LYMPHOVASCULAR INVASION IN MELANOMA AND BREAST CANCER

SABREENA SAFUAN

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy
December 2012

Abstract

The theory of metastatic cascade suggests that vasculature plays a central role in the metastatic processes by being the major route of spreading. Two main circulatory systems in the body are responsible for cancer cell dissemination; the blood vascular system and the lymphatic system. However, comparing between these circulatory systems, much less is known about lymphatic vessels, with few studies being conducted about the initial steps of metastasis.

In the first part of this project, a series of 202 formalin fixed paraffin embedded (FFPE) cutaneous melanoma sections were stained with D2-40, CD34 and CD68 to identify lymphatics, blood vessels and macrophages respectively, to examine vessel distribution and the involvement of inflammatory infiltrate in mediating vascular invasion (VI). Sections were also stained by conventional haematoxylin and eosin (H&E), to assess VI, and results compared against those obtained by immunohistochemistry (IHC) that allow discrimination of lymphatic and blood vessel invasion. It was found that lymphatics are mainly located at the peritumoural area of the tumour but intratumoural lymphatics are present and appeared to be functional based on the presence of tumour emboli in the vessels. In addition, vascular invasion in melanoma is mainly lymphatic vessel invasion with H&E assessment underestimating its incidence. Lymphatic vessel invasion were significantly associated with markers of aggressive disease which suggest their importance in melanoma. Lymphatic vessel invasion was also associated with a high macrophage count, suggesting a role for macrophage in mediating the process of metastatic via lymphatic vessels.

In the second part of this project, the adhesion pattern of melanoma and breast cancer cell lines to blood and lymphatic endothelial cell models; large vessel versus microvessel and primary versus immortalised cells were compared. In addition, the effect of macrophage secreted cytokines; TNF-α and IL-1β, tumour conditioned media and macrophage conditioned media on the adhesive process were also studied. Both melanoma and breast cancer cells exhibited a higher level of adhesion to blood compared to the lymphatic endothelial cells. IL-1β stimulation of endothelial cells, tumour cells or both together showed a

significant increased in the percentage of adhered tumour cells to the endothelial cell models with a higher increased to the lymphatic endothelial cells. A significant increased tumour cell adhesion was also observed with macrophage conditioned media and this effect seemed to be associated with the amount of IL-1β present. Interestingly, the increased adhesion effect observed with this supernatant was removed with the use of interleukin-1 converting enzyme (ICE) inhibitor.

Expression of adhesion molecules; CLEVER-1, ICAM-1 and VCAM-1 were examined to study which adhesion molecules might regulate the process of tumour-endothelial interactions. Stimulation of endothelial cell models with IL-1β did not show any significant altered CLEVER-1 expression suggesting that although CLEVER-1 is an important lymphatic specific adhesion molecule, it may not be the principle regulator of tumour-endothelial interactions. This process may be regulated by ICAM-1 and VCAM-1 in which the expression increased significantly upon stimulation with IL-1β.

In the third part of this study, the effect of TNF-α, IL-1β, tumour conditioned media and macrophage conditioned media stimulation on melanoma and breast cancer cell migration were investigated using wound healing assays. Following exposure to TNF-α and IL-1β, a significant increase in the percentage of wound closure was observed and the increase was higher in IL-1β stimulated cells. Similarly, when tumour cells were exposed to macrophage conditioned media, there was an increase in the percentage of wound closure compared to control cells. The effect of IL-1β and macrophage conditioned media on breast cancer cell migration across blood and lymphatic endothelial cells were also studied using Boyden chamber transmigration assay. Significant increased in tumour cells transmigration was observed with IL-1β stimulation, with similar affinity across both endothelial cell types. However, when cells were stimulated with macrophage supernatant from lipopolysaccharide (LPS) stimulated macrophages, an increase transmigratory effect was notably observed to the lymphatic endothelial cells. Interestingly, the increased adhesion effect was removed with the used of ICE inhibitors.

The last part of this study dealt with IL-1β expression in breast tissue samples. 1511 early stage breast cancer tissue microarray samples were stained with commercially available IL-1β antibody to examine the association with lymphatic vessel invasion, clinicopathological variables and clinical outcome. High IL-1β expression in tumour cells was significantly associated with the absence of both intra-tumoural and peri-tumoural lymphatic vessel invasion. A significant association was also observed between low IL-1β expression in tumour cells with breast cancer specific survival and disease free interval.

In conclusion, lymphatic vessels have been found to play a significant role in breast cancer and melanoma cells progression by being the major route for vascular dissemination. In the in-vitro settings, this study has shown that IL-1β, with macrophages as the main producer, could regulate tumour cell invasion especially to the lymphatic circulation. This project has yielded some important results towards understanding of the lymphatic vasculature and modulation of lymphatic vessel invasion. However, more studies are needed to enable translation of research into clinical management of cancer.

