and their outcomes

Activation of either pathway results in the constitutive expression of inflammatory mediators by the tumour cells and the subsequent recruitment of innate immune cells. These engage in cross talk with the cancer cells prompting the release of further inflammatory mediators that feedback into the disease cycle. Reprinted by permission from Springer Nature: Nature/Springer/Palgrave, Nature, Cancer related inflammation, Mantovani A, Allavena P, Sica A, Balkwill F. Copyright 2008; (Modified), (144).

Whilst it is generally true that adaptive and innate immune cells hold opposing roles in cancer: protective versus promoting, respectively, the biological reality is more complex (22). Adaptive CD4+T helper cells differentiate into a variety of subtypes depending on developmental or environmental cues. Research suggests that the Th17 subset, characterised by the secretion of IL-17, may enhance the expression of VEGF and other angiogenic chemokines, thereby promoting angiogenesis and may also induce IL-6 production in tumour cells thus augmenting the expression of pro-survival genes (reviewed in 146,147). Likewise, certain innate immune cells, specifically macrophages, MDSCs and neutrophils, demonstrate a plasticity of phenotype in the tumour micro-environment and may exert either a pro or anti-tumour phenotype depending on environmental signals (Section 1.2.5.3) (6).

1.2.5 Role of neutrophils in cancer

For many years, neutrophils were considered to have a negligible role in cancer. This is largely because they were classically viewed to be short-lived (an estimated half-life ~8 hours (148)), terminally differentiated effector cells, with their physiological function presumed to be limited to the destruction of pathogens and the orchestration of acute inflammation (149,150). However, within the last decade it has become apparent that neutrophils demonstrate a high level of plasticity and may alter their phenotype and lifespan during both homeostatic and pathological disease conditions (reviewed in 26,151,152). These properties, coupled with the knowledge that neutrophils are present within the TME, has prompted fresh investigation into their role in tumour progression, invasion and metastatic dissemination.

Without doubt, tumour-associated neutrophils or "TANs" represent a significant proportion of the immune infiltrate within many cancers, including breast cancer (25,28,29); however, there are conflicting data regarding their prognostic value. A meta-analysis (153) found a high density of intra-tumoural neutrophils to be significantly associated with poorer recurrence-free survival and overall survival for a range of cancers, including melanoma, colorectal and cervical cancer. Data suggested a 66% increased risk of death or disease recurrence when high levels of intra-tumoural neutrophils were

present (153). Likewise, neutrophil infiltration was statistically significantly associated with increasing tumour size and tumour stage in renal cell carcinoma (154) and to be associated with more aggressive (triple negative) breast tumours (29). In contrast, Galdiero *et al*. (155) found higher TAN density to be associated with improved prognosis during stages I-IV of colorectal cancer. Importantly an interaction was noted between TAN density and response to Fluorouracil based chemotherapy, further analysis subsequently demonstrating that within stage III patients TAN infiltration was associated with a better response to Fluorouracil chemotherapy, whereas it was associated with a poorer prognosis in untreated patients (155).

In addition to TANs, many patients with advanced cancers also display an elevated number of circulating neutrophils (neutrophilia), typically associated with a poorer prognosis (156). The mechanisms behind this effect are not well understood; however, research suggests that both tumour and stromal derived factors such G-CSF, GM-CSF and IL-6 may promote granulocyte production (granulopoiesis) and neutrophilia in cancer (157,158). The neutrophil to lymphocyte ratio (NLR) is frequently used as a proxy measurement for this phenomenon, again with conflicting results. A high neutrophil to lymphocyte ratio (NLR) is considered be a poor prognostic indicator for many cancers, including ovarian and breast cancer (159–161). Indeed a meta-analysis (162) of 100 studies (N = 40,599 patients) found an elevated NLR to be associated with adverse overall survival (HR = 1.81) across various cancer types and disease stages. However, others have demonstrated either no relationship or one that is cancer-type or stage-dependant; for example, a recent meta-analysis (163), found an elevated NLR was not associated with worse survival or disease recurrence in localised prostate cancer, whereas the opposite effect was found for patients with metastatic prostate cancer. It is important to note that whilst intra-tumoural TANs or a high NLR might be correlated with a poorer prognosis for certain cancers, this does not prove that neutrophils are directly involved (or not) in the pathogenesis of cancer. Indeed, it has been suggested that the NLR is a reflection of systemic inflammation, potentially explaining why a high NLR is typically associated with more advanced cancers (156).

1.2.5.1 An overview of neutrophil function

To better understand the mechanisms by which neutrophils exert their effects in cancer, it is necessary to briefly review their normal functions within the body. From an immune cell perspective, neutrophils are the first line of defence against invading pathogens (164). Neutrophils patrol the circulatory system in a resting state and become activated/primed over a series of steps in response to bacterial products, chemokines or cytokines (e.g. LPS, IL-8 or IFN-γ) secreted during infection or inflammation (165,166). These mediators prompt the rapid migration of neutrophils to the site of injury via chemotaxis, whereupon they encounter further activating signals that trigger the destruction of microbes through the following mechanisms (165,166):

i) Phagocytosis (Section 1.2.2.2), during which opsonized microbes are ingested and destroyed via respiratory burst or antimicrobial proteins (167).
ii) Degranulation, in which antimicrobial compounds including myeloperoxidase, neutrophil gelatinase-associated lipocalin and proteases such as neutrophil elastase are secreted across the plasma membrane or secreted into the phagosome to act upon extracellular and intracellular pathogens respectively (165).
iii) The release of neutrophil extracellular traps (NETs); these are structures comprised of chromatin and proteins from the granules, the former capturing and binding to pathogens, and the latter eliminating them (168).

Although these mechanisms are effective in removing micro-organisms, the toxic products released during degranulation and respiratory burst are also capable of damaging host cells. Consequently, once any pathogens have been dealt with, neutrophils undergo rapid apoptosis and are cleared from the tissue by macrophages (165). A delay in this process (Section 1.2.5.2.4), as seen in certain inflammatory diseases, such as rheumatoid arthritis (169) or inflammatory bowel disease (170), can result in their toxic contents leaking out, damaging the surrounding tissue and potentiating inflammation (171).

In addition to their direct role in antimicrobial defence, neutrophils also regulate the activities of other innate and adaptive immune cells (reviewed in 150,165). Briefly, depending on their activation, neutrophils secrete an array of cytokines

(pro/anti-inflammatory and immunoregulatory), chemokines and angiogenic factors (150,172). These initiate an acute inflammatory response and recruit additional immune cells, such as monocytes (173) and dendritic cells (174), to the site of infection, where they engage in cross talk with neutrophils. For example neutrophil derived CCL3 was demonstrated to recruit dendritic cells (DC), which in turn initiated a protective Th1 response in a mouse model of Leishmaniasis (a parasitic disease) (175). In contrast, a mouse model of Mycobacterium tuberculosis was shown to delay neutrophil apoptosis, thereby limiting antigen uptake and activation of dendritic cells and, subsequently, an adaptive CD4+ T cell response (176). These opposing effects on DC activation may prove to be site specific; stimulatory within the tissues and inhibitory within the lymph nodes (165). Neutrophils can also modulate the maturation and function of NK cells (177), activating them directly or in cooperation with dendritic cells (178). Lastly, neutrophils may also modulate the activity of B cells and T cells. For example neutrophils are a key source of "B Cell Activating Factor (BAFF)" (179) and "A proliferating-inducing ligand (APRIL)" (180); these are cytokines that regulate the survival and proliferation of B cells as well as IL-12, which may have an important role in Th1 cell differentiation (165).

1.2.5.2 Neutrophil homeostasis in health and disease

1.2.5.2.1 Neutrophil homeostasis

Neutrophil granulocytes are the most abundant leukocyte found in human blood, constituting 40-60% of the total leucocyte population (181). Approximately 1-2 x 10^{11} neutrophils are produced every day from hematopoietic stem cells (166) to form two pools of cells in the bone marrow: a mitotic group of granulocytic progenitor cells that are still undergoing proliferation and differentiation, and a post-mitotic group of mature, fully differentiated cells that are ready to be released into the circulatory system (148). A key regulator of this process, stimulating neutrophil production, proliferation and mobilisation, is the cytokine G-CSF (182). In a healthy person, neutrophil homeostasis is tightly controlled; circulating neutrophils undergo constitutive apoptosis and are

cleared by phagocytes in the liver, spleen and bone marrow (148) in order to align the rate of production with the rate of clearance (183).

1.2.5.2.2 Cell death

There are two major modes of cell death, apoptosis and necrosis (184). Apoptosis is a highly regulated, tightly controlled form of programmed cell death. It is a vital component of many essential physiological processes including cell turn over, immune defence, hormone dependant tissue atrophy (as seen in the breast following menstruation, pregnancy or lactation), embryonic development and disease or chemical induced cell death (reviewed in 3). Dysregulated apoptosis is a feature of numerous human diseases; indeed, evasion of apoptosis is considered to be the third hallmark of cancer (6). In the context of neutrophils, certain viral infections such as HIV (185) are associated with accelerated neutrophil apoptosis and impaired antimicrobial responses, whereas a delay in neutrophil apoptosis is frequently seen in inflammatory diseases and often correlates with disease severity (Section 1.2.5.2.4). In contrast, necrosis typically arises from external factors such as acute infection or trauma (186). Although classically portrayed as a passive process, research from the last few decades has demonstrated that necrosis may also function as a mode of programmed cell death (necroptosis) in response to the same death signals that trigger apoptosis (reviewed in 184).

Both apoptotic and necrotic cells demonstrate changes in their morphology. Apoptotic cells condense in size, the cytoskeleton collapses leading to small bulges or protrusions (blebbing) in the plasma membrane, and both the nuclear envelope and nuclear DNA undergo fragmentation (reviewed in 3,187). Eventually the cell may fragment, to form small apoptotic bodies comprised of an intact plasma membrane and cytoplasmic organelles (3). Apoptotic cells are recognised by phagocytes due to the presence of "eat-me" markers ("apoptotic cell-associated molecular patterns" or "ACAMPs"(188)) such as phosphatidylserine, which translocates from the inner leaflet to the outer layer of the plasma membrane during the early stages of apoptosis (189). As a consequence, apoptosis is not associated with inflammation, because the plasma membrane remains intact, preventing the release of intracellular constituents, and the cells are quickly removed by

phagocytes, thereby reducing the risk of secondary necrosis (3,190). In contrast, necrotic cells and their organelles, such as the mitochondria and endoplasmic reticulum, swell in size leading to the rapid rupture of their plasma membranes (3,186,191) and is typically associated with an inflammatory reaction (184).

1.2.5.2.3 Mechanisms of neutrophil apoptosis

There are two main apoptotic pathways within neutrophils: the extrinsic pathway, activated by death receptors located on the plasma membrane and the intrinsic pathway, mediated by the mitochondria (Figure 1.6). A third apoptosis pathways also exists, phagocytosis-induced cell death or "PICD", which is induced in neutrophils following the phagocytosis of certain bacteria and is thought to facilitate neutrophil turnover during infection (reviewed in 192). However, in each instance apoptosis is mediated by caspase cascades (cysteine proteases) that lead to the activation of molecules involved in cell death. Caspase-8 and caspase-9, respectively, are the initiator caspases for the extrinsic and intrinsic pathways (183), these in turn activate caspase-3 triggering the cleavage of downstream death substrates, chromatin condensation, DNA fragmentation and blebbing (193). Cell survival pathways, responsible for delaying apoptosis, are mediated by proteins that inhibit various stages of this caspase cascade (Figure 1.6).

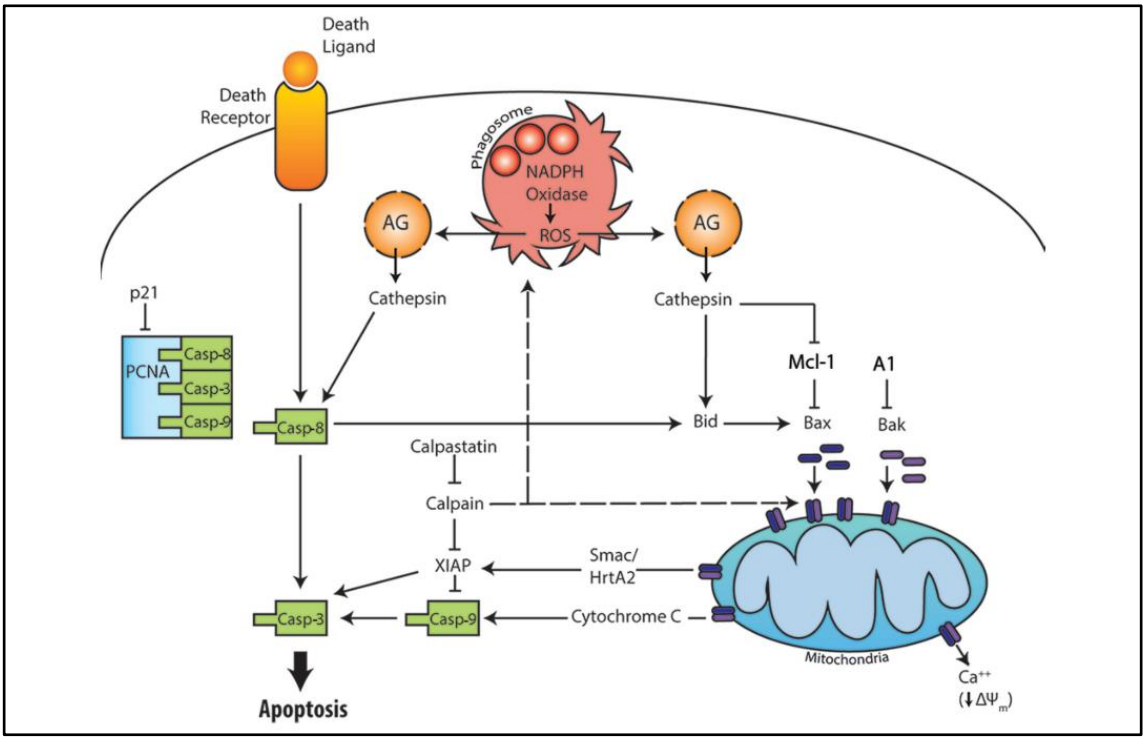


Figure 1.6: An overview of the extrinsic, intrinsic and phagocytosis-induced apoptosis pathways (PICD) within neutrophils

The extrinsic pathway is triggered by ligation of death receptors, followed by the activation of caspase 8 and caspase 3. The intrinsic pathway is triggered when the relative abundance of pro-apoptotic proteins (BAK and BAX) exceeds that of the anti-apoptotic proteins (MCL-1 and BCL2(A1)), so that BAX and BAK oligomerise and form pores within the mitochondria, releasing cytochrome C and activating caspase-9. The PICD pathway is activated by particles being taken up into the phagosome. The resulting NADPH oxidase-derived ROS prompts the release/leakage of cathepsin from the azurophilic granules and death receptor independent activation of caspase-8. Reprinted from McCracken J and Allen L, 2014; (Modified), (183). Gratis Reuse.

The extrinsic pathway is usually activated by extracellular soluble proteins that bind to death receptors located on the surface of neutrophils, such as tumour necrosis factor-α (TNF-α), FAS (CD95/APO1) and TNF related apoptosis inducing ligand (TRAIL) (194,195). Upon ligation, the receptors oligomerise to form a death-inducing complex (DISC) (196,197) which activates Caspase-8 and in turn Capase-3. However, activation of the extrinsic pathway alone is not sufficient to trigger cell death in neutrophils, the signal for apoptosis must be amplified by the intrinsic pathway (198). This process is orchestrated by BH3 interacting-domain death agonist (BID) (Figure 1.6). BID is cleaved by Caspase-8, translocates to the mitochondria and enables the oligomerisation of

Bcl-2-associated X protein (BAX) and Bcl-2 homologous antagonist/killer (BAK) thereby activating the intrinsic apoptosis pathway (199). To date, it would appear that the extrinsic apoptosis pathway does not have a role within *constitutive* neutrophil apoptosis (183). However, caspase-8 has been found to be activated during constitutive apoptosis, via mechanisms that do not involve ligation of the death receptors; for example, neutrophils isolated from healthy volunteers demonstrated spontaneous dephosphorylation (activation) of caspase-8 *in vitro* (200). The intrinsic apoptosis pathway is activated by non-receptor mediated stimuli; negative signals such as a lack of growth factors, hormones or cytokines may trigger the activation of apoptosis whereas positive signals such as hypoxia or viral infections may delay apoptosis (3). A key initiating event is "mitochondrial outer membrane permeabilization" or "MOMP" (201); this process is induced by activation of BAX and BAK, which prompts their oligomerisation and the formation of pores within the mitochondria (202,203). Proteins, such as cytochrome C, that are located within the intermembrane space of the mitochondria are subsequently released into the cytosol (203). The release of cytochrome C triggers "apoptotic protease activating actor 1" (APAF1) to oligomerise into a complex, which binds to and activates caspase-9 (204). The process of MOMP is tightly regulated by the ratio of pro versus anti apoptosis Bcl-2 proteins. Mature human neutrophils express two Bcl-2 anti-apoptosis proteins, "induced myeloid leukaemia cell differentiation protein" (MCL-1) and "Bcl-2-related protein A1" (BCL2(A1) or A1) (183); these sequester activated BAX and BAK, thereby preventing damage to the mitochondrial membrane and inhibiting apoptosis (183,205). Interestingly, TNF-α appears to have a dual role in regulating neutrophil apoptosis by acting on these two proteins; high concentrations of TNF-α are associated with cell death due to caspase-dependent MCL-1 degradation and low concentrations of TNF-α are associated with cell survival due to increased expression of BCL2(A1) (206).

A second group of proteins, the "inhibitor of apoptosis proteins" or "IAPs" regulates both the intrinsic and extrinsic pathway, primarily by inhibiting the function of caspases. X-linked inhibitor of apoptosis protein (XIAP), is thought to be the most important IAP in neutrophils, as it inhibits both procaspase-9 and procaspase-3 activity (207). However, IAPs can be inhibited by the Smac(mice)/DIABLO (human) protein (208) and Omi/HtrA2 protease (209) which are released from the mitochondria in response to

apoptotic signals. Likewise XIAP can be cleaved by calpain-1, thereby preventing caspase-3 inhibition (210). Calpain-1 has also been found to cleave BAX in spontaneous and Fas-receptor mediated neutrophil apoptosis, and is essential for the release of cytochrome C and caspase-3 activation (211). Cyclin-dependant kinases (CDKs), a group of proteins typically associated with cell cycle regulation (212) have also been implicated in regulating neutrophil apoptosis, since Roscovitine (a CDK inhibitor) was found to inhibit neutrophil survival in certain inflammatory diseases (213). Lastly, "proliferating cell nuclear antigen" (PCNA) may also regulate neutrophil survival. Although traditionally associated with nuclear functions, such as DNA synthesis and repair (214), PCNA is found within the cytosol of mature human neutrophils (215,216). Cytosolic neutrophil PCNA is constitutively associated with procaspases, and has been shown to interfere with the activation of pro-caspase-9; overexpression of PCNA in neutrophil-differentiated PLB985 myeloid cells was found to enhance their resistance to TNF-α induced apoptosis (215,216).

1.2.5.2.4 Neutrophil lifespan in health and disease

The estimated lifespan of circulating neutrophils in humans is somewhat controversial. Although classically thought to be short (approximately 8 hours) (148), research by Pillay *et al*. (217) in 2010 challenged this view by calculating lifespan to be 5.4 days. At sites of infection and inflammation, neutrophils display increased longevity. However, it is difficult to broadly quantify this response; as the influence of inflammation on lifespan appears to vary both between and within individual diseases (reviewed in 27). However, as an example, the half-life of neutrophils from liver cirrhosis patients was found to be 15h compared to 7.6h from a healthy control (cited by Tak *et al.* 27).The delay in apoptosis is mediated by a variety of cytokines and bacterial products (including GM-CSF, G-CSF, IL-1β, TNF-α, IFN-γ & LPS (218,219); Table 1.1), as well as pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) and environmental factors (220), presumably to ensure the continued presence of neutrophils at the site of inflammation and enhancing their anti-microbial actions.

Table 1.1: Survival factors associated with delayed neutrophil apoptosis in inflammation or cancer

Factor	Possible Source	Molecular Weight or Molar mass
Cytokine/Chemokine		
GCSF (220)	EnC, MF (220)	~19.6kDa (221)
GM-CSF(220)	CD4+ T, CD8+ T, Th17 cells, NK cells, MF (220)	~14-35kDA (222)
IL-1B (220)	PMN, MF, DC (220)	~17kDa (223)
IL-2 (220)	Th1 cells (220)	~15.5kDa (224)
IL-4 (220)	Th2, Tc2 cells, EO, BASO(220)	~12-20kDa (225)
IL-6 (226)	MDA-MB-231 Cells (227)	~21-28 kDa (228)
IL-8 (220)	EpC, MF, PMN (220)	~8 kDa (229)
IL-15 (220)	MF, DC(220)	~14-15kDa (230)
TNF-a (<10 ng/ml) (220)	Th1, Th17, CD8+, MF, PMN (220)	~17-26kDa (231)
Lipid Mediators		
Leukotriene B4 (220)	MF, PMN, MC, B cells (220)	336.46g/mol
Prostaglandin E2 (232,233)	Almost every cell of the body (234). MDA-MB-231 cells (235)	352.47g/mol
ECM Component		
Hyaluronic Acid (236)	Connective, neural &epithelial tissue (including MCF-7 and MDA-MB-231 cells (237))	50–200 kDa fragments associated with delayed PMN apoptosis (236)
Proton Pump		
Peptide of vacuolar ATPase 'a2'(35,238)	MCF-7 & MBA-MB-231 Cells	~20kDa (238)
DAMPS		
HMGB1 [TLR2/4] (220)	Damaged host cells (220)	~25kDa (239)
S100 [TLR4] (220)	Damaged host cells (220)	~10-12kDa (240)
ATP (220)	Damaged host cells (220)	507.18g/mol
MSU (Uric Acid) (220)	Damaged host cells (220)	168.11g/mol
Environmental		
Hypoxia (220)		NA
Acidification (220)		NA
Cell Density (220)		NA
Nitric Oxide (220)	Phagocytes (220)	NA

Abbreviations: DC, dendritic cell; EpC, epithelial cell, EnC, endothelial cell; MF, macrophage; PMN, neutrophil; BASO, basophil; EO, eosinophil; MC, mast cell.

However, a dysregulation of this process can result in a sustained inflammatory response and tissue damage. Thus enhanced neutrophil survival, as seen in chronic inflammation, may contribute towards the pathogenesis of inflammatory diseases (e.g. sepsis (241) and chronic obstructive pulmonary disease (242)), and with autoimmune diseases such as rheumatoid arthritis (169). Moreover, an extension of lifespan may allow the neutrophils time to acquire functional changes; for example, neutrophils located within the airways of cystic fibrosis patients have been shown to undergo metabolic reprogramming and display an altered phenotype (reviewed in 26).

Since chronic inflammation is a hallmark of cancer (6) many of the previously described modulators of neutrophil lifespan (218–220) are found within the TME. For example, DAMPS are released by necrotic tumour cells in a wide range of cancers, including breast cancer (reviewed in 243). Likewise, G-CSF levels, which have also been implicated within neutrophil expansion (Section 1.2.5.2.1) are higher in breast cancer patients compared to controls (244). Therefore, it is conceivable that neutrophils may demonstrate enhanced survival in cancer. This is important because a longer lifespan might allow neutrophils to contribute towards the chronic inflammation of the TME, indirectly supporting tumour development and progression (Section 1.2.4) and may enable the neutrophils to alter their phenotype (Section 1.2.5.3) and/or engage in more complex activities within tumour growth and development (Sections 1.2.5.4-5).

To date there is limited experimental evidence regarding the influence of cancer cells on neutrophil lifespan, and that which is available is from *in vitro* studies. A study by Zhu *et al*. (31) demonstrated that cross talk between cancer derived mesenchymal cells (multipotent stem cells that can differentiate into a variety of other cell types) prompted gastric cancer progression and increased the lifespan of neutrophils. In addition, Hor *et al*. (30) demonstrated that cross talk between glioma cells and neutrophils could induce a significant delay in neutrophil apoptosis mediated by IL-6 and IL-8; whilst conditioned media (media harvested from cultured glioma cells) protected the neutrophils from apoptosis the protective effect was far greater when the neutrophils were co-cultured with the cancer cells, suggesting cell-cell contact might augment the process (30). Likewise, research by Trellakis *et al*. (32) demonstrated that head and neck squamous cell

carcinoma (HNSCC) conditioned media inhibited neutrophil apoptosis, increased neutrophil chemotaxis and induced the release of MMP-9 and CCL4 (an inflammatory mediator), with a subsequent study suggesting this effect was mediated by p38-MAPK signalling (33). Furthermore, conditioned media created from hepatocellular, cervical, colorectal and gastric cancer cell lines have been shown to delay neutrophil apoptosis by increasing the expression of the anti-apoptosis peptide MCL-1 and attenuating the expression of the pro-apoptosis peptide BAX (236). Moreover, the neutrophils secreted large amounts of pro-inflammatory mediators such as TNF-α and enhanced the motility of the tumour cells (236); Hyaluronic acid (HA), an ECM component, was found to mediate this effect by activating the neutrophils (236). This is interesting because invasive breast cancer cells have been demonstrated to overexpress HA synthase *in vitro* (245), which suggests a delay in neutrophil apoptosis might be seen when neutrophils share a microenvironment with certain breast cancers. Research by Ibrahim *et al.* (published as an abstract (34)) found conditioned media from MDA-MB-231 breast cancer cells delayed neutrophil apoptosis. This delay was thought to be partially mediated by a tumour secreted peptide, derived from the N-terminal of the a2 isoform of Vacuolar ATPase (34). A subsequent study (published as an abstract (35)), showed that neutrophils treated with recombinant a2ND display a significant increase in survival; a2ND was found to modulate the intrinsic pathway, via increased expression of anti-apoptosis peptides A1 and B-cell lymphoma-extra-large ("Bcl-xL") and decreased expression of pro-apoptosis peptides BAX and Apoptotic protease activating factor 1 ("Apaf-1"), and the extrinsic pathway via decreased expression of caspase-3, -6, -7.

The current evidence suggests that neutrophil lifespan may be increased in certain cancers, and that enhanced cancer cell-neutrophil cross talk may lead to an increase in cancer cell motility and invasiveness. The mechanisms involved appear to differ according to cancer type, since cell-to-cell contact appears to be necessary in some cases but not others, suggesting that soluble factors might also be involved. Currently, the limited evidence available suggests that cross talk may occur between MDA-MB-231 breast cancer cells and neutrophils *in vitro*. However, it is not clear if such crosstalk occurs between other types of breast cancer cells and neutrophils either *in vitro* or *in vivo*. Furthermore, whilst recombinant a2ND has been demonstrated to delay neutrophil apoptosis, it does not completely account for the enhanced lifespan demonstrated by

Ibrahim *et al.* (34). This suggests that there are other, yet to be identified, soluble factors involved, which may work synergistically to delay neutrophil apoptosis. This is an important question to answer since it may provide opportunities for therapeutic intervention in breast cancer.

1.2.5.3 Neutrophil phenotype in cancer

Until recently, neutrophils were considered to be a relatively uniform population of cells, with a highly conserved, well defined function (26). However, emerging data suggests that this may not be the case, with neutrophils demonstrating plasticity and the development of distinct subsets with diverse functional and phenotypic profiles in response to both physiological and pathological conditions (reviewed in 26,152). Moreover, there is evidence to suggest that tumour associated neutrophils or "TANs" may be polarised towards either anti-tumoural (N1) or pro-tumoural (N2) activities in response to environmental signals such as IL-1β (36) and TGF-β (37) respectively.

TGF-β is expressed in many tumours and plays a significant role in tumour progression (reviewed in 246). In their seminal paper, Fridlender *et al.* (37) demonstrated that blocking TGF-β signalling can induce an influx of neutrophils within murine tumours. These neutrophils displayed an anti-tumour phenotype (N1) due to their enhanced cytotoxicity (via activation of CD8+ T cell response), increased expression of ICAM-1 (a neutrophil activation marker), higher expression of pro-inflammatory cytokines and reduced expression of arginase-1 (an immunosuppressant) (37). In contrast, TANs from control tumours displayed a pro-tumour phenotype (N2), by supressing the CD8+ T cell response and upregulating the expression of VEGF, a pro-angiogenic growth factor (37).

In contrast, neutrophils from IFN-β deficient mice displayed a tumour promoting phenotype (N2) via increased expression of pro-angiogenic factors such as VEGF and MMP-9; *in vitro* treatment of the TANs with IFN-β reduced their expression of pro-angiogenic factors back to control levels (36). Importantly, subsequent experiments demonstrated delayed apoptosis for TANs in IFN-β deficient mice (247), suggesting that

IFN-β may also regulate (by shortening) the lifespan of TANs. For reference, a simplified scheme of neutrophil polarisation as characterised by their differential expression or secretion of cytokines, chemokines and effector molecules is shown in Figure 1.7.

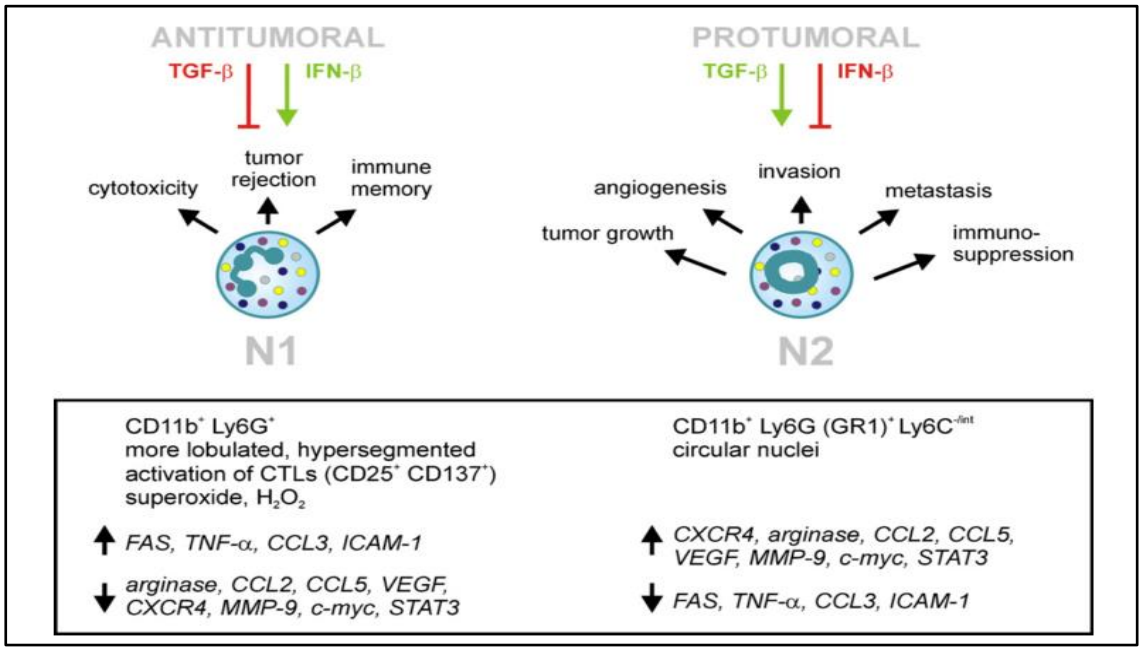


Figure 1.7: Neutrophil polarisation in cancer

As depicted by Piccard *et al*., 2012 (248), demonstrates how neutrophils might exert both anti and pro-tumoural functions within cancer development and progression. N1 (anti-tumoural) neutrophils demonstrate enhanced tumour cell cytotoxicity, assist in tumour rejection and anti-tumoral immune memory, whereas N2 (pro-tumoral) neutrophils enhance angiogenesis, invasion, metastasis and the maintenance of immunosuppression. Reprinted from Critical Reviews in Oncology/Hematology, 82 (3), Piccard H, Muschel RJ, Opdenakker G. On the dual roles and polarized phenotypes of neutrophils in tumour development and progression, Page 298, with permission from Elsevier; (Modified), (248).

In support of the N1/N2 hypothesis of neutrophil polarisation, research has demonstrated that TANs exhibit greater cytotoxicity towards tumour cells (via increased secretion of TNF-α, NO and H_20_2) during the earlier stages of tumour formation (249). These functions were subsequently down regulated within more established tumours, leading to the suggestion that neutrophils may progress from an N1-N2 phenotype as the tumour develops (249). Moreover the cytotoxic TANs isolated from the early tumours were primarily located within the tumour periphery, whereas the pro-tumour TANs from established tumours had infiltrated the tumour itself (249), correlating with studies that

suggest an association between intra-tumoural neutrophils with increasing cancer grade and/or a poorer prognosis (Section 1.2.5). It should be noted that to date, much of this research has been carried out in animal models and more research is required to determine conclusively if TAN polarisation exists in humans.

Moreover, it is unlikely that N1/N2 TANs represent two distinct classifications of cells; it is possible that neutrophils display a linear progression of activation, consequently N1 neutrophils may simply be more activated than N2 (250). This theory is supported by Fridlender *et al*. (25), who found that most of the differences in mRNA expression between N1 and N2 TANs resulted from altered expression (namely upregulation) of the same genes and pathways in N1 TANs compared to N2.

In addition to TANs, circulating peripheral neutrophils also demonstrate an altered phenotype in cancer. Sagiv *et al*. (251) identified (via density gradient centrifugation) three distinct populations of circulating neutrophils from the peripheral blood of human lung and breast cancer patients: a high density fraction of neutrophils (HDN), and a low density fraction (LDN) of neutrophils and granulocytic MDSCs that progressively accumulate throughout tumour development. The HDN fraction of mature neutrophils displayed enhanced cytotoxicity towards cancer cells and possessed an N1-like phenotype, whereas the LDN fraction were less inflammatory, were not cytotoxic and acquired immunosuppressive functions, thus suggesting an N2 phenotype. Interestingly, the LDN fraction displayed a reduced rate of apoptosis compared to the HDN fraction. Moreover a proportion of mature HDNs within late stage tumours were found to spontaneously transition into the low density (LDN) subset, possibly by a TGF-β dependent mechanism (251). Ordinarily, in a healthy person, only terminally differentiated, mature neutrophils are released from the bone marrow (158). However, in cancer many molecules that control neutrophil mobilisation are upregulated, so that immature, undifferentiated neutrophils are released prematurely from the bone marrow (158). Whilst the N1 neutrophils noted by Sagiv *et al*. (251) displayed a mature fully segmented phenotype, the N2 neutrophils contained immature ring shaped and banded neutrophils (defined by Sagiv as G-MDSCs or granulocyte-myeloid derived suppressor cells), as well as mature fully segmented neutrophils. Therefore, it is possible that a

proportion of LDN neutrophils arise from cancer related neutrophilia. However an alternative hypothesis is that LDNs are activated neutrophils that have undergone degranulation, and thus exhibit reduced density (reviewed by Mollinedo, (252) and Rosales, (253). Thus it is possible that the three distinct populations of cells identified by Sagiv *et al*. (251), are in fact all derived from mature human neutrophils, with the differential exocytosis of granule contents resulting in a change of phenotype (252).

MDSCs are a subpopulation of immature myeloid cells belonging to either granulocytic (G-MDSC) or monocytic (MDSC) lineage, characterised by their immunosuppressant and tumour promoting activities (254,255). As per neutrophils, expansion of MDSCs is frequently seen in cancer; however, they are rarely found under normal physiological conditions (158). Differentiating between G-MDSCs and neutrophils is difficult; indeed, there is controversy as to whether these represent two individual populations of cells, or whether they are in fact different phenotypes of the same cell type (reviewed in 252,256,257). A recent mRNA transcriptome analysis that compared naïve bone marrow neutrophils, TANs and G-MDSCs from tumour bearing mice found the three populations to have functionally distinct mRNA profiles, with the naïve neutrophils and G-MDSCs being more closely associated to each other than to TANs (258). This suggests that the TANs are not G-MDSC cells that have simply entered into the tumour (258).

To summarise, it would appear that neutrophil phenotype (as compared to homeostatic conditions) is altered in cancer and this may in part be dictated by cancer type and stage. Overall there is a great deal of work still to be done in this area, since the environmental signals responsible for polarising neutrophils and the underlying mechanisms that bring about this effect are not well understood. Nonetheless, it is clear that neutrophils display substantial heterogeneity in cancer, even those circulating systemically. Due to their heterogeneity, it is not surprising the role of neutrophils in cancer is controversial with both pro and anti-tumour effects being reported (reviewed in 156,164,259–261).

1.2.5.4 Neutrophils and tumour initiation

Tumour initiation is thought to commence when a single random cell incurs a genetic mutation capable of conferring a growth advantage (262). The process of tumour cell proliferation is expedited if the initial tumour cell is exposed to tumour promoters, such as soluble factors released during chronic infection and inflammation (263). In a zebrafish model of HRAS ^{G12V} melanoma, neutrophils and macrophages were recruited to the site of the transformed cells (and interacted with them) whilst they were still only singlet or doublet cells, indicating that neutrophil recruitment may occur very early in tumourigenesis (264). Both neutrophils and macrophages phagocytosed some of the healthy (non-apoptotic) cancer cells, suggesting a clearance role of the leukocytes. However, short term blocking of the immune cells reduced tumour growth, suggesting that the immune cells were also providing some form of tumour promoting factor (264).

An association has also been found between wound-induced inflammation, neutrophil recruitment and the proliferation of pre-neoplastic cells (265). Moreover, several mouse models of spontaneous and inflammation-induced cancers (including skin, and benign or invasive intestinal carcinomas (266,267)) indicate that neutrophils are recruited to premalignant inflamed tissues in response to CXCR2 (IL-8 receptor) ligands such as CXCL1, CXCL2 and CXCL5, and that their presence is required for tumour initiation and growth. Whilst it is difficult to investigate the impact of neutrophils on tumour initiation/formation in humans, a recent study found nine times more neutrophils in gastric intestinal metaplasia samples (pre-malignant) compared to control, with neutrophil density being positively correlated with gastric cell proliferation (268).

Neutrophils are thought to act as tumour promoters through the release of reactive oxygen species (ROS) or reactive nitrogen species (RNS), although these are typically released to kill pathogens (Section 1.2.5.1). ROS and RNS also cause DNA damage and increase the rate of genetic mutations within tumours (269,270). Neutrophil elastase (NE), a protease released during degranulation (Section 1.2.5.1), may also act as a tumour promoter, as it has been shown to be taken up by murine and human lung adenocarcinoma cells, prompting the degradation of insulin receptor substrate-1 (IRS-1); leading to an

increased interaction between PI3K and mitogen platelet derived growth factor receptor (PDGFR) which enhanced tumour cell proliferation (271). Similar results have been found in other types of cancer, including a human breast cancer cell line, SKBR-3 (HER2+/ER-/PR) (272). Purified NE was found to increase the growth of the SKBR-3 cells in a dose dependent manner by causing the activation and phosphorylation of epidermal growth factor receptor (EGFR), HER2+ and extracellular signal-regulated kinases (ERKs) (272). It has yet to be determined if this effect occurs under physiological conditions (i.e. via NE secreted by neutrophils), or in HER2– breast cancers.

1.2.5.5 Neutrophils, tumour growth and disease progression

Research from the last few decades has begun to demonstrate that neutrophils exert either a pro or anti-tumour effect in cancer, largely through the secretion of molecules that would ordinarily be released by neutrophils under normal physiological conditions, to destroy pathogens or modulate inflammation (Section 1.2.5.1) (273).

1.2.5.5.1 Anti-tumour roles of neutrophils in cancer

Research suggests that activated neutrophils may demonstrate direct cytotoxicity towards tumour cells by releasing ROS and myeloperoxidase (MPO), during respiratory burst and degranulation respectively (274–276). Although this may appear to contradict their role in tumour initiation (Section 1.2.5.4), it appears that small amounts of ROS are genotoxic, whereas large amounts of ROS from highly activated neutrophils are cytotoxic (250). Whilst this effect was initially demonstrated using murine models, recently neutrophils isolated from the blood of certain healthy donors were shown to be cytotoxic towards four human cancer cell lines, HeLa (cervical cancer), SKOV-3 (ovarian cancer), Capan-1 (pancreatic adenocarcinoma) and NSCLC (non-small cell lung carcinoma) (277). It is important to note that only a small subset of donors (2 out of the 27 assayed) demonstrated neutrophils with this capability, whereas the others displayed virtually no anti-cancer capability. The ability of the neutrophils to kill cells was cancer cell specific, since they demonstrated little or no cytotoxicity towards primary epithelial cells or an immortalised,

non-transformed epithelial breast cell line (MCF10A) (277). Therapies are being developed to induce neutrophils to demonstrate cytotoxicity towards cancer cells. For example antibody dependent cell-mediated cytotoxicity (ADCC) is an immunotherapy in which tumour cells are labelled with antibodies that also bind to Fc receptors on immune cells (278). Activation of the Fc receptor on neutrophils stimulates the release of ROS and other anti-tumour mediators (reviewed in 156,278). Subsequent research has demonstrated that neutrophils can function as ADCC effector cells in certain cancers, including melanoma (279), non-Hodkinson's lymphoma (280) and breast cancer (281).

Neutrophils may also stimulate an adaptive immune response within cancer. Neutrophils isolated from early stage lung cancer patients have been shown to display an activated phenotype with enhanced ROS expression and upregulated expression of pro-inflammatory cytokines, which appears to stimulate the proliferation and activation of T cells (282), Furthermore, NE has been implicated in stimulating the adaptive immune response in cancer, as uptake of NE by breast cancer cells may i) increase the expression of human leucocyte antigen (HLA) class 1, thereby enhancing antigen presentation (283) and ii) enhance tumour cell susceptibility towards CD8+ T cell lysis (284).

Neutrophils also demonstrate an anti-tumour effect through the secretion of tumour necrosis factor-related apoptosis inducing ligand (TRAIL). TRAIL was first found to be released by neutrophils in response to Mycobacterium bovis Bacillus Calmette-Guérin (BCG) (285). BCG is provided as a treatment, usually very successfully, for bladder carcinoma in situ (reviewed in 285). TRAIL was found to selectively trigger the apoptosis of carcinoma cells, without affecting the surrounding healthy cells or tissues (286,287). Importantly, the expression/release of TRAIL by neutrophils is also upregulated by IFN-γ (287) and IFN-α (172); the latter is frequently used as a treatment for haematologic cancers (288), and subsequently neutrophil derived TRAIL has been found to trigger the apoptosis of myeloid leukaemia cells (172). Lastly, neutrophil derived matrix-metalloproteinase 8 may also protect against tumour development, as it was found to reduce the number of carcinogen-induced skin cancers in mice (289).

1.2.5.5.2 Pro-tumour roles of neutrophils in cancer

Despite the anti-tumour effects described above (Section 1.2.5.5.1) the majority of studies demonstrate a pro-tumour role for neutrophils in cancer. Indeed, neutrophils have been implicated in tumour initiation (Section 1.2.5.4), tumour growth, suppression of the adaptive immune response, angiogenesis and tissue invasion.

As previously discussed (Section 1.2.5.4), neutrophil derived ROS and NE may act as tumour promoters (269–271). In addition to these, neutrophils secrete a range of growth factors, metalloproteinases and cytokines that may also support tumour development. For example, neutrophil derived hepatocyte growth factor (HGF) was found to significantly increase the invasive potential of *c-met* expressing pulmonary adenocarcinoma cells (290) and enhanced the metastases of malignant *c-met* hepatocellular carcinoma cells (291). However, HGF may also stimulate the synthesis of inducible nitric oxide synthase (iNOS) in neutrophils, enhancing nitric oxide production and cytotoxicity towards cancer cells, which suggests some form of negative feedback loop (292).

Furthermore, neutrophil derived matrix-metalloproteinase 9 (MMP-9) was found to increase the proliferation of oncogene-induced keratinocytes and enhanced the conversion of pre-malignant cells into overt carcinomas; this process was thought to be mediated by MMP-9 degradation of the ECM, which may have released tethered growth factors (such as TGF-β, see below) into the TME (293). Likewise MMP-9 was found to improve the survival and establishment of lung carcinoma cells, presumably by degrading the ECM and either i) releasing soluble factors that directly aid in tumour survival or ii) exposing the basement membrane, thereby providing a surface for the tumour cells to adhere to (294). Both NE and MMP-9 have been found to cleave latent TGF-β from the tumour stroma (295,296) and therefore may (indirectly) mediate its effects in cancer. Importantly, TGF-β is a potent chemoattractant for neutrophils (297) and may also, as previously noted, polarise neutrophils towards an N2 pro-tumour phenotype (37).

There is evidence to suggest that neutrophils may suppress both innate and adaptive immune responses within cancer. N2 neutrophils have been shown to secrete large amounts of arginase 1 (ARG1) (37) which hydrolyses arginine, a key amino acid responsible for modulating T cell metabolism and T cell mediated anti-tumour immunity (298). Research suggests that neutrophil derived ARG1 specifically depletes extracellular arginine, thereby supressing a range of T cell functions (299). Moreover, it would appear that neutrophil secretion of ARG1 is directly mediated by cytokines such as IL-8 (300) which are contained within the tumour microenvironment. Likewise, G-MDSCs were shown to inhibit CD8+ T cell responses in a murine model of fibrosarcoma, by exposing the T cells to high concentrations of H_20_2 (301). N2 TANs also secrete CCL17 during tumour development, prompting the recruitment of immunosuppressant CD4+ Treg cells to the site of the tumour (302,303). Lastly, neutrophils may inhibit innate NK cell cytotoxicity, thereby preventing tumour cell destruction in the initial phases of metastatic colonisation (304).

There is also evidence that neutrophils may have a significant role in tumour angiogenesis (reviewed in 305–307). Briefly, research has demonstrated (308) that tumour infiltrating neutrophils are responsible for mediating the angiogenic switch - the point where the balance of pro-angiogenic factors exceeds that of anti-angiogenic factors, leading to tumour vascularisation (309). Specifically, neutrophil derived MMP-9 was shown to increase the amount of bioavailable VEGF within the TME, thereby driving neovascularisation of pre-malignant lesions. Furthermore, neutrophils in IFN-β deficient mice (considered to be an N2 phenotype) expressed elevated levels of VEGF and MMP-9 and demonstrated enhanced tumour growth and vascularisation (36). Neutrophil derived MMP-9 promotes tumour vascularisation by cleaving the active form of VEGF-A and other angiogenic factors such as fibroblast growth factor (FGF) from the ECM, and once released these act upon nearby endothelial cells (306). TANS are also a direct source of angiogenic factors, as neutrophils contain a large amount of VEGF-A in their granules that can be rapidly released in response to TNF-α (310). In addition, neutrophils stimulated with G-CSF express prokinecitin-2 or "BV8" (311), a peptide that enhances the proliferation of endothelial cells and promotes tumour angiogenesis (312). Neutrophils are also a source of cytokines that can indirectly promote angiogenesis: research by Queen *et al*. (313) demonstrated that MDA-MB-231 and T47D breast cancer

cells can stimulate the production of Oncostatin M (OSM) in human neutrophils, which in turn, induced the release of VEGF from the breast cancer cells and enhanced tumour cell detachment and invasiveness. It is important to note that whilst some studies have demonstrated a pro-proliferative effect for OSM in cancer (313,314), others have found the opposite, (315), consequently its actions might depend on cancer type.

However, neutrophils have also been shown to secrete or enhance the levels of anti-angiogenic factors. For example, NE cleavage of plasminogen produces angiostatin, that in turn inhibits VEGF and FGF2 mediated endothelial cell proliferation (316), moreover NE has been shown to directly degrade VEGF and FGF2 thereby inhibiting their angiogenic activities (317). It therefore appears that neutrophils have a dual role in angiogenesis, with N2 neutrophils in particular being skewed towards a pro-tumour, pro angiogenic phenotype.

Neutrophil derived proteases, such as MMP-9, MMP-8, NE and cathepsin G have a key role in tissue invasion (reviewed in 318), primarily by degrading the structural components of the ECM, thus providing a pathway for the disseminating tumour cells (318). In addition to modulating the ECM, neutrophil derived factors may also enhance the migratory capacity of tumour cells by inducing the EMT transition; specifically this process increases tumour cell motility and degradation of the ECM/basement membrane, thereby promoting tissue invasion (129). Grosse-Steffan *et al*. (319) were the first to demonstrate this mechanism in 2012. They found neutrophil infiltration to be strongly correlated with the expression of nuclear β-catenin and ZEB1 (markers of the EMT transition) in biopsies of pancreatic ductal adenocarcinomas. Moreover, T3M4 and HuH7 pancreatic cancer cell lines co-cultured with neutrophils underwent rapid dyshesion and demonstrated altered expression of markers of the EMT transition (increased expression of TWIST, nuclear β-catenin and ZEB1, and down regulation of keratins), possibly induced by a loss of cell to cell contact, since NE was shown to degrade tumour cell E-cadherin (319). Further research (320) demonstrated an inverse association between neutrophil infiltration and E-cadherin expression in lung adenocarcinomas; co-cultures of neutrophils isolated from the blood of adenocarcinoma patients with A549 and SPC-A1 lung adenocarcinoma cell lines induced EMT in the tumour cells via a TGF-β signalling

pathway. Moreover, neutrophils were found to induce EMT in a zebrafish model of oncogene induced keratinocytes via CXCR2 signalling (321), recent research by *Li et al.* (322) found tumour associated neutrophils to induce EMT via IL-17a signalling whereas neutrophil-like HL60N cells induced EMT in human gastric cancer cells through the expression of IL-6, IL-8, IL-1β, and TNFα, which subsequently activated the ERK signalling pathway (323).

Whilst there has been recent progress in understanding how neutrophils might demonstrate a pro-tumour role in cancer there are still significant gaps in understanding how neutrophils influence the EMT transition. The results of several of the cited studies need to be viewed with caution. Specifically, one group used an immortalised "neutrophil-like" cell line "HL-60 (323); whilst HL-60 cells demonstrate many characteristics of mature neutrophils (324) their differentiation is somewhat impaired (325), consequently these cells do not fully reflect the biological activity of primary human neutrophils (325). Other researchers used a zebrafish model of cancer (321); due to their small size, large number of offspring and fast maturation time, zebrafish have become an important new cancer model over the last few decades (326). However whilst their tumours largely resemble human cancers on a histological level, they do not completely reflect human disease in terms of incidence, onset and tumour spectrum (reviewed in 326). Regardless, neutrophils appear to induce EMT by a number of mediators and signalling pathways, thus it is possible that their role in this process is dependent upon the phenotype of the neutrophils or tumour type/stage of disease progression. Throughout this project, the role the ability of neutrophils to induce EMT in breast cancer cells was unknown, as published studies during that time had only addressed this occurrence in lung (320), pancreatic (319) and gastric cancers (322,323). However, during the late stages of thesis preparation, a study was published by *Wang et al*. (327) that found intra-tumoural neutrophils (TINs) to induce EMT in MCF-7 and MDA-MB-231 cells. This paper is discussed in Section 6.4.2.