Publications resulting from the thesis

1. Storr SJ, Safuan S, Mitra A, Elliott F, Walker C, Vasko MJ, Ho B, Cook M, Mohammed RA, Patel PM, Ellis IO, Newton-Bishop JA, Martin SG; Objective assessment of blood and lymphatic vessel invasion and association with macrophage infiltration in cutaneous melanoma. Mod Pathol. 2012 April; 25(4):493-504.

2. Sabreena S, Sarah J.Storr, Poulam M.Patel, Stewart G.Martion; A comparative study of adhesion of melanoma and breast cancer cells to blood and lymphatic endothelium. (Manuscript has been submitted).

3. Sabreena S, Sarah J.Storr, Mohamed El Refaee, Andrew M.Jackson, Poulam M.Patel, Stewart G.Martin; Pro-inflammatory cytokine IL-1β secreted by macrophages promotes lymphatic vessel invasion in breast cancer and melanoma. (Manuscript in preparation)

Acknowledgement

I would like to express my greatest gratitude to my supervisors, Dr Stewart Martin and Prof Ian Ellis for all their support and guidance throughout this project.

My sincerest appreciation to the Ministry of Higher Education, Malaysia and The Science University of Malaysia for the financial support while doing my PhD at the University of Nottingham.

I am deeply grateful to Dr Sarah Storr and Dr Caroline Woolston for their technical and much needed moral support. Thank you also to all members of the Academic Unit of Oncology who have always been helpful.

My deepest and heartfelt appreciation to my family members, Sabiren, Sirhan, Safuan and Adibah for all their love and support.

Thank you all.

Abbreviations

µg	microgram
µg/ml	microgram per mililiter
µl	microliter
µm	micrometer
µM	micromolar
AJCC	the American Joint Committee on Cancer
ALMM	acral lentiginous malignant melanoma
BEC	blood endothelial cells
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CDK4	cyclin dependent kinase 4
CDKN2A	cyclin dependent kinase inhibitor 2A
CI	confidence interval
CLEVER-1	common lymphatic endothelial and vascular endothelial receptor 1
cm	centimeter
CSF-1	colony stimulating factor 1
d.f	degree of freedom
EBM	endothelial basal media
EC	endothelial cells
ECM	extracellular matrix
EGF	endothelial growth factor
ELISA	enzyme linked immunosorbent assay
ER	oestrogen receptor
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FFPE	formalin fixed paraffin embedded
FITC	fluorescein isothiocyanate
g	gram
GM	geometric mean
H&E	haematoxylin and eosin
HCl	hydrochloric acid
hDMEC	human dermal microvascular endothelial cells
HEVs	high endothelial venules
hMEC-1	human microvascular endothelial cells
hMVEC dLy Neo	human neonatal dermal lymphatic microvascular endothelial cells
HNSCC	head and neck squamous cell carcinoma

HR	hazard ratio
HRP	horseradish peroxidase
hTERT-LEC	human telomerase reverse transcriptase lymphatic endothelial cells
HUVEC	human umbilical vein endothelial cells
ICAM-1	intracellular adhesion molecule 1
ICC	intraclass correlation coefficient
ICE	interleukin 1 converting enzyme
IHC	immunohistochemistry
IL	interleukin
IL1Ra	interleukin 1 receptor antagonists
IL-1β	interleukin 1 beta
IU	international unit
JAMs	junctional adhesion molecule 1
kDa	kilodalton
L	litre
LDH	lactate dehydrogenase
LEC	lymphatic endothelial cells
LMM	lentigo maligna melanoma
LPS-γ	lipopolysaccharide gamma
LVD	lymphatic vessel density
LYVE-1	lymphatic vessel endothelial hyaluronan receptor 1
M	molar
MACS	magnetic cell sorting
MAPK	mitogen-activated protein kinase
MC1R	melanocortin 1 receptor
M-CSF	macrophage colony stimulating factor
$MgCl_2$	magnesium chloride
ml	milliliter
mm	millimeter
mM	millimolar
mm^2	millimeter squared
MR	mannose receptor
MVD	microvessel density
ng/ml	nanogram per milliliter
nm	nanometer
nM	nanomolar
NMM	nodular malignant melanoma
NPI	Nottingham prognostic index
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline

pg/ml	pictogram per milliliter
PI3K	phosphatidylinositol 3’ kinase pathway
PR	progesterone receptor
Prox-1	prospero related homeobox 1
PTEN	phosphatase with tensin homolog
rpm	rotation per minute
S1P	sphingosine-1-phosphate receptor
SBR	Scarff-Bloom-Richardson
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SLN	sentinel lymph node
SSMM	superficial spreading malignant melanoma
TCM	tumour conditioned media
TDL	tumour derived lysate
Thy-1	thymus cell antigen 1
TIL	tumour infiltrating lymphocytes
TMA	tissue microarray
TNF-α	tumour necrosis factor alpha
TNM	tumour nodes metastasis
U/ml	unit per milliliter
UV	ultraviolet
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
VEGFR-3	vascular endothelial growth factor receptor 1
VI	vascular invasion
vWf	von Willebrand factor
χ^2	Pearson Chi Square test of association

Table of Contents

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 5

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

CHAPTER 1: GENERAL INTRODUCTION

1.1 Cancer

Cancer is a class of complex diseases involving dynamic changes in the human genome (Hanahan and Weinberg, 2000). There are more than 100 distinct types of cancer that are characterised by uncontrolled cell growth and spread of abnormal cells in the body.

Cancers are classified by the types of tissue in which they develop (histological type) and by their primary location. From a histological standpoint, this disease is grouped into five major categories that are summarised in Table 1-1.

Table 1-1: Classification of cancer based on histological types. Adapted from (Weinberg, 2007)

Histological types	***Definition***
Tumours arising from epithelial tissue	
Carcinoma	- malignant neoplasm of epithelial origin - 80 to 90% of all cancer cases (breast, colon, lung) - divided into 2 major subtypes; (a) adenocarcinoma –develops in an organ or glands (b) squamous cell carcinoma –originates in the squamous epithelium
Tumours arising from non-epithelial tissue	
Sarcoma	-cancers originating in supportive or connective tissue such as bones, cartilage, tendons, muscle and fat. -named based on the tissue types from which they arise for example; (a) osteosarcoma –arises from bone (b) chondrosarcoma – arises from cartilage (c) liposarcoma –arises from smooth muscle
Haematopoietic malignancies	-cancers arise from various cell types that constitute the blood forming tissue including the cells of immune system. These includes; (a) myeloma - cancer originates from plasma cells of bone marrow which interfere with the production of normal blood cells. (b) leukemia - cancer of the blood or bone marrow associated with overproduction of immature white blood cells. (c) lymphoma - solid tumour of lymphoid cells in the lymphatic system
Neuroectodermal tumours	-cancers arise from cells that form various components of the central and peripheral nervous system -include are gliomas, glioblastomas, neuroblastomas, schwannomas and meduloblastomas.
Others	-cancers that do not fit into the major classifications for example; (a) melanomas – melanocytes, while having embryonic origin close to neuroectodermal cells, settled in the skin and eyes during development and acquire no direct connections with the nervous system. (b) small cell lung carcinoma – contains cells of unclear origins.

Cancers are usually named based on the tissue of origin with histological types of cancer as suffix. For example, hepatocarcinoma is cancer arising from liver parenchyma or liposarcoma is cancer arising from fat cells.

Of all cancers, melanoma and breast cancer are the focus of this study and will be covered in depth in the subsequent chapters.

1.2 Metastatic dissemination of cancer

The process of tumourigenesis involves multistep genetic alterations that transform normal human cells into highly malignant cells. In order for a cell to become malignant, it must successfully overcome many barriers and normal regulatory circuits that govern the human body. According to Hanahan and Weinberg (2000), there exist six essential alterations in cell physiology that collectively dictate malignant growth, which are; (1) sustained growth signalling, (2) non-sensitivity to growth suppressor signalling, (3) the ability to evade apoptosis, (4) limitless replicative potential, (5) the ability to induce angiogenesis and (6) tissue invasion and metastasis. The authors suggested that most if not all cancer cells have acquired these six capabilities during their transformation from normal cells through various mechanistic strategies. Recently, with the advancement in cancer research, Hanahan and Weinberg (2011) have suggested two emerging hallmarks to these six core hallmarks, which are reprogramming of energy metabolism to fuel cell growth and division and evading immune destruction.

Metastasis, an extension of local invasion process involved the spread of tumour cells from primary tumour site in which cancer develops to other site of the body. Metastasis remains a therapeutic challenge in the field of cancer research in which 90% of cancer patients succumb to multiple secondary tumour growths (Sporn, 1997).

There are two theories that explain the occurrence of metastasis. Late stage or clonal selection theory hypothesises that only a small fraction of cancer cells in the primary tumour will have selective advantage to cause distant metastasis through various accumulations of genetic alterations that take place as the

cancer progresses (Talmadge, 2007). Contrary to the late stage theory, the recent advances in microarray based approaches have shown that the ability of tumour cells to metastasise may not be acquired over time but is a pre-programmed event giving them an inherited general predisposition for metastasis (Weigelt et al., 2005).