1.2.5.5.3 Neutrophils in metastatic dissemination and the formation of metastases

Research from the last few decades suggests that neutrophils may actively support tumour cells during every stage of metastatic dissemination. Specifically, neutrophils may aid tumour cells to invade surrounding tissues (Section 1.2.5.5.2), intravasate and survive in the circulatory system, lodge within the microvasculature and extravasate at distal sites to form secondary tumours (reviewed in 158,305,318,328). However, whilst there is evidence to suggest a pro-metastatic role for neutrophils, this remains controversial, with opposing anti-metastatic roles also being demonstrated, even within similar experimental models (329,330).

As previously noted, (Section 1.2.5.5.2) neutrophils may support local tissue invasion by inducing EMT in tumour cells and degrading the ECM. For example, neutrophil derived proteases may enhance the bioavailability of soluble factors such as TGF-β, a cytokine demonstrated to induce EMT in murine breast carcinomas and promote intravasation (331). TGF-β may also promote intravasation by activating the LIM-homeobox gene 2 (LHX2) which acts to increase the diameter of tumour blood vessels (332). It would appear that neutrophils also indirectly support intravasation through the induction of angiogenesis (Section 1.2.5.5.2), for example neutrophil derived MMP-9 enhances angiogenesis and intravasation in fibrosarcoma and prostate carcinoma cells (333). The tumour blood vessels are thin and leaky, being comprised of highly disorganised, loosely connected endothelial cells (334). Consequently barrier function is impaired and may facilitate intravasation (334,335).

Once tumour cells are in the circulatory system they must survive the shear stress imposed by blood flow and avoid detection by immune cells (127,336). One of the mechanisms by which they may achieve this, is through the formation of clusters or aggregates of at least 2-3 cells, which are referred to as tumour microemboli, or circulating micrometastases (337). The clusters prevent the tumour cells from undergoing anoikis (programmed cell death triggered when cells detach from the ECM (338)), furthermore. platelets, which are often associated with clusters, protect the tumour cells from blood flow sheer stress and anti-tumour immune responses (339,340). It is possible that

neutrophils may have a role in this process, as cathepsin-G (a neutrophil derived protease) was recently shown to induce MCF-7 breast cancer cells to cluster *in vitro* (341). Neutrophils may also enhance the survival of murine intraluminal tumour cells by inhibiting the anti-tumour activity of circulating NK cells (304).

Neutrophils may support the arrest and extravasation of tumour cells at secondary sites by several mechanisms. Firstly, neutrophils secrete proteases and cytokines that may activate the endothelium and promote tumour cell adhesion. It has been shown that NE promoted the adhesion of pancreatic and colon cancer cell lines to human umbilical vein endothelial cells (HUVEC) by increasing the expression of E-selectin (an endothelial cell adhesion molecule) on HUVEC (342). Furthermore, neutrophils may support tumour cell extravasation through the secretion of IL-1β (which activates endothelial cells), MMP-8 and MMP-9 (304). Other studies have demonstrated that tumour cells co-localise with neutrophils at the endothelium of target organs, in order to promote their adhesion. For example, lung carcinoma cells adhere directly on top of neutrophils within the liver sinusoids, the carcinoma cells being held in place by neutrophil Mac-1 (CD11b) (343). Neutrophils have also been shown to enhance the transendothelial migration of melanoma cells by promoting Mac1 (neutrophil)/ICAM-1(melanoma cell) adhesive interactions (344). Huh *et al*. (345) demonstrated that neutrophils may increase the retention of melanoma cells in a murine model of lung metastases by 3- fold, mediated by Mac-1 (CD18) (neutrophils)/ICAM-1 (melanoma cell) adhesive interactions (346). Finally, there is evidence to suggest that neutrophil derived NETs (Section 1.2.5.1) may enhance the adhesion of circulating tumour cells, with circulating lung carcinoma cells being trapped within microvascular NETs, thereby enhancing the formation of hepatic micrometastases in mice. Likewise, the formation of NET like structures around metastatic murine breast cancer cells have been found to stimulate the invasion and migration of the breast cancer cells *in vitro* (347)..

However, there is also evidence that neutrophils may display anti-metastatic properties. Granot *et al*. (329), found an accumulation of activated neutrophils in a pre-metastatic mouse model of breast cancer (4T1). Referred to as "tumour entrained neutrophils" (329), these cells accumulated in the pre-metastatic lung and were activated (entrained) by

tumour derived CCL2, prompting the neutrophils to release ROS and acquire a cytotoxic phenotype; as a consequence, the tumour entrained neutrophils inhibited metastatic seeding. This anti-metastatic activity was questioned by Coffelt *et al*. (158), who noted a pro-metastatic role for neutrophils in other studies of the same tumour cell line. It was suggested (158) that these contradictory results might, in part, be due to experimental timing, with neutrophils being isolated from early stage tumours demonstrating different behaviour from those isolated from late stage tumours, or that the cell lines through successive culturing had undergone genetic drift so that the same cell line used by different laboratories produced divergent cytokines. However, several of the studies (348,349) cited by Coffelt *et al*. (158) were investigating the prometastatic functions of myeloid derived suppressor cells, which as previously discussed (Section 1.2.5.3) have been found to have functionally distinct genetic profiles compared to naïve neutrophils or TAN. Therefore, further work needs to be undertaken to determine the role of TANs in the development of distal metastases.

1.2.6 Conclusions from the literature

Over the last few decades it has become well recognised that the tumour micro-environment plays a pivotal role in tumour development and progression. Whilst interactions between certain cells, such as macrophages, and cancer cells have been reasonably well characterised, less is known regarding the role of neutrophils.

For a long time, neutrophils were thought to have a negligible role in cancer, mostly due to their short lifespan. However, neutrophils display an extended lifespan in certain inflammatory diseases, and there is some evidence that the same process may occur (and be associated with adverse outcomes) in cancer; however, the mechanisms and mediators behind this effect are not well understood and may in part be dependent upon the type or stage of cancer. To date only limited work has been undertaken regarding the lifespan of neutrophils in breast cancer.

The overall role of neutrophils in cancer remains controversial with both pro and anti-tumour effects demonstrated. It would appear that neutrophil phenotype can be altered in cancer, which may again be dependent upon the type or stage of cancer, which may explain their opposing roles. Thus, a great deal of work is required to fully characterise this process and determine its impact on tumour development and progression in breast cancer.

In recent years it has become clear that the epithelial-mesenchymal transition is a critical step towards malignancy. Whilst there is limited evidence that neutrophils may induce this process, some of the models used (such as zebrafish models or a neutrophil-like cell line) may not accurately reflect the biological mechanisms found in humans.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 General chemicals and reagents

General chemicals and reagents were purchased from Sigma-Aldrich (Auckland, New Zealand) unless otherwise stated.

2.1.2 Cell culture reagents

Delbucco's Modified Eagle Medium (high glucose, pyruvate DMEM) was purchased from Thermo Fisher Scientific (Auckland, New Zealand). AIM V serum free medium, penicillin, streptomycin and trypsin-EDTA were from Gibco (Thermo Fisher Scientific, Auckland, New Zealand). Ciprofloxacin was from Acros Organics (Thermo Fisher Scientific, Auckland, New Zealand). Prostaglandin E2 (Prod. No. P049) was from Sigma-Aldrich (Auckland, New Zealand). Fetal bovine serum (FBS) was from Moregate Biotech (Hamilton, New Zealand). MCF-7 cells and MDA-MB-231 cells were from the American Type Culture Collection (Manassas, Virginia, United States). Amicon Ultra-15 Centrifugal Filters were purchased from Sigma Aldrich (Merk Millipore, Darmstadt, Germany). Corning 12mm transwell plates (Prod. No. 3460) were purchased from Invitro Technologies (Auckland, New Zealand).

2.1.3 Neutrophil isolation reagents

Histopaque 1.119 and 1.077 polysucrose density gradient solutions were from Sigma Aldrich (Auckland, New Zealand). MicroBeads conjugated to monoclonal mouse anti-human CD15 antibodies (Cat. No. 130-046-601), MACS LS columns and a MidiMACS separator were from Milteny Biotec (Sydney, Australia). Bovine serum albumin (BSA) was purchased from Gibco (Thermo Fisher Scientific, Auckland, New Zealand). EDTA

anticoagulant blood collection tubes were purchased from BD-Vacutainer (Thermo Fisher Scientific, Auckland, New Zealand).

2.1.4 RT-PCR reagents

An isolate II RNA mini kit (Cat. No. BIO 52073) was from Bioline (Alexandria, NSW, AUS). High Capacity cDNA Reverse Transcription Kits and PowerUp SYBER Green Mastermix PCR reagent were from Applied Biosystems (Thermo Fisher Scientific, Auckland, New Zealand). All PCR primers were synthesised by Integrated DNA Technologies (Coralville, Iowa, USA). Mycoplasma PCR positive control and internal control was provided as a gift from Dr Cord Uphoff (Department of Human and Animal Cell Lines, DSMZ, Braunschweig, Germany). Nuclease-free water was from Thermo Fisher Scientific (Auckland, New Zealand). A QIAquick® PCR purification kit was from Qiagen (Bio-Strategy Ltd, Auckland, New Zealand).

2.1.5 Molecular biology reagents

Amplex red (Cat. No. A22177) and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (Cat. No. M6494) reagents, a 1kb DNA molecular weight ladder, 2x RNA loading Dye, 6xDNA loading dye and a prestained page-ruler protein ladder were from Thermo Fisher Scientific (Auckland, New Zealand). Trypan blue solution (0.4%) was from Gibco (Thermo Fisher Scientific, Auckland, New Zealand). A flow cytometry dead cell apoptosis kit with Annexin V FITC and PI (Cat.No. V13242), and SYBR™ Safe DNA Gel Stain (Cat. No. S33102) were from Invitrogen (Thermo Fisher Scientific, Auckland, New Zealand). BD calibrate beads were from BD Biosciences (Auckland, New Zealand). DNAse and RNAse free agarose was from Bio-Rad (Auckland, New Zealand). PVDF membrane, Immobilon P, was from Millipore (Sigma-Aldrich, Auckland, New Zealand). Ultrapure water was obtained from a Milli-Q system (Merck Millipore, Darmstadt, Germany).

2.1.6 Antibodies

Mouse monoclonal antibodies against caspase-8 (clone B.925.8) and against GAPDH (Invitrogen, clone ZG003) were from Thermo Fisher Scientific (Auckland, New Zealand). A mouse/rabbit Epithelial-Mesenchymal western blot cocktail against vimentin, β-catenin, GAPDH and B-actin (ab157392) was from Abcam (Cambridge, United Kingdom). A horse-radish peroxidase (HRP) conjugated polyclonal anti-mouse IgG secondary antibody (Cat. No. A9044) was from Sigma-Aldrich (Auckland, New Zealand).

2.2 General Methods

2.2.1 Neutrophil isolation

2.2.1.1 Identification and recruitment of study participants

This study was approved as a low risk ethics application by the Massey University Southern Ethics Committee (Southern A Application 15/08). Human neutrophils were obtained from the peripheral blood of healthy male and female volunteers. Participants were provided with an information sheet and had the study explained to them verbally, before providing written informed consent.

17 subjects (11 females, and 6 males) were selected on the basis of having good general health with no known immune system disorders, blood borne contagious diseases or disorders of bleeding and/or clotting of the blood. One of the subjects was a habitual smoker. The health screening questionnaire provided to participants for this research is provided in Appendix A.

2.2.1.2 Blood sampling and processing

Non-fasting venous blood samples were taken by a trained phlebotomist at Massey University, Albany, Auckland. Each sample (approximately 6mL of blood) was obtained in a collection tube coated with EDTA anticoagulant. Blood samples were not pooled but were used in matched pairs for experiments; i.e. a participant would donate two blood samples, with one used for the MCF-7 assay and the other for the MDA-MB-231 assay.

All blood samples were processed within 15 minutes of collection and treated in accordance with the Massey University guidelines for handling unfixed human tissue, blood body fluids in research.

2.2.1.3 Red blood cell (RBC) lysis

A 10X RBC lysis buffer (1.5M ammonium chloride, 0.1M sodium bicarbonate, 12.7mM EDTA) was prepared in ultrapure water and stored at 4ºC for up to six months. A working 1 X RBC stock solution was created by adding 10ml of 10X RBC buffer to 90ml of µultrapure water and stored at 4ºC for up to six months. RBC lysis was performed by aliquoting 3ml of whole blood into a sterile 50ml conical centrifuge tube, followed by 42ml of cold 1 x RBC buffer. The tube was gently inverted every 60 secs for ~10min, until the liquid was a clear red, then centrifuged in a Heraeus Megafuge 1.0R centrifuge (Thermo Fisher Scientific, Auckland New Zealand) (250x*g*, 5min, 4ºC). The pelleted cells were washed with 10ml cold 0.01M PBS (1X), centrifuged (250x*g*, 5min, 4ºC) and then resuspended according to the ensuing experimental protocol.

2.2.1.4 Neutrophil isolation via MACs CD15+ positive selection

Leucocytes (as prepared in Section 2.2.1.3) were resuspended in 80µl of isolation buffer (PBS (pH 7.2), 0.5% BSA, 2 mM EDTA), and 20µl CD15 Microbeads per 10^7 of total cells. The leucocyte preparation was gently inverted, incubated for 15min at 2-8ºC, then 1ml of isolation buffer was added per 10^7 cells. The sample was centrifuged (300x*g*, 10min, at room temperature or "RT"), the supernatant discarded and the pellet resuspended in 500µl of isolation buffer per 10^8 cells.

A MACS LS column was placed within the magnetic field of a MidiMACS separator and was rinsed with 3ml of isolation buffer. The cell sample was applied to the column, then the column was washed 3 times with 3ml of buffer to remove any non-labelled cells. The column was removed from the magnetic separator and placed within a fresh 15ml sterile polypropylene tube, 5mls of isolation buffer was applied to the column, after which the plunger was firmly pushed into the column to flush out the magnetically labelled neutrophils. The cells were centrifuged (250x*g*, 5min, RT), the supernatant discarded and the cells washed with PBS (pH 7.4). The purified neutrophils were pelleted (250x*g*, 5min, RT) and then resuspended according to the ensuing experimental protocols.

2.2.2 Cancer cell culture

2.2.2.1 General cell culture

MCF-7 and MDA-MB-231 cell lines were routinely cultured in Delbeccos Modified Eagle Medium (high glucose, pyruvate DMEM) supplemented with 10% v/v FBS, 100U/ml penicillin and 100µg/ml streptomycin ("complete medium"). The cell lines were maintained at 37ºC with 5% CO_2 and were routinely cultured in T 75 flasks.

Unless otherwise stated both cell-lines were passaged when 70% confluent. The media was removed, the cells washed with PBS (pH 7.4), which was subsequently removed prior to cells being incubated with 2ml of 0.05% trypsin-EDTA, for 3min at 37ºC. An aliquot of media was added to the flask to inactivate the trypsin, the cells were separated by pipetting and passaged into flasks at a ratio of ~1:3.

Frozen cell stocks were created from flasks of cells grown to ~90% confluence, the cells were washed and dissociated from the flask surface as previously described. The cell suspension was removed from the flask, centrifuged (1200rpm, 5min, RT) and the pellet resuspended in 1ml of medium supplemented with 10%v/v FBS, 30%v/v dimethyl sulphoxide (DMSO). Cells were stored immediately at -80ºC, for long term storage. Cells stocks were recovered by thawing rapidly at 37ºC. The cells were resuspended in 10ml of complete medium, then centrifuged (1200rpm, 5min, RT), the medium (containing DMSO) removed, and the cells resuspended in fresh complete medium and seeded into flasks.

2.2.2.2 Creation of conditioned media (CM) and untreated control media

Cultured cells were grown to approximately 60% confluence; the spent medium was removed, and the cells washed with PBS (pH 7.4) before fresh complete medium was added. The conditioned medium was collected 48hours later, filtered through a 0.22µm

filter and was stored for ~2hours at 37ºC before being used within experiments (Chapters 4-6)). Untreated control medium was also prepared for each conditioned medium experiment. A 20ml aliquot of medium was placed in a cell-free T 75 flask and incubated at 37ºC with 5% CO_2. The untreated medium was collected 48 hours later, filtered through a 0.22µm filter was stored for ~2hours at 37ºC before being used within experiments.

2.2.2.3 Mycoplasma testing

MCF-7 and MDA-MB-231 cell lines were routinely tested for the presence of mycoplasma contamination, according to the method of Uphoff and Drexler (350). As a precaution, to further mitigate the risk of contamination, MCF-7 and MDA-MB-231 cell lines were routinely treated with ciprofloxacin (351). Prior to the creation of frozen cell stocks; the cell lines were treated with 10µg/ml ciprofloxacin on alternate days for a duration of two weeks (350), and then frozen as previously described (Section 2.2.2.1).

2.2.3 Cell biology techniques

2.2.3.1 May-Grünwald-Giemsa staining of neutrophils

To determine the purity and composition of the isolated cells, the neutrophil preparations were subjected to May-Grünwald-Giemsa staining. A 10µl aliquot of freshly isolated neutrophils was placed on a Poly-Prep, poly-lysine coated glass slide, air dried for two hours then fixed with 100% methanol. The slides were stained in undiluted May-Grünwald solution for 3min, then placed in a working solution of May Grünwald diluted 1:1 with Sørensons buffer pH 7.0 (133mM disodium phosphate: 133mM potassium phosphate monobasic) for 5min. The slides were rinsed in Sørensons buffer for 60 secs, stained in Giemsa solution diluted 1:5 with Sørensons buffer for 25min, then gently flushed with Millipore ultrapure water. Slides were imaged using an Axiostar plus microscope (Zeiss, Oberkochen, Germany).

2.2.3.2 Determination of leucocyte populations (cell purity) via flow cytometry

A sample of leucocytes, as prepared via RBC lysis (Section 2.2.1.3) and a sample of neutrophils, as isolated via MACs CD15+ positive selection (Section 2.2.1.4) were analysed via flow cytometry to quantify their cellular composition. The leucocytes (as prepared in Section 2.2.1.3) were resuspended in 10ml of PBS (pH 7.2). The sample was split into two 5ml aliquots. These were placed in two separate 15ml sterile polypropylene tubes, one of which was centrifuged (250xg, 5min, RT), the supernatant removed, the neutrophils isolated MACs CD15+ positive selection (Section 2.2.1.4) and then resuspended in 5mls of PBS (pH 7.2). Both samples were run through a BD FACScanto II flow cytometer (BD Bioscience, Auckland, New Zealand). The samples were analysed using BD FACSDiva software; the individual populations of cells (granulocytes, monocytes and lymphocytes) were identified according to their forward scatter/side scatter characteristics. Each population was gated and the number of events (cells) determined. Sample composition was determined by dividing the number of cells within an individual population, by the total number of cells and multiplying by 100 or % composition = [1.00 – (Number of cells within a sample ÷ {Number of granulocytes + Number of lymphocytes + Number of monocytes})] × 100.

2.2.3.3 Assessment of neutrophil activation and function via the Amplex red colorimetric assay

A 10mM stock solution of Amplex Red was prepared in DMSO, and a 1000U/ml stock solution of horse radish peroxidase (HRP) was prepared in 0.25M sodium phosphate (pH 7.4). Stock solutions were divided into single use aliquots and stored at -20ºC. An Amplex red working solution, comprised of 50mM Amplex red and 1U/ml HRP in Krebs Ringer Phosphate Glucose (KRPG) buffer (145mM sodium chloride, 5.7mM sodium phosphate, 4.86mM potassium chloride, 0.54mM calcium chloride, 1.22mM magnesium sulphate and 5mM of glucose, pH 7.35) was prepared immediately prior to each experiment.

To assess the functional activity of the isolated neutrophils, as determined by their response to stimulus, a 40µg/ml stock of phorbol 12-myristate 13-acetate (PMA) was prepared in DMSO and was stored in single use aliquots at -20ºC; PMA was diluted to 60ng/ml in Amplex Red working solution immediately prior to each experiment.

Fresh H_20_2 standards were prepared each day. A 25mM stock solution of H_20_2 in KRPG was prepared, then serially diluted to create seven standards (250, 125, 62.5, 31.25, 15.63, 7.81 & 3.9 µM) of H_20_2 in KRPG. A 20µl aliquot of each standard, along with a blank, was loaded in quadruplicate into a Griener Bio-one 96 well microplate.

Neutrophils isolated via immunomagnetic positive selection (see Section 2.2.1.4) were resuspended in KPRG buffer at a final concentration of $2.5x10^6$cells per ml. A 20µl aliquot of neutrophils in KRPG ($5x10^4$ total cells) was loaded into six wells of the 96 well microplate described above. These were split into two groups; the first group were to be untreated (control) neutrophils and the second group were to be neutrophils stimulated with PMA.

A 100µl aliquot of Amplex Red working solution was added to each of the standards, blanks and control cells. A 100µl aliquot of PMA Amplex Red working solution was added to the second group of neutrophils in order to stimulate them. The plate was protected from sunlight and was incubated for 30min at RT, after which fluorescence was measured on a FLUOstar Omega Microplate reader, using wavelengths of 560nm for excitation and 590nm for emission. The plate was incubated for a further 90min at RT, and the fluorescence measured again. MARS Data analysis software (BMG Labtech 96, Ortenberg, Germany) was used to analyse the results, with the fluorescence reading obtained at 30min being used to create the standard curve and determine the baseline activation status of the neutrophils, while the reading obtained at 120min was used to determine the functional responses of the neutrophils.

2.2.3.4 Assessment of neutrophil lifespan and apoptosis

2.2.3.4.1 Trypan blue exclusion test

Neutrophil viability was determined manually via trypan blue staining according to the method of Strober (352). An 100µl aliquot of neutrophils was added to 50µl of 0.4% trypan blue solution and gently mixed. The neutrophils were manually counted on a Bright-Line haemocytometer. Cell viability was determined by dividing the number of viable cells (cells excluding trypan blue) by the total number of cells and multiplying by 100 or % viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100.

2.2.3.4.2 Flow cytometry neutrophil apoptosis assay

Control medium and MDA-MB-231 CM (day 1) or MCF-7 CM (day 2) was prepared prior to commencing each experiment (Section 2.2.2.2). Neutrophils were isolated from whole blood samples via positive selection (Section 2.2.1), resuspended in control medium, MDA-MB-231 CM (day 1) or MCF-7 CM (day 2) at a concentration of $3.33x10^5$ cells/ml, then aliquoted into a 24 well plate to a final volume of 1.5ml. A sample of neutrophils in untreated medium was stimulated with 30ng/ml phorbol 12-myristate 13-acetate (PMA) and 0.1mM H_2O_2 as a positive control for apoptosis (353). The cells were incubated at 37ºC with 5% CO_2, neutrophil viability was assessed at 0hrs, immediately post isolation and at 3, 7, 10 and 24hrs post incubation. Neutrophils were stained with Alexa Fluor® 488 Annexin V and propidium iodide according to manufacturer's instructions. The cells were analysed on a BD FACScanto II flow cytometer (BD Bioscience, Auckland, New Zealand) measuring fluorescence emission at 530nm (FITC) and 670nm (PerCP-Cy5.5). Compensation controls were set using BD FACSDiva software and BD calibrate beads according to the manufacturer's instructions. Compensation controls were confirmed using single-stained (either Annexin V or propidium iodide) sample control tubes. The data was gated and analysed using BD FACSDiva software, and exported to Microsoft Excel (2016) software for statistical analysis. Three parameters were investigated, the proportion (%) of viable cells as stained

FITC-/PI-, the proportion (%) of cells undergoing apoptosis as stained FITC+/PI- and the proportion (%) of necrotic cells as stained FITC+/PI+ and FITC-/PI+. A two-tailed paired *t* test was used to determine the difference in means for each of these parameters, comparing neutrophils incubated in untreated media, against those neutrophils incubated in either MCF-7 CM or MDA-MB-231 CM. Statistical significance was defined as $P<0.05$.

2.2.3.4.3 Neutrophils cultured in fractionated, conditioned medium, apoptosis assay

Four different sized Amicon Ultra-15 centrifugal filters were used, with molecular weight cut offs (MWCO) of 10, 30, 50 and 100kDa. Prior to use, the filters were rinsed with 12ml of Millipore ultrapure water, then centrifuged (4000x*g*, 20min, RT) to remove any residual traces of glycerine.

Control medium and MDA-MB-231 CM was prepared prior to commencing each experiment (Section 2.2.2.2). On the day of the experiment 12mls of MDA-MB-231 CM and 12mls of control medium were aliquoted into separate centrifugal filters. The filters were centrifuged at 4000x*g*, RT, for 30min (10kDa and 30kDa filters) or 20min (50kDa and 100kDa filters). A 300µl aliquot of retentate and 11.4ml aliquot of filtrate was removed from each filtration device; the filtered control medium was used to dilute the MDA-MB-231 CM retentate and the filtrate, after which the samples were filtered through a 0.22µm filter and stored at 37ºC for approximately 2hrs.

Neutrophils were isolated from whole blood samples via positive selection (Section 2.2.1) and were resuspended in the following preparations (i-vi) at a concentration of $3.33x10^5$ cells/ml:

i) control medium (negative control),
ii) unfiltered MDA-MB-231 CM (positive control)
iii) retentate from MDA-MB-231 CM
iv) filtrate from MDA-MB-231CM
v) a mixture of the retentate and filtrate from MDA-MB-231 CM

The cells were aliquoted into a 24 well plate to a final volume of 1.5ml and incubated at 37ºC with 5% CO_2. Neutrophil viability was assessed at 0hrs, immediately post isolation and at 7hrs post incubation via flow cytometry using a Dead Cell Apoptosis kit as previously described (Section 2.2.3.4.2).

2.2.3.4.4 Heat treated MDA-MB-231 conditioned medium apoptosis assay

Control medium and MDA-MB-231 CM was prepared prior to commencing each experiment (Section 2.2.2.2). A 15ml aliquot of MDA-MB-231 CM was placed into a sterile 50ml polypropylene centrifuge tube, heated for 10min at 100ºC and centrifuged (1000x*g*, 5min, RT). The supernatant filtered through a 0.22µm filter and stored at 37ºC for approximately 2hrs before use. Neutrophils were isolated from whole blood samples via positive selection (Section 2.2.1). The cells were resuspended in control medium or boiled filtered CM at a concentration of $3.33x10^5$ cells/ml, then aliquoted into a 24 well plate to a final volume of 1.5ml. The cells were incubated at 37ºC with 5% CO_2. Neutrophil viability was assessed at 0hrs, immediately post isolation and at 7hrs post incubation using a Dead Cell Apoptosis kit as previously described (Section 2.2.3.4.2).

2.2.3.4.5 Prostaglandin E2 (PGE_2) apoptosis assay

A 10mg/ml stock solution of prostaglandin E2 (PGE_2) was prepared in ethanol, the stock solutions were divided into single use aliquots and stored at -20ºC. A 1mM working stock solution of PGE_2 prepared in 0.1M PBS (10X), and a vehicle control comprised of ethanol in 0.1M PBS was prepared immediately prior to each experiment. Control medium and MDA-MB-231 CM was also prepared prior to commencing each experiment (Section 2.2.2.2).

Neutrophils were isolated from whole blood samples via positive selection (Section 2.2.1) and were resuspended in each of the following preparations (i-vi) at a concentration of $3.33x10^5$ cells/ml:

i. Untreated control medium
ii. 10µM PGE2 in control medium,
iii. 10µM PGE2 in control medium, heated for 10min at 100ºC, centrifuged (1000x*g*, 5min, RT), the supernatant removed and filtered through a 0.22µm filter.
iv. Control medium supplemented with the vehicle control (ethanol in 0.1M PBS)
v. MDA-MB-231 CM
vi. MDA-MB-231 CM, heated for 10min at 100ºC, then centrifuged (1000x*g*, 5min, RT), and the supernatant removed and filtered through a 0.22µm filter.

Each sample was aliquoted into a 24 well plate to a final volume of 1.5ml. The cells were incubated at 37ºC with 5% CO_2. Neutrophil viability was assessed at 0hrs immediately post isolation and at 7hrs post incubation using a Dead Cell Apoptosis kit as previously described (Section 2.2.3.4.2).

2.2.3.5 Tumour cell migration when co-cultured with neutrophils

The effect of neutrophils upon tumour cell migration was determined via wound scratch assay, using a modified version of the method by Liang *et al.* (354). MCF-7 and MDA-MB-231 cells were collected by trypsinisation as described for passaging (Section 2.2.2.1) and the pellet resuspended in complete medium. Each cell line was plated out into four wells of a 12 well transwell plate, to assess the effect of neutrophils or medium without cells (negative control) upon tumour cell migration in duplicate. The MCF-7 and MDA-MB-231 cells were plated out at a concentration of $3x10^5$cells and $2x10^5$cells respectively, in a volume of 1.5ml medium supplemented with 10% v/v FBS, 100U/ml Penicillin and 100µg/ml Streptomycin. The plates were incubated at 37ºC with 5% CO_2 for 24hrs to create a monolayer.

On the day of the experiment, neutrophils were isolated from whole blood samples via positive selection (Section 2.2.1) and were resuspended in DMEM supplemented with

10% v/v FBS, 100U/ml Penicillin and 100µg/ml Streptomycin. Concurrently, a "wound" was created in each well of the plates containing MCF-7 and MDA-MB-231 cells, by scraping the monolayer in a straight line with a 200µl pipette tip (354). The medium (and detached cells) was gently aspirated, the cells washed with 1ml of PBS (pH 7.4), which was subsequently removed and replaced with 1.5ml of complete medium. To obtain the same field during image acquisition two reference points were made for each well, by marking the outer bottom of the well with an ultrafine black marker pen. The plate was then placed under a phase contrast inverted microscope (CKX31, Olympus, Auckland, New Zealand). For each well the scratch/reference point was placed at the top, centred in the middle, of the eye piece field view (microscope), but outside the capture image field of the camera. A photo was taken of each well, at each reference point using a colour microscope camera (Dino-Eye AM7025X Edge Series 5.0MP, Dino-Lite, Joondalup DC Australia); images taken at this point are referred to as time zero.

The neutrophils, $1x10^5$ cells in 500µl of media, were aliquoted into transwell inserts placed above the MCF-7 and MDA-MB-231 cells. For a negative control, 500µl of medium without neutrophils was aliquoted into the transwell inserts instead. The cells were incubated at 37ºC with 5% CO_2. At 7, 24 and 30hrs the plates were removed from the incubator and the inserts, containing either neutrophils or control medium, were placed into sterile 12 well plates. A photo was then taken of each well, at each reference point as previously described. The inserts were then returned to the plate, and the plate returned to the incubator. The experiment was repeated a total of four times.

The images were analysed using ImageJ Software (355). In order to calculate surface area of the wound, the pixels of the image were converted into mm. This was achieved by measuring the scale bar located at the bottom of the image, then setting the scale to its known distance and unit of length; sample images of this method can be seen in chapter 6. To reduce the risk of measurement bias, a macro was created in ImageJ to set each image to exactly the same scale. For each image, the wound was traced using the freehand selection tool then analysed to determine the surface area of the scratch. Migration rate was expressed as the % of area reduction (wound closure) after 7, 24 and 30hrs incubation via the following formula (356)

$$\text{Wound closure } \% = \left[\frac{At\_0h - At\_\Delta h}{At\_0h}\right] X\ 100$$

Where At_0h is the area of the wound immediately after scratching ($t = 0h$), and $At\_\Delta h$ is the area of the wound measured h hours after the scratch is performed. A two tailed paired t-test was used to compare the difference in mean % wound closure between MCF-7 or MDA-MB-231 cells incubated with neutrophils compared to control.

2.2.3.6 Preparation of samples for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Attached monolayers of cultured cells were washed once with PBS (pH 7.4) and were scraped into an appropriate volume of 6 x SDS page sample buffer (7ml 0.5M Tris, pH 6.8, 40%w/v glycerol, 0.6mM dithiothreitol, 0.18mM Bromophenol Blue, 10% w/v SDS made up to 10ml w/H_2O), diluted 1:3 with ultrapure water). The cells were lysed via vigorous pipetting, then heated at 100ºC for 5min. The samples were cooled for 30min, then stored at -18ºC.

2.2.3.7 SDS PAGE

A 10% polyacrylamide gel was prepared by creating a separating mix (5ml H_20, 3ml 1.5 M Tris/pH 8.8, 4ml 30% acrylamide/0.8% bisacrylamide, 5 µl tetramethylethylenediamine (TEMED), 50 µl 10% ammonium persulphate) which was poured into the gel casting apparatus (BioRad) and overlaid with 100µl of water-saturated butanol. The gel was allowed to set before the water-saturated butanol was drained off. The separating gel was overlaid with stacking mix (6.1ml H_20, 3ml 2.5 M Tris/pH 8.8, 2ml 30% Acrylamide/0.8% Bis, 10 µl TEMED, 100 µl 10% Ammonium Persulphate), a comb was inserted, and the gel was allowed to set.

The samples were defrosted and immediately loaded onto the gel alongside a prestained page-ruler protein ladder (Thermo Fisher Scientific, Auckland, New Zealand). The proteins were separated via electrophoresis, at a constant voltage of 180V for approximately 45min. An SDS PAGE glycine buffer (25mM Tris, 180mM Glycine, 3.5mM SDS made up to 1L w/H_20) was used as the running buffer.

2.2.3.8 Coomassie Blue staining of SDS PAGE gels

Gels were stained with Coomassie stain solution (0.25% v/v Coomassie Brilliant Blue R-250, 50% v/v methanol, 10% v/v glacial acetic acid), for 30min, at RT, with gentle agitation on an orbital shaker. Surplus Coomassie Blue was removed by the application of multiple changes of de-staining solution (50% v/v methanol with 10%v/v glacial acetic acid) for approximately 2hrs at RT until the background was clear.

2.2.4 Immunological Techniques

2.2.4.1 Western-blotting

2.2.4.1.1 Transfer of proteins separated by SDS PAGE to PVDF

A PVDF membrane (Immobilon P, Millipore) was soaked with methanol, then equilibrated in transfer buffer (25mM Tris, 180mM glycine, 0.35mM SDS, 10% v/v methanol in 1L H_20) for 5min. The SDS PAGE gel was laid over the PVDF membrane, and the proteins in the gel were transferred to the PVDF membrane using a semi-dry transfer cell (BioRad, Auckland, New Zealand) at 75 mA per gel or 10V constant voltage for 1 hr.

2.2.4.1.2 Ponceau S staining of PVDF membrane

PVDF membranes were stained with Ponceau S stain (0.1% w/v Ponceau S in 5% v/v acetic acid) for one hr at RT with gentle agitation on an orbital shaker. Surplus stain was removed by rinsing the membrane in distilled water.

2.2.4.1.3 Immunostaining of PVDF membrane

After removing any remaining Ponceau S stain (by rinsing with distilled water) the PVDF membrane was rinsed with PBS-$T_{0.1}$ (0.1% v/v Tween-20 in PBS). Non-specific protein binding sites on the western blots were blocked by placing the membrane in 5% (w/v) fat-free powdered milk in PBS-$T_{0.1}$, for 60min, at RT on an orbital shaker. The membrane was washed once with PBS-$T_{0.1}$, then incubated with primary antibody diluted in 5% (w/v) fat-free powdered milk in PBS-$T_{0.1}$ for 60min, at RT on an orbital shaker then overnight at 4ºC. The primary antibody solution (see Sections 2.2.4.2-3 for dilutions) was removed and the blot was washed three times with PBS-$T_{0.1}$ over a period of 45min, after which the blot was incubated with HRP-conjugated secondary antibody diluted in 5% (w/v) fat-free powdered milk in PBS-$T_{0.1}$ for 60min, at RT, on an orbital shaker. The blot was then washed with PBS-$T_{0.1}$ three times over a period of 45min, then incubated in chemiluminescent developer comprised of 1ml luminol solution (prepared with 200 ml 0.1M Tris, pH 8.6100 mg sodium luminol), 10µl of enhancer solution (prepared with 10 ml DMSO, 11 mg Parahydroxycoumaric acid) and 3.1 µl 3% H_2O_2 for 60 secs, after which it was visualised using a Chemidoc^{TM} XRS+ Imaging system (BioRad, Auckland, New Zealand) in chemiluminescence mode.

2.2.4.1.4 Stripping of PVDF membrane for reprobing

The blot was washed with PBS-$T_{0.1}$ then incubated in stripping buffer (200mM Glycine, 3.5mM SDS, 1% v/v Tween-20 made up to 1L w/H_20, pH 2.2) for 10min at RT, after which the buffer was discarded. The blot was incubated in stripping buffer for a further 10min, and the buffer discarded. The blot was then washed with PBS twice over a period

of 20min, followed by PBS-$T_{0.1}$ twice over a period of 10min after which the blot was ready for reprobing.

2.2.4.2 Anti-Caspase 8 assay

Control (untreated) media, MDA-MB-231 CM or MCF-7 CM was prepared prior to commencing each experiment (Section 2.2.2.2). Three participants provided whole blood samples on two non-consecutive days. Neutrophils were isolated from the blood samples via positive selection (Section 2.2.1) and aliquoted into three samples of ~$7x10^6$ cells. The samples were centrifuged (250x*g*, 5min, RT) and the supernatant removed. To determine the baseline level of caspase-8 cleavage in neutrophils, one sample was immediately prepared for SDS-PAGE (Section 2.2.3.7). The two remaining samples were resuspended in control medium, MDA-MB-231 CM (day 1) or MCF-7 CM (day 2) at a concentration of $3.33x10^5$ cells/ml; the samples were aliquoted into the wells of a six well plate to a final volume of 4ml and incubated for 7hrs at 37ºC with 5% CO_2.

After incubation the medium was removed, the cells washed with 1ml PBS then dissociated in 0.05% Trypsin-EDTA, for 3min at 37ºC. An aliquot of media was added to each well to inactivate the trypsin and the cells (neutrophils in control medium and neutrophils in CM) were transferred into two 15ml sterile polypropylene tubes. The neutrophils were centrifuged (250x*g*, 5min, RT) and the supernatant discarded. The cells washed with 1ml PBS (pH 7.4), centrifuged (250x*g*, 5min, RT) and the supernatant discarded again. The cells were counted, prepared for SDS-PAGE and stored at -18ºC (Section 2.2.3.6).

Between 15-40µl of the samples were loaded onto the gels (Section 2.2.3.7). The volume loaded was determined by the number of cells contained within each sample immediately prior to preparation for SDS-PAGE; in order to load an equal number of cells across individual experiments. Following separation via SDS-PAGE (Sections 2.2.3.7-8), the proteins were transferred to a PVDF membrane (Section 2.2.4.1.1), and the transfer confirmed by Ponceau S staining (Section 2.2.4.1.2). The PVDF membrane was

immunostained (Section 2.2.4.1.3) with mouse monoclonal caspase-8 primary antibody (1:1000) then stripped (Section 2.2.4.1.4) and re-probed with mouse monoclonal anti-GAPDH (1µg/ml) as a loading control. The blots were visualised via trans UV using a Chemidoc^{TM} XRS+ Imaging system (BioRad, Auckland, New Zealand), to capture an image of the prestained protein ladder, then via chemiluminescence to visualize the western blot. The two images were superimposed using image lab^{TM} software (Bio-Rad), allowing the presence or absence of full-length caspase (~ 57 kDa) activated caspase-8 (~ 46.5kDa) and GAPDH (~ 40kDa) to be identified in each sample.

2.2.4.3 EMT western blot assay

Twenty-four hours prior to the experiment MCF-7 and MDA-MB-231 cells were collected by trypsinisation as described for passaging (Section 2.2.2.1). The MCF-7 and MDA-MB-231 cells were plated out in 12 well plates at a concentration of $3x10^5$cells and $2x10^5$cells respectively, in a volume of 1.5ml of complete medium and incubated at 37ºC with 5% CO_2 for 24hrs.

On the day of the experiment, to determine the baseline proportions of β-catenin and vimentin, one sample from each cell line was immediately prepared for SDS PAGE (Section 2.2.3.7). The medium was removed, the cells were washed with 1ml PBS then dissociated in 0.05% Trypsin-EDTA, for 3min at 37ºC. An aliquot of media was added to each well to inactivate the trypsin and the cells (MCF-7 and MDA-MB-231) were transferred into two 15ml sterile polypropylene tubes. The cells were centrifuged (250x*g*, 5min, RT) and the supernatant discarded. The cells were washed with 1ml PBS (pH 7.4), centrifuged (250x*g*, 5min, RT) and the supernatant discarded again. The cells were counted, prepared for SDS-PAGE and stored at -18ºC (Section 2.2.3.7).

Neutrophils were isolated via from whole blood samples via positive selection (Section 2.2.1), resuspended in complete medium and aliquoted ($1x10^5$ neutrophils in 500µl of media) into transwell inserts placed above the MCF-7 and MDA-MB-231 cells. For a negative control, 500µl of medium without neutrophils was used instead. The cells were

incubated at 37ºC; after 7 and 18hrs of incubation a plate was removed, the inserts discarded, the samples harvested and prepared for SDS-page as previously described and stored at -18ºC.

The samples were loaded onto the gels (Section 2.2.3.7). Following separation via SDS-PAGE (Sections 2.2.3.7-8), the proteins were transferred to a PVDF membrane (Section 2.2.4.1.1), and the transfer confirmed by Ponceau S staining (Section 2.2.4.1.2). The PVDF membrane was immunostained (Section 2.2.4.1.3) with an epithelial-mesenchymal transition (β-catenin, vimentin) western blot cocktail (1:250) which also contained smooth muscle actin and GAPDH for loading control. The blots were visualised via trans UV using a ChemidocTM XRS+ Imaging system (BioRad, Auckland, New Zealand), to capture an image of the prestained protein ladder, then via chemiluminescence to visualise the western blot. The two images were superimposed using image lab^{TM} software (Bio-Rad), allowing the presence or absence of β-catenin (~ 92 kDa), Vimentin (~ 54kDa), smooth muscle actin (~42kDa) and GAPDH (~ 36kDa) to be identified in each sample.

2.2.5 Molecular biology techniques

2.2.5.1 RNA extraction and reverse transcription into cDNA

Total cellular RNA was extracted from MCF-7 cells, MDA-DB-231 cells and human neutrophils using the Isolate II RNA Mini Kit, according to manufacturer's instructions. RNA quantity and purity were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Auckland, New Zealand). RNA integrity was confirmed via denaturing gel electrophoresis according to the method of Masek *et al.* (357). The RNA gel was visualised via a Chemidoc^{TM} XRS+ Imaging system. First strand cDNA (primed by random hexamers) was synthesised using a High Capacity cDNA Reverse Transcription Kit according to manufacturer's instructions. Aliquots of between 0.03-1µg RNA were used for each individual reaction.

2.2.5.2 qRT-PCR

Lyophilised primers were resuspended in TE buffer (10mM Tris, 0.1mM EDTA) to create 200µM stock solutions and stored at -18ºC. Aliquots of the stock primers were diluted in nuclease-free water to create a 10µM working stock solution and stored at -18ºC. Reaction mixtures were created of 1µl cDNA, 5µl of PowerUp SyberGreen Mastermix 0.4µl (each) of forward and reverse primer and 3.2µl PCR grade water. A no-template control and no-reverse transcriptase control was prepared for each PCR to ensure the reaction was free from contamination. PCRs were undertaken in duplicate on a StepOne™ Real-Time PCR cycler (Thermo Fisher Scientific, Auckland, New Zealand). Unless otherwise specified, for samples with a T_a of < 60ºC the cycling conditions comprised of 2min at 50ºC, followed by a denaturation phase of 95ºC for 2min followed by 40 amplification cycles at 95ºC for 15 secs, T_a ºC for 15 secs and 60ºC for 1min. For samples with a T_a of > 60ºC the cycling conditions were comprised of 2min at 50ºC, followed by a denaturation phase of 95ºC for 2min then 40 amplification cycles at 95ºC for 15 secs, and 60ºC for 1 min. In both instances, the instrument was set to perform a dissociation step at the end of the PCR experiment comprised of 95ºC for 15 secs, 60ºC for 1min and 95ºC for 15 secs.

Results were tabulated using Applied Biosystems StepOne Real-Time software and were exported into Microsoft Excel (2016). The delta-delta CT relative quantification method described by Livak and Schmittgen (358) was used to determine fold changes in gene expression via the following equation:

$$2^{-\Delta\Delta C_T} = [(C_T \text{ gene of interest} - C_T \text{ internal control})] \text{ sample A}$$
$$- \quad [(C_T \text{ gene of interest} - C_T \text{ internal control})] \text{ sample B}$$

Where "C_T" stands for the "cycle threshold"; the cycle number for which the fluorescence produced by the PCR product is first discernible above the background noise and "internal control" refers to the reference or "housekeeping" gene used to normalise the data.

β-actin or "ACT-B" was used as the housekeeping gene for PCR experiments undertaken with both neutrophils (359) and MCF-7/MDA-MB-231 cancer cells (360).

For each experiment sample A was the treated sample and sample B was the untreated control sample (i.e. the calibrator). A two tailed paired t-test was used to compare the difference in means between Δct Sample A (treated sample: C_T gene of interest - C_T internal control) and Δct Sample B (untreated sample: C_T gene of interest - C_T internal control). Bar charts representing fold changes in gene expression are plotted on a log2 scale using Microsoft Excel (2016).

For primer design and optimisation, please see Appendix B. To confirm the specificity of amplification all PCR products were sequenced by Massey Genome Service (Palmerston North, New Zealand). All PCR products were of the expected size and matched their targets, for results please see Appendix B.

2.2.5.3 DNA gel electrophoresis

PCR products were separated on the basis of molecular weight on a 1.5% w/v agarose gel. The agarose was melted in 100ml of TAE buffer (0.4M-Tris-acetate, 1mM EDTA), containing 10µl of SYBR™ Safe DNA Gel Stain and poured into the gel casting apparatus, a comb was inserted to create loading wells, and the gel and was allowed set. A 10µl aliquot of each PCR reaction was mixed with 2µl 6X DNA loading dye, these were loaded alongside a 3µl aliquot of DNA ladder into the wells of the gel; the samples were electrophoresed in an Owl™ Easycast™ B2 mini system (Thermo Fisher Scientific, Auckland, New Zealand) in 1X TAE Buffer at 90v for 45min, and visualised via a Chemidoc™ XRS+ Imaging system.

2.2.5.4 Expression of COX-2 in MDA-MB-231 and MCF-7 cells assay

MDA-MB-231 and MCF-7 cells were grown to 70% confluence, the media was removed, the cells washed with PBS (pH 7.4), the RNA extracted and reverse transcribed into cDNA (Section 2.2.5.1) and RT-PCR undertaken on the single target; COX-2 (for cycling conditions see Section 2.2.5.2). The PCR products were separated via DNA gel electrophoresis (Section 2.2.5.3) and visualised via a ChemidocTM XRS+ Imaging system.

2.2.5.5 Effect of MDA-MB-231 CM and MCF-7 CM on neutrophil phenotype assay

Control medium, MDA-MB-231 CM or MCF-7 CM was prepared prior to commencing each experiment (Section 2.2.2.2). Neutrophils were isolated from blood samples obtained from eight participants (provided on two non-consecutive days) via positive selection (see Section 2.2.1), the cells were centrifuged (250x*g*, 5min, RT) and the supernatant removed. The cells were resuspended in untreated medium, MDA-MB-231 CM (day 1) or MCF-7 CM (day2) at a concentration of $3.33x10^5$ cells/ml. The samples were aliquoted into six well plates to a final volume of 5ml and incubated at 37ºC with 5% CO_2. After 7hrs of incubation the medium was removed, the cells were washed with 1ml PBS (pH 7.4) then dissociated in 0.05% Trypsin-EDTA, for 3min at 37ºC. An aliquot of media was added to each well to inactivate the trypsin, after which the samples (neutrophils incubated in control media versus conditioned media) were transferred into two 15ml sterile polypropylene tubes and centrifuged (250x*g*, 5min, RT). The supernatant was discarded, the samples washed with 5ml PBS (pH 7.4) and centrifuged (250x*g*, 5min, RT). The samples were then used to prepare RNA and cDNA (Section 2.2.5.1), after which qRT-PCR was undertaken on two targets; ICAM-1 and MMP-9 (Section 2.2.5.2).

2.2.5.6. Effect of neutrophils on MDA-MB-231 cell and MCF-7 cell phenotype assay

Twenty-four hours prior to the experiment MCF-7 and MDA-MB-231 cells were collected by trypsinisation as described for passaging (Section 3.2.2.1) and the pellet resuspended in complete medium. The MCF-7 and MDA-MB-231 cells were plated out in 12 well plates at a concentration of $3x10^5$cells and $2x10^5$cells respectively, in a volume of 1.5ml of complete medium and were incubated at 37ºC with 5% CO_2 for 24hrs. Neutrophils were isolated via positive selection (Section 2.4.1), resuspended in complete medium and aliquoted ($1x10^5$ neutrophils in 500µl of media) into transwell inserts placed above the MCF-7 and MDA-MB-231 cells. For a negative control, 500µl of medium without neutrophils was used instead. The cells were incubated at 37ºC; after 7hrs the plate was removed, the inserts discarded and the cells washed with PBS (pH 7.4). The samples were used to prepare RNA and cDNA (Section 2.2.5.1), after which qRT-PCR was undertaken on the eleven targets; IL-6, IL-8, VEGF-A, TGF-β, β-catenin, Fibronectin E-Cadherin, Vimentin, ZEB22, SNAIL and SLUG (Section 2.2.5.2).

CHAPTER 3

OPTIMISATION OF NEUTROPHIL ISOLATION PROCEDURE AND CULTURE CONDITIONS

3.1 Introduction

A convenient, reliable source of neutrophils was required in order to meet the objectives of this research. Human neutrophils are typically sourced from donated whole blood samples; thus, an efficient and reproducible isolation method was required. The method needed to produce a preparation of pure, minimally activated neutrophils of high viability. Since neutrophils are very easily activated during ex-vivo manipulation, each stage of the isolation process and the ensuing experimental conditions needed to be carefully considered.

There are several different methods available for isolating human neutrophils from whole blood. These include density gradient centrifugation, in which cells are separated according to their buoyant density (361–363); immunomagnetic separation, in which antibodies coated on magnetic beads bind to cell specific surface antigens to allow their separation on magnetic columns (364) and flow cytometric cell sorting (365). Whilst immunomagnetic separation and flow cytometric sorting both produce highly pure populations of cells, the antibody technique has a limited yield (366) and both methods require costly, specialised equipment (364,365). In contrast, density gradient centrifugation is relatively inexpensive and is usually associated with a higher yield of cells (367); consequently this method is the preferred choice of many researchers and was initially selected for use in this project. However, density gradient centrifugation is technically more difficult to perform than immunomagnetic separation or flow cytometric cell sorting, and is usually associated with a lower purity of isolated cells; typically ~95% (368) as compared to >99% for neutrophils isolated by immunomagnetic selection (367).

The optimal experimental culture conditions for neutrophils also needed to be identified, in terms of both the culture medium used and the addition of supplementary factors such as fetal bovine serum (FBS). FBS contains a variety of growth factors and small molecules such as amino acids, lipids and hormones (369) that enable cultured cells to survive, grow and divide; however, these molecules may also stimulate the neutrophils during experiments. Moreover, factors known to extend neutrophil survival or alter their phenotype, such as GM-CSF, G-CSF (218), or TGF-β (37) may be present in FBS

(370,371) which may act as a confounding factor when assessing neutrophil lifespan or phenotype.