Different types of tumours have different preferential sites of metastasis. Breast cancer commonly spreads to the bones, lungs and liver whilest melanoma frequently spread to the lungs and bones (Chen et al., 2009). There have been a number of different hypotheses regarding the involvement of certain organs in the metastatic dissemination of cancer. The ‘seed and soil’ theory, proposed by Stephen Paget in 1889 (Paget, 1889), stated that metastatic process did not occur by chance but occurred in an organ-specific manner in which certain organs provided a well suited environment (soil) for the tumour cells (seeds) to grow. The tendency for certain tumours to preferentially grow in particular organs suggests that local growth factors, specific endothelial cell surface receptors (Kuwashima, 1997; Paschos et al., 2009) and specific chemotactic agents (Mochizuki et al., 2004) differentially expressed by different organs might be responsible for the distribution pattern of metastasis. Contrary to the ‘seed and soil’ theory, James Ewing in 1928 proposed that the anatomical structure of the vascular system is solely accountable for the distribution pattern of metastasis (Ribatti et al., 2006). He hypothesised that when tumour emboli, following detachment from the primary tumour site and following intravasation into the circulation got mechanically trapped in the vessel of the first organ encountered, metastasis are likely to occur in that particular organ.

Regardless of hypotheses, a malignant cell must go through a series of sequential steps, collectively known as the metastatic cascade for a successful metastasis. A tumour cell must be able to (a) detach from the primary tumour mass, (b) invade and migrate through the extracellular matrix, (c) penetrate into the local vasculature (blood and/or lymphatic vessels), (d) survive the host immune response within the circulation, (e) extravasate into the new tissue/organ, and (f) adapt and divide to form a new tumour. For these to occur,

a tumour cell must undergo various genetic and epigenetic changes to overcome protective mechanisms within the host body and immune system (Yokota, 2000). The latter steps of this metastatic cascade i.e movement of cells in the circulation, extravasation and colonisation into secondary site have been well documented. It is known that cancer cells express endothelial adhesion receptors similar to leukocytes, therefore mimicking leukocyte transmigration from the blood circulation into the tissue following inflammation. Basically, circulating leukocytes extravasate from the circulation through the open inter-endothelial junctions in a multi-step processes which involves (a) leukocyte rolling on endothelial cells, (b) activation of integrins via G-protein coupled receptors, (c) tight adhesion to the endothelial cells and (d) leukocyte diapedesis

On the contrary, there is a lack of knowledge about the initial steps of metastasis that leads to, and includes, intravasation of cancer cells into the circulation, especially in carcinomas. The initial steps of metastasis, collectively termed invasion is believed to include multiple events such as alteration in cellular adhesion and cellular motility that eventually leads to intravasation of cancer cells into the circulation. These initial steps of metastasis are the focus of this thesis and will be discuss in greater details in Chapters 2, 3, 4 and 5.

The theory of metastatic cascade suggests that the tumour vasculature plays a central role in the metastatic processes by being the major route of spreading. Tumour cells could spread via two main circulatory systems in the body (1) the blood vascular system and/or (2) the lymphatic system. The spread of cancer cells via the blood vessels is termed haematogenous spread while the spread of cancer cells via the lymphatics is known as lymphogenous spread. Currently, vascular invasion is identified as the presence of tumour cells within thin walled vessels in haematoxylin and eosin (H&E) stained tissue samples but it is difficult to distinguish between lymphatic vessel invasion and blood vessel invasion. Therefore, are there any other methods that could improve the accuracy of detection? And if so, could these methods be routinely used in clinical settings?

Detection of vascular invasion in patients' specimens will be discussed further in Chapter 2.

For a tumour cell to enter the circulation, they must first adhere to blood or lymphatic endothelial cells before penetration and migration into the vessels. What are the pathways and mechanisms used by the tumour cells in these processes? These metastatic processes i.e adhesion, migration as well as transmigration across the endothelium differ dependent upon the vessel types, the role of cell adhesion molecules, the action of chemokines or cytokines and the presence of inflammatory infiltrates in the tumour environment and will be discussed further in Chapters 3, 4 and 5.

1.3 The lymphatic system

The lymphatic system is composed of a vascular network of lymph vessels and lymphoid tissues and was initially described by Hippocrates (460-360 B.C.) as 'white blood' in an axillary node. However, Aselli, an Italian anatomist was credited with the discovery of lymphatic system when he observed milky liquid in vein-like vessels in the mesentry (Hong et al., 2004b). Since then, many new techniques have been developed to study the gross anatomical details of the lymphatic system such as mercury injection and Evan blue dye injection into the lymphatic vessels and also indirect injection methods (Johnson and Jackson, 2008)