3.2 Isolation of neutrophils via density gradient centrifugation

3.2.1 Histopaque density gradient centrifugation

Whole blood samples were obtained from healthy volunteers (Sections 2.2.1.1-2). Neutrophil isolation was performed via density gradient centrifugation, using Histopaque (polysucrose) 1.119 and 1.077 (Sigma Aldrich, Auckland, New Zealand) as reported by Freitas *et al*. (362). Briefly, a 3ml aliquot of Histopaque 1.077 was carefully layered on top of 3ml of Histopaque 1.119 in a 15ml sterile polypropylene tube (Greiner Bio-One, Kremsmünster, Austria). Six ml of whole blood was layered over the Histopaque solutions and centrifuged (700x*g*, 30min, RT), in a Heraeus Megafuge 1.0R centrifuge (Thermo Fisher Scientific, Auckland New Zealand) with the brake off. According to the manufacturer's protocol (372), this should have resulted in the erythrocytes sedimenting to the bottom of the tube, leaving a granulocyte layer at the 1.077/1.119 interface and lymphocytes/mononuclear cells at the upper plasma/1.077 interface (Figure 3.1).

Although a well-defined layer of lymphocytes was typically achieved, clear separation of granulocyte cells was never observed. Instead, they remained as a cloudy suspension beneath the lymphocyte band (Figure 3.2)

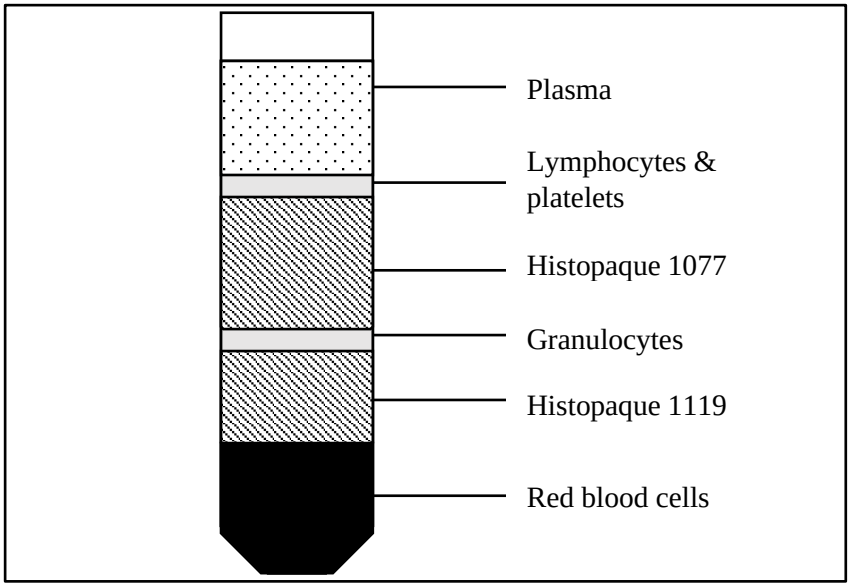


Figure 3.1: Schematic representation of leucocyte separation expected via Histopaque 1.077/1.119 density gradient centrifugation

Schematic diagram based on the manufacturers protocol (372).

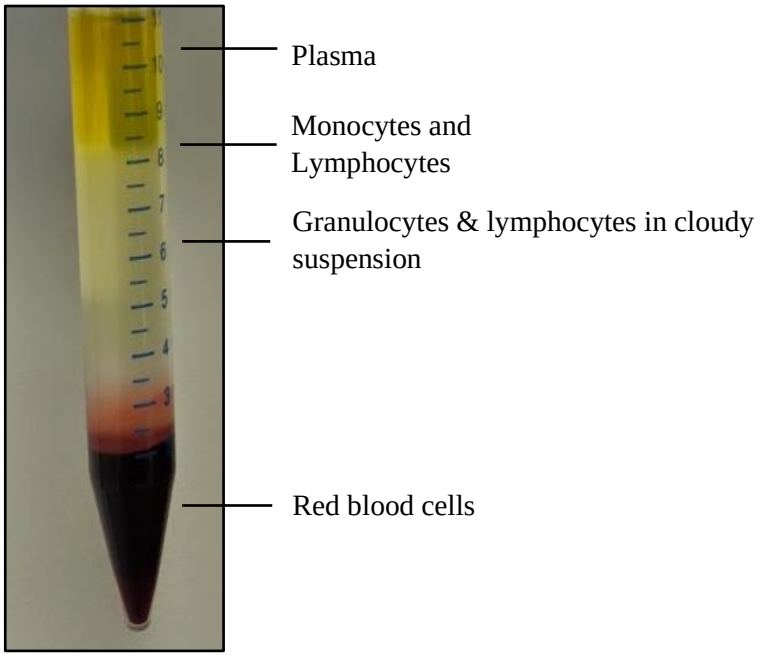


Figure 3.2: Leucocyte separation achieved using Histopaque 1.119/1.077

Leucocytes separated via Histopaque 1.119/1.077 density gradient according to manufacturer's instructions, centrifuged at 700x*g*, 30min, RT.

3.2.2 Optimisation of leucocyte separation via density gradient centrifugation

Attempts to improve the separation of the leucocytes by density gradient separation, were made by systematically trialling the manufacturer's (Sigma Aldrich) trouble shooting suggestions (373).

The following steps were undertaken:

I. The blood sample was cooled to room temperature for 30 - 45min before use and the Histopaque was warmed to room temperature prior to layering, as the separation procedure is proposed to be optimal when both blood and Histopaque are between 18-20ºC.
II. The Histopaque was examined to ensure the layering had been performed correctly as indicated by a sharp demarcation line between the boundaries of the two solutions; if this was absent/swirling was present the Histopaque was discarded.
III. The gradient solution was used immediately after its preparation, to prevent the two layers from diffusing into each other.
IV. The samples were centrifuged immediately after the blood was layered to prevent the Histopaque solutions from being tinted red by the red blood cells.

None of these factors resulted in a discernible granulocyte band.

3.2.3 Investigation of the influence of centrifugation speed and time upon leucocyte separation by density gradient centrifugation

Published papers suggest a range of centrifugation speeds and times for density gradient separation (890x*g*, 30min (362), 600x*g*, 15min (374) , 800x*g*, 50min (375), 400x*g* 30min (376)). An experiment was undertaken to determine whether altering the relative centrifugal force (RCF) or duration of centrifugation might improve the leucocyte separation.

Whole blood samples were layered over a 1119/1077 Histopaque gradient solution as previously described. The samples were centrifuged at RT at either 700x*g* or 900x*g* for 30, 45 or 60min. A discernible granulocyte band was achieved at the 1.077/1.119 interface at 700x*g* for 60min (lowest speed) (Figure 3.3) and at 900x*g* for 30min (shortest time). These samples were selected for further investigation and are referred to as sample A (700x*g* for 60min) and sample B (900x*g* for 30min) respectively.

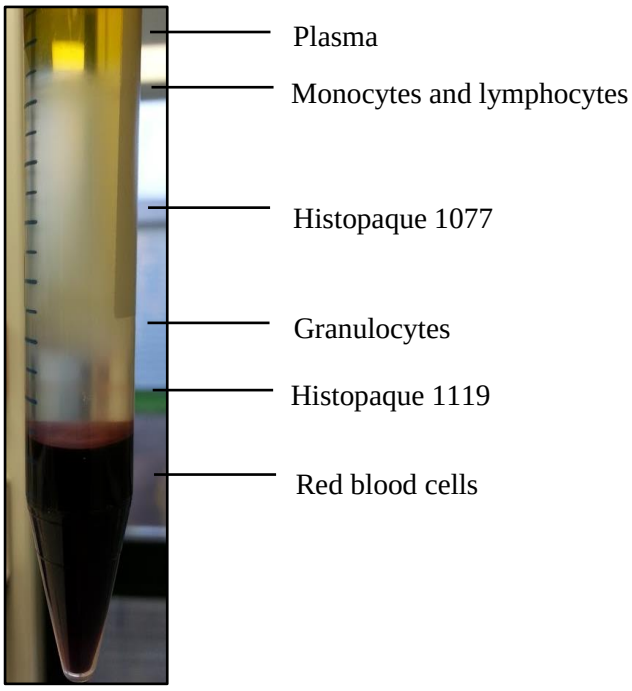


Figure 3.3: Leucocyte separation achieved using Histopaque 1.119/1.077
Leucocytes separated via Histopaque 1.119/1.077 density gradient according to manufacturer's instructions, centrifuged at 700x*g*, 60min, RT.

The plasma, monocytes, lymphocytes and density gradient material above the 1077/1119 interface of both samples were removed using a sterile plastic Pasteur pipette and discarded. Neutrophils were retrieved from the 1077/1119 interface and were transferred to a fresh 15ml centrifuge tube. The cells were washed with 10ml of PBS and centrifuged (250x*g*, 10min, RT). The supernatant was discarded, and the cell pellet gently resuspended in 100µl PBS and the cells washed a further two times with 10ml of PBS. After the final wash, the cell pellet was resuspended in 1ml of PBS. Cell yield and viability was evaluated by trypan blue exclusion (Section 2.2.3.4.1), and cell purity was assessed by May-Grünwald-Giemsa staining (Section 2.2.3.1) and microscopy.

Neutrophil yield was greatest for sample A (Table 3.1) and was similar to the yield reported by Freitas *et al*. ($1.7\times10^6\pm1.5\times10^5$ cells/ml blood)(362). However, neutrophil viability was greatest for sample B (Table 3.1). Since neutrophil lifespan is very short (an estimated circulating half-life of 6-8 hours (148)), this may be a function of the lower centrifugation time of sample B compared to sample A. Overall neutrophil viability was found to be considerably less than reported by either Freitas *et al*. at >98% (362) or Marchi *et al*. at ~85% (377).

Table 3.1: Isolation of neutrophils via Histopaque 1.077/1.119. The effect of centrifugation speed and time on neutrophil yield and viability

Sample	Total Number of Cells ($x10^6$)	Total Number of Cells per ml of Blood ($x10^6$)	Viability %
Sample A 700x*g* 60min	12.4 ± 1.0	2.1 ± 0.2	52.1 ± 2.9
Sample B 900x*g* 30min	9.2 ± 0.7	1.5 ± 1.1	58.6 ± 9.6

Comparison of neutrophil yield and viability obtained from sample A, spun at 700x*g* for 60min and sample B spun at 900x*g* for 30min. Both blood samples were obtained from a single donor, provided on the same day. Neutrophil viability was determined manually via the trypan blue exclusion test. Cell counts were undertaken in duplicate, values are expressed as mean ± sd.

May-Grünwald-Giemsa staining indicated that both samples (A and B) were heavily contaminated with lymphocytes, as can be seen in Figure 3.4. This is similar to work by Marchi *et al*. (377), who found murine neutrophils separated from peripheral blood via a Histopaque 1.119/1.077 density gradient had a purity of only 63.8%. However, the purity was very low when compared to results achieved by other researchers separating human blood that used alternative density gradient methods such as Percoll: ~ 87% purity (377) or Ficoll: ~ 96.2% purity (367).

This low purity posed a significant problem for the project, as any results achieved would have been based upon a mixed population of cells rather than a pure sample of neutrophils. Subsequently it was decided to change the isolation procedure to magnetic bead separation as research suggests (367,368) this method provides the best result in terms of cell purity.

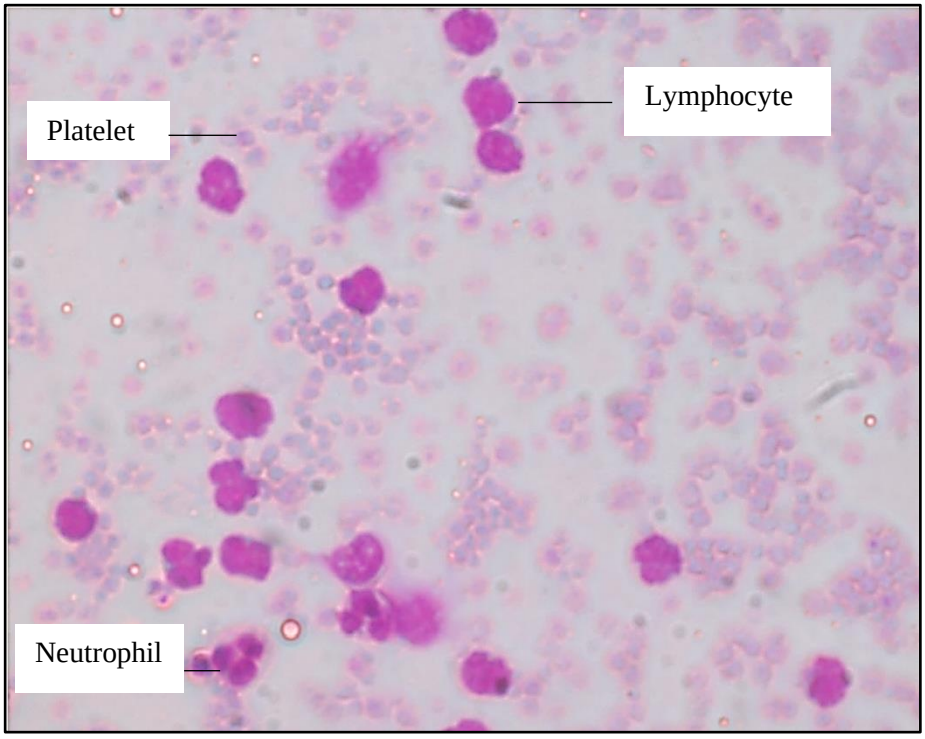


Figure 3.4: Neutrophils isolated via 1.119/1.077 density gradient demonstrate low purity

Cells obtained from the granulocyte layer of the Histopaque 1.119/1.077 gradient preparation, centrifuged at 900x*g* for 30min. Cells were fixed on poly-lysine slides and subjected to May-Grünwald-Giemsa staining. Images were captured using a 100X objective with an Axiostar plus microscope (Zeiss, Oberkochen, Germany).

3.3 Isolation of neutrophils via immunomagnetic magnetic bead separation

A positive isolation procedure using a MACS CD15 kit (Miltenyi Biotec, Cologne, Germany), was selected for use, with the microbeads being bound to CD15 antibodies and therefore recognising neutrophils and eosinophils. Since eosinophils represent between 1-4% of white blood cells (compared to 40-60% for neutrophils) contamination

from this source was likely to be minimal. Indeed, research in which CD15+ positive selection of neutrophils was compared against a density gradient isolation method, found the contamination of eosinophils (as a % of total leucocytes) to be 0% for CD15 beads and 6% for the density gradient method (378). However, even if small numbers (<5%) of contaminating leucocytes are present in the sample, research suggests that they will contribute very little to the overall transcriptome profile or the number of differentially expressed genes for cytokine stimulated neutrophils (368). The alternative option, negative selection, depletes all cell types aside from those of interest. Whilst negative selection ensures the isolated cells do not carry bead-bound antibodies, and is thus less likely to activate the neutrophils, this method is significantly more expensive and was financially beyond the scope of this project.

3.3.1 Positive selection of neutrophils via MACs CD15 beads

Venous blood samples were collected as described previously (Section 2.2.1.2) An ammonium chloride buffer was used for RBC lysis (Section 2.2.1.3), as this method does not influence the integrity or activation of isolated neutrophils (379). Neutrophils were isolated from the leucocyte preparation by positive selection using CD15 MACS beads according to the manufacturer's instructions (Section 2.2.1.4). Cell yield and viability was evaluated by trypan blue exclusion (Section 2.2.3.4.1). Cell purity was assessed immediately following RBC cell lysis and compared to neutrophils isolated by CD15+ positive selection via May-Grünwald-Giemsa staining (Section 2.2.3.1) and microscopy and re-confirmed via flow cytometry (Section 2.2.3.2)

The venous blood samples, immediately following RBC lysis contained a mixed population of cells (Figure 3.5).

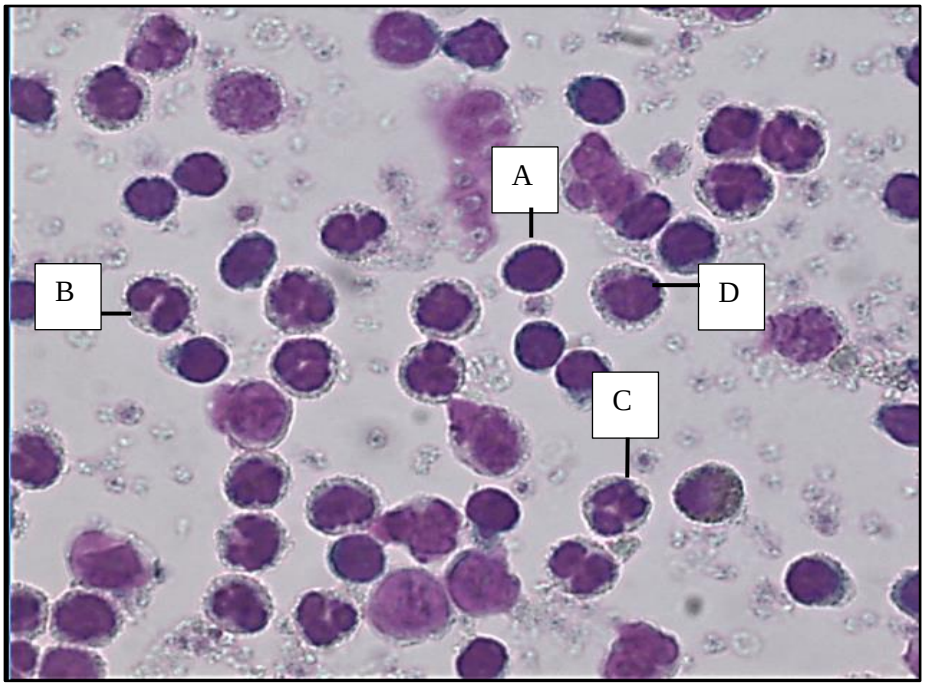


Figure 3.5: Assessment of cell purity immediately following RBC lysis

An ammonium chloride cell lysis buffer was used on venous blood samples to induce RBC lysis. The leucocytes were fixed on poly-lysine slides and subjected to May-Grünwald-Giemsa staining. Cells types are labelled as A) lymphocytes, B) eosinophils, C) neutrophils and D) monocytes. Images were captured using a using a 100X objective with an Axiostar plus microscope (Zeiss, Oberkochen, Germany).

These included lymphocytes (A), identified by their round nucleus and narrow rim of cytoplasm, eosinophils (B) identified by their two well defined lobes of equal size and pink stained granules, neutrophils (C) identified by their hyper-segmented nuclei (>2 lobes), monocytes (D) identified by their peanut shaped nucleus bound with a wide rim of cytoplasm. Basophils, large cells with poorly defined lobes and a granular appearance could not be identified amongst the cells shown in Figure 3.6. Their absence is not unexpected as basophils are the rarest of the granulocytes, representing less than 1% of circulating peripheral blood leucocytes. In contrast, histological staining of neutrophils isolated via $CD15^{+}$ positive selection indicated a homogeneous sample of cells (Figure 3.6).

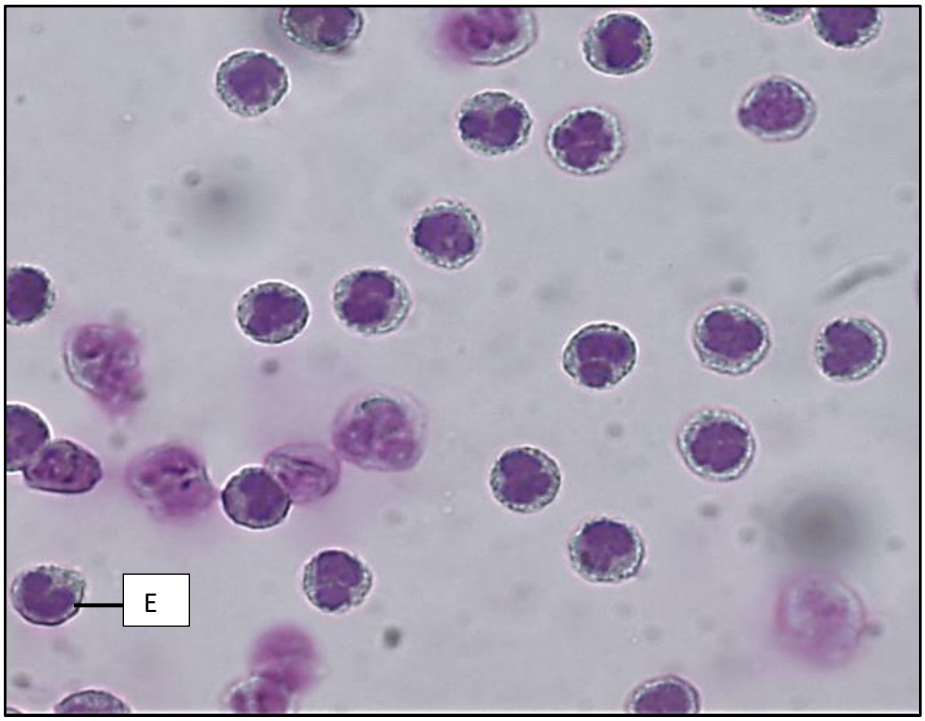


Figure 3.6: Neutrophils isolated by CD15^{+} immunomagnetic bead separation

Neutrophils were isolated by CD15+ immunomagnetic separation following RBC lysis. The cells were fixed on poly-lysine slides and subjected to May-Grünwald-Giemsa staining. Cell types are labelled as E) possible band cell. Images were captured using a 100X objective with an Axiostar plus microscope (Zeiss, Oberkochen, Germany).

Using the criteria previously described, no lymphocytes, monocytes, eosinophils or basophils were identified. The sample appears to contain only neutrophils, although based on its curved nucleus, one cell (E) is a band cell (one stage prior to the fully mature neutrophil) (Figure 3.6). Cell purity was further confirmed by flow cytometry. Leucocytes analysed immediately following RBC lysis (Sample A) comprised of 56.5% granulocytes, 10.4% monocytes and 33.1% lymphocytes (Figure 3.7, Sample A), whereas neutrophils isolated via CD15+ bead positive selection (Sample B) were comprised of 99.6% granulocytes, 0.1% monocytes and 0.3% lymphocytes (Figure 3.7, Sample B).

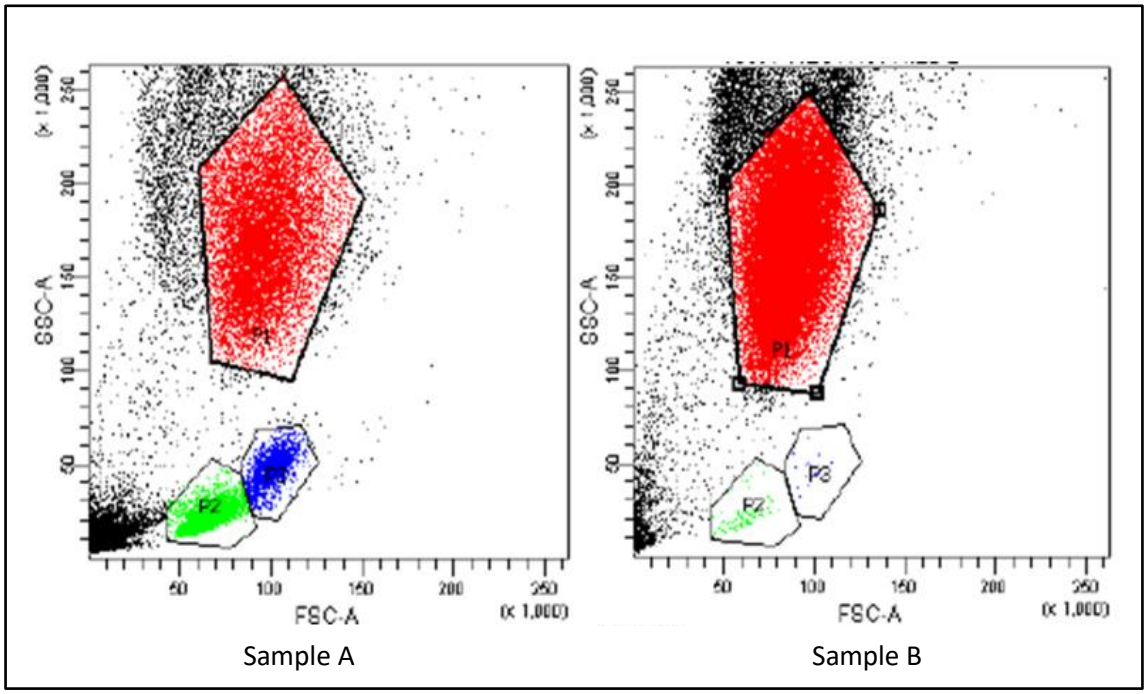


Figure 3.7: Leucocytes analysed by flow cytometry following RBC lysis (Sample A) compared to neutrophils isolated via MACs CD15$^+$ positive selection (Sample B)

Side scatter area (SSC-A) versus forward scatter area (FSC-A) density plots of light scatter obtained for leucocytes following RBC lysis compared to neutrophils isolated by positive selection. Each dot is representative of an individual particle or cell that has passed through the flow cytometer. Gates have been applied to each cell population, P1 (red) being granulocytes, P2 (green) being lymphocytes and P3 (blue) being monocytes.

Unfortunately, positive selection methods have one limitation: the microbeads cannot be removed from the cells after they have been isolated Since neutrophils are very easily activated, this has led to concern that the microbeads may inadvertently alter the phenotype of neutrophils *in vitro* or *ex vivo* (366). Activated neutrophils secrete reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-) to destroy pathogens within the body. Therefore, these compounds can be measured to determine the activation status of the freshly isolated granulocytes.

3.3.2 Functional analysis of neutrophils isolated by CD15+ positive selection

A hydrogen peroxide assay (380,381) was undertaken to determine the activation status and functional responses of neutrophils isolated by positive selection. The assay was optimised using the horseradish peroxidase (HRP) substrate, 10-acetyl-3,7-dihydroxyphenoxazine (Amplex Red). Amplex red is colourless and non-fluorescent, however, in the presence of HRP it reacts with H_2O_2 in a 1:1 stoichiometry to produce resorufin, a highly fluorescent oxidation product with an excitation maximum of 563nm and emission maximum at 587nm (380).

The H_2O_2 assay was undertaken according to the method of Mohanty *et al*. (381), (Section 2.2.3.3). An H_2O_2 standard curve was prepared (Figure 3.8), and used to calculate the amount of H_2O_2 produced by unstimulated neutrophils and neutrophils stimulated with PMA as a positive control for 120min (Figure 3.9).

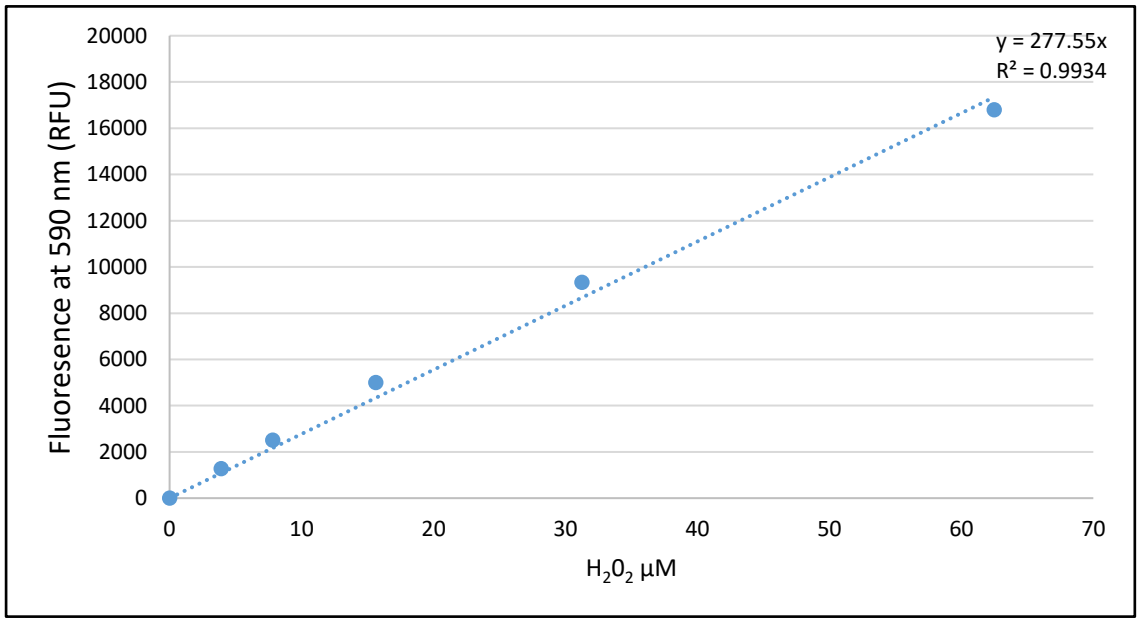


Figure 3.8: Amplex Red assay, H_2O_2 standard curve

A 25mM stock solution of H_20_2 in Krebs Ringer Phosphate Glucose (KRPG) buffer was prepared, then serially diluted in the same buffer to create seven H_20_2 standards (3.9, 7.81, 15.63, 31.25, 62.5, 125, 250 µM). A 100µl aliquot of Amplex Red working solution was added to each of the standards (50mM Amplex red and 1U/ml HRP in KRPG). The standards were incubated for 30min at RT, then the fluorescence measured on a FLUOstar Omega Microplate reader, using wavelengths of 560nm for excitation and 590nm for emission.

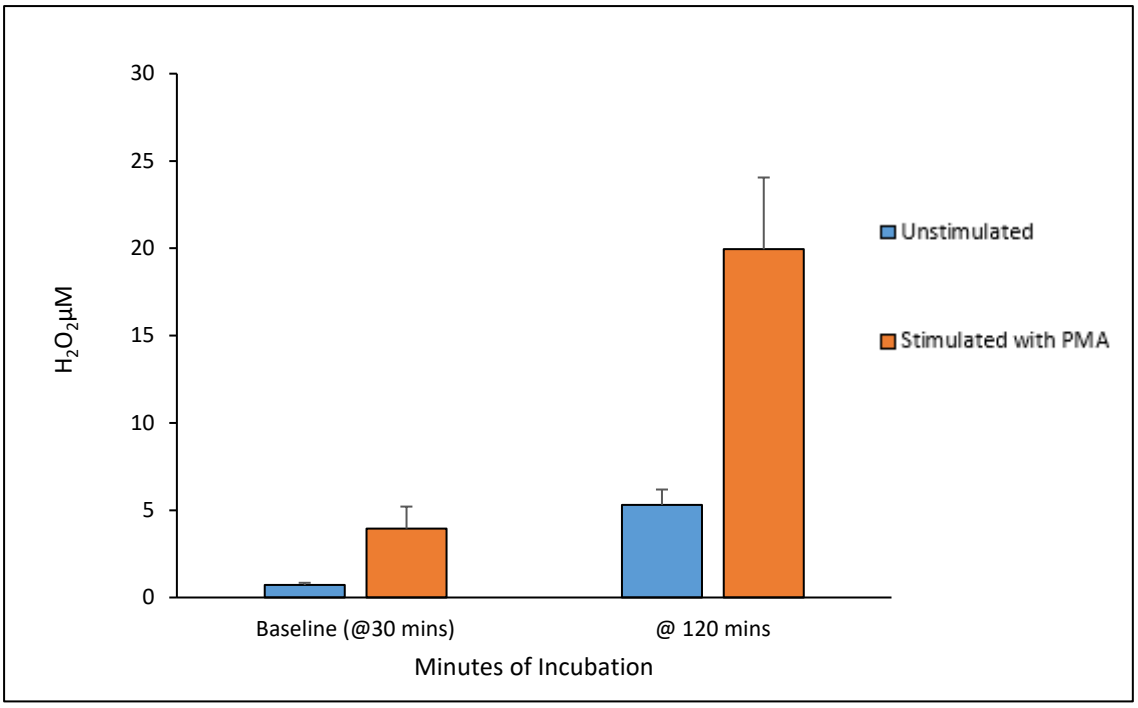


Figure 3.9: Secretion of H_2O_2 by neutrophils as calculated from H_2O_2 standard curve

Freshly isolated neutrophils were incubated in either amplex red solution (unstimulated) or amplex red solution with 50ug/ml PMA (stimulated). Flourescence was measured on a FLUOstar Omega Microplate reader after 30min (baseline reading) and 120min of incubation. H_2O_2 secretion was calculated from a standard curve prepared on the same plate. Values are expressed as mean ± S.E. (N=4).

Secretion of H_2O_2 by $5x10^4$ unstimulated neutrophils after 30min was 0.72µM. ± 0.12 (Figure 3.9). This suggests that spontaneous ROS production was minimal and that the neutrophils were not activated by the isolation procedure. Secretion of H_2O_2 by unstimulated neutrophils after 120min was 5.31µM. ± 0.88; the slight increase in H_2O_2, as compared to 30min of incubation, possibly due to the neutrophils adhering to the plastic plates and becoming mildly activated (382,383).

In contrast secretion of H_2O_2 by neutrophils stimulated with 50ng/ml PMA after 30 and 120min of incubation were 3.94 µM ± 1.26 and 19.94 µM ± 4.11 (Figure 3.9) respectively, the substantial increase in ROS production after 120min suggests the functional activity of the neutrophils was not affected by the isolation processes.

These results are in accordance with published research (378), which reported that the expression of CD11b (a cellular activation marker) after CD15+ MACS separation was not significantly different from neutrophils in whole blood samples. In contrast, neutrophils obtained from density gradient centrifugation demonstrated a significantly higher expression of CD11b (378). Other research (367) found no difference in spontaneous ROS production, the % of phagocytosing cells or the expression of the neutrophil activation marker L-selectin when comparing neutrophils isolated via two density gradient methods against CD15+ MACs positive selection. Indeed, the CD15+ MACs neutrophils demonstrated significantly greater ROS production upon stimulation with GM-CSF compared to the density gradient methods, suggesting a lower activation status post-isolation for these cells (367). However, Zhou *et al*. (367) did demonstrate a significant increase in the expression of two toll like receptors, TLR2 and TLR4 in CD15+ MACs neutrophils; these receptors primarily bind to products secreted from bacteria or fungi in order to activate the innate immune response. Therefore, positive selection methods may influence neutrophil activity involving these two receptors.

3.4 Optimisation of neutrophil culture conditions

3.4.1 The impact of culturing conditions on neutrophil adherence

Many of the experiments described in this thesis required neutrophils to be cultured in plastic flasks or plates, after which they needed to be removed in sufficient quantities for use in tests or assays. Unfortunately mature human neutrophils cultured in PBS or serum free medium readily adhere to plastic surfaces (382,383), including tissue culture plates, which may preclude their removal, activate the neutrophils and initiate respiratory burst (382). Therefore, an experiment was undertaken to determine the optimal culturing conditions for neutrophils, to prevent neutrophil adherence.

Neutrophils were isolated as previously described (Section 2.2.1) and resuspended in either AIM V; a serum free cell culture medium specifically designed for immune cells, DMEM (high glucose, pyruvate) supplemented with 10% (v/v) FBS or DMEM (high

glucose, pyruvate) without FBS. Neutrophils were plated into six well plates ($7.5x10^5$cells in 3ml of medium per well) and incubated at 37ºC, 5% CO_2.

Attempts were made to harvest the cells after 2, 20, 26 and 44h incubation using the following procedure: the medium was removed from the well and the cells washed with 2ml of PBS (pH 7.4). The cells were incubated with 0.02% Trypsin-EDTA, for approximately 5min at 37ºC to allow them to dissociate. Unfortunately, neutrophils incubated in both AIM V media and DMEM without FBS adhered very tightly to the surface of the wells and could not be removed under these conditions without a cell scraper. The experiment was repeated twice more with the same results.

In an attempt to improve the dissociation, the cells were incubated with either 0.02%, 0.03% or 0.05% Trypsin-EDTA for 5, 10, 15 or 20min, at 37ºC. The effect of temperature on cell dissociation was also assessed by comparing dissociation at 2ºC to 37ºC. It was found that the neutrophils could only be removed in quantities deemed sufficient for further experiments when cultured in DMEM with 10% (v/v) FBS and dissociated in 0.05% Trypsin-EDTA for 10min at 37 ºC. Consequently, neutrophils were cultured in DMEM with 10% v/v FBS unless stated otherwise.

3.4.2 The effect of heat inactivated FBS compared to non-heat inactivated FBS on neutrophil viability

Historically, many protocols required serum products such as FBS to be heated prior to use in order to destroy heat labile components, such as the complement system for immunoassays, or to reduce the risk of pathogen contamination. Due to improvements in the collection and processing of serum many of these reasons for heat inactivation are no longer valid (384). Moreover FBS contains only a few components of the complement system, and at much lower concentrations than that of adult or new born calf serum (385). Since heat inactivation may also degrade important biomolecules such as essential vitamin, amino acids and growth factors contained in the serum, this process is no longer required for most cell culture applications. However, since there is evidence to suggest

that certain complement fragments may delay neutrophil apoptosis *in vitro* (386) an assay was undertaken to compare the effects of culturing neutrophils with heat inactivated FBS against non-heat inactivated FBS on neutrophil lifespan.

Heat inactivated serum was prepared by incubating an aliquot of FBS for 30min at 56ºC. The FBS was cooled to room temperature, then directly added to DMEM (high glucose, pyruvate) at a concentration of 10% FBS (v/v) DMEM and stored at 4ºC. Neutrophils were isolated as previously described (Section 2.2.1.4) and resuspended ($1x10^6$ cells/ml) in either DMEM (high glucose, pyruvate) supplemented with 10% (v/v) non-heat inactivated FBS or DMEM (high glucose, pyruvate DMEM) supplemented (v/v) with 10% heat inactivated FBS. Neutrophils were seeded into a 96 well plate ($2x10^5$ cells/well) and cultured for 3, 5, 9, 20, 28 and 45hrs, after which the medium was removed from the wells and the cells washed with 100µl of PBS (pH 7.4). The cells were incubated with 0.05% Trypsin-EDTA, for approximately 5min at 37ºC to allow them to dissociate; viability was then assessed by trypan blue staining (Section 2.2.3.4.1).

No difference in viability was found between heat inactivated and non-heat inactivated FBS at any of the time points (Figure 3.10). Therefore, for convenience, non-heat inactivated FBS was used for the remainder of the experiments.

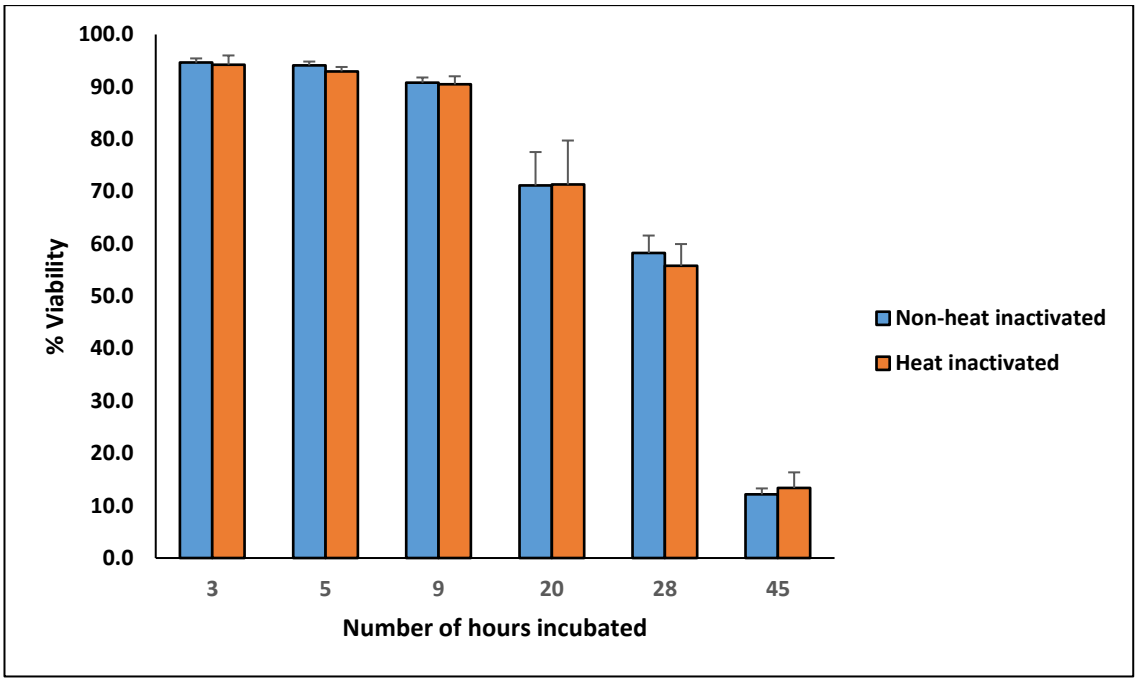


Figure 3.10: Effect of heat inactivated FBS compared to non-heat inactivated FBS on neutrophil viability

Neutrophils were incubated in medium containing either 10% heat inactivated FBS or 10% non-heat inactivated FBS. The cells were harvested after 3, 5, 9, 20, 28 and 45hrs and viability were assessed by trypan blue staining. Values are expressed as mean ± S.E. (N=2-4).

CHAPTER 4

THE EFFECT OF CANCER-CELL-CONDITIONED MEDIUM ON NEUTROPHIL LIFESPAN

4.1 Introduction

For many years neutrophils were classically viewed as short-lived effector cells, with an estimated half-life of approximately 8 hours (148). However, recent studies have challenged this view (217). At sites of infection and inflammation neutrophils display enhanced longevity (27), prompting a sustained inflammatory response that may contribute towards the pathogenesis of inflammatory disease (169,241,242). Since chronic inflammation is a hallmark of cancer (6), it is conceivable that neutrophils might also have an increased lifespan in cancer. This is important because it may allow the neutrophils to contribute towards the inflammation within the tumour microenvironment (TME) and/or allow the neutrophils time to acquire functional changes that may assist with tumour development and progression. Whilst there is limited evidence that certain cancers may enhance neutrophil lifespan *in vitro* (30,31,236,387), it is not clear if such a relationship exists between breast cancer cells and neutrophils.

To resolve the question of whether soluble factors secreted by breast cancer cells alter neutrophil lifespan, a series of experiments were carried out in which neutrophils were incubated in cancer-cell-conditioned medium (CM), containing all of the factors secreted by the cancer cells. Two human breast cancer lines were chosen for use in these experiments, MDA-MB-231 and MCF-7, both of which were established from pleural infusions of metastatic breast adenocarcinomas (388,389). These cell lines were selected because they reflect the features of cancer cells *in vivo* (390) and more importantly, because they are functionally distinct from each other and demonstrate very different phenotypes, thus enabling us to compare and contrast the influence of their individual phenotypes on neutrophil biology/activity. The MDA-MB-231 cell-line is a highly aggressive, highly invasive, poorly differentiated, triple negative breast cancer cell line (ER-PR-HER2-) and displays markers of the EMT transition, whereas the MCF-7 cell line is less aggressive, non-invasive (ER+PR+ HER2-) and does not display markers of EMT (391,392).

4.2 Determination of the effect of cancer cell conditioned medium (CM) on neutrophil viability

4.2.1 Assessment of neutrophil viability via trypan blue staining

To determine if cancer cell conditioned medium could alter neutrophil lifespan, neutrophils were isolated by positive selection (Section 2.2.1) and resuspended in either control medium, MCF-7 CM or MDA-MB-231 CM (Section 2.2.2.2) and their viability over a 26-hr period assessed via trypan blue exclusion (Section 2.2.3.4.1). This initial data set, although small, suggested a trend towards increased survival for neutrophils cultured in MCF-7 CM and MDA-MB-231 CM compared to control medium (see Figure 4.1).

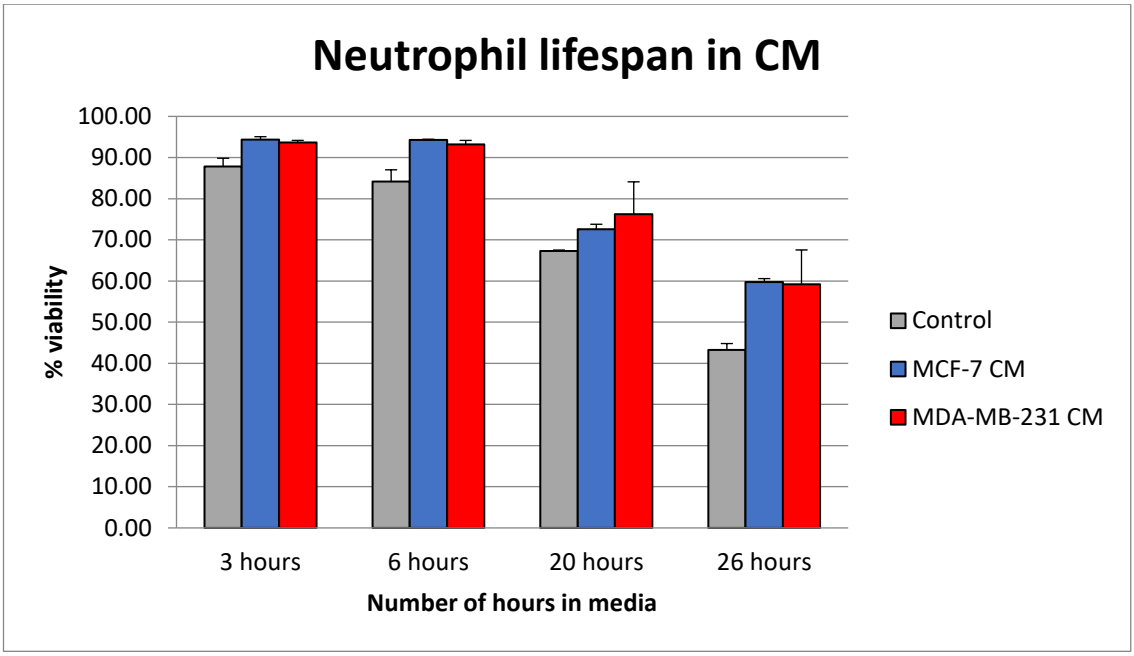


Figure 4.1: Preliminary data indicating that neutrophils incubated in MCF-7 conditioned medium (CM) and MDA-MM-231 CM demonstrate a trend towards increased survival compared to control medium

Neutrophils were incubated in control medium, undiluted MCF-7 conditioned medium (MCF-7 CM) or undiluted MDA-MB-231 conditioned medium (MDA-MB-231 CM) at a concentration of $3.33x10^5$ cells/ml and incubated at 37ºC. Neutrophil viability was assessed after 3, 6, 20 and 26hrs of incubation by trypan blue exclusion (N=2).

4.2.2 Assessment of neutrophil viability by fluorescein isothiocyanate (FITC) labelled Annexin V and propidium iodide (PI) fluorescence staining

To confirm whether cancer-cell CM could alter neutrophil life span, neutrophils were isolated by positive selection (Section 2.2.1) and resuspended in either control medium, MCF-7 CM or MDA-MB-231 CM (Section 2.2.2.2) and their viability assessed over a 24 hr period by fluorescein isothiocyanate (FITC) labelled Annexin V and propidium iodide (PI) fluorescence staining (Section 2.2.3.4.2).

The results of the dead cell apoptosis assay (Table 4.1) show that compared to the control, neutrophils incubated in MDA-MB-231 CM had a significantly higher proportion of viable cells ($P=0.043$, $P<0.001$ and $P=0.005$ respectively), and a significantly lower proportion of apoptotic cells ($P=0.044$, $P<0.001$ and $P=0.005$ respectively) after 3, 7 and 10hrs of incubation. Since the preparation time for the experiment, from blood sampling

to incubating the plates, was ~2.5-3h, these results suggest that approximately half of the neutrophils cultured in MDA-MB-231 CM remained viable (non–apoptotic) 13hrs after they were removed from the circulatory system. In contrast neutrophils incubated in MCF-7 CM demonstrated a significantly lower proportion of apoptotic cells compared to control medium at 3hrs ($P=0.033$), however this result should be viewed with caution as samples from only two subjects were able to be assayed at this time point. An example of the flow cytometry profiles produced for neutrophils immediately after isolation, and after 7hrs of incubation in control medium, MDA-MB-231 CM and MCF-7 CM is shown in Figure 4.2. This shows that the majority of cells (>98%) were viable (non-apoptotic) immediately post isolation. After 7hrs of incubation the majority of neutrophils in control medium and MCF-7 CM were apoptotic, whereas the majority of neutrophils in MDA-MB-231 CM remained viable. These data (Table 4.1) suggest that some form of soluble factor secreted by MDA-MB-231 cells, but not MCF-7 cells, might delay neutrophil apoptosis after 7 or 10hrs of incubation in CM. Since the difference in viability (MDA-MB-232 CM versus control) was most significant at 7hrs ($P<0.001$) this incubation time was used for all further experiments. It is important to note that the prolongation of lifespan for neutrophils cultured in MDA-MB-231 CM was fairly short lived (10hrs). Indeed after 24 hours of incubation the proportion of apoptotic neutrophils cultured in MDA-MB-231 CM was close to that of control levels. It is possible that the soluble factor responsible for the delay in apoptosis had either been used up or degraded. Alternatively, the neutrophils may have reached the point of “no return”, with their lifespan unable to be extended any further.

Table 4.1: Proportion of viable, apoptotic and necrotic neutrophils when cultured in MCF-7 or MDA-MB-231 conditioned medium (CM)

			% Viable (FITC-/PI-)			% Apoptotic (FITC+/PI-)			% Necrotic (FITC+/PI+ & FITC-/PI+)		
Cell line	Hrs in medium	N	Control	CM	P. Value	Control	CM	P. Value	Control	CM	P. Value
MCF-7	0	4	98.1 ± 0.8			0.5 ± 0.0			1.5 ± 0.8		
	3	2	85.0 ± 5.2	88.0 ± 4.9	0.075	14.6 ± 5.1	11.7 ± 4.9	0.033*	0.4 ± 0.2	0.4 ± 0.1	1.000
	7	4	34.9 ± 15.2	40.4 ± 15.5	0.389	64.0 ±15.5	59.2 ±15.5	0.455	1.2 ± 0.4	0.4 ± 0.1	0.174
	10	4	10.2 ± 4.0	10.3 ± 2.1	0.983	88.0 ± 4.2	88.5 ± 2.3	0.912	1.6 ± 0.4	1.2 ± 0.4	0.421
	24	3	0.8 ± 0.3	0.5 ± 0.1	0.272	90.9 ± 2.3	84.5 ± 6.6	0.496	8.2 ± 2.5	14.9 ± 6.5	0.487
MDA-MB-231	0	4	98.7 ± 0.2			0.68 ± 0.2			0.6 ± 0.1		
	3	4	67.8 ± 3.0	89.6 ± 5.6	0.043*	31.2 ± 3.1	10.1 ± 5.5	0.044*	1.0 ± 0.6	0.3 ± 0.1	0.351
	7	4	11.7 ± 4.1	79.3 ± 5.4	0.000***	87.4 ± 4.3	20.4 ± 5.3	0.000***	1.0 ± 0.3	0.3 ± 0.1	0.045*
	10	4	6.6 ± 2.6	56.7 ± 9.1	0.005**	91.2 ± 3.0	42.9 ± 9.2	0.005**	2.3 ± 0.6	0.4 ± 0.1	0.048*
	24	3	0.7 ± 0.1	11.2 ± 4.6	0.149	84.4 ± 2.0	75.1 ± 3.5	0.171	14.8 ± 2.0	13.7 ± 1.9	0.071

Neutrophils were cultured in control medium, MCF-7 conditioned medium (CM) or MDA-MB-231 conditioned medium (CM), at a concentration of $3.33x10^5$ cells/ml and incubated at 37ºC. Neutrophil viability was assessed after 3, 7, 10 and 24hrs of incubation (hrs in medium) via flow cytometry using a Dead Cell Apoptosis kit with Annexin V FITC and PI. Viable cells were identified as FITC-/PI-, Apoptotic as FITC+/PI- and necrotic as FITC+/PI+ and FITC-/PI+. Values are expressed as means ±SEM. Statistical significance was determined by Student's paired t-test. *, $P \leq 0.05$; ** $P \leq 0.01$; ***, $P \leq 0.001$.

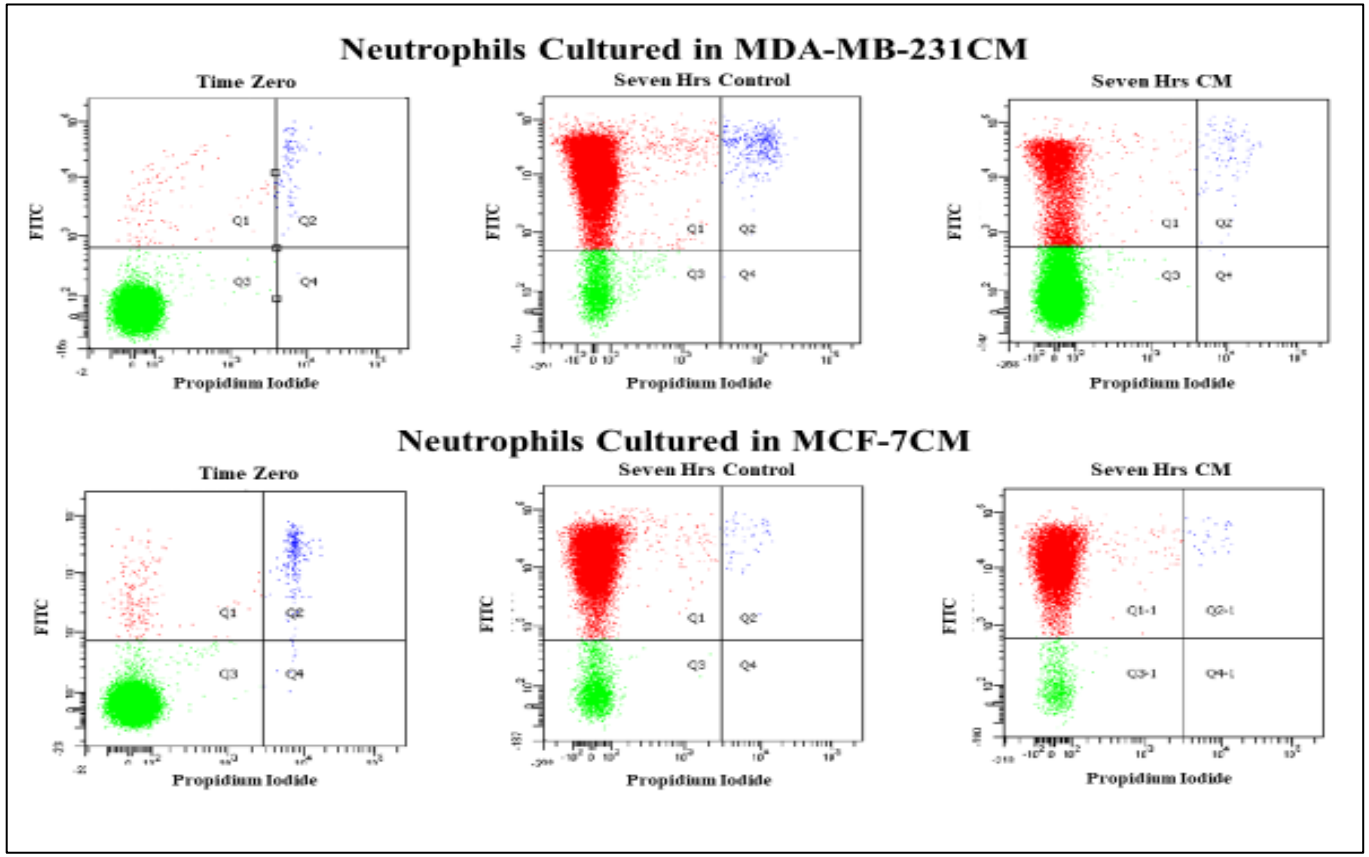


Figure 4.2: Representative dot plots showing proportions of viable, apoptotic and necrotic neutrophils when cultured in MCF-7 or MDA-MB-231 conditioned medium (CM)

Neutrophils were cultured in control medium, MCF-7 CM or MDA-MB-231 CM. Neutrophil viability was assessed immediately post isolation and after 7hrs of incubation via flow cytometry using a Dead Cell Apoptosis kit with Annexin VFITC and PI. Viable cells are defined as FITC-/PI-(green), Apoptotic cells are defined as FITC+/PI-(red), Necrotic cells are defined as FITC+/PI+ and FITC-/PI+ (blue).

4.3 Neutrophil apoptosis pathways

4.3.1 The effect of cancer cell conditioned medium (CM) on caspase–8 activation in neutrophils

To determine which apoptotic pathways were affected by the conditioned medium a series of experiments were carried out in which neutrophils were incubated in control medium, MDA-MB-231 CM or MCF-7 CM, after which the proportion of caspase–8 cleavage (activation) was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (Section 2.2.4.2). Full length caspase-8 and cleaved (activated) caspase-8 were quantified in each lane via computerised image analysis (Image lab^{TM} software, Bio-Rad laboratories Inc, Grand Junction, CO, USA). Multiple band are to be expected on the blot, as at least eight isoforms of caspase - 8 are known to exist, caspase-8a (~55 kDa) and caspase- 8b (~54kDa) are both present in most normal human tissues (393).

Western blotting of extracts prepared from freshly isolated neutrophils (time zero) and from neutrophils cultured for 7hrs with MDA-MB-231 CM showed a major band at ~ 57 kDa (Figure 4.3, A), which corresponds to full length caspase-8. In contrast, extracts prepared from neutrophils incubated for 7hrs in control medium or MCF-7 CM showed a major band at ~ 46.5kDa which corresponds to cleaved caspase-8 (Figure 4.3, B) with little or none of the intact caspase-8 remaining.

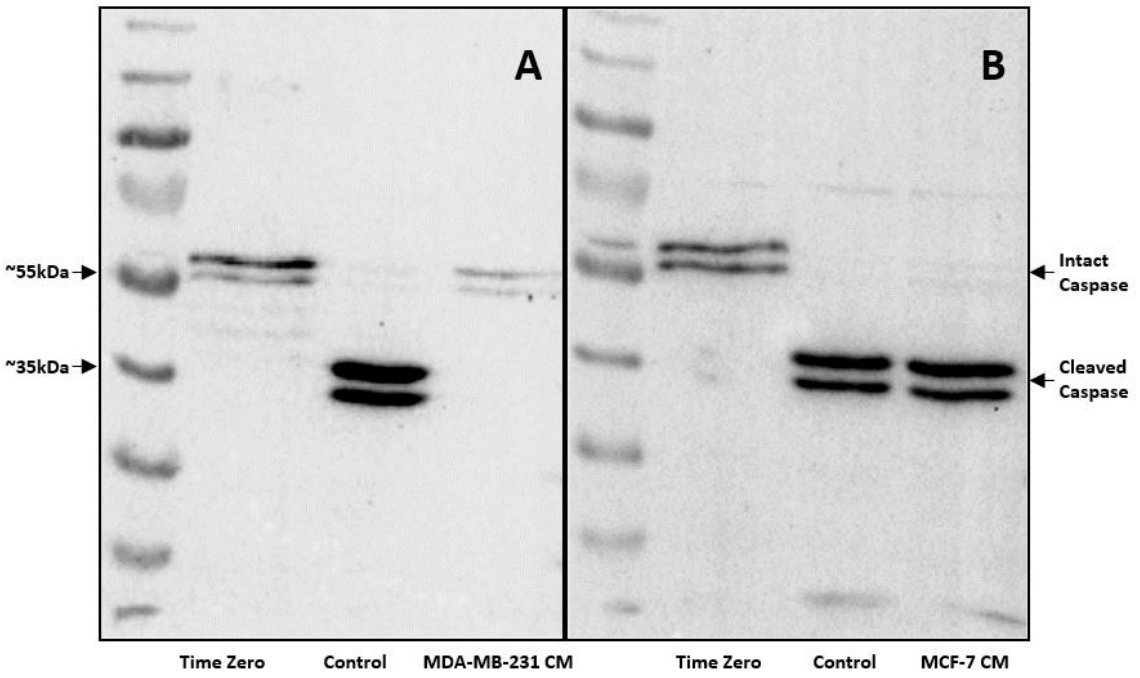


Figure 4.3: Relative proportions of full length and cleaved caspase-8 in freshly isolated neutrophils, compared to neutrophils incubated for 7hrs in control medium, MDA-MB-231 CM (A) and MCF-7 CM (B)

Cell lysates of freshly isolated neutrophils and neutrophils cultured in control medium, MDA-MB-231 conditioned medium (CM) or MCF-7 conditioned medium (CM) for 7hrs were subjected to SDS-PAGE (10% polyacrylamide gels) and western blotted with anti-caspase-8 (diluted 1:1000) (N=3). Full length caspase-8 is detected at ~57kDa, and cleaved caspase-8 at ~46.5kDa.

The results of the caspase-8 assay, obtained by quantifying the proportion of active or inactive caspase-8 in each lane of the western blot, (Figure 4.4) show that neutrophils incubated in control medium for 7hrs demonstrated a significantly higher proportion of caspase-8 cleavage compared to freshly isolated neutrophils, $P < 0.001$ for the MCF-7 assay and $P = 0.006$ for the MDA-MB-231 assay. Furthermore, neutrophils incubated in control medium or MCF-7 CM demonstrated a significantly higher proportion of caspase-8 cleavage (93.8% and 90.4% respectively) compared to MDA-MB-231 (5.9%), $P = 0.007$ and $P = 0.008$. These results suggest that some form of soluble factor secreted by MDA-MB-231 cells but not MCF-7 cells modulates caspase-8 cleavage in neutrophils after 7hrs of incubation.

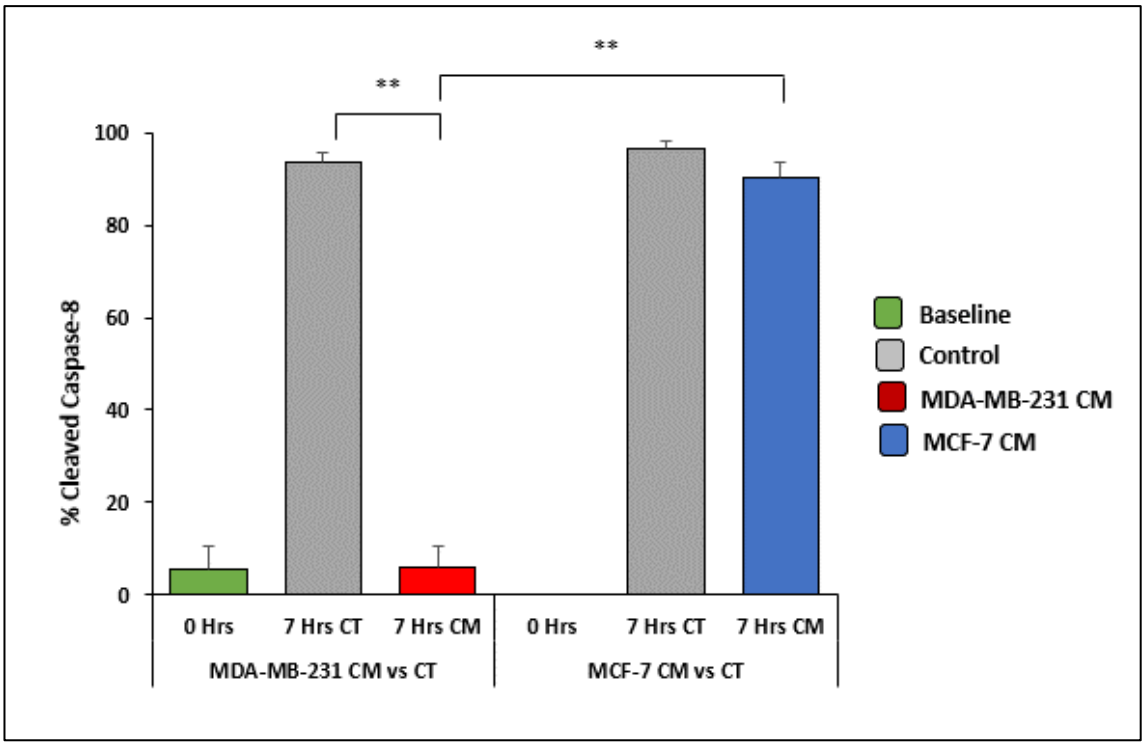


Figure 4.4: Proportion of cleaved caspase-8 in neutrophils incubated in control medium, MDA-MB-231 conditioned medium (CM) or MCF-7 CM for 7hrs, quantified via western blot computerised image analysis

Neutrophils were cultured in control medium, MCF-7 conditioned medium (CM) or MDA-MB-231 CM for 7hrs. The samples were subjected to SDS-PAGE and western blotting. Blots were probed with anti-caspase-8 (1:1000). A band analysis, to determine the % of full length versus cleaved caspase within each lane was undertaken using Image Lab™ software. Values are means ± SEM. (N=3). Statistical significance, comparing the % cleaved caspase for neutrophils cultured in MDA-MB-231 CM, against neutrophils cultured in control medium or MCF-7 CM was determined by Student's paired t-test. **, $P \leq 0.01$.

4.3.2 Expression of FAS, MCL, BAX, BCL2(A1), BAK

To resolve the question of whether the intrinsic or extrinsic pathways might be regulating the delay in neutrophil apoptosis, quantitative RT-PCR (qRT-PCR) was used to measure the expression of two anti-apoptosis proteins, MCL-1 and BCL2(A1), two pro-apoptosis proteins, BAX and BAK in the intrinsic pathway, and the extrinsic pathway component, FAS, for neutrophils cultured in MCF-7 CM, MDA-MB-231 CM or control medium for 7hrs (Section 2.2.5.5).

Results from the qRT-PCR assay for neutrophils cultured in MDA-MB-231 CM showed a significant increase in the relative mRNA expression of the anti-apoptosis peptide BCL2(A1) (P= 0.004) and a significant decrease in the relative mRNA expression of the pro-apoptosis peptide BAK (P=0.021) compared to control (Figure 4.5, A & C), however no difference was noted in the expression of MCL (P=0.299) or BAX (P=0.199), (Figure 4.5, B & D). In contrast, neutrophils cultured in MCF-7 CM for 7hrs showed no difference in the relative expression of BCL2(A1) (P=0.762) MCL (P=0.191), BAK (P=0.231) or BAX (P=0.318) compared to control (Figure 4.5, A-D). With regards to the extrinsic apoptosis pathway, neutrophils cultured in both MDA-MB-231 CM and MCF-7 CM demonstrated a trend towards decreased expression of FAS compared to the control (P=0.066 and P=0.085, respectively), (Figure 4.6).

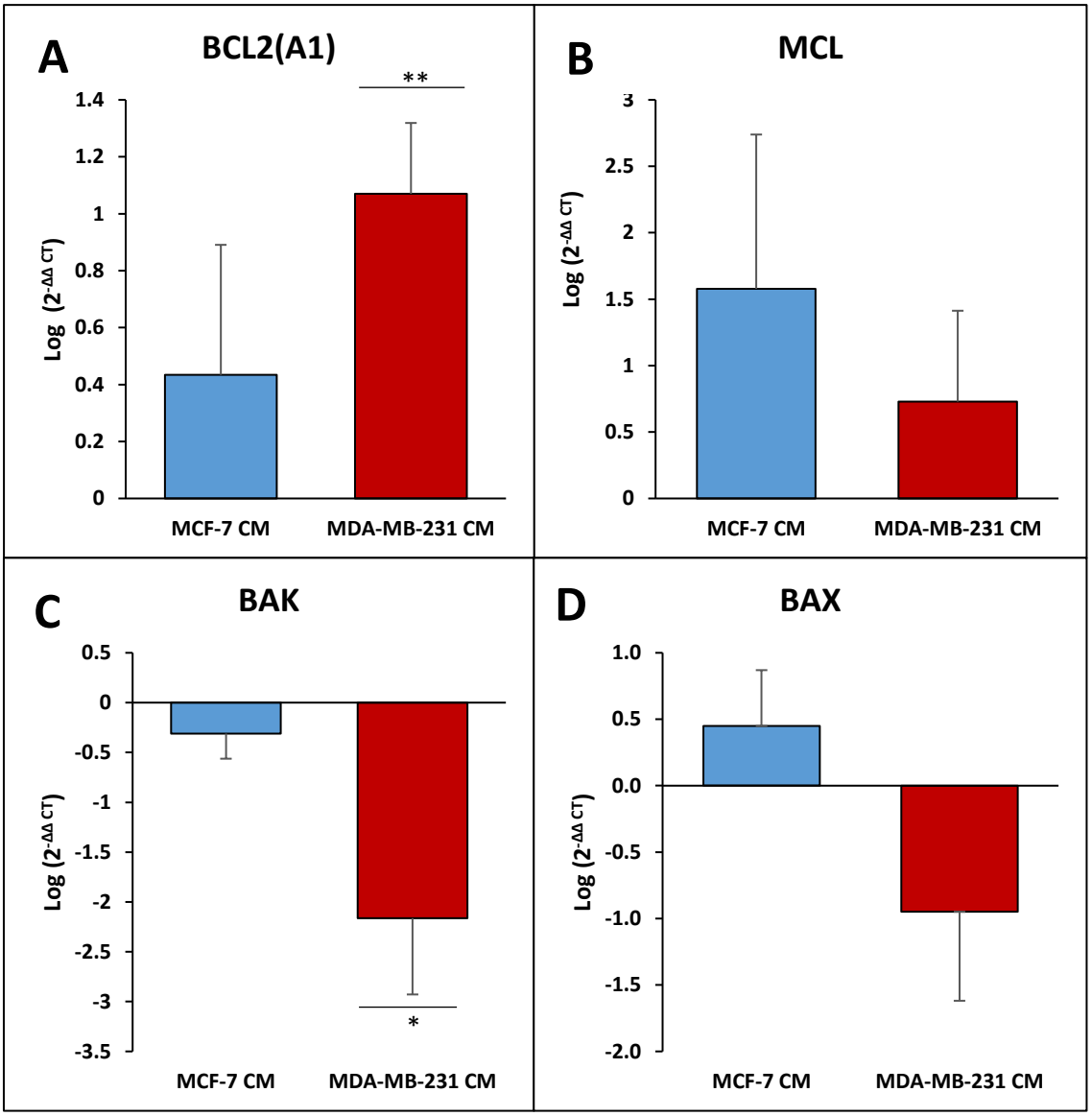


Figure 4.5: Relative expression of BCL2 homologue mRNA in neutrophils cultured in MCF-7 conditioned medium (CM) or MDA-MB-231 CM compared to control

Relative mRNA expression of anti-apoptosis peptides BCL2(A1) (A) and MCL (B) and pro-apoptosis peptides BAK (C) and BAX (D). Freshly isolated human neutrophils were cultured in control medium, MCF-7 CM or MDA-MB-231 CM at a concentration of $3.33x10^5$ cells/ml, for 7hrs. RNA was isolated from the samples, cDNA prepared and gene expression measured using qRT-PCR. β-actin was used as the housekeeping gene. Values are means ± SEM of Log (2-ΔΔCT) (N=8 participants). Statistical significance, comparing ΔCT of neutrophils in CM versus ΔCT neutrophils in control medium via Student's paired t-test.*, P ≤0.05, **, P≤0.01.

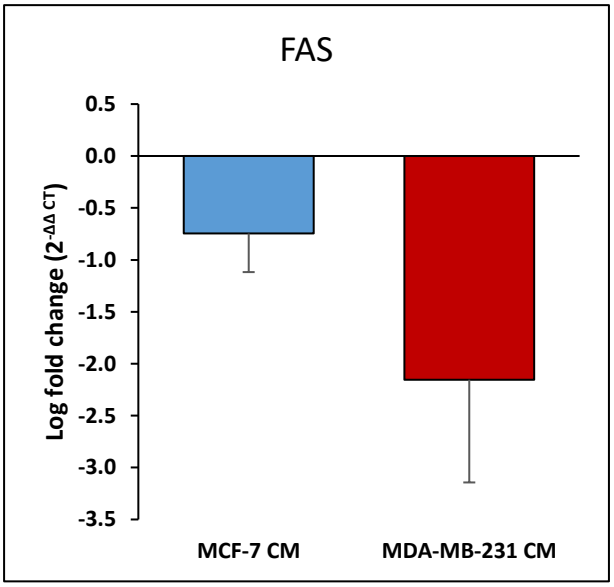


Figure 4.6: Relative expression of FAS receptor mRNA in neutrophils cultured in MCF-7 CM or MDA-MB-231 CM compared to control

Freshly isolated human neutrophils were cultured in control medium MCF-7 CM or MDA-MB-231 CM at a concentration of $3.33x10^5$ cells/ml, for 7hrs. RNA was isolated from the samples, cDNA prepared and FAS gene expression measured using qRT-PCR. β-actin was used as the housekeeping gene. Values are means ±SEM of Log ($2^{-\Delta\Delta CT}$). (N=8). Statistical significance, comparing ΔCT of neutrophils in CM versus ΔCT neutrophils in control medium via Student's paired t-test.

These results suggest that some form of soluble factor secreted by MDA-MB-231 cells and not MCF-7 cells increased the expression of the anti-apoptosis peptide BCL2(A1) and decreased the expression of the pro-apoptosis peptide BAK in neutrophils after 7hrs of incubation in CM, leading to an increased lifespan.

4.4 Discussion

4.4.1 Neutrophils cultured in MDA-MB-231 cell conditioned medium (CM) demonstrate a delay in apoptosis

A preliminary experiment suggested a trend towards delayed apoptosis for neutrophils cultured in both MCF-7 and MDA-MB-231CM. In this initial experiment viability (%) was assessed via the trypan blue exclusion method (352) (Figure 4.1). Whilst this is one of the most common techniques used to determine cell viability (394), the dye has been found to overestimate viability when compared to fluorescence methods (395,396). Subsequently for all remaining experiments, viability (%) was assessed by flow cytometry using fluorescein isothiocyanate (FITC) labelled Annexin V and propidium iodide (PI) fluorescence staining (397). This assay has an advantage over the trypan blue exclusion method, in that it can discriminate between apoptotic (FITC+/PI-) and necrotic cells (FITC+/PI+ and FITC-/PI+). Results from the dead cell apoptosis assay (Section 4.2.2) subsequently demonstrated a significant delay in apoptosis for neutrophils cultured in MDA-MB-231 CM, and not MCF-7 CM after 7 or 10hrs of incubation (Table 4.1). It is of course possible that the soluble factor(s) contained within MDA-MB-231 CM that are responsible for the delay in neutrophil apoptosis are also produced by MCF-7 cells, albeit at a concentration too low to effectively delay neutrophil apoptosis under these experimental conditions. However, overall these results are in accordance with those presented by Ibrahim *et al.* (34), who reported enhanced survival of neutrophils cultured in MDA-MB-231 CM, and with those presented by Trellakis *et al*. (32) and Wu *et al.*(236) who found enhanced survival for neutrophils cultured in conditioned medium prepared from head and neck squamous cell carcinomas and hepatocellular, cervical, colorectal and gastric cancer cell lines respectively.

4.4.2 Neutrophils cultured in MDA-MB-231 cell conditioned medium (CM) demonstrate a decreased caspase-8 cleavage compared to control

Results from the capase-8 assay obtained by quantifying the proportion of active or inactive caspase-8 in each lane of the western blots, showed that neutrophils cultured in MDA-MB-231 CM, and not MCF-7 CM, demonstrate a significantly lower proportion of caspase-8 activation compared to control. This is important because caspase-8 plays a pivotal role in mediating neutrophil apoptosis. As previously indicated (Section 1.2.5.2.3) caspase-8 regulates extrinsic apoptosis via caspase-3, either directly by cleaving caspase-3 (398), or indirectly, by activating the intrinsic mitochondrial pathway via BH3 interacting-domain death agonist (BID) (196,197). However, there is conflicting evidence as to whether caspase-8 may also have a role in regulating constitutive neutrophil apoptosis, caspase-8 has been found to be spontaneously activated in peripheral neutrophils isolated from healthy volunteers, as demonstrated by increased caspase-8 catalytic activity and increased pro-caspase-8 cleavage (200). The neutrophils were shown to be constitutively apoptotic, and inhibition of caspase-8 using IETD-CHO (a reversible tetrapeptide inhibitor) induced a dose dependant delay in apoptosis (200). However, other researchers (399) found that neutrophils exposed to a broad spectrum caspase-8 inhibitor (zVAD-fmk) did not demonstrate a reduction in constitutive apoptosis. Given that only neutrophils cultured in MDA-MB231 CM showed a significant delay in apoptosis, and that this was associated with a significantly lower proportion of caspase-8 cleavage (activation), it is possible that the delay in apoptosis was at least partly mediated via altered caspase-8 signalling, either directly via inhibition of the extrinsic apoptosis pathway or indirectly through reduced activation of BID. Furthermore, these results may corroborate an abstract published by Ibrahim *et al.* (35), who found that neutrophils treated with a2 isoform V-ATPase (a2NTD) (a cleaved peptide expressed on the surface of invasive breast cancer cells (238)) demonstrate delayed apoptosis, with both the intrinsic (via decreased gene expression and activity of caspase-3,-6,-7) and extrinsic (via decreased gene expression and activity of caspase-8) apoptosis pathways being modulated.

4.4.3 Neutrophil expression of intrinsic and extrinsic apoptosis regulating peptides

The intrinsic apoptosis pathway in neutrophils is primarily regulated by B cell lymphoma (Bcl)-2 proteins, the relative ratios of which (pro-apoptosis versus anti-apoptosis peptides) determine the overall permeability of the mitochondrial membrane (183). Neutrophil expression of two anti-apoptosis peptides Bcl-2-related protein A1 (BCL2(A1)) and induced myeloid leukaemia cell differentiation protein (MCL-1) and two pro-apoptosis peptides, Bcl-2-associated X protein (BAX), Bcl-2 homologous antagonist killer (BAK) were investigated via qRT-PCR, as detectable levels of all four peptides have been identified previously in freshly isolated neutrophils (400–402). BCL2(A1) and BAK both belong to the BCL2 protein family; BCL2 proteins regulate the intrinsic apoptosis pathway via the release of cytochrome c from mitochondria (Section 1.2.5.2.3). Specifically, BH3 proteins (such as BID) activate BAK and BAX which in turn mediate cytochrome C release by forming pores in the mitochondrial membrane (referred to as mitochondrial membrane permeabilisation or "MOMP"), whereas the anti-apoptosis BCL2 proteins such as BCL2(A1) and MCL sequester and inhibit the activating BH3 proteins and the pore formers BAK and BAX (403,404). However the anti-apoptosis proteins themselves are also subject to sequestration and inhibition via sensitising BH3 proteins such as BAD and NOXA (403), consequently it is the sum of all of these interactions that will determine if BAK or BAX are able to mediate cytochrome C release. Given that only neutrophils cultured in MDA-MB-231 CM demonstrated a significant delay in apoptosis, and that this was associated with a significantly higher expression of the anti-apoptosis BCL2(A1) and a significantly lower expression of anti-apoptosis BAK, it is possible the delay in apoptosis was partly mediated via reduced pore formation (MOMP), either directly due to the reduced concentration of BAK or indirectly via increased BCL2(A1) associated sequestration of activating BH3 proteins and pore forming BAK and BAX. This hypothesis could be investigated further via a cytochrome C release assay; should neutrophils cultured in MDA-MB-231 CM demonstrate reduced cytochrome C release this could indicate a reduction in MOMP permeability. These results may partially corroborate the abstract published by Ibrahim *et al.* (35), who found that neutrophils treated with a2 isoform V-ATPase (a2NTD) demonstrate delayed apoptosis via increased expression of the anti-apoptosis peptides BCL2(A1) and B-cell

lymphoma-extra-large (Bcl-xL) and decreased expression of the pro-apoptotic factors; BAX and Apoptotic protease activating factor 1 (APAF-1).

In contrast the extrinsic pathway, activated by cell death receptors, is thought to have little or no role in constitutive neutrophil apoptosis (194,405). However FAS (CD95/APO1) receptor mediated apoptosis, is thought to be important during inflammation (406,407) and was therefore explored in these experiments. Although there was a trend towards reduced expression of FAS receptor in neutrophils cultured in MDA-MD-231 CM compared to control, it was not significant. This does not necessarily exclude the extrinsic apoptosis pathway from further study, as several other cell death receptors such as TRAIL-R1 and TRAIL-R2 have been identified on neutrophils, however, much like the FAS receptor they do not appear to regulate constitutive neutrophil apoptosis and may only be important during inflammation (194).

CHAPTER 5

CHARACTERISATION OF SOLUBLE FACTORS IN MDA-MB-231 CONDITIONED MEDIUM RESPONSIBLE FOR DELAYED NEUTROPHIL APOPTOSIS

5.1 Introduction

Results from the experiments in the preceding chapter, strongly suggest that some form of soluble factor(s) secreted by MDA-MB-231 cells, and not MCF-7 cells, is capable of delaying neutrophil apoptosis by more than seven hours. Over the last few decades a number of soluble factors have been implicated in delayed neutrophil apoptosis (reviewed in 218,220) several of which, including IL-6 & IL-8 (30), hyaluronic acid (HA) (236) and an N-terminal peptide from the a2 isoform of vacuolar ATPase (a2NTD) (35) have been associated with delayed neutrophil apoptosis in various cancers, and *in vitro* experiments with cancer cell lines, including MDA-MB-231 breast cancer cells (34) (Table 1.1, Section 1.2.5.2.4). This latter study in accordance with the results presented in the previous chapter, demonstrated enhanced survival of neutrophils cultured in MDA-MB-231 CM. Ibrahim *et al.* (34,35) reported that neutrophils stimulated with recombinant a2NTD showed enhanced survival; however, this was to a lesser extent compared to neutrophils cultured in MDA-MB-231 CM (34), leading the authors to suggest that a2NTD is only partly responsible for the delay in apoptosis. However, as both studies (34,35) have only been published as abstracts this prevents a full examination of the methods undertaken and results achieved. However, Ibrahim *et al.* have published papers demonstrating the ability of a2ND to modulate neutrophil function (238) and promote neutrophil migration (408) *in vitro*. High concentrations of recombinant a2NTD (from 200-500ng/ml) were used in all of the experiments which may not be comparable to the amount secreted by breast cancer cells. Overall, this suggests that the identity and mechanisms of action of the soluble factor(s) responsible for enhanced neutrophil lifespan in MDA-MB-231 CM have yet to be fully established. In order to resolve this gap in understanding, an attempt was made to characterise the basic properties of the soluble factor(s) responsible for enhanced neutrophil lifespan in MDA-MB-231 CM, including molecular weight and heat stability.

5.2 The effect of molecular weight fractionated MDA-MB-231 conditioned medium (CM) on neutrophil viability

To determine the approximate molecular weight of the soluble factor responsible for the delay in neutrophil apoptosis, normal control medium and MDA-MB-231 conditioned medium was fractionated by centrifugation using 10kDa, 30kDa, 50kDa and 100kDa molecular weight cut off (MWCO) filters (Section 2.2.3.4.3). These filters were selected as many of the mediators known to delay neutrophil apoptosis are proteins or lipids with molecular weights that range from 8-200kDa (Table 1.1, Section 1.2.5.2.4). Neutrophils were cultured in each of the molecular weight fractions for 7hrs, after which viability was assessed using a Dead Cell Apoptosis kit with Annexin V FITC and PI (Section 2.2.3.4.2.)

The results of the neutrophil viability assay using size fractionated MDA-MB-231 CM are shown in Figure 5.1. As per previous experiments (Section 4.2.2) neutrophils incubated in unfiltered MDA-MB-231 CM for 7hrs demonstrated a greater proportion of viable cells compared to control (89.6% ± 2.4 versus 12.9% ± 2.4, $P \leq 0.001$). Importantly, neutrophils incubated in a mixture of MDA-MB-231 filtrate and retentate recombined demonstrated a similar proportion of viable cells to those incubated in unfiltered MDA-MB-231 CM (84.5% ± 5.6 versus 89.6% ± 2.4, $P = 0.279$); thus indicating that the soluble factor(s) responsible for delayed apoptosis were unaffected by the filtration process.

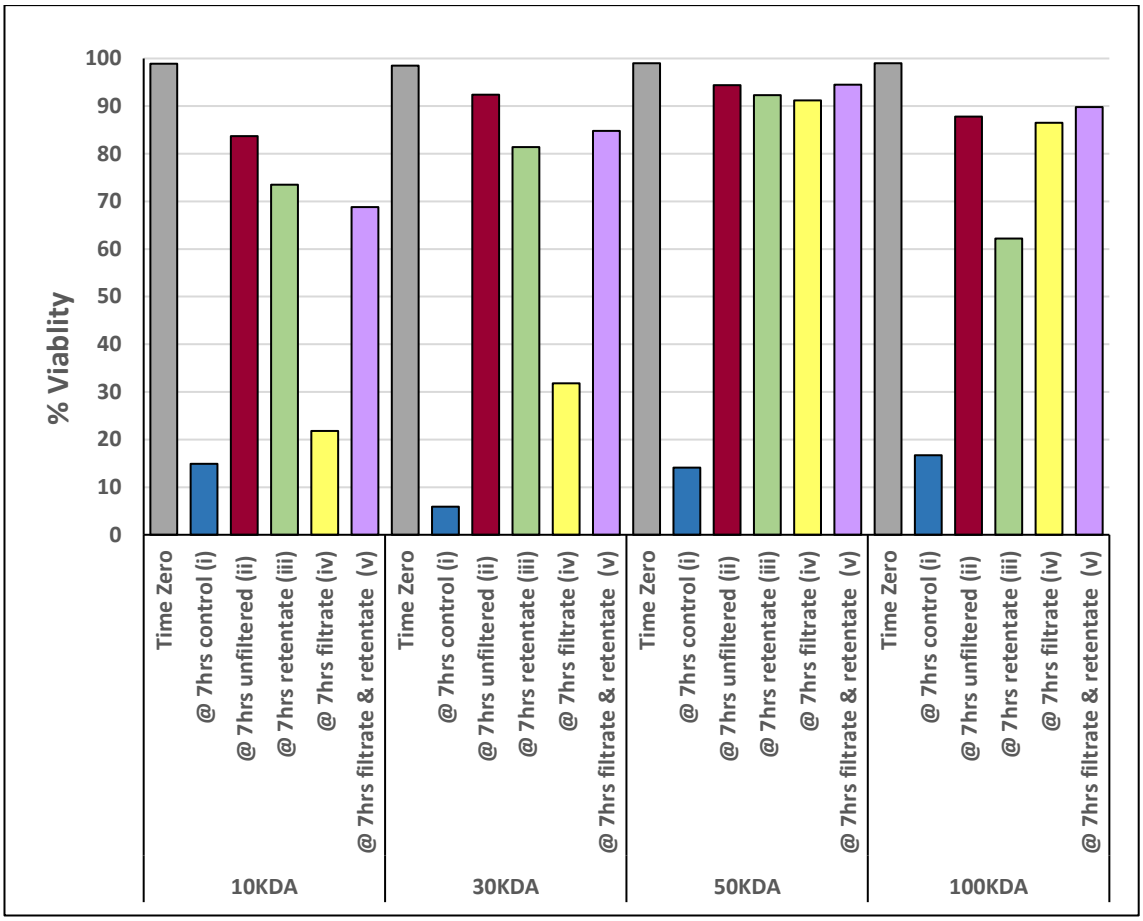


Figure 5.1: Effect of MW fractionation of MDA-MB-231 conditioned medium (CM) on neutrophil viability

Control medium and MDA-MB-231 CM was prepared prior to commencing each experiment and were fractionated by centrifugation using either 10kDa, 30kDa, 50kDa or 100kDa molecular weight cut off (MWCO) filters. Neutrophils were cultured in i) control medium (negative control), ii) unfiltered MDA-MB-231 CM (positive control), iii) retentate from MDA-MB-231 CM, iv) filtrate from MDA-MB-231CM v) a mixture of the retentate and filtrate from MDA-MB-231 CM, at a concentration of $3.33x10^5$ cells/ml and incubated at 37ºC. Neutrophil viability was assessed after 7hrs of incubation via flow cytometry using a Dead Cell Apoptosis kit with Annexin V FITC and PI. Viable cells were identified as FITC-/PI-, Apoptotic as FITC+/PI- and necrotic as FITC+/PI+ and FITC-/PI+.

Since the proportion of viable cells appeared to be somewhat lower for neutrophils incubated in the 10kDa and 30kDa filtrate (21.8% and 31.8% respectively) compared to the retentate (73.5% and 81.4%), this initially suggested that the soluble factor(s) could have a molecular weight ≥30kDa. Likewise, since the proportion of viable cells seemed to be slightly lower for neutrophils incubated in the 100kDa retentate compared to the filtrate (62.2% versus 86.5%), this suggested that a proportion of the soluble factor(s) could have a molecular weight ≤100kDa.

In an attempt to confirm these results, the 30kDa and 100kDa filter experiments were repeated utilising freshly prepared CM. The 100kDa filter experiment demonstrated a similar result as before (Figure 5.1), with neutrophils incubated in retentate showing a lower proportion of viable cells (47.9%) compared to those incubated in the filtrate (79.6%). However, the 30kDa filter experiment produced conflicting results (Figure 5.2), as similar proportions of viable cells were found in the retentate and filtrate (92.3% versus 89%, respectively).

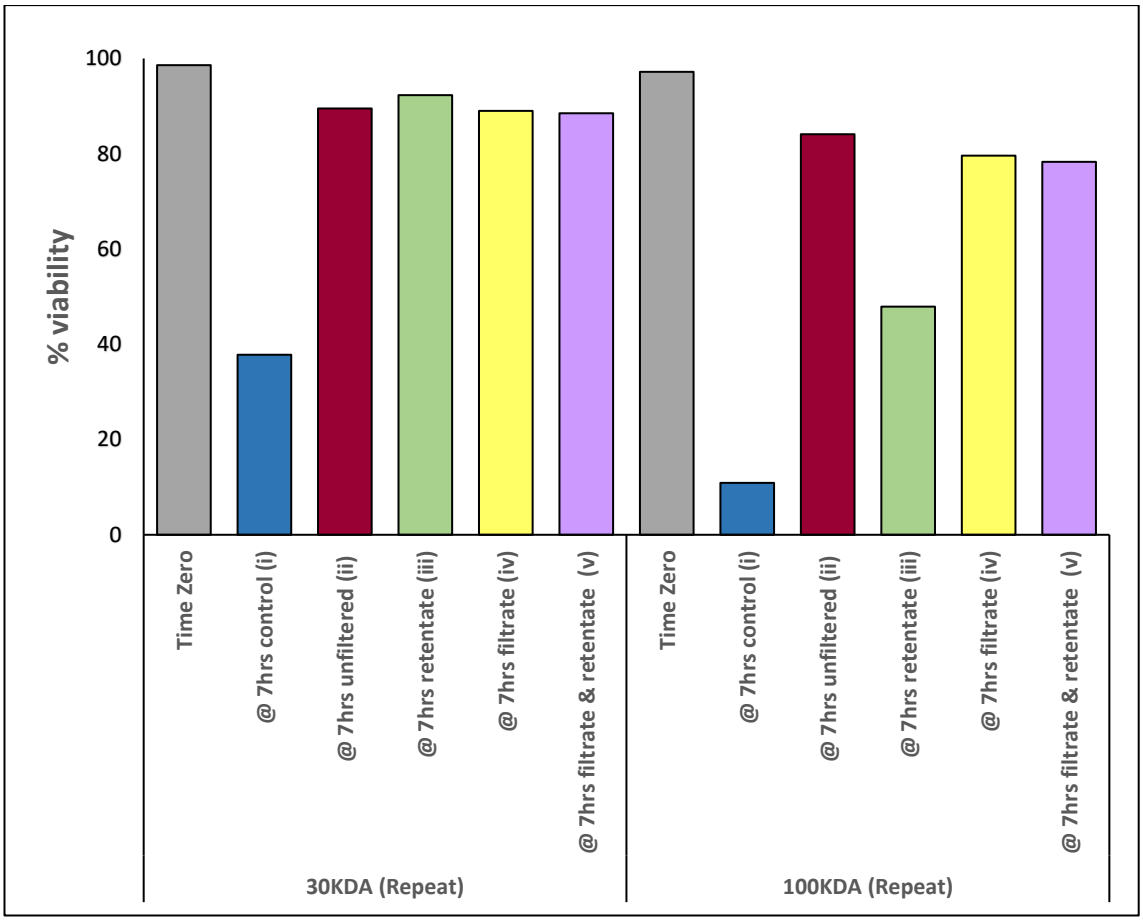


Figure 5.2: Repeat experiment examining the effect of MW fractionation on MDA-MB 231 conditioned medium (CM) on neutrophil viability

Control medium and MDA-MB-231 CM was prepared prior to commencing each experiment and was fractionated by centrifugation using either 30kDa or 100kDa molecular weight cut off (MWCO) filters. Neutrophils were cultured in i) control medium (negative control), ii) unfiltered MDA-MB-231 CM (positive control), iii) retentate from MDA-MB-231 CM, iv) filtrate from MDA-MB-231CM v) a mixture of the retentate and filtrate from MDA-MB-231 CM at a concentration of $3.33x10^5$ cells/ml and incubated at 37ºC. Neutrophil viability was assessed after 7hrs of incubation via flow cytometry using a Dead Cell Apoptosis kit with Annexin V FITC and PI. Viable cells were identified as FITC-/PI-, Apoptotic as FITC+/PI- and necrotic as FITC+/PI+ and FITC-/PI+.

This variation in results is perhaps not surprising given the complexity in the range of compounds secreted by cancer cells, the variation in their sizes and the potential for batch to batch variation in the composition of the CM. For example, MDA-MB-231 cells are known to secrete a variety of substances associated with delayed neutrophil apoptosis including GM-CSF (409), IL-6 & IL-8 (227), a2ND (238) HA (237) and Prostaglandin

E_2 (PGE_2) (410) and these compounds range in molecular weight from 0.3kDa to 200kDa (Table 1.1, Section 1.2.5.2.4). In addition, some of these substances, demonstrate a range of molecular weights depending upon their degree of glycosylation or fragmentation. For example, HA fragments can vary from just a few disaccharides in length to more than 700kDa (411). Consequently, the conflicting results generated from the 30kDa filters might simply have been due to the differential secretion and processing of these soluble factors, by the MDA-MB-231 cells, in the different batches of culture media produced.

Results shown here (Figure 5.1 and Figure 5.2) suggest that the soluble factor(s) responsible for the delay in neutrophil apoptosis have a molecular weight of at least 10kDa; two of the soluble factors known to be secreted by MDA-MB-231 cells, as identified in the literature, IL-6 (21-28kDa depending upon post translational processing) (228) and a2ND (~20kDa) (238) fit this criterion. A further soluble factor PGE_2, does not immediately fit the criteria as the individual molecules are very small (~352.5 Da). However, due to their hydrophobic nature, PGE_2 molecules are likely to form micelles or bind to proteins contained in FBS. For example, a study in which low to moderate concentrations of PGE_2 were added to human plasma demonstrated 73% binding of PGE_2 to plasma proteins, with 42% binding specifically to human serum albumin (412). This is important because bovine serum albumin constitutes 60-67% of the total protein found in FBS (413), and has a molecular weight of ~66kDa (414). Thus, PGE_2 is also a possible candidate for the soluble factor, as when bound to bovine serum albumin it would be present in the retentate obtained from the 50kDa filters and the filtrate obtained from the 100kDa filters, both of which were associated with enhanced neutrophil lifespan. Moreover, another important consideration is that the results from the previous chapter demonstrated a significant delay in neutrophil apoptosis after 7hrs of incubation in MDA-MB-231 CM, but not MCF-7 CM. This suggests that the soluble factor is either not secreted, or is only secreted at comparatively low levels by MCF-7 cells. Information in the literature indicates that PGE_2 (410) fits the criteria of being secreted by MDA-MB-231 cells, but not (or only in low amounts) by MCF-7 cells, which supports the hypothesis that PGE_2.could be at least partly responsible for the delay in neutrophil apoptosis.

5.3 The effect of heat-treated MDA-MB-231 conditioned medium (CM) on neutrophil viability

To further characterise the soluble factor responsible for the delay in apoptosis, an experiment was designed to determine whether the soluble factor was most likely to be a protein or a lipid. Whilst a number of methods can be used to separate and extract macromolecules from biological samples; such as the depletion of lipids from serum via solvent based chromatography (415), these are time consuming and challenging to undertake. Therefore, to address this question in the simplest manner, neutrophils were cultured in heat treated MDA-MB-231 CM (Section 2.2.3.4.4), as heat treatment should denature the proteins contained in the CM thereby preventing their function.

The results from the heat treatment assay (Table 5.3) show that neutrophils incubated for 7hrs in heat treated CM, as per neutrophils incubated in non-heat-treated CM (Section 4.2 & Section 5.2) demonstrated a greater proportion of viable cells compared to those incubated in control media.

Table 5.1: Effect of heat-treated MDA-MB-231 conditioned medium (CM) on neutrophil viability

Sample	Viable %	Apoptotic %	Necrotic %
Control	15.3	83.8	0.8
Heat-treated filtered CM	79.7	18.4	1.95

Control medium and MDA-MB-231 CM were prepared prior to commencing each experiment. An aliquot of MDA-MB-231 CM was heated for 10min at 100ºC, then centrifuged (1000xg, 5min, RT), the supernatant was filtered through a 0.22µm filter and stored at 37ºC for approximately 2hrs before use. Neutrophils were cultured in control medium or heat treated, filtered CM at a concentration of $3.33x10^5$ cells/ml and incubated at 37ºC. Neutrophil viability was assessed after 7hrs of incubation via flow cytometry using a Dead Cell Apoptosis kit with Annexin V FITC and PI. Viable cells were identified as FITC-/PI-, Apoptotic as FITC+/PI- and necrotic as FITC+/PI+ and FITC-/PI+. (N=2).

This is a significant result, as it suggests that at least one of the soluble factors(s) responsible for enhanced neutrophil lifespan in MDA-MB-231 CM is heat stable. Most proteins are usually denatured following heat treatment, irreversibly losing their structure and function (416), consequently the results from this experiment suggest that one of the soluble factor(s) is not a protein. This is important because of the candidate molecules, PGE_2 is a heat stable lipid; with one study finding commercially available PGE_2 retained its biological activity even after boiling for 2hrs at 100ºC (417). However, it is possible that a small peptide, with little structure, might be heat stable, and hence may contribute to the enhancement of neutrophil lifespan. Nonetheless, the results from the preceding section suggest that the molecule has a molecular weight of at least 10-30kDa, and as described previously (Section 5.2), PGE_2 would meet this criteria when bound to bovine serum albumin (~66kDa (414)).

5.4 The effect of heat-treated PGE_2 on neutrophil viability

As discussed in the preceding sections, based on its molecular weight when bound to serum proteins, differential secretion by MDA-MB-231 cells compared to MCF-7 cells (410) and heat stability (417), PGE_2 is a candidate for the soluble factor responsible for enhanced neutrophil lifespan. However, whilst other studies have indicated that PGE_2 is associated with enhanced neutrophil lifespan (232,233) it is not known if PGE_2 exerts the same effect when it has been heat treated. Consequently, an experiment was undertaken to determine if heat treated PGE_2 could enhance neutrophil lifespan (Section 2.2.3.4.5).

As in previous experiments, neutrophils incubated in either MDA-MB-CM (v) or heat-treated MDA-MB-CM (vi) demonstrated a significantly greater proportion of viable cells compared to those incubated in control medium ($P \leq 0.001$ and $P \leq 0.001$ respectively) (Figure 5.1). Likewise, neutrophils incubated in 10μM PGE_2 control (ii) and 10μM PGE_2 control, heated (iii) also demonstrated a significantly greater proportion of viable cells compared to those incubated in control medium ($P = 0.031$ and $P \leq 0.001$) (Figure 5.3). No difference in viability was demonstrated for cells incubated in the vehicle control (control medium supplemented with ethanol in 0.1M PBS) (iv) compared to control medium ($P=0.846$).

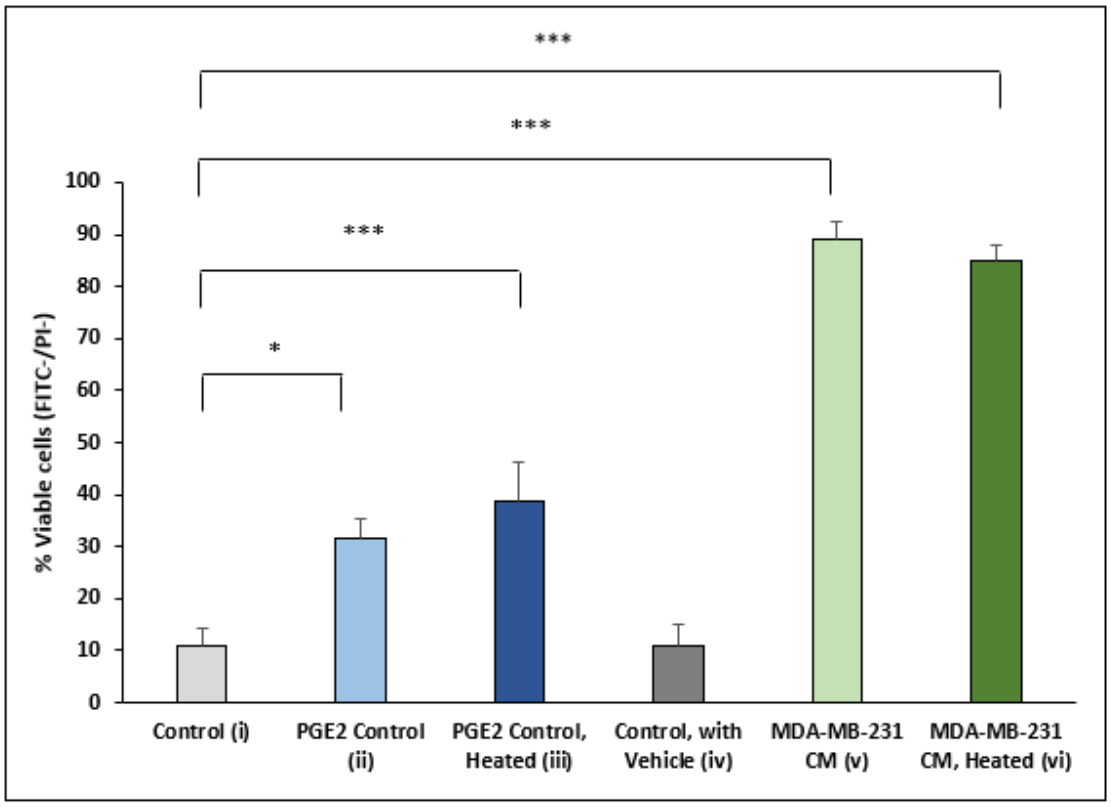


Figure 5.3: Proportion of viable neutrophils when cultured in control medium, control medium with PGE2, heat-treated control medium with PGE2, MDA-MB-231 conditioned medium (CM) or heat-treated MDA-MB-231 CM for 7hrs

Neutrophils were cultured in i) Control medium ii) 10µM PGE2 Control iii) 10µM PGE2 Control, Heated, iv) Control with vehicle control, v) MDA-MB-231 CM and vi) MDA-MB-231 CM, heated, at a concentration of $3.33x10^5$ cells/ml and incubated at 37ºC for 7hrs. Neutrophil viability was determined via flow cytometry using a Dead Cell Apoptosis kit with Annexin V FITC and PI. Viable cells were identified as FITC-/PI-, Apoptotic as FITC+/PI- and necrotic as FITC+/PI+ and FITC-/PI+. Values are mean ± SEM of 4 (N=4). Statistical significance was determined by Student's paired t test. *, $P \leq 0.05$, ***, $P \leq 0.001$.