The lymphatic system has many properties in common with the blood vascular system; however, they also have very distinct structural features. This system consists of (1) lymphatic capillaries, (2) collecting afferent and efferent lymphatics, (3) lymph nodes, (4) lymphatic trunks and (5) lymphatic ducts with diameters ranging between 10µm to 2mm. Lymphatic capillaries possess a more irregular and wider lumen than blood capillaries. They lack supporting structure such as pericytes and have incomplete basement membrane. Under normal physiological conditions, the lymphatic capillaries remain in a partially or fully collapsed stage. In blood vessels, tight and adherens junctions maintain firm cell to cell adhesion while focal point adhesion are the main intracellular

junction in lymphatic vessels. Lymph vessels attached to extracellular matrix by anchoring filaments which are elastic fibres opposed to collagen and laminin which attached blood vessels to extracellular matrix (Nathanson, 2003; Stacker et al., 2002). Figure 1-1 shows the structural and functional differences between blood and lymphatic vasculatures.

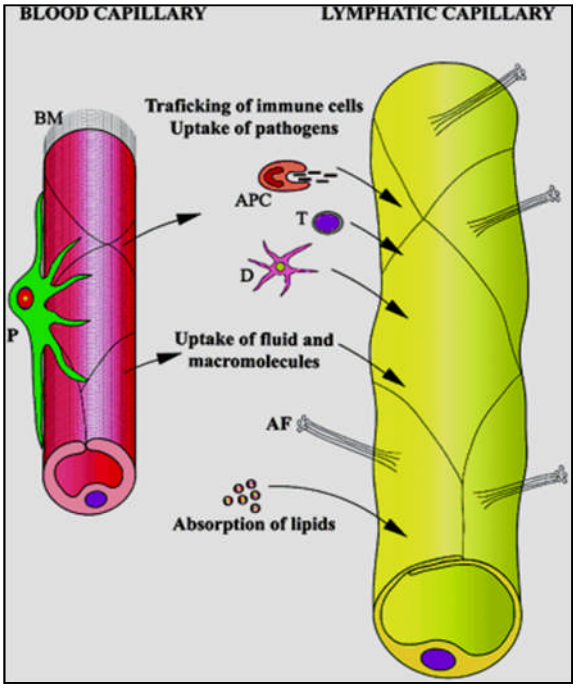


Figure 1-1: Structure of blood and lymphatic capillaries. The lymphatic capillaries differ in that they are wider and possess a more irregular and wider lumen which lack continuous basal lamina and pericytes which are supporting cells that cover the blood endothelial cell in blood capillaries. Adapted from (Pepper and Skobe, 2003)

The lymphatic system plays an important role in the maintenance of tissue homeostasis. The blind-ending vessels of lymphatics collect intercellular fluid from the interstitial compartment and eventually drain it back into the blood circulation via a combination of osmotic and hydrostatic pressure, thus maintaining normal fluid balance within the body (Karpanen et al., 2006). When interstitial pressure within the tissue compartment rises, the overlapping intracellular junctions will be forced to open, permitting the entry of fluid into the lymph vessels. Once inside the lumen, the interstitial fluid is known as the lymph. The lymph fluid is moved along the lymphatic circulation, through the lymph nodes and ultimately into the right lymphatic duct and the thoracic duct where they are returned to the blood circulation via the right and left subclavian

veins (Oliver, 2004). In addition to this, the lymphatic system is also involved in the body's immune defence by directing antigen presenting cells from tissue to the lymph nodes. This system is also responsible for the transportation of fatty acids and fats in the form of milky fluid called chyle (Podgrabinska et al., 2002). Impaired function of lymphatic, congenital or acquired, can result in the formation of lymphedema, a condition of localised fluid retention and tissue swelling which is often caused by inflammatory or neoplastic obstruction (Pepper, 2001). Lymphatic vessels are also involved in metastatic dissemination of cancer cells. Evidence has shown that the extent of lymphangiogenesis i.e. the formation of lymphatic vessels from pre-existing lymphatics create a favourable environment for tumour cells dissemination. Increased lymphatic vessels density in and around the tumour is associated with lymphatic metastasis and reduced patient survival (Straume and Akslen, 2004; Zhang et al., 2009) –this topic would be discussed further in Chapter 2.

Although it was long suggested that the metastatic spread of tumour cells via the lymphatics follows the routes of natural lymph drainage, the exact mechanisms of how tumour cells enter the lymphatic capillaries is still uncertain. The entry of tumour cells into the lymphatic vessels was thought to be a passive process facilitated by a complex interaction between interstitial pressure and the structure of the lymphatics which would not offer a significant barrier for the entry of tumour cells (Gershenwald and Fidler, 2002; Mandriota et al., 2001). However, recent works have suggested that lymphatic endothelium may play a more active role in the recruitment of tumour cells. They secreted chemokines, which are small molecular size protein that attracted tumour cells towards lymphatic capillaries (Achen and Stacker, 2008; Nathanson, 2003). Chemokines receptor-ligand relationships are important to regulate leukocytes trafficking and this relationship was hypothesised to be exploited by cancer cells to modulate entry into the lymphatic circulation (Pepper, 2001). The role of lymphatic specific adhesion molecules is also of particular important because in order for tumour cells to migrate and transmigrate into the lymphatic vessels, they first required an adhesive interaction with lymphatic endothelial cells. To date, many

adhesion molecules preferentially expressed on lymphatic endothelial cells have been discovered and will be discussed further in Section 1.5.

In comparison with vascular circulation, lymphatic research has progressed slowly, mainly because of the lack of histological and immunohistochemical markers that could reliably distinguish between blood and lymphatic endothelial cells. However, in recent years, the identification of lymphatic vessels has been made possible by the discovery of specific lymphatic markers such as (1) D2-40/podoplanin (Breiteneder-Geleff et al., 1999; Kahn and Alexander, 2002), (2) LYVE-1 (Banerji et al., 1999b), (3) Prox-1 (Wigle et al., 2002), and (4) VEGFR-3 (Joukov et al., 1996). Since then, research related to the lymphatics has greatly expanded.

1.4 Molecular markers of lymphatic vessels

1.4.1 D2-40/podoplanin

D2-40 is a monoclonal antibody first developed against an oncofetal antigen (M2A) associated with germ cell neoplasias (Marks et al., 1999). This antibody binds to an O-linked sialoglycoprotein (molecular weight -40kDa) found on lymphatic endothelial cells but not on the endothelium of arteries and veins. Since D2-40 staining is immunonegative in blood endothelium, this antibody has been used as a reliable marker to evaluate lymphatic invasion in many cancer types. However, this antibody is not specific to endothelial cells as it can, in certain settings, stain mesothelial cells, myoepithelial cells, basal and follicular dendritic cells (Kaiserling, 2004).

The commercially available D2-40 antibody binds to a fixation resistant epitope on podoplanin molecules (Schacht et al., 2005b), an integral transmembrane glycoprotein selectively expressed on the apical surface of alveolar type I cells in rat lung. Podoplanin was first reported by Wetterwald et al. (1996) as E11 antigen. It is homologous to T1α, which was detected in choroid plexus, intestine, kidney, thyroid and esophagus of foetal rat (Wetterwald et al., 1996). On endothelial cells, podoplanin is expressed on the lymphatic endothelial cells but not on blood endothelial cells. Its expression has been observed on the

small lymphatic capillaries but not on larger lymphatic trunks or high endothelial venules of the lymph nodes (Breiteneder-Geleff et al., 1999). Ultrastructural analysis revealed its predominant localisation to the luminal surface of lymphatic vessels. Podoplanin is also implicated in the development of lymphatic vessels. Podoplain$^{-/-}$ mice have defects in lymphatic vessels but not blood vessels resulting in the formation of dilated, disorganised lymphatic capillaries (Ramirez et al., 2003; Schacht et al., 2003).

1.4.2 LYVE-1

Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) is a homolog to CD44, a cell surface glycoprotein involved in cell-cell interactions, cell adhesion and migration. LYVE-1 is expressed on luminal and abluminal surfaces of lymphatic endothelial cells of both normal and neoplastic tissue. LYVE-1 is also expressed on tissue especially where high level of hyaluronan absorption and degradation occurs such as activated tissue macrophage and sinusoidal epithelium of the liver and spleen (Banerji et al., 1999a; Banerji et al., 1999b; Hong et al., 2004b; Schledzewski et al., 2006). Although a reliable marker to study lymphatics in normal tissue, LYVE-1 expression is altered in inflammatory conditions. Studies are currently underway to determine how pro-inflammatory cytokines could influence LYVE-1 function and expression (Carreira et al., 2001; Jackson, 2004).

1.4.3 Prox-1

Prospero-related homeobox-1 (Prox-1) is a member of the homeobox gene family and was originally cloned by homology to the *Drosophila gene prospero,* a gene required for normal differentiation of neuronal lineage (Hong et al., 2004a). In early embryonic development, Prox-1 expression in the cardinal vein promotes and maintains the budding of venous endothelial cells that will differentiate into lymphatic endothelium. It was shown that the endothelial cells in Prox-1 deficient mice remain as blood vascular endothelium as they failed to acquire the lymphatic phenotype. In humans, Prox-1 is detected in the nuclei of lymphatic endothelial cells in all stages of development; early embryonic, foetal

and adult tissue but not detected in the blood endothelial cells (Wilting et al., 2002).