These results confirm that PGE_2 has a modulatory effect on neutrophil lifespan, albeit to a lesser extent compared to that of MDA-MB-231 CM. However, more significantly, they show that heat treated PGE_2, retains the same level of biological activity as native PGE_2. This is important because heat treated MDA-MB-CM retains the same biological activity as untreated CM. Taken together these results continue to support the hypothesis that PGE_2 is a candidate for the soluble factor responsible for the delay in apoptosis. Nonetheless, it is important to note that whilst this research has focussed on characterising a single candidate molecule it is possible that other, yet to be identified, molecules may

also be involved. From these experiments alone, it is impossible to determine what proportion of this effect (the delay in neutrophil apoptosis) might be attributed to PGE_2 compared to any other molecules involved.

5.5 Expression of COX-2 in MDA-MB-231 and MCF-7 cells

One of the important findings from this study was that neutrophils incubated in MDA-MB-231 CM for 7hrs demonstrated a significant delay in apoptosis, however, the same effect was not observed in neutrophils incubated in MCF-7 CM. An early study by Schrey *et al*. (410), which investigated PGE_2 production in seven breast cancer cell lines, was unable to detect PGE_2 production in MCF-7 cells and only demonstrated constitutive cyclo-oxygenase (COX-2) activity in MDA-MB-231 cells. This is important because COX-2 is required for the first step of PGE_2 synthesis (see discussion Section 5.6.3). To verify these results, an experiment was undertaken to determine the expression of COX-2 in MDA-MB-231 and MCF-7 cells under normal growth conditions by RT-PCR (Section 2.2.5.4).

Results from the COX-2 RT-PCR assay show that only the MDA-MB-231 cells and not the MCF-7 cells demonstrate a discernible PCR product of the correct size (144b.p.) on the agarose gel (Figure 5.4). This suggests that only MDA-MB-231 cells and not MCF-7 cells were expressing the COX-2 gene.

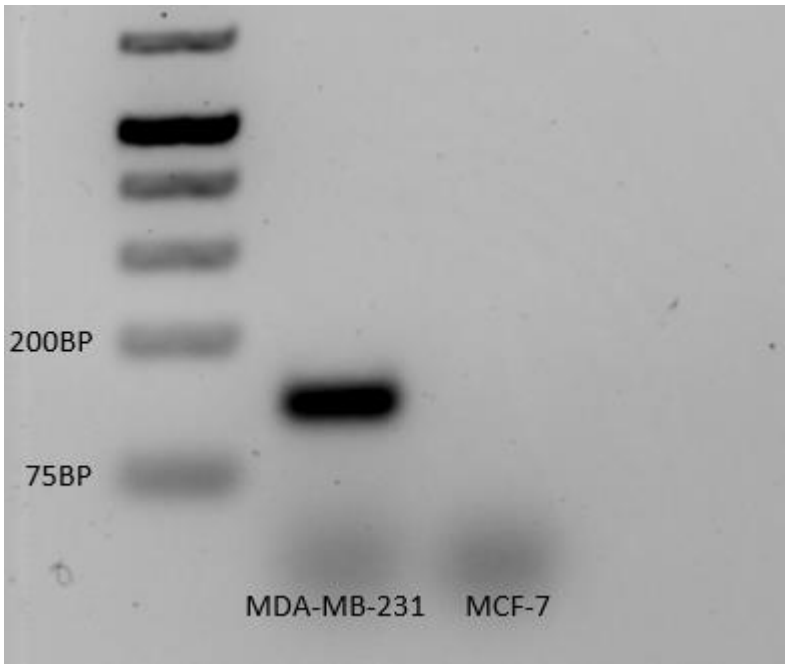


Figure 5.4: Expression of COX-2 in MDA-MB-231 cells versus MCF-7 cells

MCF-7 cells and MDA_MB-231 cells were grown to 70% confluence, RNA was extracted using an Isolate II RNA Mini Kit (Bioline, Alexandria, NSW, AUS) and reverse transcribed into cDNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Auckland, New Zealand) according to manufacturer's instructions. PCRs were undertaken on a StepOne™ Real-Time PCR cycler (Thermo Fisher Scientific, Auckland, New Zealand). β-actin was used as the housekeeping gene and acted as a positive control. PCR products were separated via DNA gel electrophoresis and visualised via a ChemidocTM XRS+ Imaging system. A 1kb DNA ladder (Thermo Fisher Scientific, Auckland, New Zealand) is found in lane 1 and PCR products for COX-2 mRNA in MDA MD-231 and MCF-7 cells are found in lanes 2 and 3 respectively. (N=6).

5.6 Discussion

This chapter has documented a body of work that attempts to characterise the soluble factor(s) contained in MDA-MB-231 CM responsible for delayed neutrophil apoptosis.

5.6.1 Molecular weight and biological class of the candidate molecule

The aim of the first experiment was to identify the approximate molecular weight of the soluble factor(s) responsible for delayed apoptosis. Overall the results achieved in Section 5.2 suggest that the soluble factor(s) responsible for the delay in neutrophil apoptosis have a molecular weight of at least 10kDa, and less than 100kDa. A search of the literature identified three molecules known to be secreted by MDA-MB-231 cells that have been associated with delayed neutrophil apoptosis and fit this molecular weight criteria; IL-6 (30) and a2ND (238) (both proteins) and PGE_2 (a lipid) when bound to serum proteins (414). However, there could be other molecules, yet to be identified by research that also meet these criteria. The next aim (Section 5.3) was to determine the biological class of the candidate molecule(s); a goal achieved by culturing neutrophils in heat treated MDA-MB-231 CM. Results showed (Section 5.3, Table 5.3) that at least one of the soluble factors(s) responsible for delayed apoptosis is heat stable, which suggests the molecule is not a protein. This data contrasts with the work of Ibrahim *et al.* (34) who suggest that a peptide (a2ND) cleaved from the a2 isoform of vacuolar ATPase (a2V-ATPase) and secreted by MDA-MB-231 cells might be partially responsible for enhanced neutrophil lifespan; a2V-ATPase has been shown to be overexpressed in certain cancers, including ovarian (418) and breast cancers (238) and a recombinant version of its peptide, a2ND, has been found to have an immunomodulatory, pro-inflammatory effect on neutrophils (238) and monocytes (419). The data presented in this thesis also contrasts with the work of Hor *et al*. (30) who found two peptides, IL-6 and IL-8, secreted by Glioma cells were able to induce a significant delay in apoptosis. To conclusively rule out the possibility that a heat stable peptide was responsible for the delay in apoptosis an experiment should be carried out in which neutrophils are cultured in heat treated CM that has been depleted of lipids. However, the difficulty with such experiments is that the reagents used during the extraction process (such as organic solvents and detergents

(415)) can be quite toxic and may have an adverse effect on neutrophil function thereby affecting the results achieved.

5.6.2 Heat-treated PGE_2 modulates neutrophil lifespan

The results of the heat-treated PGE_2 assay confirmed that PGE_2, and more critically heat-treated PGE_2, has a modulatory effect upon neutrophil lifespan. It is important to note that the delay in apoptosis was less for neutrophils cultured in CM containing PGE_2 compared to MDA-MB-231 CM. Due to time constraints a dose response relationship test was not undertaken for neutrophils incubated with PGE_2. Consequently, for the experiment described in Section 5.4, neutrophils were incubated in control medium supplemented with 10μM of PGE_2, as this concentration has been shown to be associated with delayed apoptosis in other studies (232). The concentration of PGE_2 reported to be present in breast cancer tissue varies considerably. One study of 78 patients with benign and malignant tumours identified two groups of malignant tissues the first (high PGE_2) demonstrated a mean tissue concentration of PGE_2 55.4pg/ml (range 25.3-101.9pg/ml), the second group (low PGE_2) demonstrated a mean tissue concentration of 10.7pg/ml (range 6.7-16.7pg/ml) (420). In contrast, another study found the concentration of PGE_2 in nipple aspirates of breast cancer patients to be ~10.7ng/ml ± 12 (421). In both instances, the concentration of PGE_2 reported was considerably lower than concentration of PGE_2 used in this study. Whilst other researchers have measured PGE_2 concentrations in MDA-MB-231 CM (235,410) the conditions and cell concentrations are too disparate from the current study for any meaningful comparisons to be made. However, the concentration of PGE_2 in MDA-MB-231 or MCF-7 CM was not measured in this study.

5.6.3 COX-2 is expressed in MDA-MB-231 cells

Prostaglandins are members of the eicosanoid family and are primarily synthesised from the eicosanoid precursor, arachidonic acid (AA). The first step of prostaglandin synthesis occurs when AA is presented to the enzyme cyclooxygenase to form an intermediate product prostaglandin H_2 (PGH_2) (422). PGH_2 is subsequently metabolised by

downstream enzymes to produce a final prostaglandin or leukotriene product; with the enzyme PGE_2 synthase being responsible for the terminal step of PGE_2 synthesis (423). Whilst two isoforms of cyclooxygenase are known to exist, COX-2 is the most important source of prostaglandin formation in inflammatory and proliferative diseases such as cancer (424). Consequently, to confirm the activity of cyclooxygenase in MDA-MB-231 cells, and by inference PGE_2 production, an experiment was undertaken to examine the expression of COX-2 in MDA-MB-231 and MCF-7 cells. Results from the COX-2 assay demonstrated COX-2 expression only in the MDA-MB-231 cells, which is in accordance with the results demonstrated by Schrey *et al.* (410). However, these results do not conclusively prove that PGE_2 was secreted by the MDA-MB-231 cells, since the concentration of PGE_2 in MDA-MB-231 CM was not measured.

5.6.4 PGE_2 as a candidate for the soluble factor responsible for delayed neutrophil apoptosis

Taken together, the data contained in this chapter points towards the possibility that PGE_2 is at least one of the candidate(s) molecules responsible for the delay in neutrophil apoptosis; as when bound to plasma proteins it fits the approximate molecular weight identified in Section 5.2, it is a heat stable lipid and not a protein (Section 5.3), neutrophils incubated in control medium supplemented with PGE_2 (in native form or heat-treated) demonstrate delayed apoptosis (Section 5.4) and COX-2 expression (the enzyme required for the first step of PGE_2 synthesis) was detected in MDA-MB-231 cells and not MCF-7 cells (Section 5.5). However, these results are preliminary, and do not conclusively prove that PGE_2 is the soluble factor present in MDA-MB-231 CM responsible for delaying neutrophil apoptosis.

To further investigate if neutrophil apoptosis is regulated by MDA-MB-231 cell derived PGE_2, the next logical step would be to measure the concentration of PGE_2 in MDA-MB-231 CM and MCF-7 CM. There are a number of techniques that could facilitate this goal, such as enzyme-linked immunosorbent assay (ELISA) or high-performance liquid chromatography (HPLC) (425). If PGE_2 is only found in MDA-MB-231 CM and not MCF-7 CM, this would validate the results of the COX-2 RT-PCR assay (Section 5.5).

That being the case, neutrophils should then be cultured in heat treated CM prepared from MDA-MB-231 cells that have been treated with a selective COX-2 inhibitor drug such as Celecoxib or MDA-MB-231 cells that have had the COX-2 gene silenced; if the CM is unable to delay neutrophil apoptosis this would suggest that at least one of the eicosanoids produced by COX-2, is likely responsible for delaying neutrophil apoptosis. Due to the time and financial constraints on this project, the focus was placed on PGE_2 as there was evidence in the literature that PGE_2 was produced by MDA-MB-231 cells (410) and it has been shown to be a heat stable lipid (417). However, research suggests that two other metabolites of PGH_2; 11-deoxy prostaglandin E_1 (11-deoxyPGE_1), and prostaglandin F_2 (PGF_2) may also delay neutrophil apoptosis, albeit to a lesser extent than PGE_2 (426) and should also be investigated in future research.

To definitively determine if PGE_2 is responsible, at least partially, for the delay in apoptosis the final step would be to culture neutrophils in heat treated CM prepared from MDA-MB-231 cells that have had the PGE_2 synthase gene silenced. If the conditioned media is unable to delay neutrophil apoptosis this would suggest that PGE_2 is the soluble factor responsible for delayed apoptosis, whereas if the CM is only able to partially delay apoptosis (compared to control) this would suggest other, yet to be identified molecules, in addition to PGE2 are involved. That being the case, an attempt could be made to identify any additional soluble factors by identifying all of the compounds present in MCF-7 and MDA-MB-231 CM via either gas chromatography – mass spectroscopy (GC/MS) or liquid chromatography – mass spectroscopy (LC/MS). The composition of the two samples could then be compared to identify any differences between the two, any lipids that are present in MDA-MB-231 CM but are not present (or present only in low amounts) in MCF-7 CM would then be investigated as to their ability to delay neutrophil apoptosis.

In conclusion, these experiments indicate that PGE_2 may be partly responsible for the delay in neutrophil apoptosis. However, it is important to note that there are likely to be other, yet to be identified compounds, in the conditioned medium which may be contributing to the observed delay in apoptosis. These compounds may work

synergistically, along with PGE_2, such that their cumulative effect is greater than that demonstrated by a single molecule, such as PGE_2, alone.

CHAPTER 6

PHENOTYPIC INTERACTIONS BETWEEN NEUTROPHILS AND MCF-7 AND MDA-MB-231 BREAST CANCER CELL LINES

6.1 Introduction

During the last decade research has shown (reviewed in 26,151,152) that neutrophils can alter their phenotype in response to physiological and pathological conditions including cancer (Section 1.2.5.3). Due to their apparent heterogeneity, both pro and anti-tumour functions of neutrophils have been reported (Sections 1.2.5.5.1-2). The work discussed in Chapters 4 and 5 showed that neutrophils cultured in MDA-MB-231 conditioned medium for 7hrs (CM) display a significant delay in neutrophil apoptosis. This is important because an increase in lifespan may allow the neutrophils time to acquire changes in phenotype that can either enhance or hinder tumour progression.

There is evidence (reviewed in 248,427) that neutrophils may be polarised towards either an "N1" (anti-tumour) or an "N2" (pro-tumour) phenotype in cancer, characterised by changes in morphology and gene expression. For example, N1 neutrophils are proposed to show increased expression of intercellular adhesion molecule 1 (ICAM-1) and decreased expression of matrix metallopeptidase 9 (MMP-9), with decreased expression of ICAM-1 and increased expression of MMP-9, proposed for N2 TANs (reviewed in 248). Research suggests that this polarisation ("N1" versus "N2") is brought about by environmental signals such as IL-1β (36) and TGF-β (37) respectively. However, the underlying mechanisms that bring about this effect are not well understood. Furthermore, it is possible the phenotype of neutrophils in cancer may, in part, be dictated by cancer type, and stage. Subsequently there remain significant gaps in understanding how cancer cells alter neutrophil phenotype, and in turn, how neutrophils might influence tumour progression particularly with regards to the epithelial mesenchymal transition or "EMT" (Section 1.2.5.5.2). Whilst there is limited *in vitro* evidence that neutrophil derived factors such as TGF- β (320) and neutrophil elastase (319) may promote EMT in certain cancer cells (pulmonary adenocarcinoma and pancreatic ductal adenocarcinoma respectively), until very recently (327), less was known regarding the role of neutrophils in breast cancer EMT. This is/was an important question to answer because EMT is thought to be a critical mechanism by which epithelial cancer cells gain an invasive phenotype (428).

Thus, in order to fill some of the gaps in the literature the aim of this research was to determine whether culturing neutrophils in conditioned medium (CM) prepared from specific types of breast cancer cells, in this instance poorly invasive MCF-7 cells versus highly invasive MDA-MB-231 cells, might alter the phenotype of the neutrophils. An additional aim of this research was to determine whether neutrophils indirectly co-cultured with MCF-7 and MDA-MB-231 cells via segregated transwell plates could induce EMT in "epithelial-like" MCF-7 cells and/or enhance or reverse the process of EMT in "mesenchymal-like" MDA-MB-231 cells.

6.2 The effect of cancer cell conditioned medium (CM) on neutrophil phenotype

To determine whether cancer cell CM could alter neutrophil phenotype, qRT-PCR was used to measure the expression of intracellular adhesion molecule 1 (ICAM-1) and matrix metallopeptidase 9 (MMP-9) for neutrophils cultured in MCF-7 CM, MDA-MB-231 CM or control medium for 7hrs (Section 2.2.5.5).

Results from the qRT-PCR assay for neutrophils cultured in MDA-MB-231 CM (Figure 6.1, A-B) showed a trend towards an increase in the relative expression of ICAM-1 ($P=0.052$) compared to control, however no difference was noted in the expression of MMP-9 ($P=0.783$). In contrast, neutrophils cultured in MCF-7 CM showed no difference in the relative expression of ICAM-1 ($P=0.107$) or MMP-9 ($P=0.344$) compared to control (Figure 6.1, A-B).

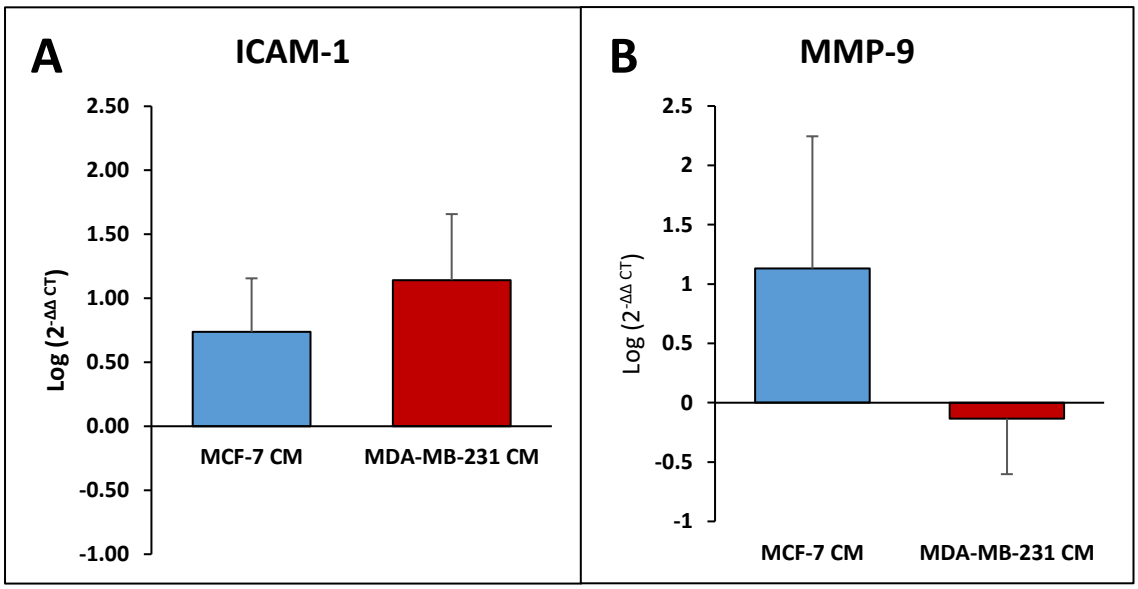


Figure 6.1: Relative expression of ICAM-1 (A) and MMP-9 (B) mRNA in neutrophils cultured in MCF-7 or MDA-MB-231 conditioned medium (CM) compared to control

Freshly isolated human neutrophils were cultured in control medium, MCF-7 CM or MDA-MB-231 CM at a concentration of $3.33x10^5$ cells/ml, for 7hrs. RNA was isolated from the samples, cDNA prepared and gene expression measured using qRT-PCR. β-actin was used as the housekeeping gene. Values are means ± SEM of Log (2-ΔΔCT); (N=8 participants). Statistical significance, comparing ΔCT of neutrophils in CM versus ΔCT neutrophils in control medium, was carried out via Student's paired t-test.

These results suggest that soluble factors secreted by MDA-MB-231 cells or MCF-7 cells do not alter the expression of ICAM-1 or MMP-9 mRNA in human neutrophils. However, a number of additional "N1" versus "N2" polarisation markers have been proposed (as reviewed in 233), (Section 1.2.5.3), and should be reviewed in future research. A full exploration of all the proposed polarisation markers was beyond the scope of this thesis.

6.3 Neutrophils and the EMT transition

6.3.1 The effect of neutrophils on the expression of β-catenin and vimentin

To determine if neutrophils could influence the process of EMT, a series of three experiments were carried out in which MCF-7 cells and MDA-MB-231 cells were indirectly cultured, using segregated transwell plates, with neutrophils or control medium. In each instance the cancer cells were seeded into the bottom wells of the transwell plate and the neutrophils (or control medium) were seeded into the inserts above. This ensured that the neutrophils and cancer cells were segregated from each other to prevent physical contact, whilst allowing interactions to occur between any soluble factors secreted by the two cell types.

Under normal growth conditions, MCF-7 cells demonstrate an epithelial-like phenotype and express cell-to-cell adhesion molecules such as E-Cadherin, whereas MDA-MB-231 cells demonstrate a mesenchymal phenotype and express markers of EMT such as vimentin. The expression of β–catenin; which is typically more abundant in epithelial cells, and a mesenchymal marker; vimentin, was determined after 7 and 18hrs of incubation via sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting (Section 2.2.4.3). The 7 hr time point was selected for consistency with previous experiments (Chapters 4 and 5), which found a substantial proportion of neutrophils to be non-apoptotic after 7hrs of incubation in MDA-MB-231 CM and may therefore, potentially, still be releasing soluble factors capable of influencing EMT; it is not known whether such factors would be released by apoptotic neutrophils. It was also unclear how long it may take for the soluble factors to influence the process of EMT in the cancer cells, consequently the 18hr time point was selected to assess any slower acting or delayed effects upon EMT.

Western blotting of extracts prepared from MCF-7 cells at time zero, 7hrs and 18hrs showed major bands at ~92kDA, ~ 42kDa and ~36kDa (Figure 6.2) corresponding to β-catenin, smooth muscle actin and GAPDH respectively. No vimentin was detected in any of the MCF-7 cell extracts (treated or control).

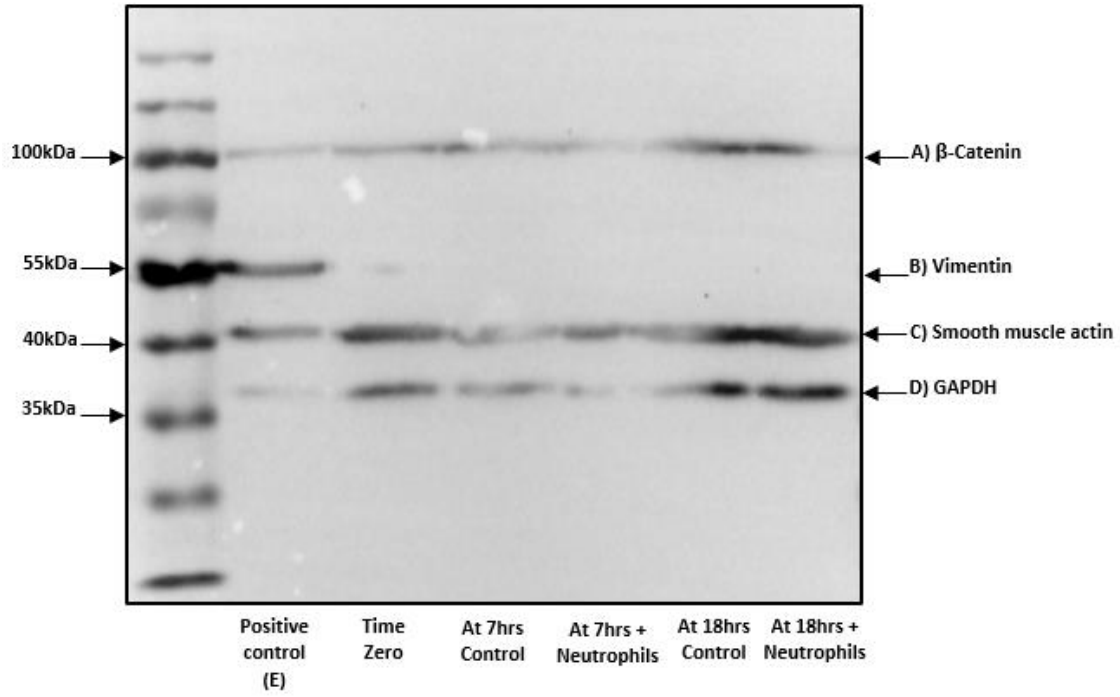


Figure 6.2: Expression of β-catenin, smooth muscle actin and GAPDH in MCF-7 cells cultured with neutrophils or control medium

Cell lysates of freshly harvested MCF-7 cells (time zero) and MCF-7 cells indirectly cultured with neutrophils or control medium in segregated transwell plates for 7 and 18hrs, were subjected to SDS-PAGE (10% polyacrylamide gels) and western blotted with epithelial-mesenchymal transition western blot cocktail (anti-β catenin, vimentin; diluted 1:250), (N=3) β-catenin was detected at A) ~ 92 kDa), Vimentin at B) ~ 54kDa), smooth muscle actin at C) ~42kDa and GAPDH at D) ~ 36kDa. Cell lysates of freshly harvested MDA-MB-231 cells were included as a positive control for vimentin (E).

In contrast western blotting of extracts prepared from MDA-MB-231 cells (treated and control) at time zero, 7hrs and 18hrs detected major bands at ~92kDA, ~54kDa, ~ 42kDa and ~36kDa (Figure 6.3) corresponding to β-catenin, vimentin, smooth muscle actin and GAPDH respectively. Unfortunately, the western blots were of insufficient quality to allow an accurate densitometry analysis to be performed.

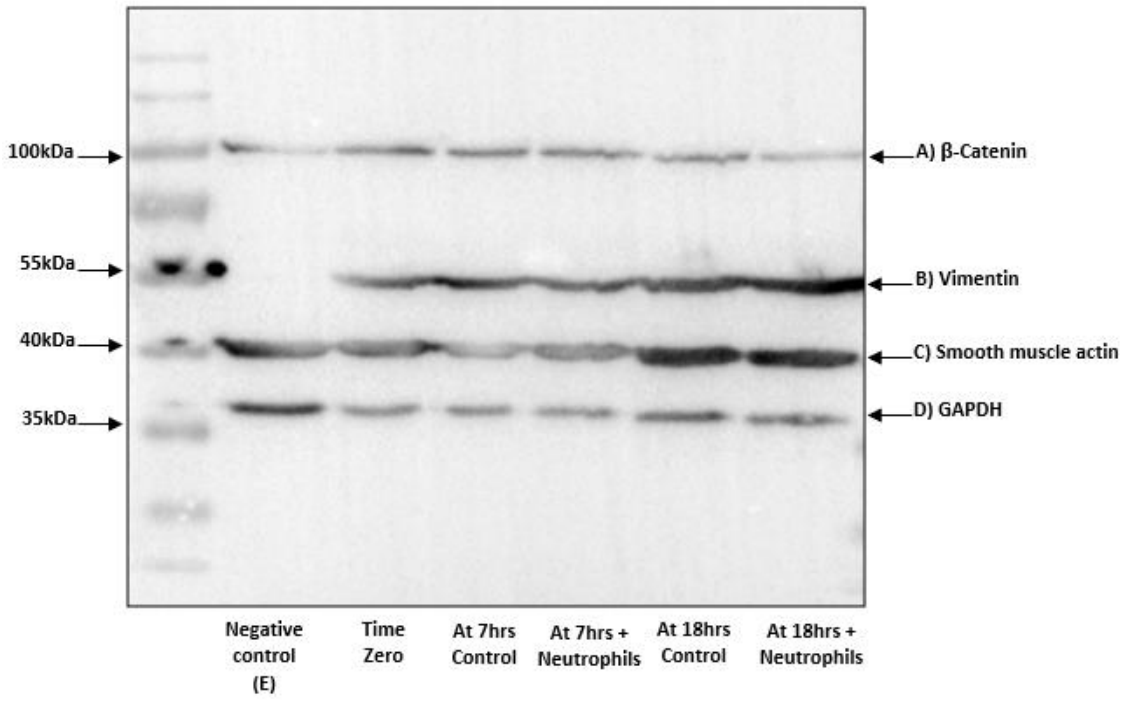


Figure 6.3: Expression of β-catenin, vimentin, smooth muscle actin and GAPDH in MDA-MB-231 cells cultured with neutrophils or control medium

Cell lysates of freshly harvested MDA-MB-231 cells (time zero) and MDA-MB-231 cells indirectly cultured with neutrophils or control medium in segregated transwell plates for 7 and 18hrs were subjected to SDS-PAGE (10% polyacrylamide gels) and western blotted with epithelial-mesenchymal transition western blot cocktail (anti-β catenin, vimentin; diluted 1:250), (N=3) β-catenin was detected at A) ~ 92 kDa), Vimentin at B) ~ 54kDa), smooth muscle actin at C) ~42kDa and GAPDH at D) ~ 36kDa. Cell lysates of freshly harvested MCF-7 cells were included as a negative control for vimentin (E).

The results shown here suggest that neutrophils do not induce the expression of vimentin or inhibit the expression of β-catenin in MCF-7 cells after 7 or 18hrs of incubation. Likewise, neutrophils do not appear to inhibit the expression of either vimentin or β-catenin in MDA-MB-231 cells. However, it is possible that an increase or decrease in the expression of these proteins occurred in the cell lysates, but at a level of change which was undetectable on the western blot. Subsequently, qRT-PCR was undertaken on both of these targets as this method is more sensitive than western blotting and can be used to quantify gene transcription (429).

6.3.2 qRT-PCR analysis of E-Cadherin, β-catenin, Fibronectin, SNAIL, SLUG, Vimentin and ZEB2 expression

To confirm and further investigate whether neutrophils might influence the process of EMT, qRT-PCR was used to measure the relative expression of β-catenin, one epithelial marker: E-Cadherin; and five mesenchymal markers: fibronectin, Snail family transcriptional repressor 2 (SLUG), Snail family transcriptional repressor 1 (SNAIL), vimentin and Zinc Finger E-Box Binding Homeobox 2 (ZEB2) for MCF-7 and MDA-MB-231 cells via segregated co-culture with neutrophils or control medium for 7 hrs (Section 2.2.5.6).

qRT-PCR analysis showed no expression of the mesenchymal makers vimentin or Zeb2 for MCF-7 cells incubated with control medium or cultured with neutrophils (data not shown). MCF-7 cells cultured with neutrophils showed no difference in the relative expression of E-cadherin (P=0.530) or β-catenin (P=0.209) compared to control (Figure 6.4). Low levels of three mesenchymal markers; fibronectin, SLUG and SNAIL were identified in the MCF-7 cells; however, MCF-7 cells cultured with neutrophils demonstrated no difference in the relative expression of these targets compared to control (P=0.991, P=0.680 and P=0.093 respectively).

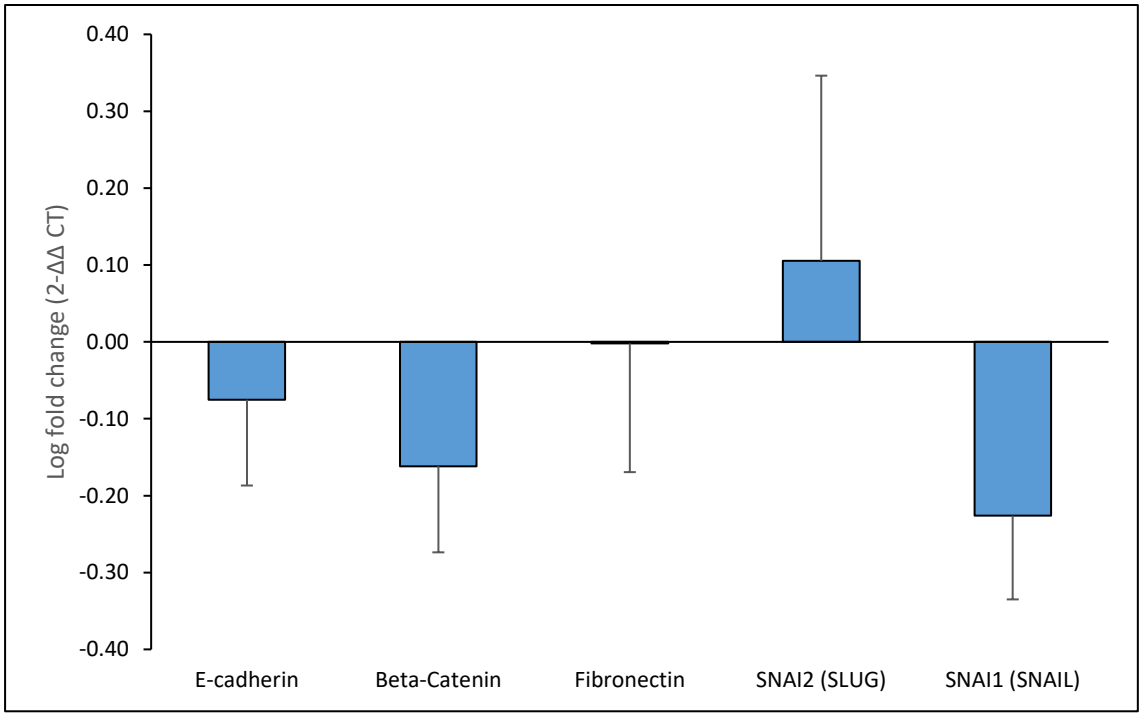


Figure 6.4: Relative expression of EMT markers in MCF-7 cells cultured with neutrophils compared to control medium

Relative mRNA expression of β-catenin, one epithelial marker: E-Cadherin and three mesenchymal markers: fibronectin, SLUG and SNAIL in MCF-7 cells cultured, via segregated co-culture, with neutrophils or control medium for 7hrs. RNA was isolated from the samples, cDNA prepared and gene expression measured using qRT-PCR. β-actin was used as the housekeeping gene. Values are means ± SE of Log ($2^{-\Delta\Delta CT}$); (N=6 participants). Statistical significance, comparing ΔCT MCF-7 cells + neutrophils versus ΔCT MCF-7 cells + control medium was carried out using the Student's paired t-test.

qRT-PCR analysis showed no expression of the epithelial marker E-Cadherin for MDA-MB-231 cells incubated with control medium or cultured with neutrophils. All five mesenchymal markers: fibronectin, SNAIL, SLUG, vimentin and ZEB2 were expressed in the MDA-MB-231 cells. No difference was noted in the relative expression of β-catenin (P=0.105), fibronectin (P=0.486), SLUG (P=0.117), SNAIL (P = 0.867) or ZEB2 (P=0.312) for MDA-MB-231 cells cultured with neutrophils compared to control (Figure 6.5). A significant decrease was demonstrated in the relative expression of vimentin (P=0.001) for MDA-MB-231 cells cultured in neutrophils compared to control (see Figure 6.5, however the fold change ($2^{-\Delta\Delta CT}$) was relatively small at only 0.93. Given that no difference was noted in the relative expression of any of the other markers of

EMT, this suggests that whilst the decrease in vimentin expression was statistically significant, it may not be of biological significance.

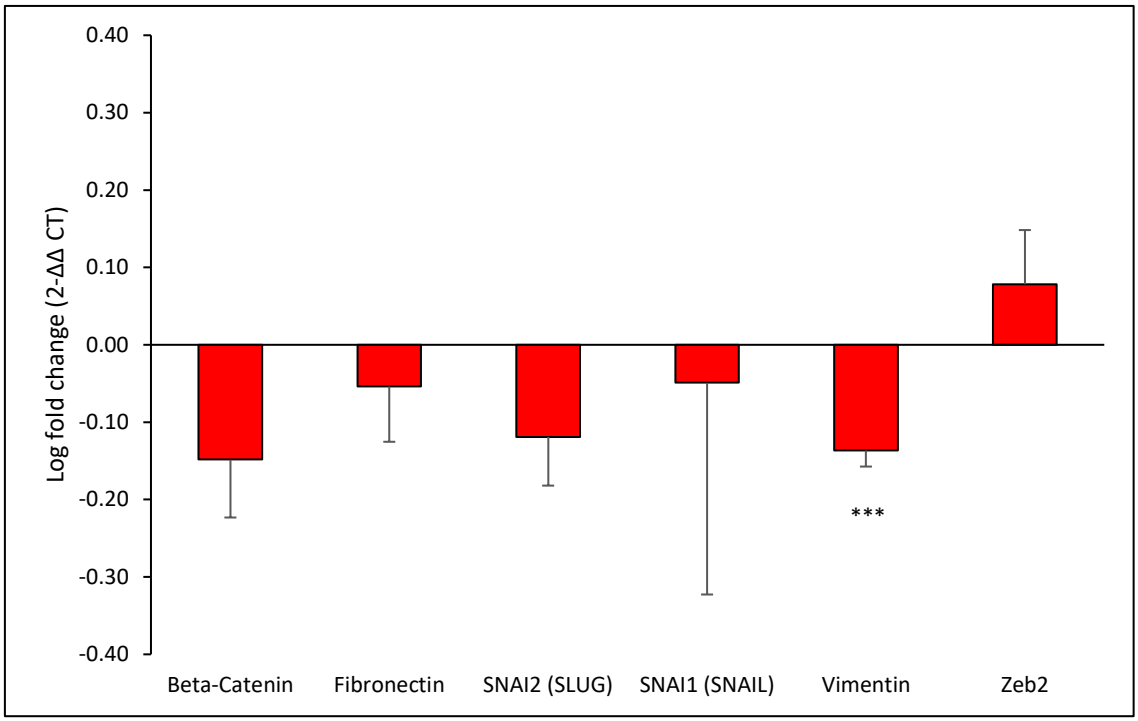


Figure 6.5: Relative expression of EMT markers in MDA-MB-231 cells cultured with neutrophils compared to control medium

Relative expression of β-catenin and five mesenchymal markers: fibronectin, SLUG, SNAIL, vimentin and Zeb2 in MDA-MB-231 cells cultured, via segregated co-culture, with neutrophils or control medium for 7hrs. RNA was isolated from the samples, cDNA prepared and gene expression measured using qRT-PCR. Values are means ± SE of Log ($2^{-\Delta\Delta CT}$); (N=5-6 participants). Statistical significance, comparing ΔCT MDA-MB-231 cells with neutrophils versus ΔCT MDA-MB-231 cells with control medium was carried out using the Student's paired t-test. ***, P≤0.001

The results shown for the MCF-7 cell qRT-PCR assay corroborate those demonstrated for the MCF-7 cell western blot assay (Section 6.3.1) which demonstrated no expression of vimentin for MCF-7 cells cultured with either neutrophils or control medium. In contrast, whilst the results of the MDA-MB-231 cell western blot assay (Section 6.3.1) clearly demonstrate the expression of vimentin for MDA-MB-231 cells cultured with both neutrophils and control medium, results from the qRT-PCR assay showed a

significant decrease in expression of vimentin for the MDA-MB-231 cells cultured with neutrophils compared to incubated with control medium.

6.4. qRT-PCR of IL-6, IL-8, TGF- β and VEGF-A

To investigate whether neutrophils might alter the expression of growth factors or cytokines associated with tumour development and or/progression, qRT-PCR was used to measure the expression of IL-6, IL-8, TGF- β and VEGF-A for MCF-7 and MDA-MB-231 cells via segregated co-culture with neutrophils or control medium for 7hrs (Section 2.2.5.6). Il-6 and Il-8 were selected as both cytokines have been shown to demonstrate multiple tumour promoting effects including enhanced proliferation, differentiation and survival of cancer cells (reviewed in 430,431), TGF-β was selected due to its important signalling role in EMT (reviewed in 432) and VEGF-A because it is a key mediator of angiogenesis in cancer (reviewed in 433).

qRT-PCR analysis of MCF-7 cells showed no difference in the relative expression of IL-6 (P=0.257), IL-8 (P=0.279), TGF-β (P=0.105) or VEGF-A (P=0.167) for MCF-7 cultured with neutrophils compared to control (Figure 6.6, A-D). Likewise, MDA-MB-231 cells cultured with neutrophils demonstrated no difference in the relative expression of IL-6 (P=0.142), IL-8 (P=0.437), TGF-β (P=0.177) or VEGF-A (P=0.977) compared to control (Figure 6.6, A-D).

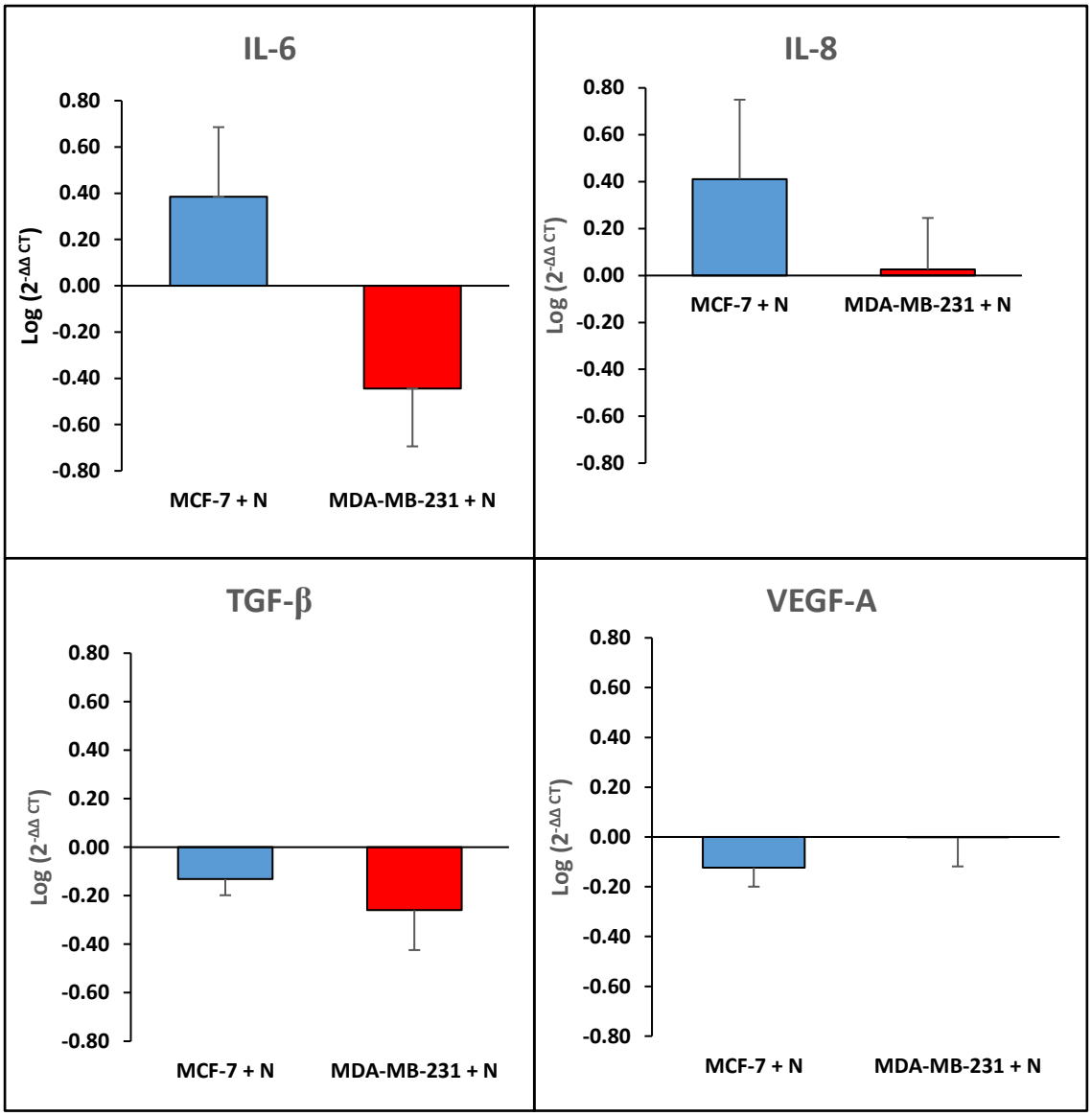


Figure 6.6: Relative expression of inflammatory cytokines and growth factor mRNA in MCF-7 and MDA-MB-231 cells cultured with neutrophils compared to control medium

Relative mRNA expression of IL-6, IL-8, TGF-β and VEGF-A in MCF-7 cells and MDA-MB-231 cells cultured, via segregated co-culture, with neutrophils or control medium for 7hrs. RNA was isolated from the samples; cDNA was prepared and gene expression measured using qRT-PCR. β-actin was used as the housekeeping gene Values are means ±SE of Log ($2^{-\Delta\Delta CT}$) (N=6 participants). Statistical significance, comparing ΔCT of MCF-7 cells with neutrophils (+N) versus ΔCT MCF-7 cells with control medium and ΔCT of MDA-MB-231 cells with neutrophils (+N) versus ΔCT MDA-MB-231 cells with control medium was carried out using the Student's paired t-test.

6.5 The effect of neutrophils on MCF-7 and MDA-MB-231 cell migration

The effect of neutrophils on MCF-7 and MDA-MB-231 cell migration was investigated using a modified version of the wound scratch assay described by Laing *et al.* (354) (Section 2.2.3.5.1). MCF-7 and MDA-MB-231 cells were plated into 12 well plates for 24hrs to create a monolayer, after which a "wound" or "scratch" was created in each well of the plates by scraping the monolayer in a straight line with a sterile 200µl pipette tip (354). Neutrophils or control medium were aliquoted into transwell inserts above the cells and the plates incubated at 37ºC with 5% CO_2. After 0, 7, 24 and 30hrs of incubation the wound was photographed at specific points along the scratch, in order to assess the migration of the cells into the wound or "wound closure". The surface area of the wound, at each of these points, was analysed using image J software (355) (Figures 6.7 & 6.8). Wound closure at 7, 24 and 30hrs, was expressed as a % compared to time zero.

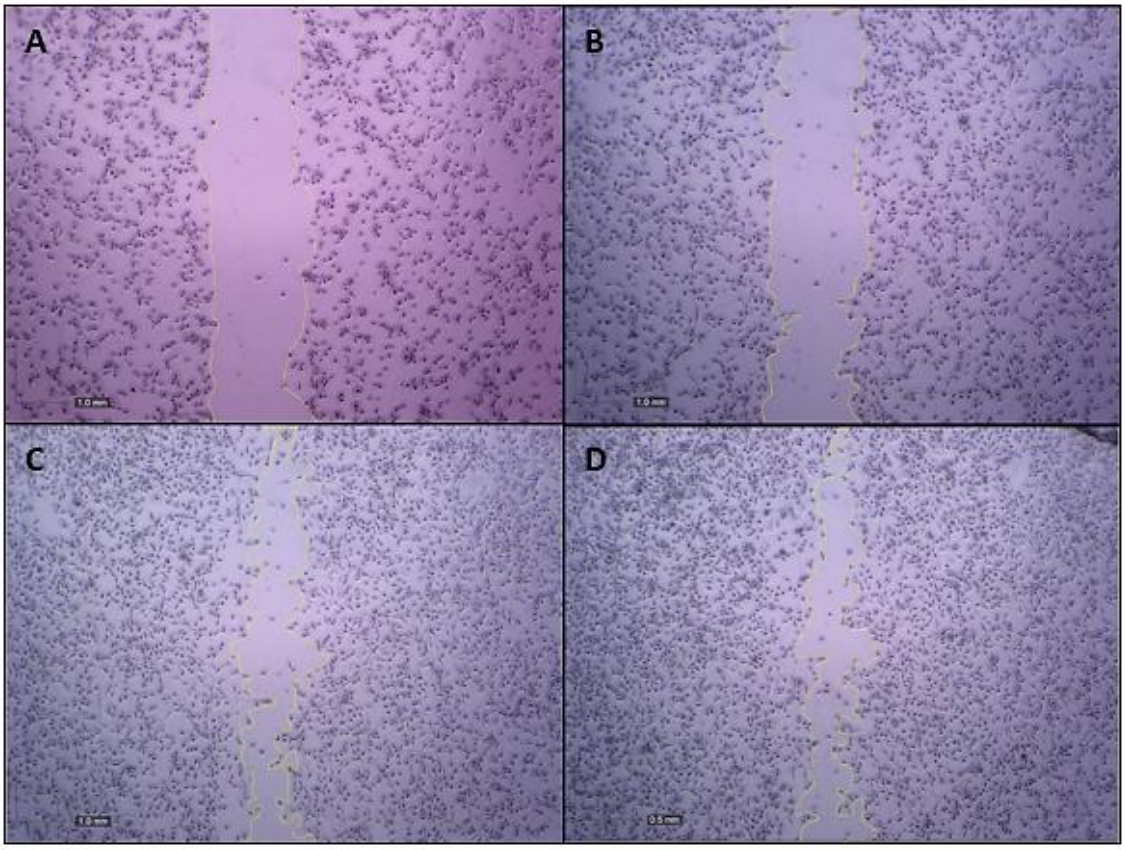


Figure 6.7: Wound created in MDA-MB-231 cells at 0hrs and wound closure due to cell migration after 7, 24 and 30hrs of incubation

MDA-MB-231 cells were incubated for 24hrs to create a monolayer, after which a scratch or "wound" was created in the monolayer with a sterile 200µl pipette tip. At time zero (A); immediately prior to adding the neutrophils or control medium, and after 7 (B), 24 (C) and 30hrs (D) of incubation the wound was visualised using a phase contrast inverted microscope (CKX31, Olympus, Auckland, New Zealand) at 40X magnification and a photo taken using a colour camera (Dino-Eye AM7025X Edge Series 5.0MP, Dino-Lite, Joondalup DC Australia). The surface area of the wound was analysed at specific points along the scratch using image J software. The scale on the lower left of the image was used to convert the pixels of the image into mm in order to calculate the surface area of the wound; the outline of the wound, as used to determine surface area, is highlighted in yellow.