1.4.4 VEGFR-3

Vascular endothelial growth factor receptor 3 (VEGFR3) also known as FLT4 is a tyrosine kinase receptor expressed specifically on lymphatic endothelial cells in normal adult tissues. However, it is also expressed on blood endothelium during wound healing and on some tumour blood endothelium as well as the myoepithelial cells. VEGFR-3 binds to vascular endothelial growth factor-C and –D and is associated with lymphangiogenic processes and tumour angiogenesis (Laakkonen et al., 2008). Activated VEGFR-3 stimulates lymphatic endothelial cell proliferation, increasing the ability of cancer cells to metastasise to the lymphatic vessels (Podgrabinska et al., 2002).

1.4.5 Thy-1

Thymus cell antigen-1 (Thy-1) or CD90 has been used as murine T-cell marker for over three decades (Saalbach et al., 2005). However, its expression on lymphatic endothelial cells has only recently been discovered. Thy-1 is strongly expressed by tumour associated lymphatic vessels at much higher levels than blood endothelial cells. Importantly, Thy-1 was up-regulated in lymphatic endothelial cells but not on blood endothelial cells when samples were treated with tumour necrosis factor (TNF) (Jurisic et al., 2010)

In addition to the more commonly used markers discussed above, there are other lymphatic specific markers available. CC chemokine ligand 21 (CCL21) is expressed on lymphatic endothelial cells and high endothelial venules as well as T cell areas of the spleen, lymph node and Peyer’s patches (Gunn et al., 1998). Neurophilin-2 and Angiopoietin-2 are non-tyrosine and tyrosine kinase receptor respectively and have been implicated in lymphangiogenic signalling pathways (Baldwin et al., 2002; Maisonpierre et al., 1997).

On top of these commonly used and commercially available lymphatic specific markers, a number of additional lymphatic specific adhesion molecules are discussed below.

1.5 Adhesion molecules of lymphatic endothelium

1.5.1 Junctional Adhesion Molecules

Junctional adhesion molecules (JAMs) are members of the immunoglobulin superfamily characterised by immunoglobulin folds at their extracellular domain. These 20-30 kDa proteins can be found localised at the tight junctions of endothelial and epithelial cells as well as on the leukocytes and platelets (Ghislin et al., 2011). JAMs are molecules with dual main functions; regulating cell and membrane polarity, in doing so organising the tight junction's formation and also involved in cell-cell and cell-leukocyte interactions.

There are three different homologous molecules in the JAM family, JAM-A, JAM-B and JAM-C (Cera et al., 2004); each has shown differential human tissue expression profiles. JAM-A/JAM-1 has a wider distribution compared to the others. This molecule is present on both epithelial and endothelial cells and mainly localises at the tight junctions of these cells. It is also expressed on circulating leukocytes (neutrophils, monocytes, lymphocytes) and platelets. Knockdown of JAM-A protein expression in MCF7 cells was shown to significantly reduced the breast cancer cell adhesion and migration (McSherry et al., 2011).

Human JAM-B/JAM-2 on the other hand is localised at the tight junctions of both vascular and lymphatic endothelial cells with a more prominent expression on the high endothelial venules (HEVs). As with JAM-A, JAM-C expression can be observed on various human leukocytes subsets, dendritic cells, natural killer cells and platelets. In addition to HEVs, vascular expression of JAM-C can also be found on blood vessels and lymphatic vessels. Regarding their involvement in cancer cell dissemination, JAM-C has been shown as a novel player in melanoma metastasis to the lung which promotes the transendothelial migration of melanoma cells. Endothelial specific JAM-C deficient mice displayed

significantly decreased B16 melanoma cell metastasis to the lung and soluble JAM-C treatment in mice was shown to prevent melanoma lung metastasis (Langer et al., 2011).

1.5.2 Sphingosine-1-phosphate receptor

Sphingosine-1-phosphate receptor (S1P) was thought as an end product of sphingolipids, a class of lipid molecules present on plasma membranes of all eukaryotic cells. For the last fifteen years, research has been focusing on this protein molecule due to findings that S1P can regulate both cell growth and apoptosis. To date, S1P has been shown to be involved in various cellular processes such as angiogenesis, wound healing and tumourigenesis as well as autoimmune diseases such as multiple sclerosis (Kim et al., 2009). However, the role of S1P receptors in controlling lymphocytes trafficking in and out of the lymph nodes is relatively novel with studies reporting that both lymphocytes and lymphatic endothelium strongly express S1P receptors (Matloubian et al., 2004). Furthermore, it was shown that the level of sphingosine kinase 1 (SphK1), which phosphorylates sphingosine to S1P, is overexpressed in various cancer types (van Brocklyn, 2011; Young and van Brocklyn, 2007) and inhibition of SphK1 with either a SphK1 inhibitor or siRNA reduced human liver cancer cell migration and invasion (Bao et al., 2012).