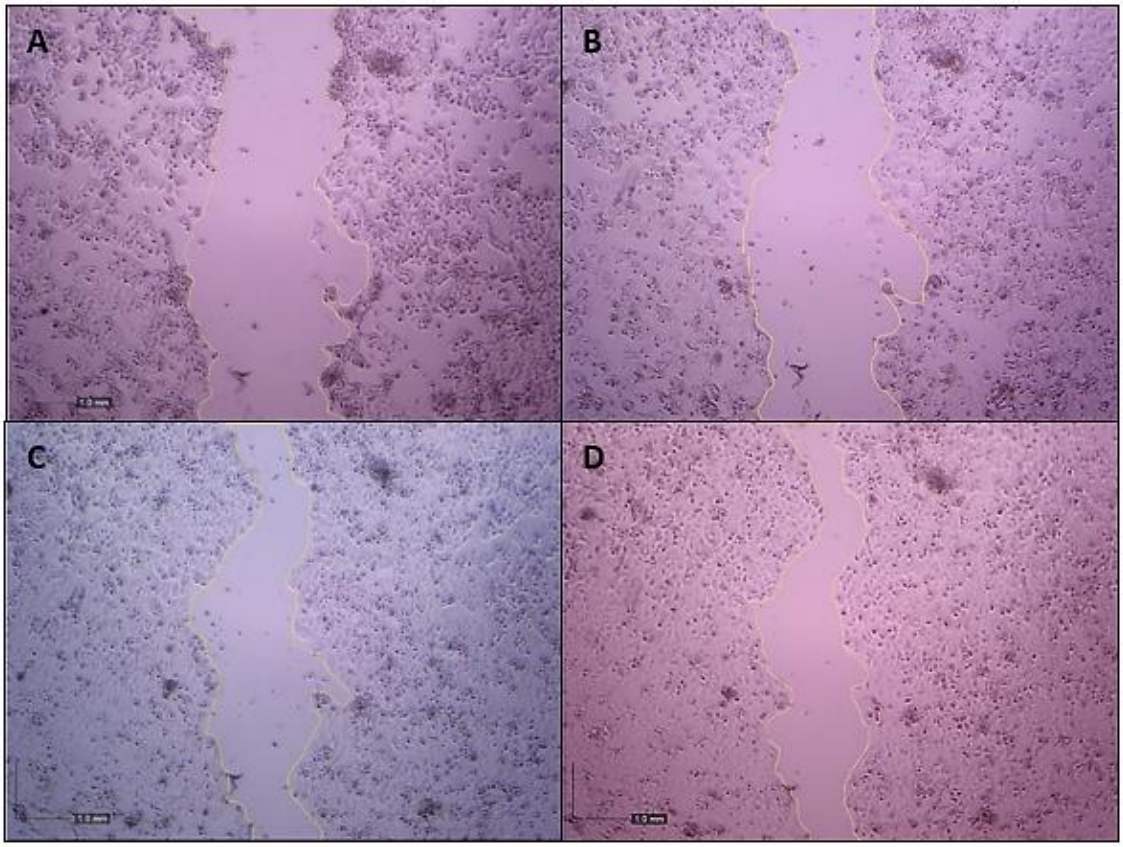


Figure 6.8: Wound created in MCF-7 cells at 0hrs and wound closure due to cell migration after 7, 24 and 30hrs of incubation

MCF-7 cells were incubated for 24hrs to create a monolayer, after which a scratch or "wound" was created in the monolayer with a sterile 200µl pipette tip. At time zero (A); immediately prior to adding the neutrophils or control medium, and after 7 (B), 24 (C) and 30hrs (D) of incubation the wound was visualised using a phase contrast inverted microscope (CKX31, Olympus, Auckland, New Zealand) at 40X magnification and a photo taken using a colour camera (Dino-Eye AM7025X Edge Series 5.0MP, Dino-Lite, Joondalup DC Australia). The surface area of the wound was analysed at specific points along the scratch using image J software. The scale on the lower left of the image was used to convert the pixels of the image into mm in order to calculate the surface area of the wound; the outline of the wound, as used to determine surface area, is highlighted in yellow.

6.5.1 Reproducibility (precision) of wound scratch assay

The repeatability (precision) of the surface area measurements was determined by a single person analysing the surface area of wound, at a single reference point 10 times, on the same day. Results shown (Table 6.1) suggest this method of analysis has a high level of repeatability as the co-efficient of variance in each instance was <5%.

Table 6.1: Repeatability of wound surface area measurements for wound created at 0hrs and wound closure due to cell migration at 7, 24 and 30hrs of incubation

		Time Point			
		T0	At 7hrs	At 24hrs	At 30hrs
MDA-MB-231 Cells	Surface area (mm^2)	13.94	12.46	10.37	9.69
	St. Dev	0.19	0.34	0.22	0.29
	% CV	1.34	2.75	2.10	3.01
MCF-7 Cells	Surface area (mm^2)	14.62	11.09	4.70	3.48
	St. Dev	0.30	0.17	0.13	0.16
	% CV	2.07	1.53	2.72	4.46

An aliquot of $3x10^5$ MCF-7 cells and $2x10^5$ MDA-MB-231 cells were incubated for 24hrs to create a monolayer, after which a "wound" was created in the monolayer with a 200µl pipette tip. The cells were indirectly incubated with $1x10^5$ neutrophils via segregated transwell plates. At time 0 and after 7, 24 and 30hrs the wound was visualised under a phase contrast inverted microscope (CKX31, Olympus, Auckland, New Zealand) at 100X magnification and a photo taken using a colour camera (Dino-Eye AM7025X Edge Series 5.0MP, Dino-Lite, Joondalup DC Australia). The wound was analysed at a single reference point, x10 to check for precision, using image J Software.

6.5.2 Effect of neutrophil presence on wound scratch assay

Having established the reproducibility of the measurement system at a single reference point under controlled conditions, the surface area of wound was then assessed in the presence or absence of neutrophils over time.

Results for the wound scratch assay of MCF-7 cells cultured with neutrophils showed no difference in % wound closure after 7, 24 or 30hrs of incubation compared to control ($P=0.374$, $P=0.787$ and $P=0.736$ respectively), Figure 6.9.

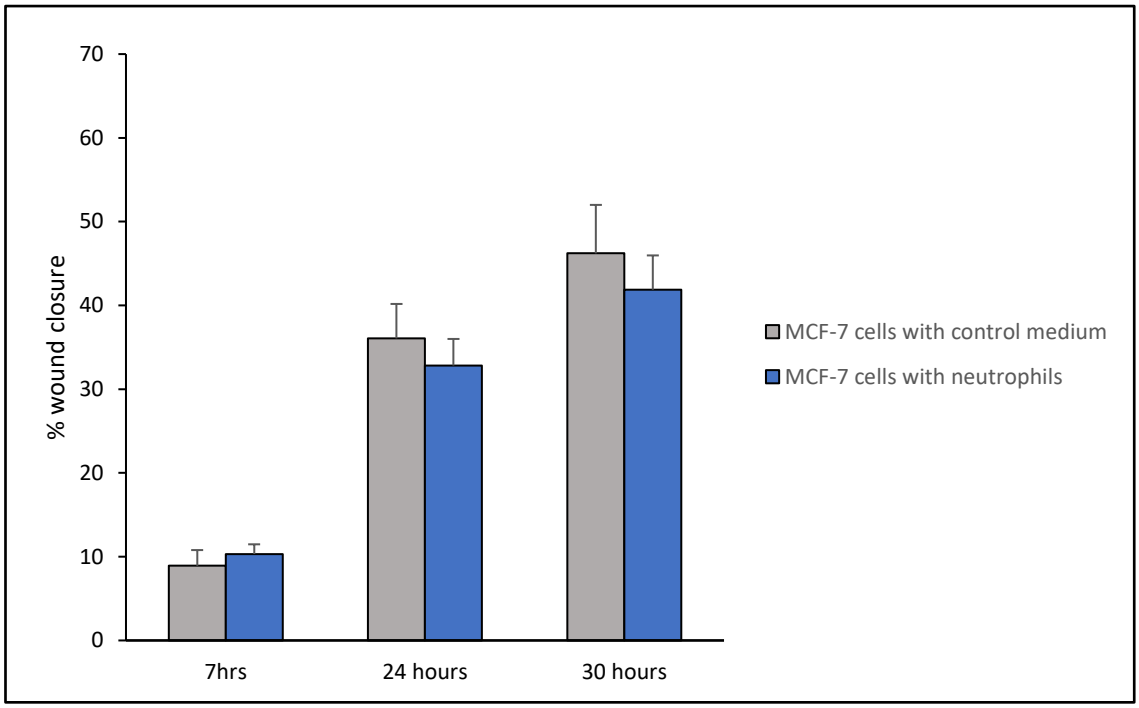


Figure 6.9: % Wound closure for MCF-7 cells indirectly co-cultured with neutrophils or control medium

A modified version of a wound scratch assay was undertaken in which MCF-7 cells were indirectly co-cultured with neutrophils or control medium for 7, 24 and 30hrs. The wound was visualised under a phase contrast inverted microscope (CKX31, Olympus, Auckland, New Zealand) at 40X magnification, photos were taken with a colour camera (Dino-Eye AM7025X Edge Series 5.0MP, Dino-Lite, Joondalup DC Australia). The surface area of the wound, at each reference point, was determined via image J software. Wound closure at 7, 24 and 30hrs are expressed as a % compared to time zero. The images shown are representative of four independent experiments, two - four replicates were undertaken for each experiment. Values are means ± SEM. Statistical significance was determined by Student's paired t test.

Likewise, MDA-MB-231 cells cultured with neutrophils showed no difference in % wound closure after 7, 24 or 30hrs of incubation compared to control (P=0.690, P=0.866 and P=0.649 respectively), Figure 6.10.

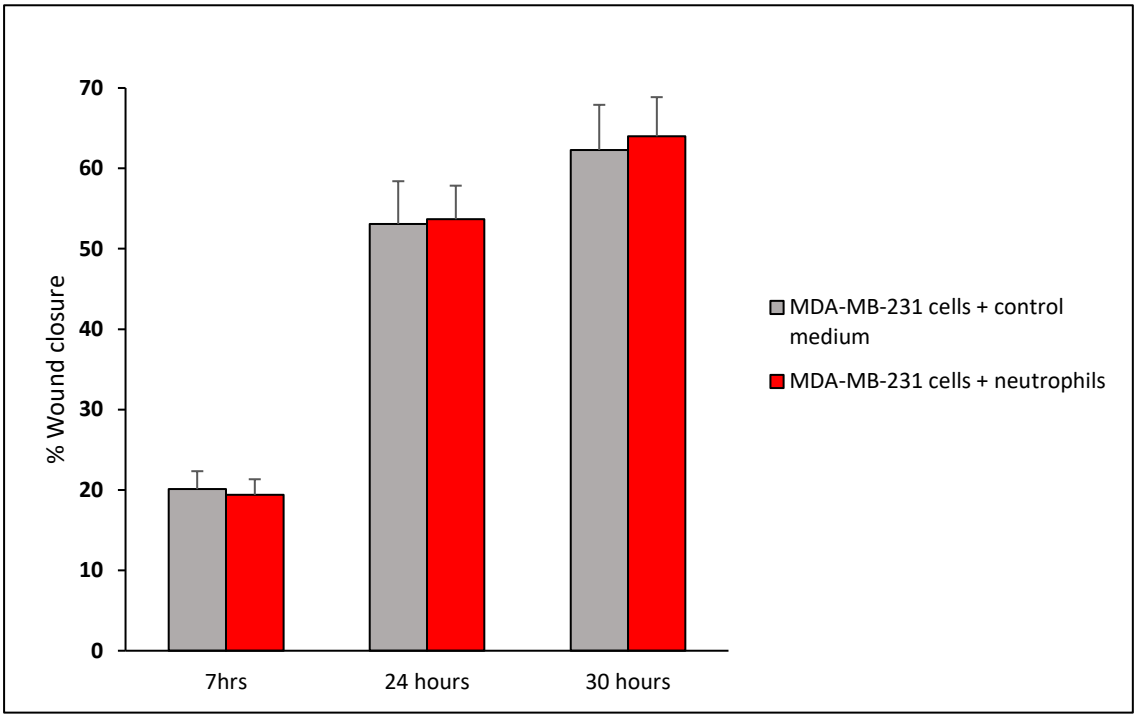


Figure 6.10: % Wound closure by MDA-MB-231 cells indirectly co-cultured with neutrophils or control medium

A modified version of a wound scratch assay was undertaken in which MDA-MB-231 cells were indirectly co-cultured with neutrophils or control medium for 7, 24 and 30hrs. The wound was visualised under a phase contrast inverted microscope (CKX31, Olympus, Auckland, New Zealand) at 40X magnification, photos were taken with a colour camera (Dino-Eye AM7025X Edge Series 5.0MP, Dino-Lite, Joondalup DC Australia). The surface area of the wound, at each reference point, was determined via image J software. Wound closure at 7, 24 and 30hrs are expressed as a % compared to time zero. The images shown are representative of four independent experiments, four replicates were undertaken for each experiment. Values are means ± SEM. Statistical significance was determined by Student's paired t test.

6.6 The effect of neutrophils upon MCF-7 and MDA-MB-231 cell morphology

Results from the qRT-PCR assay (Section 6.3.2) suggest that neutrophils may decrease the expression of vimentin in MDA-MB-231 cells. As there is evidence that the level of vimentin expression within a cell correlates to mesenchymal cell shape (434), the wound scratch assay (Section 2.2.3.5.1) was also used to investigate the effect of neutrophils on the morphology of MCF-7 and MDA-MB-231 cells. Research suggests reviewed in (129) that mesenchymal carcinoma cells are typically found at the invasive, migrating front of tumours. Therefore, migrating cells located immediately adjacent to the wound created in the MCF-7 and MDA-MB-231 cells were photographed and examined for any changes in morphology.

Results for the MCF-7 morphology assay show that MCF-7 cells indirectly co-cultured with either control medium or neutrophils (Figure 6.11) both demonstrate the classic "cobble-stone" appearance and strong cell to cell adhesion characteristic of epithelial cells (129) with no obvious difference in morphology at any of the time points or over time. Likewise results for the MDA-MB-231 morphology assay show that MDA-MB-231 cells cultured with either control medium or neutrophils (Figure 6.12) both demonstrate an elongated spindle shaped morphology with pronounced scattering (129) with no obvious difference in morphology at any of the time points or overtime.

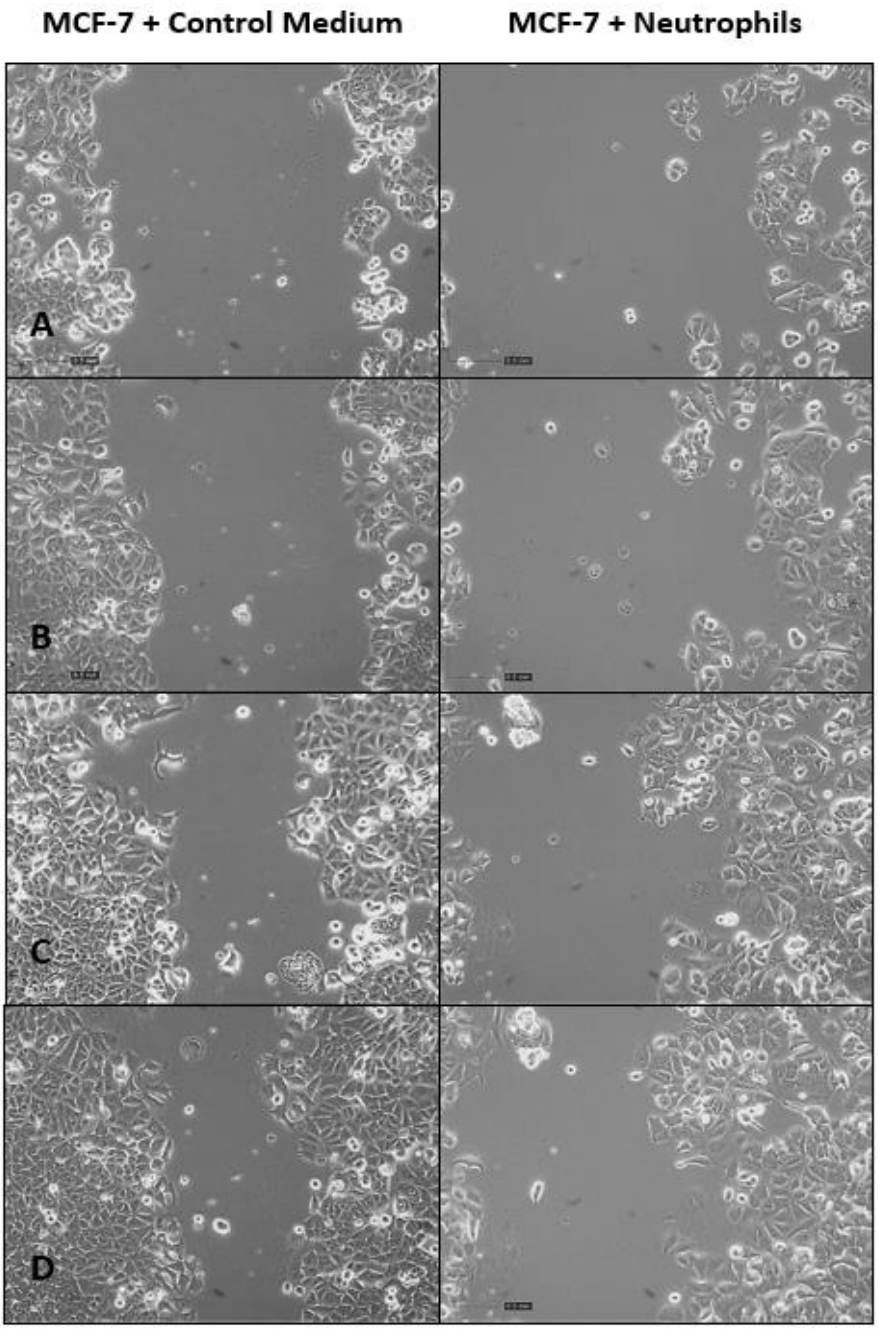


Figure 6.11: Phase contrast image of MCF-7 cells indirectly co-cultured with control medium or neutrophils at time 0 and after 7, 24 and 30hrs of incubation

MCF-7 cells ($3x10^5$cells) were plated out into 12 well plates. After 24hrs a wound was created in each well and control medium or neutrophils ($1x10^5$ cells) were placed above the MCF-7 cells in transwell inserts. The plates were incubated at 37ºC for a total of 30hrs. The plates were visualised under a phase contrast inverted microscope (CKX31, Olympus, Auckland, New Zealand) at 100X magnification. Photographs were taken at the same location in each well at time 0 (A), 7hrs (B), 24hrs (C) and 30hrs (D) using a colour camera (Dino-Eye AM7025X Edge Series 5.0MP, Dino-Lite, Joondalup DC Australia). The images shown are representative of four independent experiments, four replicates were undertaken for each experiment.

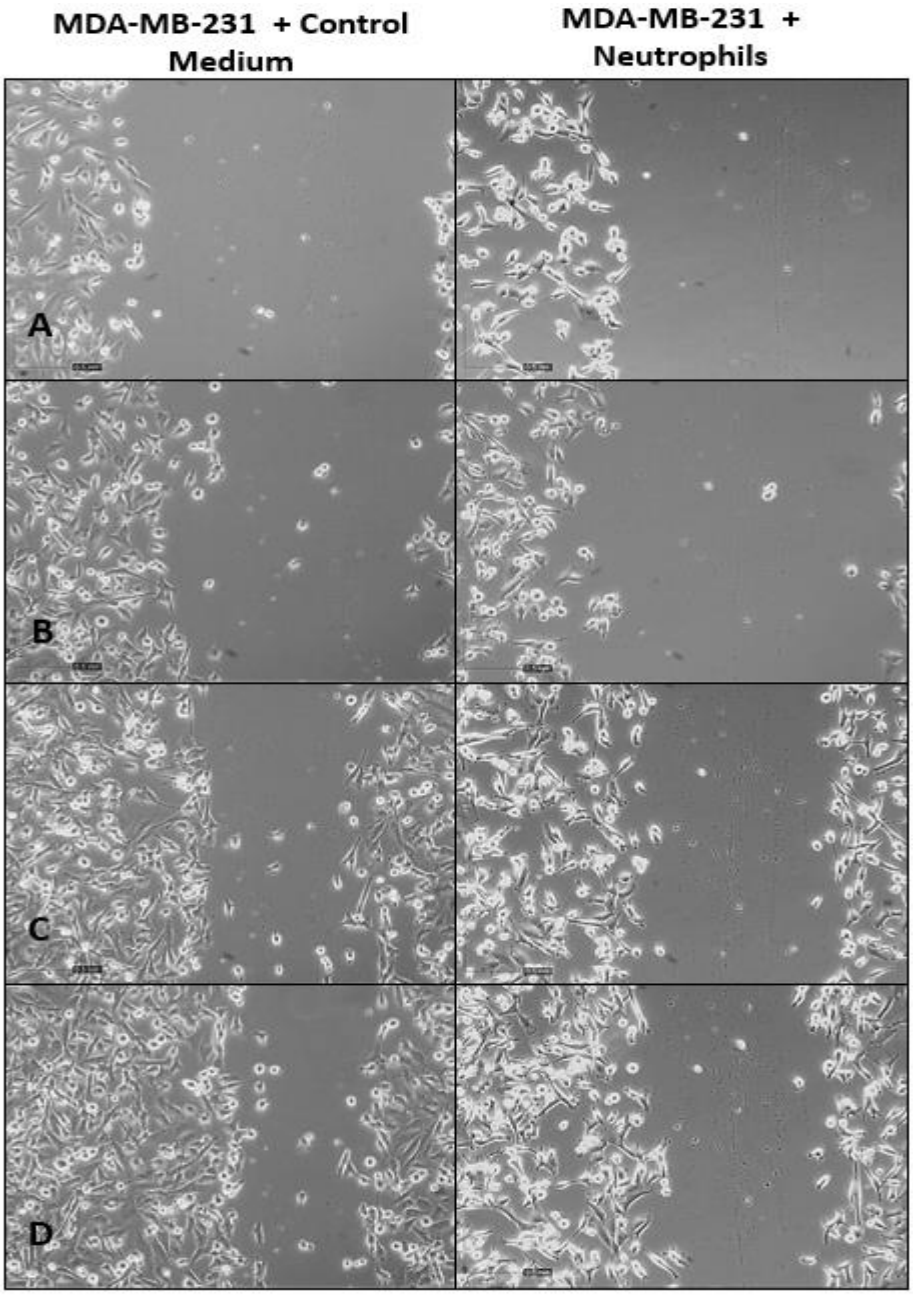


Figure 6.12: Phase contrast image of MDA-MB-231 cells indirectly co-cultured with control medium or neutrophils at time 0 and after 7, 24 and 30hrs of incubation

MDA-MB-231 cells ($2x10^5$cells) were plated out into 12 well plates. After 24hrs a wound was created in each well and control medium or neutrophils ($1x10^5$ cells) were placed above the MDA-MB-231 cells in transwell inserts. The plates were incubated at 37ºC for a total of 30hrs. The plates were visualised under a phase contrast inverted microscope (CKX31, Olympus, Auckland, New Zealand) at 100X magnification. Photographs were taken at the same location in each well at time 0 (A), 7hrs (B), 24hrs (C) and 30hrs using a colour camera (Dino-Eye AM7025X Edge Series 5.0MP, Dino-Lite, Joondalup DC Australia). The images shown are representative of four independent experiments, four replicates were undertaken for each experiment.

These results suggest that under these experimental conditions, neutrophils do not alter the morphology of MCF-7 or MDA-MB-231 cancer cells. This suggests that neutrophils do not induce EMT in MCF-7 cells or its reversal – "Mesenchymal-epithelial-transition" or "MET" in MDA-MB-231 cells. Whilst the results from Section 6.3.2 indicate that co-culture with neutrophils may decrease the expression of vimentin in MDA-MB-231 cells, no discernible change in their morphology was observed. However, a limitation of this assay was that the cells were only analysed at specific points along the scratch. As the entire scratch boundary was not observed it is possible that neutrophils demonstrated a change in morphology elsewhere on the plate. As EMT is a key mechanism by which cancer cells acquire an invasive, migratory phenotype, taken together the results from the EMT western blot (Section 6.3.1) and qRT-PCR assay (Section 6.3.2) correlate with the data obtained from the wound scratch assay, which found no difference in the % wound closure for MCF-7 and MDA-MB-231 cells indirectly co-cultured with neutrophils compared to control (428).

6.7 Discussion

6.7.1 Influence of cancer cell conditioned medium on neutrophil phenotype

Although a number of "N1" versus "N2" polarisation markers have been proposed (as reviewed in Section 1.2.5.3), only two markers, ICAM-1 and MMP-9, were selected for this preliminary investigation regarding the influence of MCF-7 and MDA-MB-231 CM on neutrophil phenotype. Whilst ICAM-1 is generally absent or expressed at very low levels on circulating human neutrophils (435), elevated levels have been demonstrated on peripheral neutrophils in certain inflammatory conditions (436,437). In the context of cancer, *in vitro* research (302,438) has shown that N1 or "anti-tumour" neutrophils demonstrate significantly higher levels of ICAM-1 and enhanced tumour cytotoxicity compared to N2 or "pro-tumour" neutrophils. However, it is pertinent to note that a recent study (439), in which intratumoural gastric cancer neutrophils demonstrated a prolonged lifespan and high levels of ICAM-1(similar to the neutrophils cultured in MDA-MB-231

CM in this study), were in fact associated with disease progression and reduced patient survival as opposed to an anti-tumour response.

To date, the majority of the published *in vitro* research suggests that freshly isolated neutrophils cultured in cancer cell conditioned medium, or malignant pleural effusions and ascites (fluid build-up surrounding the organs of the abdomen) acquire an immunosuppressant or pro-tumour phenotype (236,440–442). Whilst it is tempting to speculate that neutrophils cultured in MDA-MB-231 CM may have been polarised, if only slightly, towards an "N1" phenotype, further research assessing additional polarisation markers is required. For example N1 TANs are also proposed to demonstrate increased expression of TNFα and decreased expression of VEGF, with decreased expression of TNFα and increased expression of VEGF proposed for N2 TANs (reviewed in 248). Likewise, assays should be undertaken to determine if neutrophils cultured with MCF-7 or MDA-MB-231 cells demonstrate tumour cell cytotoxicity. An attempt was made to determine the influence of neutrophils on the cellular proliferation of MCF-7 and MDA-MB-231 cells using the MTT assay (443); however, in each instance the washing process caused cells to be lost, making the data inaccurate and unusable.

6.4.2 The effect of neutrophils on cancer cell phenotype

Neutrophils indirectly co-cultured with MCF-7 cells did not alter the expression of key growth factors or cytokines associated with cancer progression (VEGF, TGF-β, IL-6 or IL-8) in MCF-7 cells. Moreover, they were not found to induce EMT or alter the migration of MCF-7 cells. Likewise, neutrophils indirectly co-cultured with MDA-MB-21 cells did not alter the migration or the expression of key growth factors or cytokines (VEGF, TGF-β, IL-6 or IL-8) in MDA-MB-231-cells. However, results from the qRT-PCR assay (Section 6.3.2) suggest that MDA-MB-231 cells cultured with neutrophils demonstrate a decrease in the relative expression of vimentin mRNA compared to control. Vimentin is an intermediate filament generally found in non-epithelial, mesenchymal cells (444) and is a key marker for EMT (129,445). Vimentin expression is thought to be regulated by growth factors such as TGF-β (432) and (as identified for MDA-MB-231

cells (446)), transcription factors such as ZEB2. Although no difference was noted in the expression of ZEB2 or TGF-β for MDA-MB-231 cells cultured with neutrophils, other regulators of vimentin, such as hypoxia inducible factor 1 (HIF-1) and ZBP-89 are thought to exist (447,448) and should be investigated in future research. Moreover, given that MDA-MB-231 cells did not appear to alter their morphology when cultured with neutrophils, experiments should be undertaken to determine if the decrease in vimentin mRNA expression translates into a functional difference in vimentin protein concentration; as the quantity of mRNA present does not always directly correlate to the final amount of a functional protein (reviewed in 449). It should be noted that the morphology assay was somewhat subjective, however alternative methods of EMT quantification such as Atomic Force Microscopy (450) were beyond the scope of this project

Overall the results of this study suggest that soluble factors secreted by peripheral neutrophils obtained from healthy volunteers, do not influence EMT in MCF-7 cells and may only have a minor, yet to be substantiated effect on EMT in MDA-MB-231 cells. This data is in agreement with very recent research (327) published whilst this thesis was being written. Wang *et al*. (327) demonstrated that tumour infiltrating neutrophils (TINs) isolated from invasive ductal carcinoma samples from patients with stage 1-111 primary breast carcinomas, but not peripheral neutrophils isolated from the same patients, significantly increased the migration and proliferation of MCF-7 and MDA-MB-231 cells. Moreover MCF-7 cells cultured with the conditioned media of TINs, but not the conditioned media of the peripheral neutrophils, underwent EMT, as demonstrated by changes in cellular morphology (cells became elongated and stretched), decreased expression of the epithelial marker E-Cadherin and increased expression of the mesenchymal marker vimentin (327). The results from this study, taken in conjunction with the findings of Wang *et al*. (327) suggest that soluble factors secreted by peripheral neutrophils do not alter the phenotype of breast cancer cells. Future research should determine if there is an association between the type or stage of breast cancer and the ability of TINs to induce EMT, as research by Mishalian *et al.* (249) suggests that TANs may acquire their pro-tumour phenotype over time with tumour progression.

In contrast to the results described in this chapter, research has demonstrated that peripheral neutrophils can induce EMT in other cancer cell types. For example Hu *et al.* (320) found that neutrophils derived from the venous blood of lung adenocarcinoma patients both directly and indirectly co-cultured with A549 and SPC-A1 lung adenocarcinoma lines could induce EMT; the concentration of TGF-β was significantly increased in the supernatant for neutrophils co-cultured with both cell lines, moreover there was increased nuclear localisation of Smad4 which suggests the TGF-β/Smad signalling pathway was required to induce EMT. Likewise Grosse-Steffan *et al*. (319), showed that freshly isolated neutrophils from healthy volunteers directly co-cultured with T3M4 and HuH7 pancreatic cancer cells could induce EMT, in this instance via neutrophil derived neutrophil elastase which was found to cleave E-Cadherin in monolayers of T3M4 and HuH7 cells. Whilst these results may differ from the current study due to the different cancer cell types used, it is also possible that the difference may, at least in part, be influenced by the different experimental conditions used. For example Grosse-Steffan *et al*. (319), directly co-cultured neutrophils with the cancer cells, thus it is possible that cell-cell contact signalling mechanisms were required to induce EMT in the pancreatic cancer cells.

A limitation of the work described in this chapter is that, in each instance, neutrophils were indirectly co-cultured with the cancer cells. This is important because, as described above, other researchers (319), have shown that peripheral neutrophils from healthy volunteers can induce EMT when directly co-cultured with T3M4 and HuH7 pancreatic cancer cells. Likewise recent work by Rodriguez *et al.* (451) demonstrated that freshly isolated peripheral human neutrophils from healthy volunteers could increase the migration and invasion of MCF-7 cells, when the neutrophils and MCF-7 cells were co-injected into zebrafish embryos. The neutrophils co-migrated with the cancer cells and promoted intravasation which suggests that cell-cell contact was required for this process; however it should be noted that the migration of MDA-MB-231 cells was not affected by the presence of neutrophils, prompting speculation that this mechanism may only be of importance in low grade cancers (451). Therefore, future research should include experiments in which neutrophils are directly co-cultured with breast cancer cells to determine if neutrophils enhance the invasiveness, or induce EMT, within breast cancer cells via mechanisms that require cell-cell contact. And, finally, it must be acknowledged

that cancer cells may indirectly influence neutrophil behaviour via an intermediary cell, such as macrophages. The microenvironment of the tumour (TME) is complex and includes many cell types, not just cancer cells and neutrophils, thus a multi-cellular coculture investigating interaction between cancer cells, neutrophils and other cells known to inhibit the TME may provide additional information.

In conclusion the influence of neutrophils on EMT in cancer cells appears to be extremely complex, and likely to be specific to cancer type, as in each of the published studies a different cytokine or growth factor was shown to induce EMT(319,320,322,323,327). Furthermore, for some of the published studies only TINs were able to induce EMT (320,327), whereas for others (unlike in this project) neutrophils derived from healthy volunteers were able to activate EMT in the cancer cells (319).

CHAPTER 7

DISCUSSION

7.1 General Discussion

Over the last few decades it has become clear that the microenvironment of the tumour plays a pivotal role in tumour development and progression. Whilst interactions between certain immune cells, such as macrophages, and cancer cells have been reasonably well characterised, until recently, less was known regarding the role of neutrophils in cancer. To date, there is still a great deal of work to be done at the cellular and molecular level in order to elucidate the role of neutrophils within the tumour micro-environment. Consequently, the purpose of this research was to determine how interactions between breast cancer cells and neutrophils might influence tumour progression. This project will add to the current body of work investigating the mechanisms and molecular pathways underpinning tumour development and metastatic dissemination. Long term, such knowledge will contribute to the identification of potential new drug targets for cancer and ultimately, the development of targeted treatments for breast cancer and MBC.

To meet the objectives of this research (Section 1.1), a convenient, reliable source of neutrophils needed to be found. Chapter 3 described the optimisation of the neutrophil isolation procedure and culture conditions; this work was critical to the experiments described in subsequent chapters. Neutrophils are notoriously difficult to work with, primarily because they have short lifespans and are terminally differentiated cells that have lost the ability to divide, which prevents their growth in tissue culture (165). Whilst methods are being developed to mass-produce neutrophils *in vitro* or *ex vivo* from human embryonic or haemopoietic stem cells for clinical therapeutic purposes, these techniques are time consuming and complex (452,453). Consequently, for *in vitro* work, neutrophils are commonly isolated from the peripheral blood of healthy volunteers. However, they are hard to isolate from whole blood samples in a quiescent (resting) state as mechanical perturbations can induce their activation (454). The experiments described in Chapter 3 demonstrated that neutrophils isolated by positive selection using immunomagnetic beads produced a highly pure population of cells, and examination of H_2O_2 secretion found spontaneous ROS production to be minimal suggesting the neutrophils were not activated by the isolation procedure, which is in accordance with other research (367,378).

The influence of cancer cell conditioned medium on neutrophil apoptosis and investigation of the possible molecular pathways responsible

The work described in Chapter 4 demonstrated that some form of soluble factor secreted by MDA-MB-231 cells, but not MCF-7 cells, delayed neutrophil apoptosis after 7 or 10hrs of incubation in conditioned medium. Consistent with this delay in apoptosis the work described in Chapter 4 demonstrated that neutrophils cultured in MDA-MB 231 CM and not MCF-7 CM showed a decrease in the activation of caspase-8 as well as increased expression of the anti-apoptosis peptide BCL2 (A1) and decreased expression of BAK. This indicates that the delay in apoptosis is at least partly mediated via altered caspase-8 signalling (extrinsic pathway) and reduced mitochondrial membrane permeabilization (intrinsic apoptosis pathway).

Characterisation of the soluble factor responsible for the delay in neutrophil apoptosis

Chapter 5 described the partial characterisation of the soluble factor(s) responsible for delayed neutrophil apoptosis in MDA-MB-231 CM. The molecular weight of the soluble factor was found to be greater than 10kDa and less than 100kDa. Three candidate molecules were identified according to this criterion; PGE_2 (a lipid) when bound to serum proteins (412), IL-6 (30) and a2ND (238) (both proteins). The soluble factor was shown to be heat stable, which led to the hypothesis that the candidate molecule was a lipid. Consistent with this, neutrophils cultured with native or heat treated PGE_2 demonstrated delayed apoptosis. Furthermore, COX-2 expression was found only in MDA-MB-231 cells and not MCF-7 cells, which is in agreement with the results that neutrophils cultured in MDA-MB-231 CM but not MCF-7 CM showed delayed apoptosis (Chapter 4). Taken together these data indicate that MDA-MB-231 cell-derived PGE_2 could be responsible for the observed delay in neutrophil apoptosis.

These results are novel, as to the author's knowledge no papers have reported the potential role of PGE_2 on delayed neutrophil apoptosis in cancer. Interestingly however, research does exist suggesting that COX-2 and PGE_2 are associated with resistance to apoptosis by cancer cells themselves (reviewed in 455,456). As previously noted, (Section 5.6.3),

COX-2 catalyses the initial conversion of arachidonic acid into prostaglandin G_2, which is subsequently converted into PGE_2 by PGE_2 synthase. COX-2 is overexpressed in many types of cancers, including breast cancer, with the expression of COX-2 shown to be increased in 63-85% of premalignant breast cancers (as cited by Regulski *et al.* 438). In addition to the inhibition of cancer cell apoptosis, COX-2 expression is associated with the proliferation, angiogenesis, invasion and metastatic dissemination of cancer cells (reviewed in 455,456). As a consequence, COX-2 is a target for anti-cancer therapies, and research suggests (reviewed in 456,457) that selective COX-2 inhibitor drugs, such as Celecoxib, could assist in the management of breast cancer. The work described in Chapter 5 (Section 5.4) clearly demonstrates the modulatory effect of PGE_2 on neutrophil lifespan. Thus, it is possible that neutrophils recruited to the site of COX-2 expressing tumours may demonstrate a delay in apoptosis that is at least partly mediated by PGE_2. If found to be correct, this discovery has important implications. Should COX-2 inhibitor drugs be provided as an adjuvant therapy for breast cancer they could also, incidentally, inhibit the effect of PGE_2 on neutrophil lifespan, thus reducing or limiting any adverse effects attributed to neutrophils in cancer.

The influence of cancer cell conditioned medium on neutrophil phenotype

The work described in Chapter 6 found that neutrophils cultured in MDA-MB-231 CM showed a trend towards increased expression of ICAM-1, which indicates they were mildly activated. No difference was noted in the expression of MMP-9 for neutrophils cultured in MDA-MB-231 or MCF-7 CM, which suggests they were not polarised, under the experimental conditions used, towards a pro-tumour phenotype. This suggests that soluble factors released by breast cancer cells do not alter the phenotype of neutrophils obtained from the peripheral blood of healthy volunteers.

The influence of neutrophils on cancer cell phenotype

Additional experiments in Chapter 6 showed that neutrophils, indirectly co-cultured with MCF-7 or MDA-MB-231 cells, did not alter the expression of IL-6, IL-8, TGF-β or VEGF-A in either cell line. Likewise, neutrophils indirectly co-cultured with MCF-7 or

MDA-MB-231 cells did not influence the EMT in either cell line, as determined by the absence of change to cellular morphology, the presence or absence of vimentin or β-catenin on western blots and expression of E-cadherin, β-catenin, fibronectin, Snail family transcriptional repressor 2 (SLUG), Snail family transcriptional repressor 1 (SNAIL) and Zinc Finger E-Box Binding Homeobox 2 (ZEB2). However, the expression of one EMT marker, vimentin, was found to be decreased in MDA-MB-231 cells indirectly co-cultured with neutrophils; given that no difference was noted in the relative expression of any of the other markers of EMT and no changes were demonstrated in cellular morphology, this suggests that whilst the decrease in vimentin expression was statistically significant, it may not be of biological significance.

Lastly, neutrophils indirectly co-cultured with MDA-MB-231 cells and MCF-7 cells were not found to influence the invasiveness of either cell line. These observations suggest that soluble factors released by neutrophils obtained from the peripheral blood of healthy volunteers do not influence EMT or alter the invasiveness of aggressive, triple negative (ER-PR-HER2-) breast cancer cells or less aggressive non-invasive (ER+ PR+HER2-) breast cancer cells.

The significance of delayed neutrophil apoptosis upon neutrophil phenotype and cancer progression.

The first objective of this project was to determine whether soluble factors released by breast cancer cells could delay neutrophil apoptosis, as an increase in longevity might allow neutrophils sufficient time to alter their phenotype or participate in activities that could either help or hinder tumour progression. Taken together the results from experiments in Chapters 4 & 5 suggest that soluble factors secreted by certain types of breast cancers may delay neutrophil apoptosis. However, whilst MDA-MB-231 CM was found to delay neutrophil apoptosis, this did not coincide with a significant alteration in neutrophil phenotype. Indeed, the work described in Chapter 6 suggests that neutrophils cultured in MDA-MB-231 CM only demonstrate a trend towards increased ICAM-1 expression, which suggests that they were only mildly activated. As previously discussed (Section 1.2.5) neutrophil mobilisation is upregulated in cancer, so that immature,

undifferentiated neutrophils are released prematurely from the bone marrow (158). It is possible that these immature neutrophils are more readily polarised in the tumour micro-environment. In this context, where neutrophils have been shown to develop a pro-tumour phenotype in parallel with tumour progression (249), a delay in apoptosis may enhance tumour growth and development. However, the neutrophils used in this project were obtained from the peripheral blood of healthy volunteers, therefore it is possible that these cells which would have been mature terminally differentiated neutrophils, were less able to be polarised by the breast cancer cells towards either an "N1" (anti-tumour) or "N2" (pro-tumour) phenotype. Likewise, co-cultures of neutrophils with MDA-MB-231 or MCF-7 cells were not found to enhance the invasiveness or influence the process of EMT for either cell line, which suggests that under these experimental conditions, the delay in apoptosis neither helped nor hindered tumour progression.

This project has clearly demonstrated that soluble factors released by certain types of breast cancer cells can delay neutrophil apoptosis. In addition, this project has also identified some of the underlying molecular mechanisms responsible for this delay in neutrophil apoptosis (Section 4.3). In recent years research has demonstrated both pro and anti-tumour roles of neutrophils in cancer (reviewed in Section 1.2.5.5); consequently, a delay in apoptosis, as demonstrated in this project, may allow the neutrophils additional time to participate in activities that either help or hinder tumour progression. Thus, targeted treatments that are capable of preventing delayed apoptosis in pro-tumour neutrophils, may prove beneficial for the management of breast cancer. Lastly, this research also identified PGE_2 as at least one of the likely mechanistic factor(s) responsible for the observed delay in neutrophil apoptosis and thus highlighted a novel target for the development of new cancer therapies.

7.2 Future Research

The research described in this thesis could be developed in several key directions.

As discussed in Chapter 5, additional work is required to characterise the soluble factor(s) responsible for the delay in neutrophil apoptosis. To this end, a series of experiments should be undertaken to conclusively determine whether PGE_2 is at least partially responsible for the delay in neutrophil apoptosis. Firstly, as discussed in Section 5.6.4, the concentration of PGE_2 contained in MDA-MB-231 CM and MCF-7 CM should be measured (via ELISA or HPLC (425)). Secondly, an experiment should be undertaken to determine if neutrophils cultured in medium from PGE_2 synthase gene silenced MDA-MB-231 cells demonstrate delayed apoptosis. Failure to delay apoptosis would indicate the importance of PGE_2 in enhanced neutrophil lifespan in certain types of breast cancers.

A further area of study could be to examine the effect of selective COX-2 inhibitors, such as those currently used in cancer therapy, on neutrophil lifespan. For example, neutrophils could be cultured in CM prepared from MDA-MB-231 cells treated with a COX-2 inhibitor such as Celecoxib. If the CM was unable to delay neutrophil apoptosis, this would suggest a novel beneficial effect for COX-2 inhibitor drugs when used for cancer treatment. This work could be extended to both *in vivo* and *ex vivo* models; by investigating the lifespan of tumour associated neutrophils (TANs) in a COX-2 murine model of breast cancer treated with Celecoxib compared to control, or by investigating the lifespan of TANs dissected from human breast tumours obtained from patients treated with Celecoxib compared to those who were not treated with selective COX-2 inhibitors. Furthermore, this work could also be extended to rheumatoid arthritis since this condition is associated with a delay in neutrophil apoptosis (458), or to other inflammatory conditions for which prostaglandins have been shown to contribute towards the pathogenesis of disease (459) and for which Celecoxib is currently provided as a treatment option (460).

The question of whether soluble factors released by breast cancer cells might alter neutrophil phenotype also requires further investigation. Results from Chapter 6 showed that neutrophils cultured in MDA-MB-231 or MCF-7 cell CM did not demonstrate a significant alteration in phenotype, however this work was limited in that it only addressed two polarisation markers (ICAM-1 and MMP-9). As discussed in Section 1.2.5.3, "N1" versus "N2" neutrophils are characterised by the differential expression or secretion of a wide range of cytokines, chemokines and effector molecules. It is possible that the exact phenotype of N1 versus N2 neutrophils is variable, as the microenvironment of a tumour (and the soluble factors it contains) will vary according to tumour type, stage and the presence or absence of other cells. Therefore, the expression of additional "N1 versus N2" polarisation markers for neutrophils cultured in MDA-MB-231 or MCF-7 cell CM should be investigated by qRT-PCR. As discussed above, it is possible that mature, freshly isolated neutrophils obtained from the peripheral blood of healthy volunteers (as used in this research) are less able to be polarised than tumour associated neutrophils which are believed, as a result of being released from the bone marrow early, to complete their maturation process within the tumour micro-environment. To test this hypothesis an experiment could be undertaken to compare the expression of "N1" versus "N2" polarisation markers in freshly isolated peripheral murine neutrophils versus immature neutrophils obtained from the bone marrow of the same mice, following culture in the conditioned medium prepared from a murine breast cancer cell line.

It would also be important to determine whether neutrophils are able to enhance the invasiveness of breast cancer cells via direct cell to cell contact mechanisms. This is relevant because the work described in Chapter 6 and that of Wang *et al.* (327) used a non-contact method to investigate the influence of neutrophils on EMT. To fulfil this aim the majority of the experiments described in Chapter 6, apart from the wound scratch assay, could be replicated. However, in this instance the neutrophils would be directly cultured with the cancer cells, then removed via positive selection to allow the relevant protein and mRNA EMT targets to be investigated in the MDA-MB-231 and MCF-7 cells. If the neutrophils were found to enhance or hinder the invasiveness of either cell line, this would indicate that cell to cell contact mechanisms are required to facilitate the process.

7.3 Conclusion

The work in this thesis has successfully met the first three research objectives of this project (Section 1.1) by demonstrating the ability of MDA-MB-231 CM to alter the lifespan and phenotype of peripheral blood neutrophils obtained from the venous blood of healthy volunteers and by investigating the potential molecular pathways and soluble factors involved. In particular, this work showed that soluble factors released by certain types of cancer cells can delay neutrophil apoptosis by modulating both the extrinsic and intrinsic apoptosis pathways. This work also met the fourth research objective (Section 1.1) of this project by demonstrating that peripheral neutrophils indirectly co-cultured with either MCF-7 cells or MDA-MB-231 cells do not alter the invasiveness or influence the process of EMT in MCF-7 or MDA-MB-231 cells, which substantiates the recent work of Wang *et al.* (327). Lastly this work has identified a novel candidate molecule, PGE_2 that may be at least partially responsible for delayed neutrophil apoptosis in certain breast cancers. This new and valuable data is important because COX-2 inhibitor drugs such as Celecoxib are currently being trialled as adjuvant treatments for the management of breast cancer. Future work should therefore investigate whether administration of Celecoxib could also, incidentally, inhibit the effect of PGE_2 upon neutrophil lifespan, and as a consequence, limit or prevent the adverse activities of pro-tumour neutrophils in breast cancer.

APPENDIX A

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

School of Food and Nutrition
Massey University
Private Bag 102-904
North Shore Mail Centre
Auckland, New Zealand

Health Screening Questionnaire

Name:.....

Address:.....

.....

.....

Phone:.....

Email:.....

Emergency Contact:.....

Name of GP:.....

Address of GP:.....

.....

.....

Name of the investigator:.....

Please answer the following questions. This questionnaire has been designed to identify the small number of individuals (aged 18 years or older) for whom volunteering might be inappropriate. If you have any doubts or difficulty with the questions, please ask the investigator for guidance.

1	What is your date of birth.....(DD/MM/YY)		
2	Do you suffer from any disorders of the immune system? If yes, please provide details here:.....	Yes	No
3	Do you suffer from any blood borne contagious diseases?	Yes	No
4	Do you suffer from any disorder of bleeding or clotting of the blood?	Yes	No
5	Have you ever experience difficulties (such as dizziness) when providing blood samples? If yes, please provide details here:.....	Yes	No

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

Signed:.....

Date:.....

APPENDIX B

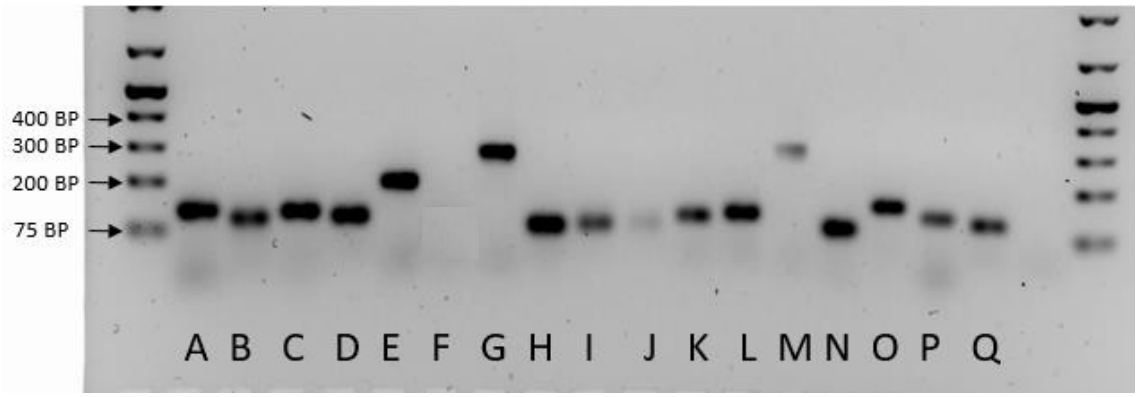
Primer design

A vast number of validated primer sequences are available in the literature or from primer databases such as PrimerBank (461), however oligonucleotides that are synthesised from these sequences still require optimisation when used in a laboratory for the first time. As a consequence, it was decided to design the majority primers required for this project in house, since it took little extra time and ensured that the sequences were specific to the target gene of interest. Furthermore it also ensured that the sequences were designed according to specific parameters (such as an annealing temperature of ~60 ºC) so that multiple assays could be run concurrently using the same cycling conditions (462). Sequences of the individual genes of interest were obtained from GenBank (https://www.ncbi.nlm.nih.gov/genbank) (463). Primers were designed using Primer-Blast software (464); all primers were designed to span exon-exon boundaries or be separated by an intron >1000BP to mitigate the risk of genomic DNA amplification (465). Candidate primers were analysed for potential hairpin structures and/or primer dimers by NetPrimer analysis software (http://www.premierbiosoft.com/netprimer). To confirm the specificity of amplification all PCR products were sequenced by Massey Genome Service (Palmerston North, New Zealand).

Primer Sequences

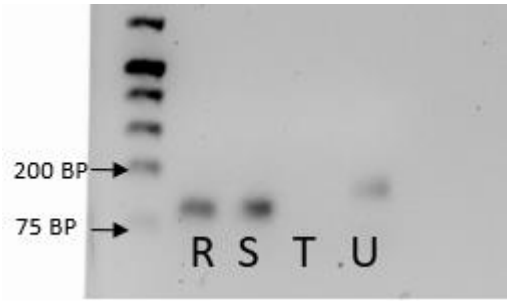
Target	Ref Seq	Location	amplicon size	Forward primer 5-3	Reverse primer 5-3
IL-8	NM_000584.3	4q13.3	89BP	ACTCCAAACCTTTCCACCCC	CTTTACAATAATTTCTGTGTTGGCG
VEGF-A	NM_001025366.2	6p21.1	110BP	TTCAAGCCATCCTGTGTGCC	ATCCGCATAATCTGCATGGTG
TGF-B1	NM_000660.6	19q13.2	122BP	TACAGCAACAATTCCTGGCG	ATTTCCCCTCCACGGCTCAA
IL-6	NM_001318095.1	7p15.3	89BP	TGGCAGAAAACAACCTGAACC	TTTCACCAGGCAAGTCTCCTCAT
MMP-9	NM_004994.2	20q13.12	82BP	GGCAGCTGGCAGAGGAATAC	GGCCCCAGAGATTTCGACTC
ICAM1	NM_000201	19p13.3-p13.2	204BP	CTTCGTGTCCTGTATGGCCC	ACAGAGGTAGGTGCCCTCAA
COX 2	NM_000963.3	1q31.1	144BP	TCCCTTGGGTGTCAAAGGTAAAA	AACTGATGCGTGAAGTGCTG
β-Catenin	NM_001904.3	3p22.1	150BP	GGAGCTAAAATGGCAGTGCG	AGCCAGTATGATGAGCTTGC
SNAIL	NM_005985.3	20q13.13	116BP	CCAGTGCCTCGACCACTATG	CTGCTGGAAGGTAAACTCTGGA
SLUG	NM_003068.4	8q11.21	106BP	TCGGACCCACACATTACCTTG	AAAAGGCTTCTCCCCCGTGT
ZEB2	NM_001171653.1	2q22.3	125BP	CAAGTACCGCCACGAGAAGA	TTTGGTGCTGATCTGTCCCTG
Vimentin	NM_003380.4	10p13	105BP	CGGGAGAAATTGCAGGAGGA	AAGGTCAAGACGTGCCAGAG
E-Cadherin	NM_001317184.1	16q22.1	308BP	TTTGACGCCGAGAGCTACAC	CCAGAAACGGAGGCCTGATG
Fibronectin	XM_005246404.1	2q35	83BP	AGCCGAGGTTTTAACTGCGA	CCCACTCGGTAAGTGTTCCC
BCL2(A1)	NM_004049.3	15q25.1	116BP	AAATTGCCCCGGATGTGGAT	TACAAAGCCATTTTCCCAGCC
BAX	NM_001291428.1	19q13.33	103BP	TCGCCCTTTTCTACTTTGCCA	CGGAGGAAGTCCAATGTCCA
BAK	NM_001188.3	6p21.31	124BP	CACAGAGGAGGTTTTCCGCA	CATGGTGCTGCTAGGTTGC
FAS	NM_000043.4	10q24.1	288 BP	GGCTTTTCGTGAGCTCGTCT	GTAAGAACCAGAGGTAGGAGGG
MCL-1	NM_001197320.1	1q21.2	121 BP	CCAGTGCCTCGACCACTATG	CGAAGCATGCCTTGGAA
β-Actin (359)	NM_001101.4	7p22.1	104 BP	CACTCTTCCAGCCTTCCTTC	GTACAGGTCTTTGCGGATGT

PCR products are of correct size



L-R:

A) BAK, 124 BP; B) BAX, 103 BP; C) MCL, 121 BP; D) BCL2(A1), 116 BP, E) ICAM-1, 204 BP; F) N/A; G) FAS, 288 BP; H) MMP-9, 82 BP; I) IL-8, 89 BP; J) IL-6, 89 BP; K) VEGF-A, 110 BP, L) TGF-β, 122 BP, M) E-Cadherin, 308 BP, N) Fibronectin, 83 BP, O) β-Catenin, 150 BP, P) SNAIL, 116 BP, Q) SLUG 106 BP.



L-R:

R) Vimentin, 105 BP; S) β-Actin, 104BP; T) N/A; U) COX-2, 144 BP (for a clearer gel image of this PCR product see Section 5.5)

Sequencing results:

BAK:

GGAGGCTGAAGGGGTGGCTGCCCCTGCCGACCCAGAGATGGTCACCTTACCTCTGCAACCTAGCAGCA
CCATGA

BAX:

AACCATCATGGGCTGGACATTGGACTTCCTCCG

MCL:

CTGGAGACCTTACGACGGGTTGGGGATGGCGTGCAGCGCAACCACGAGACGGCCTTCCAAGGATGCT
TCG

BCL2(A1):

GAGAATGGATAAGGCAAAACGGAGGCTGGGAAAATGGCTTTGTAA

ICAM-1:

CCAGAAAATTCCCAGCAGACTCCAATGTGCCAGGCTTGGGGGAACCCATTGCCCGAGCTCAAGTGTCT
AAAGGATGGCACTTTCCCACTGCCCATCGGGGAATCAGTGACTGTCACTCGAGATCTTGAGGGCACCTA
CCTCTGT

FAS:

CGGTTTACGAGTGACTTGGCTGGAGCCTCAGGGGCGGGCACTGGCACGGAACACACCCTGAGGCCAG
CCCTGGCTGCCCAGGCGGAGCTGCCTCTTCTCCCGCGGGTTGGTGGACCCGCTCAGTACGGAGTTGGG
GRAGCTCTTTCACTTCGGAGGATTGCTCAACAACCATGCTGGGCATCTGGACCCTCCTACCTCTGGTTCT
TAC

MMP-9:

TCGGGTGGCAGAGATGCGTGGAGAGTCGAAATCTCTGGGGCC

IL-8:

GATTGAGAGTGGACCACACTGCGCCAACACAGAAATTATTGTAAAGA

IL-6:

CCAATCTGGATTCAATGAGGAGACTTGCCTGGTGAAAA

VEGFA:

TGCATGACGAGGGCCTGGAGTGTGTGCCCACTGAGGAGTCCAACATCACCATGCAGATTATGCGGAT

TGF-β:

GAGTGGTTATCTTTTGATGTCACCGGAGTTGTGCGGCAGTGGTTGAGCCGTGGAGGGGAAAT

E-Cadherin:

CTATTTTTCCCTCGACACCCGATTCAAAGTGGGCACAGATGGTGTGATTACAGTCAAAAGGCCTCTACG
GTTTCATAACCCACAGATCCATTTCTTGGTCTACGCCTGGGACTCCACCTACAGAAAGTTTTCCACCAAA
GTCACGCTGAATACAGTGGGGCACCACCACCGCCCCCCGCCCCATCAGGCCTCCGTTTCTGG

Fibronectin:

TTGCTTTGACAGTACACTGGGAACACTTACCGAGTGGGA

β-Catenin:

CAAATGTTAAATTCTTGGCTATTACGACAGACTGCCTTCAAATTTTAGCTTATGGCAACCAAGAAAGCAA
GCTCATCATACTGGCT

SNAIL:

AATCGGAAGCCTAACTACAGCGAGCTGCAGGACTCTAATCCAGAGTTTACCTTCCAGCAGA

SLUG:

CCCTGGTTGCTTCAAGGACACATTAGAACTCACACGGGGGAGAAGCCTTT

Vimentin:

CCCTGCAATCTTTCAGACAGGATGTTGACAATGCGTCTCTGGCACGTCTT

β-Actin:

CTCCATCATGAAGTGTGACGTGGACATCCGCAAAGACCTGTAC

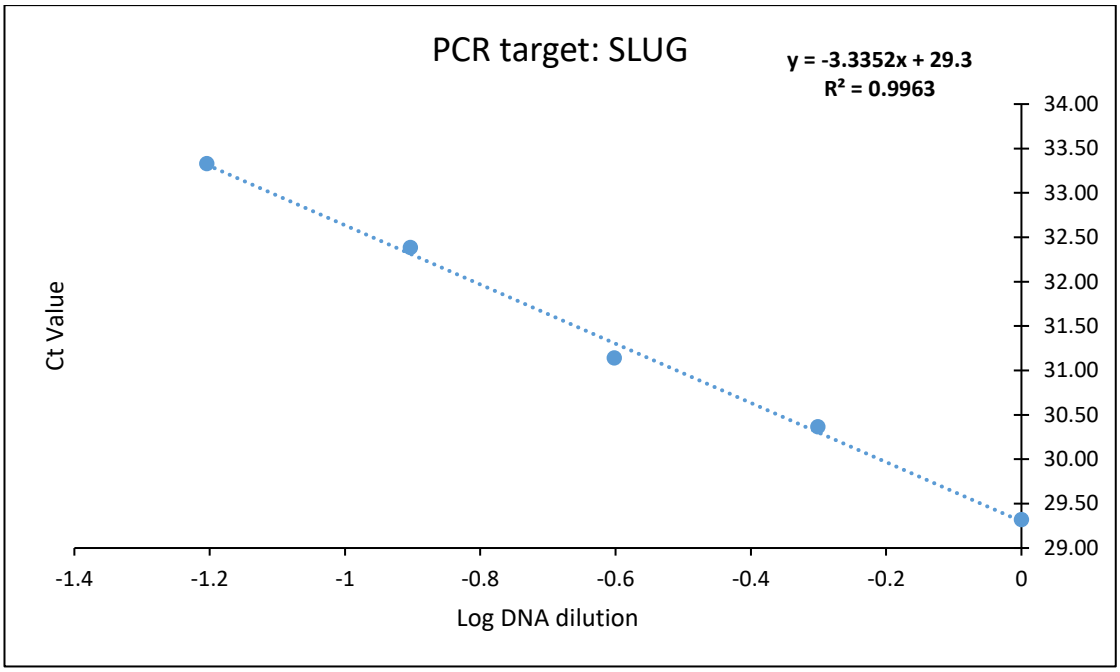
COX-2:

TTGCTTCTAAGAAGAAAGTTCATCCCTGATCCCCAGGGCTCAAACATGATGTTTGCATTCTTTGCCCAGC
ACTTCACGCATCAGTT

Calculation of Amplification efficiency

The two main methods to quantify DNA or cDNA in qRT-PCR are the standard curve method or the $\Delta\Delta C_T$ Method, the latter being used within this project due to its cost effectiveness. The $\Delta\Delta C_T$ Method is a relative quantification method that assumes the PCR reactions are 100% efficient, so that the amount of template should double at the end of each cycle. In reality the amount of template increases by a factor of (1+n), where n = the cycle/amplification efficiency (466). Typically, an amplification efficiency of between 80-110% is considered acceptable (467), with assays of 95-105% being deemed to have a high amplification efficiency (462) and therefore being well optimised.

The amplification efficiency of each assay was calculated using two- or four-fold serial dilutions of cDNA. The mean cycle threshold or "CT" value (where the fluorescence exceeded the threshold) was plotted against the log of the DNA dilution, using Microsoft Excel (2016) software (see example overleaf). Linear regression was performed, and the amplification efficiency was calculated using the following equation: Efficiency = $10^{(-1/slope)}$-1 (468). Only primers with an efficiency of between 85-110% were selected for use.



Plot of Ct versus log DNA dilution for SLUG (SNAI2). Y = -3.3352x + 29.3. The efficiency of the assay, as calculated via the equation Efficiency = $10^{(-1/\text{slope})}$-1 was found to be 0.99 or 99%.

APPENDIX C

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Figure 1.1: Anatomy of the female breast

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Figure 1.2: Haematopoiesis of myeloid and lymphoid cells

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Figure 1.3: The process of cellular transformation and immunoediting

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Figure 1.4: Tumour stroma during disease progression

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Figure 1.5: The two pathways of inflammation in cancer and their outcomes

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Figure 1.6: An overview of the extrinsic and intrinsic apoptosis pathways within neutrophils

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Figure 1.7: Neutrophil polarisation in cancer

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REFERENCES

1. Bianconi E, Piovesan A, Facchin F, Beraudi A, Casadei R, Frabetti F, et al. An estimation of the number of cells in the human body. Ann Hum Biol. 2013;40(6):463–71.

2. Pellettieri J, Sánchez Alvarado A. Cell turnover and adult tissue homeostasis: from humans to planarians. Annu Rev Genet. 2007;41:83–105.

3. Elmore S. Apoptosis: a review of programmed cell death. Toxicol Pathol. 2007;35(4):495–516.

4. Medzhitov R, Janeway Jr CA. Decoding the pattern of self and nonself by the innate immune system. Science. 2002;296(5566):298–300.

5. Soo You J, Jones PA. Cancer genetics and epigenetics: Two sides of the same coin? Cancer Cell. 2012;22(1):9–20.

6. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144(5):646–74.

7. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. Annu Rev Immunol. 2011;29:235–71.

8. Mittal D, Gubin MM, Schreiber RD, Smyth MJ. New insights into cancer immunoediting and its three component phases - elimination, equilbrium and escape. Curr Opin Immunol. 2014;27:16–25.

9. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting : integrating immunity's roles in cancer suppression and promotion. Science. 2011;331(6024):1565–70.

10. The World Health Organisation. The top 10 causes of death [Internet]. World Health Organization; 2017 [cited 2018 Mar 21]. Available from: http://www.who.int/mediacentre/factsheets/fs310/en/

11. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.

12. Ferlay J, Ervick M, Lam F, Colombet M, Mery L, Piñeros M, et al. Global Cancer Observatory: Cancer Tomorrow. [Internet]. Lyon, France: International Agency for Research on Cancer. 2018 [cited 2019 Feb 24]. Available from:

https://gco.iarc.fr/tomorrow

13. Jemal A, Center MM, DeSantis C, Ward EM. Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol biomarkers Prev. 2010;19(8):1893–907.

14. Anand P, Kunnumakkara AB, Kunnumakara AB, Sundaram C, Harikumar KB, Tharakan ST, et al. Cancer is a preventable disease that requires major lifestyle changes. Pharm Res. 2008;25(9):2097–116.

15. American Cancer Society. Global Cancer Facts and Figures 3rd Edition. Atlanta; 2015.

16. Preston-Martin S, Pike MC, Ross RK, Jones P a, Henderson BE. Increased cell division as a cause of human cancer. Cancer Res. 1990;50(23):7415–21.

17. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer. 2012;9(4):239–52.

18. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med. 2013;19(11):1423–37.

19. Kitamura T, Qian B, Pollard JW. Immune cell promotion of metastasis. Nat Publ Gr. 2015;15(2):73–86.

20. Hanahan D, Coussens LM. Accessories to the crime: functions of cells recruited to the tumor microenvironment. Cancer Cell. 2012;21(3):309–22.

21. Biswas SK, Allavena P, Mantovani A. Tumor-associated macrophages: functional diversity, clinical significance, and open questions. Semin Immunopathol. 2013;35(5):585–600.

22. Allavena P, Mantovani A. Immunology in the clinic review series; focus on cancer: tumour-associated macrophages: undisputed stars of the inflammatory tumour microenvironment. Clin Exp Immunol. 2012;167(2):195–205.

23. Mantovani A, Bottazzi B, Colotta F, Sozzani S, Ruco L. The origin and function of tumor-associated macrophages. Immunol Today. 1992;13(7):265–70.

24. Mantovani A, Marchesi F, Malesci A, Laghi L. Tumor-associated macrophages as treatment targets in oncology. Nat Rev Clin Oncol. 2017;14(7):399–416.

25. Fridlender ZG, Albelda SM. Tumor-associated neutrophils: Friend or foe?

Carcinogenesis. 2012;33(5):949–55.

26. Silvestre-Roig C, Hidalgo A, Soehnlein O. Neutrophil heterogeneity: implications for homeostasis and pathogenesis. Blood. 2016;127(18):2173–81.

27. Tak T, Tesselaar K, Pillay J, Borghans JAM, Koenderman L. What's your age again? Determination of human neutrophil half-lives revisited. J Leukoc Biol. 2013;94(4):595–601.

28. Shen M, Hu P, Donskov F, Wang G, Liu Q, Du J. Tumor-associated neutrophils as a new prognostic factor in cancer: a systematic review and meta-analysis. PLoS One. 2014;9(6):e98259.

29. Soto-Perez-de-Celis E, Chavarri-Guerra Y, Leon-Rodriguez E, Gamboa-Dominguez A. Tumor-associated neutrophils in breast cancer subtypes. Asian Pacific J Cancer Prev. 2017;18(10):2689–93.

30. Hor W-S, Huang W-L, Lin Y-S, Yang B-C. Cross-talk between tumor cells and neutrophils through the Fas (APO-1, CD95)/FasL system: human glioma cells enhance cell viability and stimulate cytokine production in neutrophils. J Leukoc Biol. 2003;73(3):363–8.

31. Zhu Q, Zhang X, Zhang L, Li W, Wu H, Yuan X, et al. The IL-6-STAT3 axis mediates a reciprocal crosstalk between cancer-derived mesenchymal stem cells and neutrophils to synergistically prompt gastric cancer progression. Cell Death Dis. 2014;5:e1295.

32. Trellakis S, Bruderek K, Dumitru CA, Gholaman H, Gu X, Bankfalvi A, et al. Polymorphonuclear granulocytes in human head and neck cancer: enhanced inflammatory activity, modulation by cancer cells and expansion in advanced disease. Int J cancer. 2011;129(9):2183–93.

33. Dumitru CA, Fechner MK, Hoffmann TK, Lang S, Brandau S. A novel p38-MAPK signaling axis modulates neutrophil biology in head and neck cancer. J Leukoc Biol. 2012;91(4):591–8.

34. Ibrahim S., Amin M, Beaman K. Abstract 1176: Tumor derived peptide from a2 isoform of Vacuolar ATPase immunomodulates tumor associated neutrophils [abstract]. Cancer Res. 2014;74(19 Supplement):1176–1176.

35. Ibrahim S., Kulshrestha A, Katara G., Beaman K. Delayed neutrophil apoptosis is

regulated by cancer associated a2 isoform vacuolar ATPase [abstract]. J immunolgy. 2017;198(1 (Supplement)):76.15.

36. Jablonska J, Leschner S, Westphal K, Lienenklaus S, Weiss S. Neutrophils responsive to endogenous IFN-β regulate tumor angiogenesis and growth in a mouse tumor model. J Clin Invest. 2010;120(4):1151–64.

37. Fridlender ZG, Sun J, Kim S, Kapoor V, Cheng G, Worthen GS, et al. Polarization of tumor-associated neutrophil (TAN) phenotype by TGF-β: "N1" versus "N2" TAN. Cancer Cell. 2009;16(3):183–94.

38. National Breast and Ovarian Cancer Centre. Breast cancer risk factors: a review of the evidence. Surry Hills, NSW.: National Breast and Ovarian Cancer Centre; 2009.

39. Weir R, Day P, Ali W. Risk factors for breast cancer in women. NZHTA Rep [Internet]. 2007;10(2). Available from: https://www.nsu.govt.nz/system/files/resources/risk-factors-breast-cancer-report-final.pdf

40. Nagy R, Sweet K, Eng C. Highly penetrant hereditary cancer syndromes. Oncogene. 2004;23(38):6445–70.

41. Zilfou JT, Lowe SW. Tumor suppressive functions of p53. Cold Spring Harb Perspect Biol. 2009;1(5):1–12.

42. Masciari S, Dillon DA, Rath M, Robson M, Weitzel JN, Balmana J, et al. Breast cancer phenotype in women with TP53 germline mutations : a Li Fraumeni syndrome consortium effort. Breast Cancer Res Treat. 2013;133(3):1125–30.

43. Malkin D. Li-Fraumeni syndrome. Genes Cancer. 2011;2(4):475–84.

44. Easton DF, Bishop DT, Ford D, Crockford GP. Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. The Breast Cancer Linkage Consortium. Am J Hum Genet. 1993;52(4):678–701.

45. Struewing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, et al. The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi jews. N Engl J Med. 1997;336(20):1401–8.

46. International Agency for Research on Cancer. World Cancer Report. Lyon,

France: World Health Organization; 2003. 84–88 p.

47. Henderson BE, Feigelson HS. Hormonal carcinogenesis. Carcinogenesis. 2000;21(3):427–33.

48. Bezemer ID, Rinaldi S, Dossus L, Van Gils CH, Peeters PHM, Van Noord PAH, et al. C-peptide, IGF-I, sex-steroid hormones and adiposity: A cross-sectional study in healthy women within the European Prospective Investigation into Cancer and Nutrition (EPIC). Cancer Causes Control. 2005;16(5):561–72.

49. Gill J. The effects of moderate alcohol consumption on female hormone levels and reproductive function. Alcohol Alcohol. 2000;35(5):417–23.

50. Smith AJ, Phipps WR, Thomas W, Schmitz KH, Kurzer MS. The effects of aerobic exercise on estrogen metabolism in healthy premenopausal women. Cancer Epidemiol Biomarkers Prev. 2013;22(5):756–64.

51. Howlander N, Noone A, Krapcho M, Miller D, Bishop K, Kosary C, et al. SEER Cancer Statistics Review, 1975-2014 [Internet]. National Cancer Institute. Bethesda, MD. [cited 2018 Dec 18]. Available from: https://seer.cancer.gov/archive/csr/1975_2014/

52. Makki J. Diversity of breast carcinoma: Histological subtypes and clinical relevance. Clin Med Insights Pathol. 2015;8:23–31.

53. OpenStax. 27.2 Anatomy and Physiology of the Female Reproductive System. In: OpenStax CNX [Internet]. 2019 [cited 2019 Jun 18]. Available from: http://cnx.org/contents/9cccba49-6490-4e5b-a366-9991b7dbc56c@9

54. Rahman M, Mohammed S. Breast cancer metastasis and the lymphatic system. Oncol Lett. 2015;10(3):1233–9.

55. Ran S, Volk L, Hall K, Flister MJ. Lymphangiogenesis and lymphatic metastasis in breast cancer. Pathophysiology. 2010;17(4):229–51.

56. Wong SY, Hynes RO. Lymphatic or hematogenous dissemination: how does a metastatic tumour decide? Cell Cycle. 2006;5(8):812–7.

57. Nagashima T, Sakakibara M, Nakano S, Tanabe N, Nakamura R, Nakatani Y, et al. Sentinel node micrometastasis and distant failure in breast cancer patients. Breast Cancer. 2006;13(2):186–91.

58. Sharma GN, Rahual D, Sanadya J, Sharma P, Sharma KK. Various types and management of breast cancer: an overiew. J Adv Pharm Technol Res. 2010;1(2):109–26.

59. Weigelt B, Geyer FC, Reis-Filho JS. Histological types of breast cancer: How special are they? Mol Oncol. 2010;4(3):192–208.

60. Rowell MD, Perry RR, Hsiu JG, Barranco SC. Phyllodes tumors. Am J Surg. 1993;165(3):376–9.

61. American Cancer Society. Breast Cancer Survival Rates [Internet]. 2017 [cited 2017 Dec 14]. Available from: https://www.cancer.org/cancer/breast-cancer/understanding-a-breast-cancer-diagnosis/breast-cancer-survival-rates.html

62. Nguyen DX, Bos PD, Massagué J. Metastasis: from dissemination to organ-specific colonization. Nat Rev Cancer. 2009;9(4):274–84.

63. Mariotto AB, Etzioni R, Hurlbert M, Penberthy L, Mayer M. Estimation of the number of women living with metastatic breast cancer in the United States. Cancer Epidemiol Biomarkers Prev. 2017;26(6):809–15.

64. Roth MY, Elmore JG, Yi-Frazier JP, Reisch LM, Oster N V., Miglioretti DL. Self-detection remains a key method of breast cancer detection for U.S. women. J Women's Heal. 2011;20(8):1135–9.

65. Centers for Disease Control and prevention. What Are the Symptoms of Breast Cancer? [Internet]. 2017 [cited 2017 Dec 14]. Available from: https://www.cdc.gov/cancer/breast/basic_info/symptoms.htm

66. Breast Cancer Foundation New Zealand. Breast Cancer in New Zealand [Internet]. 2017 [cited 2017 Dec 14]. Available from: https://www.breastcancerfoundation.org.nz/breast-awareness/breast-cancer-facts/breast-cancer-in-nz

67. Ministry of Health NZ. Tatau Kahukura: Māori health statistics > Ngā mana hauora tūtohu: Health status indicators > Cancer [Internet]. Available from: http://www.health.govt.nz/our-work/populations/maori-health/tatau-kahukura-maori-health-statistics/nga-mana-hauora-tutohu-health-status-indicators/cancer#1

68. Wiemann B, Starnes CO. Coley's toxins, tumor necrosis factor and cancer research: A historical perspective. Pharmacol Ther. 1994;64(3):529–64.

69. Ribatti D. The concept of immune surveillance against tumors: the first theories. Oncotarget. 2017;8(4):7175–80.

70. Burnet M. Cancer - a biological approach. III. Viruses associated with neoplastic conditions. Br Med J. 1957;1(5023):841–7.

71. Burnet FM. The concept of immunological surveillance. Prog Exp tumour Res. 1970;13:1–27.

72. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer. 2004;4(1):11–22.

73. OpenStax. 3.6 Cellular Differentiation. In: OpenStaxCNX [Internet]. 2019 [cited 2019 May 26]. Available from: http://cnx.org/contents/966c32cc-3d6f-4f4e-af4f-ea0c975e825c@8.

74. Buchmann K. Evolution of innate immunity: Clues from invertebrates via fish to mammals. Front Immunol. 2014;5:459.

75. Flannagan RS, Jaumouillé V, Grinstein S. The cell biology of phagocytosis. Annu Rev Pathol Mech Dis. 2012;7:61–98.

76. Rosales C, Uribe-Querol E. Phagocytosis: a fundamental process in immunity. Biomed Res Int. 2017;2017:9042851.

77. Stone KD, Prussin C, Metcalfe DD. IgE, mast cells, basophils and eosinophils. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S73–80.

78. Topham NJ, Hewitt EW. Natural killer cell cytotoxicity: How do they pull the trigger? Immunology. 2009;128(1):7–15.

79. Malmberg KJ, Carlsten M, Björklund A, Sohlberg E, Bryceson YT, Ljunggren HG. Natural killer cell-mediated immunosurveillance of human cancer. Semin Immunol. 2017;31:20–9.

80. Garcia-Lora A, Algarra I, Garrido F. MHC class I antigens, immune surveillance, and tumor immune escape. J Cell Physiol. 2003;195(3):346–55.

81. Peterson TR, Dickgreber N, Hermans IF. Tumour antigen presentation by dendritic cells. Crit Rev Immunol. 2010;30(4):345–86.

82. Fehres CM, Unger WWJ, Garcia-Vallejo JJ, van Kooyk Y. Understanding the biology of antigen cross-presentation for the design of vaccines against cancer.

Front Immunol. 2014;5:149.

83. Medzhitov R. Origin and physiological roles of inflammation. Nature. 2008;454(7203):428–35.

84. Zhang J-M, An J. Cytokines, Inflammation and Pain. Int Anesth Clin. 2007;45(2):27–37.

85. Delves PJ, Roitt IM. The Immune System. First of two parts. N Engl J Med. 2000;343(1):37–49.

86. Choy, Earnest H., Panayi, Gabriel S. Cytokine pathways and joint inflammation in rheumatoid arthritis. N Engl J Med. 2007;344(12):907–16.

87. Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. Lancet. 2007;369(9753):1627–40.

88. Chow MT, Möller A, Smyth MJ. Inflammation and immune surveillance in cancer. Semin Cancer Biol. 2012;22(1):23–32.

89. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420(6917):860–7.

90. Balkwill F, Mantovani A. Inflammation and cancer: back to Virchow? Lancet. 2001;357(9255):539–45.

91. Lu H, Ouyang W, Huang C. Inflammation, a key event in cancer development. Mol Cancer Res. 2006;4(4):221–33.

92. Hoffman W, Lakkis FG, Chalasani G. B cells, antibodies and more. Clin J Am Soc Nephrol. 2016;11(1):137–54.

93. Reuschenbach M, Doeberitz MVK. A systematic review of humoral immune responses against tumor antigens. Cancer Immunol. 2009;58(10):1535–44.

94. Yuen GJ, Demissie E, Pillai S. B lymphocytes and cancer: a love–hate relationship. Trends in Cancer. 2016;2(12):747–57.

95. Barry M, Bleackley R. Cytotoxic T lymphocytes: all roads lead to death. Nat Rev Immunol. 2002;2(6):401–9.

96. Kara EE, Comerford I, Fenix KA, Bastow CR, Gregor CE, McKenzie DR, et al. Tailored immune responses: novel effector helper T cell subsets in protective immunity. PLoS Pathog. 2014;10(2):e1003905.

97. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Helper T cells and lymphocyte activation. In: Molecular Biology of the Cell. 4th ed. New York: Garland Science; 2002.

98. Corthay A. How do regulatory t cells work? Scand J Immunol. 2009;70(4):326–36.

99. Haabeth OAW, Tveita AA, Fauskanger M, Schjesvold F, Lorvik KB, Hofgaard PO, et al. How do CD4+ T cells detect and eliminate tumor cells that either lack or express MHC class II molecules? Front Immunol. 2014;5:174.

100. Schüler T, Blankenstein T. Cutting edge: CD8+ effector T cells reject tumors by direct antigen recognition but indirect action on host cells. J Immunol. 2003;170(9):4427–31.

101. Roithmaier S, Hayden AM, Loi S, Esmore D, Griffiths A, Bergin P, et al. Incidence of malignancies in heart and/or lung tranplant recipients: a single-institution experience. J Heat Lung Transplant. 2007;26(8):845–9.

102. Mortaz E, Tabarsi P, Mansouri D, Khosravi A, Garssen J, Velayati A, et al. Cancers related to immunodeficiencies: Update and perspectives. Front Immunol. 2016;7:365.

103. Silverberg MJ, Chao C, Leyden WA, Xu L, Horberg MA, Klein D, et al. HIV infection, immunodeficiency, viral replication, and the risk of cancer. Cancer Epidemiol Biomarkers Prev. 2011;20(12):2551–9.

104. Dunn GP, Old LJ, Schreiber RD. The three Es of cancer immunoediting. Annu Rev Immunol. 2004;22(4):329–60.

105. V.Shankaran, H.Ikeda, AT.Bruce, JM.White, PE.Swanson, LJ.Old, et al. IFNγ and lymphocytes prevent primary tumor development and shape tumor immunogenecity. Nature. 2001;410(6832):1107–11.

106. Dunn GP, Old LJ, Schreiber RD. The immunobiology of cancer immunosurveillance and immunoediting. Immunity. 2004;21(2):137–48.

107. Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity. 2008;28(4):571–80.

108. Dunn GP, Bruce AT, Ikeda H, Old LJ, Schrieber RD. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3(11):991–8.

109. Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ. HMGB1 and RAGE in inflammation and cancer. Annu Rev Immunol. 2010;28:367–88.

110. Koebel CM, Vermi W, Swann JB, Zerafa N, Rodig SJ, Old LJ, et al. Adaptive immunity maintains occult cancer in an equilibrium state. Nature. 2007;450(7171):903–7.

111. Greaves M, Maley CC. Clonal evolution in cancer. Nature. 2012;481(7381):306–13.

112. Seliger B, Maeurer MJ, Ferrone S. Antigen-processing machinery breakdown and tumor growth. Trends Immunol. 2000;21(9):455–64.

113. Adams JM, Cory S. Bcl-2-regulated apoptosis: mechanism and therapeutic potential. Curr Opin Immunol. 2007;19(5):488–96.

114. Mimura K, Kono K, Takahashi A, Kawaguchi Y, Fujii H. Vascular endothelial growth factor inhibits the function of human mature dendritic cells mediated by VEGF receptor-2. Cancer Immunol Immunother. 2007;56(6):761–70.

115. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol. 2007;56(6):761–70.

116. Connolly J, Schnitt S, Wang H, Longtine J, Dvorak A, Dvorak H. Tumor structure and tumor stroma generation. In: Kufe WD, Pollack R, Weichselbaum R, Bast R, Gansler T, Holland J, et al., editors. Holland-Frei Cancer Medicine. 6th ed. Hamilton (ON): BC Decker; 2003.

117. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer. 2004;4(11):839–49.

118. Clark AG, Vignjevic DM. Modes of cancer cell invasion and the role of the microenvironment. Curr Opin Cell Biol [Internet]. 2015;36:13–22. Available from: http://dx.doi.org/10.1016/j.ceb.2015.06.004

119. Jain RK, Martin JD, Stylianopoulos T. The role of mechanical forces in tumor growth and therapy. Annu Rev Biomed Eng. 2014;16:321–46.

120. Ariffin AB, Forde PF, Jahangeer S, Soden DM, Hinchion J. Releasing pressure in

tumors: What do we know so far and where do we go from here a review. Cancer Res. 2014;74(10):2655–62.

121. Friedl P, Alexander S. Cancer invasion and the microenvironment: Plasticity and reciprocity. Cell [Internet]. 2011;147(5):992–1009. Available from: http://dx.doi.org/10.1016/j.cell.2011.11.016

122. Friedl P, Wolf K. Tumour-cell invasion and migration: Diversity and escape mechanisms. Nat Rev Cancer. 2003;3(5):362–74.

123. Krakhmal N V, Zavyalova M V, Denisov E V, Vtorushin S V, Perelmuter VM. Cancer Invasion: Patterns and Mechanisms. Acta Naturae [Internet]. 2015;7(2):17–28. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26085941%5Cnhttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4463409

124. Häger A, Alexander S, Friedl P. Cancer invasion and resistance. Eur J Cancer, Suppl. 2013;11(2):291–3.

125. Rintoul RC, Sethi T. The role of extracellular matrix in small-cell lung cancer. Lancet Oncol. 2001;2(7):437–42.

126. Khalil AA, Ilina O, Gritsenko PG, Bult P, Span PN, Friedl P. Collective invasion in ductal and lobular breast cancer associates with distant metastasis. Clin Exp Metastasis. 2017;34(6–7):421–9.

127. Zijl F van, Krupitza G, Wolfgang M. Initial steps of metastasis: Cell invasion and endothelial transmigration. Mutat Res. 2011;728(1–2):23–34.

128. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nat Rev Cancer. 2002;2(6):442–54.

129. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Invest. 2009;119(6):1420–8.

130. Chockley PJ, Keshamouni VG. Immunological Consequences of Epithelial-Mesenchymal Transition in Tumor Progression. J Immunol [Internet]. 2016;197(3):691–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/27431984

131. Pei D, Shu X, Gassama-diagne A, Thiery JP. Mesenchymal-epithelial transition

in development and reprogramming. Nat Cell Biol. 2019;21(1):44–53.

132. Nieto MA, Huang RY, Jackson RA, Thiery JP. Review EMT : 2016. Cell. 2016;166(1):21–45.

133. Joyce JA, Pollard JW. Microenvironmental regulation of metastasis. Nat Rev Cancer. 2009;9(4):239–52.

134. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene. 2008;27(45):5904–12.

135. Polyak K, Haviv I, Campbell IG. Co-evolution of tumor cells and their microenvironment. Trends Genet. 2009;25(1):30–8.

136. Ungefroren H, Sebens S, Seidl D, Lehnert H, Hass R. Interaction of tumor cells with the microenvironment. Cell Commun Signal. 2011;9:18.

137. Bussard KM, Mutkus L, Stumpf K, Gomez-Manzano C, Marini FC. Tumor-associated stromal cells as key contributors to the tumor microenvironment. Breast Cancer Res. 2016;18:84.

138. Pagès F, Galon J, Dieu-Nosjean MC, Tartour E, Sautès-Fridman C, Fridman WH. Immune infiltration in human tumors: A prognostic factor that should not be ignored. Oncogene. 2010;29(8):1093–102.

139. Barnes TA, Amir E. HYPE or HOPE: The prognostic value of infiltrating immune cells in cancer. Br J Cancer. 2017;117(4):451–60.

140. Dieci M, Mathieu M, Guarneri V, Conte P, Delaloge S, Andre F. Prognostic and predictive value of tumor-infiltrating lymphocytes in two phase III randomized adjuvant breast cancer trials. Ann Oncol. 2015;26(8):1698–704.

141. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol. 2011;29(15):1949–55.

142. Denkert C, von Minckwitz G, Darb-Esfahani S, Lederer B, Heppner BI, Weber KE, et al. Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy. Lancet Oncol. 2018;19(1):40–50.

143. Denkert C, Loibl S, Noske A, Roller M, Müller BM, Komor M, et al. Tumor-

associated lymphocytes as an independent predictor of response to neoadjuvant chemotherapy in breast cancer. J Clin Oncol. 2010;28(1):105–13.

144. Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature. 2008 Jul 24;454(7203):436–44.

145. Grivennikov SI, Greten FR, Karin M. Immunity, inflammation, and cancer. Cell. 2011;140(6):883–99.

146. Kim H-J, Cantor H. CD4 T-cell subsets and tumor immunity: the helpful and the not-so-helpful. Cancer Immunol Res. 2014;2(2):91–8.

147. Dobrzanski MJ. Expanding roles for CD4 T Cells and their subpopulations in tumor immunity and therapy. Front Oncol. 2013;3:63.

148. Summers C, Rankin SM, Condliffe AM, Singh N, Peters M, Chilvers ER. Neutrophil kinetics in health and disease. Trends Immunol. 2010;31(8):318–24.

149. Galdiero MR, Garlanda C, Jaillon S, Marone G, Mantovani A. Tumor associated macrophages and neutrophils in tumor progression. J Cell Physiol. 2013;228(7):1404–12.

150. Mantovani A, Cassatella M a, Costantini C, Jaillon S. Neutrophils in the activation and regulation of innate and adaptive immunity. Nat Rev Immunol. 2011;11(8):519–31.

151. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013;13(3):159–75.

152. Beyrau M, Bodkin J V, Nourshargh S. Neutrophil heterogeneity in health and disease: a revitalized avenue in inflammation and immunity. OpenBiol. 2012;2(11):120134.

153. Shen M, Hu P, Donskov F, Wang G, Liu Q, Du J. Tumor-associated neutrophils as a new prognostic factor in cancer: a systematic review and meta-analysis. PLoS One. 2014;9(6):e98259.

154. Jensen HK, Donskov F, Marcussen N, Nordsmark M, Lundbeck F, Von Der Maase H. Presence of intratumoral neutrophils is an independent prognostic factor in localized renal cell carcinoma. J Clin Oncol. 2009;27(28):4709–17.

155. Galdiero MR, Bianchi P, Grizzi F, Di Caro G, Basso G, Ponzetta A, et al.

Occurrence and significance of tumor-Associated neutrophils in patients with colorectal cancer. Int J Cancer. 2016;139(2):446–56.

156. Treffers LW, Hiemstra IH, Kuijpers TW, van den Berg TK, Matlung HL. Neutrophils in cancer. Immunol Rev. 2016;273(1):312–28.

157. Casbon A-J, Reynaud D, Park C, Khuc E, Gan DD, Schepers K, et al. Invasive breast cancer reprograms early myeloid differentiation in the bone marrow to generate immunosuppressive neutrophils. Proc Natl Acad Sci. 2015;112(6):E566–75.

158. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. Nat Rev Cancer. 2016;16(7):431–46.

159. Chen J, Deng Q, Pan Y, He B, Ying H, Sun H, et al. Prognostic value of neutrophil-to-lymphocyte ratio in breast cancer. FEBS Open Bio. 2015;12(5):502–7.

160. Wei B, Yao M, Xing C, Wang W, Yao J, Hong Y, et al. The neutrophil lymphocyte ratio is associated with breast cancer prognosis: an updated systematic review and meta-analysis. Onco Targets Ther. 2016;9:5567–75.

161. Elyasinia F, Keramati MR, Ahmadi F, Rezaei S, Ashouri M, Parsaei R, et al. Neutrophil-lymphocyte ratio in different stages of breast cancer. Acta Med Iran. 2017;55(4):228–32.

162. Templeton AJ, McNamara MG, Šeruga B, Vera-Badillo FE, Aneja P, Ocaña A, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. J Natl Cancer Inst. 2014;106(6):dju124.

163. Yin X, Xiao Y, Li F, Qi S, Yin Z, Gao J. Prognostic role of neutrophil-to-lymphocyte ratio in prostate cancer: a systematic review and meta-analysis. Medicine (Baltimore). 2016;95(3):e2544.

164. Powell DR, Huttenlocher A. Neutrophils in the Tumor Microenvironment. Trends Immunol. 2016;37(1):41–52.

165. Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. Neutrophil function: from mechanisms to disease. Annu Rev Immunol. 2012;30:459–89.

166. Borregaard N. Neutrophils, from marrow to microbes. Immunity.

2010;33(5):657–70.

167. Dahlgren C, Karlsson A. Respiratory burst in human neutrophils. J Immunol Methods. 1999;232(1–2):3–14.

168. Brinkmann V. Neutrophil extracellular traps kill bacteria. Science. 2004;303(5663):1532–5.

169. Cascão R, Rosário HS, Souto-Carneiro MM, Fonseca JE. Neutrophils in rheumatoid arthritis: more than simple final effectors. Autoimmun Rev. 2010;9(8):531–5.

170. Brannigan AE, O'Connell R, Hurley H, O'Neill A, Brady HR, Fitzpatrik JM, et al. Neutrophil apoptosis is delayed in patients with inflammatory bowel disease. Shock. 2000;13(5):361–6.

171. Fox S, Leitch AE, Duffin R, Haslett C, Rossi AG. Neutrophil apoptosis: relevance to the innate immune response and inflammatory disease. J Innate Immun. 2010;2(3):216–27.

172. Tecchio C, Micheletti A, Cassatella MA. Neutrophil-derived cytokines: facts beyond expression. Front Immunol. 2014;5:508.

173. Soehnlein O, Lindbom L, Weber C. Mechanisms underlying neutrophil-mediated monocyte recruitment. Blood. 2009;114(21):4613–24.

174. Schuster S, Hurrell B, Tacchini-Cottier F. Crosstalk between neutrophils and dendritic cells: a context-dependent process. J Leukoc Biol. 2013;94(4):671–5.

175. Charmoy M, Brunner-Agten S, Aebischer D, Auderset F, Launois P, Milon G, et al. Neutrophil-derived CCL3 is essential for the rapid recruitment of dendritic cells to the site of Leishmania major inoculation in resistant mice. PLoS Pathog. 2010;6(2):e1000755.

176. Blomgran R, Desvignes L, Briken V, Ernst JD. Mycobacterium tuberculosis inhibits neutrophil apoptosis, leading to delayed activation of naïve CD4 T cells. Cell Host Microbe. 2013;11(1):81–90.

177. Jaeger BN, Donadieu J, Cognet C, Bernat C, Ordoñez-Rueda D, Barlogis V, et al. Neutrophil depletion impairs natural killer cell maturation, function, and homeostasis. J Exp Med. 2012;209(3):565–80.

178. Costantini C, Micheletti A, Calzetti F, Perbellini O, Pizzolo G, Cassatella M a. Neutrophil activation and survival are modulated by interaction with NK cells. Int Immunol. 2010;22(10):827–38.

179. Scapini P, Bazzoni F, A.Cassatella M. Regulation of B-cell-activating factor (BAFF)/B lymphocyte stimulator (BLyS) expression in human neutrophils. Immunol Lett. 2008;116(1):1–6.

180. Huard B, Mckee T, Bosshard C, Durual S, Matthes T, Myit S, et al. APRIL secreted by neutrophils binds to heparan sulfate proteoglycans to create plasma cell niches in human mucosa. J Clin Invest. 2008;118(8):2877–95.

181. Wright HL, Moots RJ, Bucknall RC, Edwards SW. Neutrophil function in inflammation and inflammatory diseases. Rheumatology. 2010;49(9):1618–31.

182. Bendall LJ, Bradstock KF. G-CSF: from granulopoietic stimulant to bone marrow stem cell mobilizing agent. Cytokine Growth Factor Rev. 2014;25(4):355–67.

183. Mccracken JM, Allen LH. Regulation of human neutrophil apoptosis and lifespan in health and disease. J Cell Death. 2014;7:15–23.

184. Nikoletopoulou V, Markaki M, Palikaras K, Tavernarakis N. Crosstalk between apoptosis, necrosis and autophagy. Biochim Biophys Acta. 2013;1833(12):3448–59.

185. Elbim C, Katsikis PD, Estaquier J. Neutrophil apoptosis during viral infections. Open Virol J. 2009;3:52–9.

186. Challa S, Ka-Ming Chan F. Going up in flames: necrotic cell injury and inflammatory diseases. Cell Mol life Sci. 2010;67(19):3241–53.

187. Majno G, Joris I. Apoptosis, Oncosis, and Necrosis. Am J Pathol. 1995;146(1):3–15.

188. Franc NC, White K, Ezekowitz RA. Phagocytosis and development back to the future. Curr Opin Immunol. 1999;11(1):47–52.

189. Fadok V, Voelker D, Campbell P, Cohen J, Bratton D, Henson P. Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J immunolgy. 1992;148(7):2207–16.

190. Krause S, Maffini M V, Soto AM, Sonnenschein C. The microenvironment determines the breast cancer cells' phenotype: organization of MCF7 cells in 3D cultures. BMC Cancer. 2010;10:263.

191. Silva MT. Secondary necrosis : the natural outcome of the complete apoptotic program. FEBS Lett. 2010;584(22):4491–9.

192. Kennedy AD, Deleo FR. Neutrophil apoptosis and the resolution of infection. Immunol Res. 2009;43(1–3):25–61.

193. Porter AG, Ja RU. Emerging roles of caspase-3 in apoptosis. Cell Death Differ. 1999;6(2):99–104.

194. Renshaw SA, Parmar JS, Singleton V, Rowe SJ, Dockrell DH, Dower SK, et al. Acceleration of human neutrophil apoptosis by TRAIL. J immunolgy. 2003;170:1027–33.

195. Wallach D, Varfolomeev EE, Malinin NL, Goltsev Y V, Kovalenko A V, Boldin MP. Tumour necrosis factor receptor and Fas signalling mechanisms. Annu Rev Immunol. 1999;17:331–67.

196. Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, et al. Cytotoxicity-dependent APO-1 (Fas / CD95) - associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J. 1995;14(22):5579–88.

197. Guicciardi ME, Gores GJ. Life and death by death receptors. FASEB J. 2009;23(6):1625–37.

198. Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, et al. Two CD95 (APO-1/Fas) signaling pathways. EMBO J. 1998;17(6):1675–87.

199. Korsmeyer SJ, Wei MC, Saito M, Weiler S, Oh KJ, Schlesinger PH. Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. Cell Death Differ. 2000;7(12):1166–73.

200. Jia SH, Parodo J, Kapus A, Rotstein OD, Marshall JC. Dynamic regulation of neutrophil survival through tyrosine phosphorylation or dephosphorylation of caspase-8. J Biol Chem. 2007;283(9):5402–13.

201. Chipuk JE, Bouchier-Hayes L, Green DR. Mitochondrial outer membrane

permeabilization during apoptosis: the innocent bystander scenario. Cell Death Differ. 2006;13(8):1396–402.

202. Westphal D, Kluck RM, Dewson G. Building blocks of the apoptotic pore: how Bax and Bak are activated and oligomerize during apoptosis. Cell Death Differ. 2014;21(2):196–205.

203. Zhang M, Zheng J, Nussinov R, Ma B. Release of cytochrome c from Bax pores at the mitochondrial membrane. Sci Rep. 2017;7:2635.

204. Riedl SJ, Salvesen GS. The apoptosome: signalling platform of cell death. Nat Rev Mol Cell Biol. 2007;8(5):405–13.

205. Vier J, Groth M, Sochalska M, Kirschnek S. The anti-apoptotic Bcl-2 family protein A1/Bfl-1 regulates neutrophil survival and homeostasis and is controlled via PI3K and JAK/STAT signaling. Cell Death Dis. 2016;7:e2103.

206. Cross A, Moots RJ, Edwards SW. The dual effects of TNFalpha on neutrophil apoptosis are mediated via differential effects on expression of Mcl-1 and Bfl-1. Blood. 2008;111(22):878–84.

207. van Raam B, Drewniak A, Groenewold V, van den Berg T, Kuijpers TW. Granulocyte colony-stimulating factor delays neutrophil apoptosis by inhibition of calpains upstream of caspase-3. Blood. 2008;112(5):2046–54.

208. Adrain C, Creagh EM, Martin SJ. Apoptosis-associated release of Smac / DIABLO from mitochondria requires active caspases and is blocked by Bcl-2. EMBO J. 2001;20(23):6627–36.

209. Walle L Vande, Lamkanfi M, Vandenabeele P. The mitochondrial serine protease HtrA2/Omi:an overview. Cell Death Dis. 2008;15(3):453–60.

210. Kobayashi S, Yamashita K, Takeoka T, Ohtsuki T, Suzuki Y, Takahashi R, et al. Calpain-mediated X-linked inhibitor of apoptosis degradation in neutrophil apoptosis and its impairment in chronic neutrophilic leukemia. J Biol Chem. 2002;277(37):33968–77.

211. Altznauer F, Conus S, Cavalli A, Folkers G, Simon H. Calpain-1 regulates Bax and subsequent Smac-dependent caspase-3 activation in neutrophil apoptosis. J Biol Chem. 2004;279(7):5947–57.

212. Sherr CJ. Cancer cell cycles. Science. 1996;274(5293):1672–7.

213. Rossi AG, Sawatzky DA, Walker A, Ward C, Sheldrake TA, Riley NA, et al. Cyclin-dependent kinase inhibitors enhance the resolution of inflammation by promoting inflammatory cell apoptosis. Nat Med. 2006;12:1056–64.

214. Kelman Z. PCNA: Structure, functions and interactions. Oncogene. 1997;14(6):629–40.

215. Witko-Sarsat V, Ohayon D. Proliferating cell nuclear antigen in neutrophil fate. Immunol Rev. 2016;273(1):344–56.

216. Witko-Sarsat V, Mocek J, Bouayad D, Tamassia N, Ribeil J-A, Candalh C, et al. Proliferating cell nuclear antigen acts as a cytoplasmic platform controlling human neutrophil survival. J Exp Med. 2010;207(12):2631–45.

217. Pillay J, den Braber I, Vrisekoop N, Kwast LM, de Boer RJ, Borghans JAM, et al. In vivo labeling with 2H2O reveals a human neutrophil lifespan of 5.4 days. Blood. 2010;116(4):625–7.

218. Colotta F, Re F, Polentarutti N, Sozzani S, Mantovani A. Modulation of granulocyte survival and programmed cell death by cytokines and bacterial products. Blood. 1992;80(8):2012–20.

219. Derouet M, Thomas L, Cross A, Moots RJ, Edwards SW. Granulocyte macrophage colony-stimulating factor signaling and proteasome inhibition delay neutrophil apoptosis by increasing the stability of Mcl-1. J Biol Chem. 2004;279(26):26915–21.

220. Geering B, Stoeckle C, Conus S, Hans Uwe S. Living and dying for inflammation: neutrophils, eosinophils, basophils. Trends Immunol. 2013;34(8):398–409.

221. Hill CP, Osslund TD, Eisenberg D. The structure of granulocyte-colony-stimulating factor and its relationship to other growth factors. Proc Natl Acad Sci. 1993;90(11):5167–71.

222. Kurzrock R. Granulocyte-macrophage colony-stimulating factor. In: Kufe D., Pollock R., Weichselbaum R., Bast R., Gansler T., Holland J., et al., editors. Holland-Frei Cancer Medicine. 6th ed. Hamilton (ON): BC Decker; 2003.

223. Sipka S, Kovács I, Szántó S, Szegedi G, Brugós L, Bruckner G, et al. Adenosine inhibits the release of interleukin-1β in activated human peripheral mononuclear cells. Cytokine. 2005;31(4):258–63.

224. Smith KA. The structure of IL2 bound to the three chains of the IL2 receptor and how signaling occurs. Med Immunol. 2006;5:3.

225. Luzina IG, Keegan AD, Heller NM, Rook GA, Shea-Donohue T, Atamas SP. Regulation of inflammation by interleukin-4: a review of "alternatives." J Leucoc Biol. 2012;92(4):753–64.

226. Kettritz R, Gaido ML, Haller H, Luft FC, Jennette CJ, Falk RJ. Interleukin-8 delays spontaneous and tumor necrosis factor- α-mediated apoptosis of human neutrophils. Kidney Int. 1998;53(1):84–91.

227. Ortiz-Montero P, Londoño-Vallejo A, Vernot J-P. Senescence-associated IL-6 and IL-8 cytokines induce a self- and cross-reinforced senescence/inflammatory milieu strengthening tumorigenic capabilities in the MCF-7 breast cancer cell line. Cell Commun Signal. 2017;15(1):17.

228. Keller ET, Wanagat J, Ershler WB. Molecular and cellular biology of interleukin-6 and its receptor. Front Biosci. 1996;1:d340-357.

229. Baggiolini M, Clark-Lewis I. Interleukin-8, a chemotactic and inflammatory cytokine. FEBS Lett. 1992;307(1):97–101.

230. Fehniger TA, Caligiuri MA. Interleukin 15 : biology and relevance to human disease. Blood. 2001;97(1):14–32.

231. Horiuchi T, Mitoma H, Harashima SI, Tsukamoto H, Shimoda T. Transmembrane TNF-α: Structure, function and interaction with anti-TNF agents. Rheumatology. 2010;49(7):1215–28.

232. Ward C, Dransfield I, Murray J, Farrow SN, Haslett C, Rossi AG. Prostaglandin D2 and its metabolites induce caspase-dependent granulocyte apoptosis that is mediated via inhibition of IκBα degradation using a peroxisome proliferator-activated receptor-γ-independent mechanism. J Immunol. 2002;168(12):6232–43.

233. Ottonello L, Gonella R, Dapino P, Sacchetti C, Dallegri F. Prostaglandin E2 inhibits apoptosis in human neutrophilic polymorphonuclear leukocytes: role of

intracellular cyclic AMP levels. Exp Hematol. 1998;26(9):895–902.

234. Smith W. The eicosanoids and their biochemical mechanisms of action. Biochem J. 1989;259(2):315–24.

235. Gimenes SNC, Lopes DS, Alves PT, Azevedo FVP V, Yoneyama KAG, Oliveira RA, et al. Antitumoral effects of γ CdcPLI , a PLA 2 inhibitor from Crotalus durissus collilineatus via PI3K / Akt pathway on MDA-MB-231 breast cancer cell. Sci Rep. 2017;7:7077.

236. Wu Y, Zhao Q, Peng C, Sun L, Li XF, Kuang DM. Neutrophils promote motility of cancer cells via a hyaluronan-mediated TLR4/PI3K activation loop. J Pathol. 2011;225(3):438–47.

237. Wu M, Cao M, He Y, Liu Y, Yang C, Du Y, et al. A novel role of low molecular weight hyaluronan in breast cancer metastasis. FASEB J. 2015;29(4):1290–8.

238. Ibrahim SA, Katara GK, Kulshrestha A, Jaiswal MK, Amin MA, Beaman KD. Breast cancer associated a2 isoform vacuolar ATPase immunomodulates neutrophils: potential role in tumor progression. Oncotarget. 2015;6(32):33033–45.

239. Shang D, Peng T, Gou S, Li Y, Wu H, Wang C, et al. High mobility group box protein 1 boosts endothelial albumin transcytosis through the RAGE/Src/Caveolin-1 pathway. Sci Rep. 2016;6:32180.

240. Abraha HD, Noble PL, Nicolaides KH, Sherwood RA. Maternal serum S100 protein in normal and down syndrome pregnancies. Prenat Diagn. 1999;19(4):334–6.

241. Shen XF, Cao K, Jiang JP, Guan WX, Du JF. Neutrophil dysregulation during sepsis: an overview and update. J Cell Mol Med. 2017;21(9):1687–97.

242. Hoenderdos K, Condliffe A. The neutrophil in chronic obstructive pulmonary disease: too little, too late or too much, too soon? Am J Respir Cell Mol Biol. 2013;48(5):531–9.

243. Hernandez C, Huebener P, Schwabe RF. Damage-associated molecular patterns in cancer: a double-edged sword. Oncogene. 2016;35(46):5931–41.

244. Mouchemore KA, Anderson RL, Hamilton JA. Neutrophils, G-CSF and their

contribution to breast cancer metastasis. FEBS J. 2017;285(4):665–79.

245. Li Y, Li L, Brown T, Heldin P. Silencing of hyaluronan synthase 2 suppresses the malignant phenotype of invasive breast cancer cells. Int J Cancer. 2007;120(12):2557–67.

246. Massagué J. TGFβ in cancer. Cell. 2008;134(2):215–30.

247. Andzinski L, Wu CF, Lienenklaus S, Kröger A, Weiss S, Jablonska J. Delayed apoptosis of tumor associated neutrophils in the absence of endogenous IFN-β. Int J Cancer. 2014;136(3):572–83.

248. Piccard H, Muschel RJ, Opdenakker G. On the dual roles and polarized phenotypes of neutrophils in tumor development and progression. Crit Rev Oncol Hematol. 2012;82(3):296–309.

249. Mishalian I, Bayuh R, Levy L, Zolotarov L, Michaeli J, Fridlender ZG. Tumor-associated neutrophils (TAN) develop pro-tumorigenic properties during tumor progression. Cancer Immunol Immunother. 2013;62(11):1745–56.

250. McGarry Houghton A. The paradox of tumor-associated neutrophils: Fueling tumor growth with cytotoxic substances. Cell Cycle. 2014;9(9):1732–7.

251. Sagiv JY, Michaeli J, Assi S, Mishalian I, Kisos H, Levy L, et al. Phenotypic diversity and plasticity in circulating neutrophil subpopulations in cancer. Cell Rep. 2015;10(4):562–73.

252. Mollinedo F. Neutrophil degranulation , plasticity , and cancer metastasis. Trends Immunol. 2019;40(3):228–42.

253. Rosales C. Neutrophil : A cell with many roles in inflammation or several cell types ? Front Physiol. 2018;9:Article 113.

254. Gabrilovich D, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. Nat Rev Immunol. 2012;12(4):253–68.

255. Brandau S, Moses K, Lang S. The kinship of neutrophils and granulocytic myeloid-derived suppressor cells in cancer: cousins, siblings or twins? Semin Cancer Biol. 2013;23(3):171–82.

256. Moses K, Brandau S. Human neutrophils: their role in cancer and relation to myeloid-derived suppressor cells. Semin Immunol. 2016;28(2):187–96.

257. Mishalian I, Granot Z, Fridlender ZG. The diversity of circulating neutrophils in cancer. Immunobiology. 2017;222(1):82–8.

258. Fridlender ZG, Sun J, Mishalian I, Singhal S, Cheng G, Kapoor V, et al. Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. PLoS One. 2012;7(2):e31524.

259. Singel KL, Segal BH. Neutrophils in the tumor microenvironment: trying to heal the wound that cannot heal. Immunol Rev. 2016;273(1):329–43.

260. Shaul ME, Fridlender ZG. Neutrophils as active regulators of the immune system in the tumor microenvironment. J Leukoc Biol. 2017;102(2):343–9.

261. Galdiero MR, Varricchi G, Loffredo S, Mantovani A, Marone G. Roles of neutrophils in cancer growth and progression. J Leukoc Biol. 2017;103(3):457–64.

262. Nowell PC. The clonal evolution of tumor cell populations. Science. 1976;194(4260):23–8.

263. Coussens LM, Werb Z. Inflammation and cancer. Nature. 2002;420(6917):860–7.

264. Feng Y, Santoriello C, Mione M, Hurlstone A, Martin P. Live imaging of innate immune cell sensing of transformed cells in zebrafish larvae: Parallels between tumor initiation and wound inflammation. PLoS Biol. 2010;8(12):e1000562.

265. Antonio N, Bonnelykke-Behrndtz ML, Ward LC, Collin J, Christensen IJ, Steiniche T, et al. The wound inflammatory response exacerbates growth of pre-neoplastic cells and progression to cancer. EMBO J. 2015;34(17):2219–36.

266. Jamieson T, Clarke M, Steele CW, Samuel MS, Neumann J, Jung A, et al. Inhibition of CXCR2 profoundly suppresses inflammation-driven and spontaneous tumorigenesis. J Clin Invest. 2012;122(9):3127–44.

267. Shang K, Bai YP, Wang C, Wang Z, Gu HY, Du X, et al. Crucial involvement of tumor-associated neutrophils in the regulation of chronic colitis-associated carcinogenesis in mice. PLoS One. 2012;7(12):e51848.

268. Fu H, Ma Y, Yang M, Zhang C, Huang H, Xia Y, et al. Persisting and increasing

neutrophil infiltration associates with gastric carcinogenesis and e-cadherin downregulation. Sci Rep. 2016;6:29762.

269. Güngör N, Knaapen AM, Munnia A, Peluso M, Haenen GR, Chiu RK, et al. Genotoxic effects of neutrophils and hypochlorous acid. Mutagenesis. 2010;25(2):149–54.

270. Sandhu JK, Privora HF, Wenckebach G, Birnboim HC. Neutrophils, nitric oxide synthase, and mutations in the mutatect murine tumor model. Am J Pathol. 2000;156(2):509–18.

271. McGarry Houghton A, Rzymkiewicz DM, Hongbin J, Gregory AD, Egea EE, Metz HE, et al. Neutrophil elastase-mediated degradation of IRS-1 accelerates lung tumor growth. Nat Med. 2010;16(2):219–23.

272. Nawa M, Osada S, Morimitsu K, Nonaka K, Futamura M, Kawaguchi Y, et al. Growth effect of neutrophil elastase on breast cancer: Favorable action of sivelestat and application to anti-HER2 therapy. Anticancer Res. 2012;32(1):13–9.

273. Uribe-Querol E, Rosales C. Neutrophils in cancer: two sides of the same coin. J Immunol Res. 2015;2015:983698.

274. Clark RA, Klebanoff SJ. Neutrophil-mediated tumor cell cytotoxicity: role of the peroxidase system. J Exp Med. 1975;141(6):1442–1447.

275. Dissemond J, Weimann TK, Schneider LA, Schneeberger, A Scharffetter-Kochanek, K, Goos M, Wagner SN. Activated neutrophils exert antitumor activity against human melanoma cells: reactive oxygen species-induced mechanisms and their modulation by granulocyte-macrophage-colony-stimulating factor. J Invest Dermatol. 2003;121(4):936–8.

276. Zivkovic M, Poljak-Blazi M, Zarkovic K, Mihaljevic D, Schaur RJ, Zarkovic N. Oxidative burst of neutrophils against melanoma B16-F10. Cancer Lett. 2007;246(1–2):100–8.

277. Yan J, Kloecker G, Fleming C, Bousamra M, Hansen R, Hu X, et al. Human polymorphonuclear neutrophils specifically recognize and kill cancerous cells. Oncoimmunology. 2014;3(7):e950163.

278. van Egmond M, Bakema JE. Neutrophils as effector cells for antibody-based

immunotherapy of cancer. Semin Cancer Biol. 2013;23(3):190–9.

279. Challacombe JM, Suhrbier A, Parsons PG, Jones B, Hampson P, Kavanagh D, et al. Neutrophils are a key component of the antitumor efficacy of topical chemotherapy with Ingenol-3-angelate. J Immunol. 2006;177(11):8123–32.

280. Hernandez-ilizaliturri FJ, Jupudy V, Ostberg J, Oflazoglu E, Huberman A, Repasky E, et al. Neutrophils contribute to the biological antitumor activity of Rituximab in a Non-Hodgkin's lymphoma severe combined immunodeficiency mouse model. Clin Cancer Res. 2003;9(16 Pt 1):5866–73.

281. Stockmeyer B, Beyer T, Neuhuber W, Repp R, Kalden JR, Valerius T, et al. Polymorphonuclear granulocytes induce antibody-dependent apoptosis in human breast cancer cells. J Immunol. 2003;171(10):5124–9.

282. Eruslanov EB, Bhojnagarwala PS, Quatromoni JG, Stephen TL, Ranganathan A, Deshpande C, et al. Tumor-associated neutrophils stimulate T cell resposnses in early-stage human lung cancer. J Clin Invest. 2014;124(12):5466–80.

283. Chawla A, Alatrash G, Philips A V, Qiao N, Sukhumalchandra P, Kerros C, et al. Neutrophil elastase enhances antigen presentation by upregulating human leukocyte antigen class I expression on tumor cells. Cancer Immunol Immunother. 2016;65(6):741–51.

284. Mittendorf EA, Alatrash G, Qiao N, Wu Y, Sukhumalchandra P, St. John LS, et al. Breast cancer cell uptake of the inflammatory mediator neutrophil elastase triggers an anticancer adaptive immune response. Cancer Res. 2012;72(13):3153–62.

285. Brincks EL, Risk MC, Griffith TS. PMN and anti-tumor immunity-the case of bladder cancer immunotherapy. Semin Cancer Biol. 2013;23(3):183–9.

286. Zhang XD, Nguyen T, Thomas WD, Sanders JE, Hersey P. Mechanisms of resistance of normal cells to TRAIL induced apoptosis vary between different cell types. FEBS Lett. 2000;482(3):193–9.

287. Koga Y, Matsuzaki A, Suminoe A, Hattori H, Hara T. Neutrophil-derived TNF-related apoptosis-inducing ligand (TRAIL): a novel mechanism of antitumor effect by neutrophils. 2004;64(3):1037–43.

288. Kirkwood J. Cancer immunotherapy: The interferon alpha experience. Semin

Oncol. 2002;29(3 supplement 7):18–26.

289. Balbín M, Fueyo A, Tester AM, Pendás AM, Pitiot AS, Astudillo A, et al. Loss of collagenase-2 confers increased skin tumor susceptibility to male mice. Nat Genet. 2003;35(3):252–7.

290. Wislez M, Rabbe N, Marchal J, Milleron B, Crestani B, Mayaud C, et al. Hepatocyte growth factor production by neutrophils infiltrating bronchioloalveolar subtype pulmonary adenocarcinoma: role in tumor progression and death. Caner Res. 2003;63(6):1405–12.

291. He M, Peng A, Huang XZ, Shi DC, Wang JC, Zhao Q, et al. Peritumoral stromal neutrophils are essential for c-Met-elicited metastasis in human hepatocellular carcinoma. Oncoimmunology. 2016;5(10):e1219828.

292. Finisguerra V, Conza G Di, Matteo M Di, Serneels J, Thompson AAR, Wauters E, et al. MET is required for the recruitment of anti-tumoural neutrophils. Nature. 2015;522(7556):349–53.

293. Coussens LM, Tinkle CL, Hanahan D, Werb Z. MMP-9 supplied by bone marrow-derived cels contributes to skin carcinogenesis. Cell. 2000;103(3):481–90.

294. Acuff HB, Carter KJ, Fingleton B, Gorden DL, Matrisian LM. Matrix metalloproteinase-9 from bone marrow-derived cells contributes to survival but not growth of tumor cells in the lung microenvironment. Cancer Res. 2006;66(1):259–66.

295. Wada Y, Yoshida K, Tsutani Y, Shigematsu H, Oeda M, Sanada Y, et al. Neutrophil elastase induces cell proliferation and migration by the release of TGF-alpha, PDGF and VEGF in esophageal cell lines. Oncol Rep. 2007;17(1):161–7.

296. Costanza B, Umelo I, Bellier J, Castronovo V, Turtoi A. Stromal modulators of TGF-β in cancer. J Clin Med. 2017;6(1):7.

297. Brandes ME, Mai UE, Ohura K, Wahl SM. Type I transforming growth factor-beta receptors on neutrophils mediate chemotaxis to transforming growth factor-beta. J Immunol. 1991;147(5):1600–6.

298. Geiger R, Rieckmann JC, Wolf T, Basso C, Feng Y, Fuhrer T, et al. L-arginine

modulates T cell metabolism and enhances survival and anti-tumor activity. Cell. 2016;167(3):829–42.

299. Munder M, Schneider H, Luckner C, Giese T, Langhans CD, Fuentes JM, et al. Suppression of T-cell functions by human granulocyte arginase. Blood. 2006;108(5):1627–34.

300. Rotondo R, Barisione G, Mastracci L, Grossi F, Orengo AM, Costa R, et al. IL-8 induces exocytosis of arginase 1 by neutrophil polymorphonuclears in nonsmall cell lung cancer. Int J Cancer. 2009;125(4):887–93.

301. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J Immunol. 2004;172(2):989–99.

302. Shaul ME, Levy L, Sun J, Mishalian I, Singhal S, Kapoor V, et al. Tumor-associated neutrophils display a distinct N1 profile following TGFβ modulation: A transcriptomics analysis of pro- vs. antitumor TANs. Oncoimmunology. 2016;5(11):e1232221.

303. Mishalian I, Bayuh R, Eruslanov E, Michaeli J, Levy L, Zolotarov L, et al. Neutrophils recruit regulatory T-cells into tumors via secretion of CCL17 - a new mechanism of impaired antitumor immunity. Int J Cancer. 2014;135(5):1178–86.

304. Spiegel A, Brooks MW, Houshyar S, Reinhardt F, Ardolino M, Fessler E, et al. Neutrophils suppress intraluminal NK-mediated tumor cell clearance and enhance extravasation of disseminated carcinoma cells. Cancer Discov. 2016;6(6):630–49.

305. Liang W, Ferrara N. The complex role of neutrophils in tumor angiogenesis and metastasis. Cancer Immunol Res. 2016;4(2):83–91.

306. Tazzyman S, Lewis CE, Murdoch C. Neutrophils: key mediators of tumour angiogenesis. Int J Exp Pathol. 2009;90(3):222–31.

307. De Palma M, Biziato D, Petrova T V. Microenvironmental regulation of tumour angiogenesis. Nat Rev Cancer. 2017;17(8):457–74.

308. Nozawa H, Chiu C, Hanahan D. Infiltrating neutrophils mediate the initial angiogenic switch in a mouse model of multistage carcinogenesis. Proc Natl Acad Sci. 2006;103(33):12493–8.

309. Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer. 2003;3(6):401–10.

310. Gaudry M, Brégerie O, Andrieu V, El Benna J, Pocidalo M-AA, Hakim J. Intracellular pool of vascular endothelial growth factor in human neutrophils. Blood. 1997;90(10):4153–61.

311. Qu X, Zhuang G, Yu L, Meng G, Ferrara N. Induction of Bv8 Expression by granulocyte colony-stimulating factor in CD11b+Gr1+ cells: Key role of Stat3 signaling. J Biol Chem. 2012;287(23):19574–84.

312. Shojaei F, Wu X, Zhong C, Yu L, Liang X, Yao J, et al. Bv8 regulates myeloid-cell-dependent tumour angiogenesis. Nature. 2007;450(7171):825–31.

313. Queen MM, Ryan RE, Holzer RG, Keller-Peck CR, Jorcyk CL. Breast cancer cells stimulate neutrophils to produce oncostatin M: Potential implications for tumor progression. Cancer Res. 2005;65(19):8896–904.

314. Lauber S, Wong S, Cutz JC, Tanaka M, Barra N, Lhotak S, et al. Novel function of Oncostatin M as a potent tumour-promoting agent in lung. Int J Cancer. 2015;136(4):831–43.

315. Zarling J, Shoyab M, Marquardt H, Hanson M, Lioubin M, Todaro G. Oncostatin M: a growth regulator produced by differentiated histiocytic lymphoma cells. Proc Natl Acad Sci. 1986;83(24):9739–43.

316. Scapini P, Nesi L, Morini M, Tanghetti E, Belleri M, Noonan D, et al. Generation of biologically active angiostatin kringle 1-3 by activated human neutrophils. J Immunol. 2002;168(11):5798–804.

317. Ai S, Cheng XU, Inouea A, Nakamuraa K, Okumura K, Iguchia A, et al. Angiogenic activity bFGF and VEGF supressed by proteolytic cleavage of neutrophil elastase. Biochem Biophys Res Commun. 2007;364(2):395–401.

318. Leach J, Morton JP, Sansom OJ. Neutrophils: Homing in on the myeloid mechanisms of metastasis. Mol Immunol. 2017;

319. Grosse-Steffen T, Giese T, Giese N, Longerich T, Schirmacher P, Hänsch GM, et al. Epithelial-to-mesenchymal transition in pancreatic ductal adenocarcinoma and pancreatic tumor cell lines: the role of neutrophils and neutrophil-derived elastase. Clin Dev Immunol. 2012;2012:720768.

320. Hu P, Shen M, Zhang P, Zheng C, Pang Z, Zhu L, et al. Intratumoral neutrophil granulocytes contribute to epithelial-mesenchymal transition in lung adenocarcinoma cells. Tumour Biol. 2015;36(10):7789–96.

321. Freisinger CM, Huttenlocher A. Live imaging and gene expression analysis in zebrafish identifies a link between neutrophils and epithelial to mesenchymal transition. PLoS One. 2014;9(11):e1121183.

322. Li S, Cong X, Gao H, Lan X, Li Z, Wang W, et al. Tumor-associated neutrophils induce EMT by IL-17a to promote migration and invasion in gastric cancer cells. J Exp Clin Cancer Res. 2019;38(1):6.

323. Zhang W, Gu J, Chen J, Zhang P, Ji R, Qian H, et al. Interaction with neutrophils promotes gastric cancer cell migration and invasion by inducing epithelial-mesenchymal transition. Oncol Rep. 2017;38(5):2959–66.

324. Gallagher R, Collins S, Trujillo J, McCredie K, Ahearn M, Tsai S, et al. Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. Blood. 1979;54(3):713–33.

325. Yaseen R, Blodkamp S, Lüthje P, Reuner F, Völlger L, Naim HY, et al. Antimicrobial activity of HL-60 cells compared to primary blood-derived neutrophils against Staphylococcus aureus. J Negat Results Biomed. 2017;16(1):2.

326. Yen J, White RM, Stemple DL. Zebrafish models of cancer: progress and future challenges. Curr Opin Genet Dev. 2014;24:38–45.

327. Wang Y, Chen J, Yang L, Li J, Wu W, Huang M, et al. Tumor-contacted neutrophils promote metastasis by a CD90-TIMP-1 juxtacrine–paracrine loop. Clin Cancer Res. 2019;25(6):1957–70.

328. Lou X-L, Sun J, Gong S-Q, Yu X-F, Gong R, Deng H. Interaction between circulating cancer cells and platelets: clinical implication. Chin J Cancer Res. 2015;27(5):450–60.

329. Granot Z, Henke E, Comen E, King T, Norton L, Benezra R. Tumor entrained neutrophils inhibit seeding in the pre-metastatic lung. Cancer Cell. 2011;20(3):300–14.

330. Wculek S, Malanchi I. Neutrophils support lung colonization of metastasis-

initiating breast cancer cells. Nature. 2015;528(7582):413–7.

331. Giampieri S, Manning C, Hooper S, Jones L, Hill CS, Sahai E. Localized and reversible TGFβ signalling switches breast cancer cells from cohesive to single cell motility. Nat Cell Biol. 2009;11(11):1287–96.

332. Kuzmanov A, Hopfer U, Marti P, Meyer-Schaller N, Yilmaz M, Christofori G. LIM-homeobox gene 2 promotes tumor growth and metastasis by inducing autocrine and paracrine PDGF-B signaling. Mol Oncol. 2014;8(2):401–16.

333. Bekes EM, Schweighofer B, Kupriyanova T a, Zajac E, Ardi VC, Quigley JP, et al. Tumor-recruited neutrophils and neutrophil TIMP-free MMP-9 regulate coordinately the levels of tumor angiogenesis and efficiency of malignant cell intravasation. Am J Pathol. 2011;179(3):1455–70.

334. McDonald DM, Foss AJE. Endothelial cells of tumor vessels: abnormal but not absent. Cancer Metastasis Rev. 2000;19(1–2):109–20.

335. Valastyan S, Weinberg RA. Tumor metastasis: molecular insights and evolving paradigms. Cell. 2011;147(2):275–92.

336. Chiang SPH, Cabrera RM, Segall J. Tumour cell intravasation. Am journla Physiol Physiol. 2016;311(1):C1–114.

337. Hou JM, Krebs MG, Lancashire L, Sloane R, Backen A, Swain RK, et al. Clinical significance and molecular characteristics of circulating tumor cells and circulating tumor microemboli in patients with small-cell lung cancer. J Clin Oncol. 2012;30(5):525–32.

338. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. Biochim Biophys Acta - Mol Cell Res. 2013;1833(12):3481–98.

339. Sharma D, Brummel-Ziedins KE, Bouchard BA, Holmes CE. Platelets in tumor progression: a host factor that offers multiple potential targets in the treatment of cancer. J Cell Physiol. 2014;229(8):1005–15.

340. Hong Y, Fang F, Zhang Q. Circulating tumor cell clusters: what we know and what we expect (Review). Int J Oncol. 2016;49(6):2206–16.

341. Morimoto-Kamata R, Mizoguchi S, Ichisugi T, Yui S. Cathepsin G induces cell

aggregation of human breast cancer MCF-7 cells via a 2-step mechanism: catalytic site-independent binding to the cell surface and enzymatic activity-dependent induction of the cell aggregation. Mediators Inflamm. 2012;2012:456462.

342. Nozawa F, Hirota M, Okabe A, Shibata M, Iwamura T, Haga Y, et al. Elastase activity enhances the adhesion of neutrophil and cancer cells to vascular endothelial cells. J Surg Res. 2000;94(2):153–8.

343. Spicer JD, McDonald B, Cools-Lartigue JJ, Chow SC, Giannias B, Kubes P, et al. Neutrophils promote liver metastasis via Mac-1-mediated interactions with circulating tumor cells. Cancer Res. 2012;72(16):3919–27.

344. Slattery MJ, Dong C. Neutrophils influence melanoma adhesion and migration under flow conditions. Int J Cancer. 2003;106(5):713–22.

345. Cools-Lartigue J, Spicer J, McDonald B, Gowing S, Chow S, Giannias B, et al. Neutrophil extracellular traps sequester circulating tumor cells and promote metastasis. J Clin. 2013;123(8):3446–58.

346. Huh SJ, Liang S, Sharma A, Dong C, Robertson GP. Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. Cancer Res. 2010;70(14):6071–82.

347. Park J, Wysocki RW, Amoozgar Z, Maiorino L, Fein R, Jorns J, et al. Cancer cells induce metastasis-supporting neutrophil extracellular DNA traps. Sci Transl Med. 2016;8(361):361ra138.

348. Yang L, Edwards CM, Mundy GR. Gr-1+CD11b+myeloid-derived suppressor cells: Formidable partners in tumor metastasis. J Bone Miner Res. 2010;25(8):1701–6.

349. Bodogai M, Moritoh K, Lee-Chang C, CM H, Sherman-Baust C, Wersto R, et al. Immune suppressive and pro-metastatic functions of myeloid-derived suppressive cells rely upon education from tumor-associated B cells. Cancer Res. 2015;75(17):3456–65.

350. Uphoff C, Drexler H. Elimination of Mycoplasma from infected cell lines using antibiotics. In: Langdon SP, editor. Cancer cell culture, methods and protocols. Totowa, New Jersey: Humana Press; 2004. p. 327–34.

351. Mowles JM. The use of ciprofloxacin for the elimination of mycoplasma from naturally infected cell lines. Cytotechnology. 1988;1(4):355–8.

352. Strober W. Tryan blue exclusion test of cell viability. Curr Protoc Immunol. 2001;Appendix 3:Appendix3B.

353. Tsurubuchi T, Aratani Y, Maeda N, Koyama H. Retardation of early-onset PMA-induced apoptosis in mouse neutrophils deficient in myeloperoxidase. J Leucoc Biol. 2001;70:52–8.

354. Liang C-C, Park AY, Guan J-L. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc. 2007;2(2):329–33.

355. Schneider C, Rasband W, Eliceiri K. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9(7):671–5.

356. Grada A, Otero-Vinas M, Prieto-Castrillo F, Obagi Z, Falanga V. Research techniques made simple: analysis of collective cell migration using the wound healing assay. J Invest Dermatol. 2017;137(2):e11–6.

357. Masek T, Vopalensky V, Suchomelova P, Pospisek M. Denaturing RNA electrophoresis in TAE agarose gels. Anal Biochem. 2005;336(1):46–50.

358. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2-delta delta CT method. Methods. 2001;25:402–8.

359. Ledderose C, Heyn J, Limbeck E, Kreth S. Selection of reliable reference genes for quantitative real-time PCR in human T cells and neutrophils. BMC Res Notes. 2011;4(427).

360. Liu L-L, Zhao H, Ma T-F, Ge F, Chen C-S, Zhang Y-P. Identification of valid reference genes for the normalization of RT-qPCR expression studies in human breast cancer cell lines treated with and without transient transfection. PLoS One. 2015;10(1):e0117058.

361. Ferrante A, Thong YH. A rapid one-step procedure for purification of mononuclear and polymorphonuclear leukocytes from human blood using a modification of the hypaque-ficoll technique. J Immunol Methods. 1978;24(3–4):389–93.

362. Freitas M, Porto G, Lima JLFC, Fernandes E. Isolation and activation of human neutrophils in vitro. The importance of the anticoagulant used during blood collection. Clin Biochem. 2008;41:570–5.

363. Kuhns DB, Priel DAL, Chu J, Zarember KA. Isolation and functional analysis of human neutrophils. Curr Protoc Immunol. 2016;111(7.23):1–16.

364. Miltenyi S, Müller W, Weichel W, Radbruch A. High gradient magnetic cell separation with MACS. Cytometry. 1990;11(2):231–8.

365. Ibrahim SF, Van den Eng G. Flow cytometry and cell sorting. Adv Biochem Eng. 2007;106:19–39.

366. Hasenberg M, Köhler A, Bonifatius S, Borucki K, Riek-Burchardt M, Achilles J, et al. Rapid immunomagnetic negative enrichment of neutrophil granulocytes from murine bone marrow for functional studies in vitro and in vivo. PLoS One. 2011;6(2):e17314.

367. Zhou L, Somasundaram R, Nederhof RF, Dijkstra G, Faber KN, Peppelenbosch MP, et al. Impact of human granulocyte and monocyte isolation procedures on functional studies. Clin vaccine Immunol. 2012;19(7):1065–74.

368. Thomas HB, Moots RJ, Edwards SW, Wright HL. Whose gene is it anyway? the effect of preparation purity on neutrophil transcriptome studies. PLoS One. 2015;10(9):e0138982.

369. Johnson M. Fetal Bovine Serum. Mater methods. 2012;2:117.

370. Oida T, Weiner HL. Depletion of TGF-B from fetal bovine serum. J Immunol Methods. 2010;362(1–2):195–8.

371. Zheng X, Baker H, Hancock W, Fawaz F, McCamen M, PungorE. Proteomic analysis for the assessment of different lots of fetal bovine serum as a raw material for cell culture. Part IV. Application of proteomics to the manufacture of biological drugs. Biotechnol Prog. 2006;22(5):1294–300.

372. Sigma Aldrich. Product Information. Histopaque 1119 [Internet]. St.Lois, MO; Available from: https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Sigma/Product_Information_Sheet/1/11191pis.pdf

373. Frei M. Histopaque troubleshooting guide [Internet]. Biofiles. p. v6 n5. Available

from: https://www.sigmaaldrich.com/technical-documents/articles/biofiles/histopaque-troubleshooting-guide.html

374. Maqbool M, Vidyadaran S, George E, Ramasamy R. Optimisation of laboratory procedures for isolating human peripheral blood derived neutrophils. Med J Malaysia. 2011;66(4):296–9.

375. Harris PC. Effect of density gradient material upon ex-vivo neutrophil behaviour. University of Birmingham; 2012.

376. Marchi LF, Sesti-Costa R, Ignacchiti MDC, Chedraoui-Silva S, Mantovani B. In vitro activation of mouse neutrophils by recombinant human interferon-gamma: Increased phagocytosis and release of reactive oxygen species and pro-inflammatory cytokines. Int Immunopharmacol. 2014;18(2):228–35.

377. Marchi LF, Sesti-Costa R, Chedraoui-Silva S, Mantovani B. Comparison of four methods for the isolation of murine blood neutrophils with respect to the release of reactive oxygen and nitrogen species and the expression of immunological receptors. Comp Clin Path. 2013;23(5):1469–76.

378. Zahler S, Kowalski C, Brosig A, Kupatt C, Becker BF, Gerlach E. The function of neutrophils isolated by a magnetic antibody cell separation technique is not altered in comparison to a density gradient centrifugation method. J Immunol Methods. 1997;200(1–2):173–9.

379. Vuorte J, Jansson SE, Repo H. Evaluation of red blood cell lysing solutions in the study of neutrophil oxidative burst by the DCFH assay. Cytometry. 2001;43(4):290–6.

380. Zhou M, Diwu Z, Panchuk-voloshina N, Haugland RP. A stable non fluorescent derivative of Resorufin for the fluorometric determination of trace hydrogen peroxide: Applications in detecting the activity of phagocyte NADPH oxidase and other oxidases. Anal Biochem. 1997;253(2):162–8.

381. Mohanty JG, Jaffe JS, Schulman ES, Raible DG. A highly sensitive fluorescent micro-assay of H2O2 release from activated human leukocytes using a dihydroxyphenoxazine derivative. J Immunol Methods. 1997;202(2):133–41.

382. Ginis I, Tauber a I. Activation mechanisms of adherent human neutrophils. Blood. 1990;76(6):1233–9.

383. Videm V. Endpoint-attached heparin blocks neutrophil sticking and spreading. Biomaterials. 2004;25(1):43–51.

384. Hyclone. Heat inactivation - are you wasting your time? Vol. 15, Art to Science. 1996.

385. Triglia RP, Linscott WD. Titers of nine complement components, conglutinin and C3b-inactivator in adult and fetal bovine sera. Mol Immunol. 1980;17(6):741–8.

386. Perianayagam MC, Balakrishnan VS, King AJ, Pereira BJG, Jaber BL. C5a delays apoptosis of human neutrophils by a phosphatidylinositol 3-kinase-signaling pathway. Kidney Int. 2002;61(2):456–63.

387. Trellakis S, Farjah H, Bruderek K, Dumitru CA, Hoffmann TK, Lang S, et al. Peripheral blood neutrophil granulocytes from patients with head and neck squamous cell carcinoma functionally differ from their counterparts in healthy donors. Int J Immunopathol Pharmacol. 2011;24(3):683–93.

388. Cailleau R, Young R, Olivé M, Reeves WJ. Breast tumor cell lines from pleural effusions. J Natl Cancer Inst. 1974;53(3):661–74.

389. Soule HD, Vazquez J, Long A, Albert S, Brennan M. A human cell line from a pleural effusion derived from a breast carcinoma. J Natl Cancer Inst. 1973;51(5):1409–16.

390. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat. 2004;83(3):249–89.

391. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, et al. A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell. 2006;10(6):515–27.

392. Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. Breast Cancer Res. 2011;13(4):215.

393. Himeji D, Horiuchi T, Tsukamoto H, Hayashi K, Watanabe T, Harada M. Characterization of caspase-8L: A novel isoform of caspase-8 that behaves as an inhibitor of the caspase cascade. Blood. 2002;99(11):4070–8.

394. Piccinini F, Tesei A, Arienti C, Bevilacqua A. Cell counting and viability

assessment of 2D and 3D cell cultures: expected reliability of the Trypan Blue assay. Biol Proc online. 2017;19:8.

395. Mascotti K, McCullough J, Burger S. HPC viability measurement: trypan blue versus acridine orange and propidium iodide. Transfusion. 2000;40(6):693–6.

396. Jones K, Senft J. An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide. J Histochem Cytochem. 1985;33(1):77–9.

397. Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis flow cytometric detection of phosphatidylserine early apoptotic cells using fluorescein labelled expression on Annexin V. J Immunol Methods. 1995;184(1):39–51.

398. Stennicke HR, Jürgensmeier JM, Shin H, Deveraux Q, Wolf BB, Yang X, et al. Pro-caspase-3 is a major physiologic target of caspase-8. J Biol Chem. 1998;273(42):27084–90.

399. Zhu D, Hattori H, Jo H, Jia Y, Subramanian KK, Loison F, et al. Deactivation of phosphatidylinositol 3,4,5-triphospahte/Akt signalling mediates neutrophil spontaneous death. PNAS. 2006;103(40):14836–41.

400. Hamasaki BA, Sendo F, Nakayama K, Ishida N, Negishi I, Nakayama K, et al. Accelerated neutrophil apoptosis in mice lacking A1-a, a Subtype of the bcl-2 related A1 gene. J Exp Med. 1985;188(11):1985–92.

401. Moulding DA, Akgul C, Derouet M, White MRH, Edwards SW. BCL-2 family expression in human neutrophils during delayed and accelerated apoptosis. J Leukoc Biol. 2001;70(5):783–92.

402. Bazzoni F, Giovedi S, Kiefer MC, Cassatella MA. Analysis of the Bak protein expression in human polymorphonuclear neutrophils. Int J Clin Lab Res. 1999;29(1):41–5.

403. Kale J, Osterlund EJ, Andrews DW. BCL-2 family proteins: changing partners in the dance towards death. Cell Death Differ. 2018;25(1):65–80.

404. Vogler M. BCL2A1: the underdog in the BCL2 family. Cell Death Differ. 2012;19(1):67–74.

405. Fecho K, Cohen PL. Fas ligand (gld)- and Fas (lpr)-deficient mice do not show alterations in the extravasation or apoptosis of inflammatory neutrophils. J Leucoc Biol. 1988;64(3):373–83.

406. Renshaw SA, Timmons SJ, Eaton V, Usher LR, Akil M, Bingle CD, et al. Inflammatory neutrophils retain susceptibility to apoptosis mediated via the Fas death receptor. J Leucoc Biol. 2000;67(5):662–8.

407. Jonsson H, Allen P, Peng S. Inflammatory arthritis requires Foxo3a to prevent Fas ligand-induced neutrophil apoptosis. Nat Med. 2005;11(6):666–71.

408. Ibrahim SA, Kulshrestha A, Katara GK, Amin MA, Beaman KD. Cancer derived peptide of vacuolar ATPase “a2” isoform promotes neutrophil migration by autocrine secretion of IL-8. Sci Rep. 2016;6:36865.

409. Bautista-Lopez N, Galipeau J, Cuerquis J, Lalu M, Eliopoulos N. Induction of increased levels of matrix metalloproteinase-2 (MMP-2) and -9 in human breast cancer cell lines by activation of GM-CSF receptor Bc via C-Fos-ERK 1/2 Signaling. J Clin Exp Oncol. 2017;6(2).

410. Schrey MP, Patel K V. Prostaglandin E2 production and metabolism in human breast cancer cells and breast fibroblasts. Regulation by inflammatory mediators. Br J Cancer. 1995;72(6):1412–9.

411. Cyphert JM, Trempus CS, Garantziotis S. Size matters: molecular weight specificity of hyaluronan effects in cell biology. Int J Cell Biol. 2015;2015:563818.

412. Raz A. Interaction of prostaglandins with blood plasma proteins. Comparative binding of prostaglandins A 2 , F 2 and E 2 to human plasma proteins. J Biochem. 1972;130(2):631–6.

413. Hong X, Meng Y, Kalkanis SN. Serum proteins are extracted along with monolayer cells in plasticware and interfere with protein analysis. J Biol Methods. 2016;3(4):e51.

414. Babcock J., Brancaleon L. Bovine serum albumin oligomers in the E-and B-forms at low protein concentration and ionic strength. Int J Biol Macromol. 2013;53:42–53.

415. Ferraz TP., Fiuza M., dos Santos MI., Pontes de Carvalhoa L, Soares N.

Comparison of six methods for the extraction of lipids from serum in terms of effectiveness and protein preservation. J Biochem Biophys Methods. 2004;58(3):187–93.

416. Bull HB, Breese K. Thermal transitions of proteins. Arch Biochem Biophys. 1973;156(2):604–12.

417. Kvirkvelia N, Vojnovic I, Warner TD, Free P, Rayment N, Chain BM. Placentally derived prostaglandin E2 acts via the EP4 receptor to inhibit IL-2-dependent proliferation of CTLL-2 T cells. Clin Exp Immunol. 2002;127(2):263–9.

418. Kulshrestha A, Ibrahim S, Pamarthy S, Sachs AG, Beaman KD, Jaiswal MK, et al. Vacuolar ATPase “a2” isoform exhibits distinct cell surface accumulation and modulates matrix metalloproteinase activity in ovarian cancer. Oncotarget. 2015;6(6):3797–810.

419. Kwong C, Gilman-Sachs A, Beaman K. Tumor-associated a2 vacuolar ATPase acts as a key mediator of cancer-related inflammation by inducing pro-tumorigenic properties in monocytes. J Immunol. 2011;186(3):1781–9.

420. Malachi T, Chaimoff C, Feller N, Halbrecht I. Prostaglandin E2 and cyclic AMP in tumor and plasma of breast cancer patients. J Cancer Res Clin Oncol. 1981;102(1):71–9.

421. Sauter ER, Qin W, Schlatter L, Hewett JE, Flynn JT. Celecoxib decreases prostaglandin E2 concentrations in nipple aspirate fluid from high risk postmenopausal women and women with breast cancer. BMC Cancer. 2006;6:248.

422. Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science. 2001;294:1871–5.

423. Jakobsson P-J, Thoren S, Morgenstern R, Samuelsson B. Identification of human prostaglandin E synthase: A microsomal, glutathione-dependent, inducible enzyme, constituting a potential novel drug target. Proc Natl Acad Sci. 2002;96(13):7220–5.

424. Ricciotti E, FitzGeralad G. Prostaglandins and inflammation. Arter Thromb Vasc Biol. 2011;31(5):986–1000.

425. Terragno A, Rydzik R, Terragno NA. High performance liquid chromatography and UV detection for the separation and quantitation of prostaglandins. Prostaglandins. 1981;21(1):101–12.

426. Ward C, Dransfield I, Murray J, Farrow SN, Haslett C, Rossi AG. Prostaglandin D2 and its metabolites induce caspase-dependent granulocyte apoptosis that is mediated via inhibition of IKBA degradation using a peroxisome proliferator-activated receptor-independent mechanism. J Immunol. 2002;168(12):6232–43.

427. Granot Z, Jablonska J. Distinct functions of neutrophil in cancer and its regulation. Mediators Inflamm. 2015;2015:701067.

428. Thiery JP, Acloque H, Huang RYJ, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139(5):871–90.

429. Freeman WM, Walker SJ, Vrana KE. Quantitative RT-PCR: Pitfalls and potential. Biotechniques. 1999;26(1):112–25.

430. Todorović-Raković N, Milovanović J. Interleukin-8 in breast cancer progression. J Interf Cytokine Res. 2013;33(10):563–70.

431. Setrerrahmane S, Xu H. Tumor-related interleukins: old validated targets for new anti-cancer drug development. Mol Cancer. 2017;16(1):153.

432. Xu J, Lamouille S, Derynck R. TGF-β-induced epithelial to mesenchymal transition. Cell Res. 2009;19(2):156–72.

433. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. Oncology. 2005;69(Suppl 3):4–10.

434. Mendez M, Kojima S, Goldman R. Vimentin induces changes in cell shape, motility, and adhesion during the epithelial to mesenchymal transition. FASEB J. 2010;24(6):1838–51.

435. Woodfin A, Beyrau M, Voisin M-B, MA B, Whiteford JR, Hordijk PL, et al. ICAM-1 expressing neutrophils exhibit enhanced effector functions in murine models of endotoxemia. Blood. 2016;127(7):898–907.

436. Elsner J, Sach M, Knopf H, Norgauer J, Kapp A, Schollmeyer P, et al. Synthesis and surface expression of ICAM-1 in polymorphonuclear neutrophilic leukocytes in normal subiects and during inflammatory disease. Immunobiology.

1995;193(5):456–64.

437. Dabrowski A, Osada J, Dabrowska MI, Wereszczynska-Siemiatkowska U, Siemiatkowski A. Increased expression of the intercellular adhesion molecule-1 (ICAM-1) on peripheral blood neutrophils in acute pancreatitis. Adv Med Sci. 2014;59(1):102–7.

438. Andzinski L, Kasnitz N, Stahnke S, Wu C-F, Gereke M, von Köckritz-Blickwede M, et al. Type I IFNs induce anti-tumor polarization of tumor associated neutrophils in mice and human. Int J Cancer. 2016;138(8):1982–93.

439. Wang T, Zhao Y, Peng L, Chen N, Chen W, Lv Y, et al. Tumour-activated neutrophils in gastric cancer foster immune suppression and disease progression through GM-CSF-PD-L1 pathway. Gut. 2017;66(11):1900–11.

440. Zhang X, Shi H, Yuan X, Jiang P, Qian H, Xu W. Tumor-derived exosomes induce N2 polarization of neutrophils to promote gastric cancer cell migration. Mol Cancer. 2018;17(1):146.

441. Dumitru C, Gholaman H, Trellakis S, Bruderek K, Dominas N, Gu X, et al. Tumor-derived macrophage migration inhibitory factor modulates the biology of head and neck cancer cells via neutrophil activation. Int J Cancer. 2011;129(4):859–69.

442. Singel KL, Emmons TR, Khan A, Mayor PC, Shen S, Wong JT, et al. Mature neutrophils suppress T cell immunity in ovarian cancer microenvironment. JCI Insight. 2019;4(5):e122311.

443. Mosmann T. Rapid colorimetric assay for cellular growth and survival : application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65(1–2):55–63.

444. Franke WW, Schmid E, Osborn M, Weber K. Different intermediate-sized filaments disntiguished by immunofluorescence microscopy. Proc Natl Acad Sci. 1978;75(10):5034–8.

445. Lamouille S, Xu J, Derynck R. Molecular mechanisms of epithelial-mesenchymal transition. Nat Rev Mol Cell Biol. 2014;15(3):178–96.

446. Bindels S, Mestdagt M, Vandewalle C, Jacobs N, Volders L, Noël A, et al. Regulation of vimentin by SIP1 in human epithelial breast tumor cells.

Oncogene. 2006;25(36):4975–85.

447. Zhang XI, Diab IH, Zehner ZE. ZBP-89 represses vimentin gene transcription by interacting with the transcriptional activator, Sp1. Nucleic Acids Res. 2003;31(11):2900–14.

448. Krishnamachary B, Berg-Dixon, SKelly B, Agani F, Feldser D, Ferreira G, Iyer N, et al. Regulation of colon carcinoma cell invasion by hypoxia-inducible factor 1. Cancer Res. 2003;63(5):1138–43.

449. de Sousa Abreua R, Penalvaa LO, Marcotte EM, Vogel C. Global signatures of protein and mRNA expression levels. Mol Biosyst. 2009;5(12):1512–26.

450. Schneider D, Baronsky T, Pietuch A, Rother J, Oelkers M, Fichtner D, et al. Tension monitoring during epithelial-to-mesenchymal transition links the switch of phenotype to expression of moesin and cadherins in NMuMG Cells. PLoS One. 2013;8(12):e0080068.

451. Vazquez Rodriguez G, Abrahamsson A, Jensen LDE, Dabrosin C. Estradiol promotes breast cancer cell migration via recruitment and activation of neutrophils. Cancer Immunol Res. 2017;5(3):234–47.

452. Lieber JG, Webb S, Suratt BT, Young SK, Johnson GL, Keller GM, et al. The in vitro production and characterization of neutrophils from embryonic stem cells. Blood. 2004;103(3):852–9.

453. Jie Z, Zhang Y, Wang C, Shen B, Guan X, Ren Z, et al. Large-scale ex vivo generation of human neutrophils from cord blood CD34+ cells. PLoS One. 2017;12(7):e0180832.

454. Chilvers ER, Li W, Summers C, Cojoc G, Ekpenyong AE, Guck J, et al. Mechanical deformation induces depolarization of neutrophils. Sci Adv. 2017;3(6):e1602536.

455. Grösch S, Maier TJ, Schiffmann S, Geisslinger G. Cyclooxygenase-2 (COX-2) - Independent anticarcinogenic effects of selective COX-2 inhibitors. J Natl Cancer Inst. 2006;98(11):736–47.

456. Goradel NH, Najafi M, Salehi E, Farhood B, Mortezaee K. Cyclooxygenase-2 in cancer: A review. J Cell Physiol. 2019;234(5):5683–99.

457. Regulski M, Regulska K, Prukała W, Piotrowska H, Stanisz B, Murias M. COX-2 inhibitors: A novel strategy in the management of breast cancer. Drug Discov Today. 2016;21(4):598–615.

458. Ottonello L, Cutolo M, Frumento G, Arduino N, Bertolotto M, Mancini M, et al. Synovial fluid from patients with rheumatoid arthritis inhibits neutrophil apoptosis: role of adenosine and proinflammatory cytokines. Rheumatology. 2002;41(11):1249–60.

459. Fattahi MJ, Mirshafiey A. Prostaglandins and Rheumatoid Arthritis. Arthritis. 2012;2012:23910.

460. Fidahic M, Jelicic Kadic A, Radic M, Puljak L. Celecoxib for rheumatoid arthritis. Cochrane Database Syst Rev. 2017;2017(6).

461. Spandidos A, Wang X, Wang H, Seed B. PrimerBank: a resource of human and mouse PCR primer pairs for gene expression detection and quantification. Nucleic Acids Res. 2010;38(suppl 1):D792-9.

462. Bustin S, Huggett J. qPCR primer design revisited. Biomol Detect Quantif. 2017;14:19–28.

463. Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. GenBank. Nucleic Acids Res [Internet]. 2005 [cited 2018 Mar 23];33((Database Issue)):D34–8. Available from: https://www.ncbi.nlm.nih.gov/nucleotide/

464. Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics [Internet]. 2012 Jan [cited 2014 Nov 8];13:134. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3412702&tool=pmcentrez&rendertype=abstract

465. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nat Protoc. 2006;1(3):1559–82.

466. Booth CS, Pienaar E, Termaat JR, Whitney SE, Louw TM, Viljoen HJ. Efficiency of the polymerase chain reaction. Chem Eng Sci. 2011;65(17):4996–5006.

467. Sigma. qPCR Technical Guide [Internet]. [cited 2018 Jul 5]. Available from:

https://www.sigmaaldrich.com/technical-documents/articles/biology/pcr-technologies-table-of-contents.html

468. Svec D, Tichopad A, Novosadova V, Pfaffl MW, Kubista M. How good is a PCR efficiency estimate: recommendations for precise and robust qPCR efficiency assessments. Biomol Detect Quantif. 2015;3:9–16.