1.5.3 Mannose Receptor

Mannose receptor (MR) is a calcium dependent carbohydrate binding protein. This 180-kDa protein is expressed on macrophages; hence is also known as macrophage mannose receptor, plays a role in cell-cell recognition, serum glycoprotein turnover and in the phagocytosis/endocytosis of pathogens through its recognition of complex carbohydrate structures on the surface of cells (Linehan, 2005).

Besides macrophages, MR is also expressed on the afferent and efferent lymphatics of lymph nodes with no expression observed on HEVs (Irjala et al., 2003), suggesting that the vascular expression of MR is strictly on lymphatic endothelial cells. Functionally, MR has been shown to be involved with

lymphocyte binding to lymphatic endothelium via L-selectin dependent mechanisms. L-selectin is a cell adhesion molecule found on leukocytes that act as receptors for leukocytes to enter lymphoid tissues. Hypothetically, tumour cells that express L-selectin may spread from lymph vessels using the same L-selectin mediated binding to the MR as in the case of lymphocytes. In fact, Irjala et al. (2003) has shown that the binding of head and neck carcinoma cells to the lymphatic endothelium is MR dependent. In their in vitro adhesion assay, a statistically significant reduction (>50%) in the adhesion of squamous cell carcinoma cells was obtained using anti-MR antibody. Another study using MR-deficient mice has shown that the lack of MR in these mice not only leads to faster growth of primary tumours but tumours possessed a smaller metastatic lesion in the draining lymph nodes (Marttila-Ichihara et al., 2008). Their results suggested that, in the absence of MR, tumour cells cannot metastasise efficiently to the lymph nodes.

1.5.4 CLEVER-1

Common lymphatic endothelial and vascular endothelial receptor-1 (CLEVER-1) is a large glycoprotein molecule ranging from 270- to 300-kDa in size. The structure of CLEVER-1 consists of 7 fasciclin domains, 22 epidermal growth factor (EGF)-like repeats and 2 laminin-type EGF-like domains (Shetty et al., 2011). This protein has independently been characterized as FEEL-1, MS-1, Stabilin-1/STAB-1 depending on the detection antibodies used.

The use of these antibodies on fresh frozen tissues revealed some disagreement in the staining pattern as different antibodies recognise different epitopes on the same molecule. FEEL-1 was found to be highly expressed in the spleen and lymph nodes as well as in CD14+ mononuclear cells (Adachi and Tsujimoto, 2002). MS-1 antigen is expressed in macrophages and in the non-continuous sinusoidal endothelial cells in human spleen, liver, lymph nodes and adrenal cortex but no expression was detected in nonsinusoidal continuous endothelium of the same organ (Goerdt et al., 1991). CLEVER-1 on the other hand is expressed on lymphatic vessels and inflamed blood vessels and also on

macrophages and has been used as a marker for regulatory/suppressive macrophages besides a pan-macrophage marker, CD68 (Algars et al., 2011).

In a study comparing the expression of CLEVER-1 between lymphatic and vascular endothelial cells, it was shown that the expression of CLEVER-1 was mainly in lymphatic but not in the normal vascular endothelial cells (Salmi et al., 2004). However, vascular expression of CLEVER-1 can be observed in inflamed vascular vessels surrounded by inflammatory infiltrates and this observation was in accordance with the other studies (Ammar et al., 2011; Irjala et al., 2003; Kzhyshkowska et al., 2006) all of which, using different antibodies, demonstrated vascular expression of CLEVER-1 during wound healing, tumour vascularisation and chronic inflammatory condition of the skin. In addition, using formalin fixed paraffin embedded (FFPE) breast cancer samples, CLEVER-1 expression in vascular vessels was shown to correlate significantly with the density of inflammatory infiltrate, primarily macrophages (Ammar et al., 2011).

Regarding the expression of CLEVER-1 in lymphatic vessels, Ammar et al. (2011) observed that although both hTERT-LEC and HUVEC expressed CLEVER-1, surface expression was only detected on lymphatic endothelial cells. This finding is of special importance in relation to the role of CLEVER-1 as a lymphatic adhesion molecule. An in vitro study utilising flow chamber adhesion assay demonstrated that CLEVER-1 facilitates peripheral blood mononuclear cell (PBMC) transmigration through lymphatic endothelium (Salmi et al., 2004). CLEVER-1 was shown to facilitate lymphocyte binding not only to the lymphatic vessels but also to the HEVs (Irjala et al., 2003). A significant decrease in lymphocyte binding to lymphatic endothelium was observed when an inhibition assay was carried out with anti-CLEVER-1 antibody. CLEVER-1/Stabilin-1 was also shown to support regulatory T cells (Tregs) transendothelial migration across human hepatic sinusoidal endothelial cells under flow conditions (Shetty et al., 2011) .

In a study focusing on the adhesion of tumour cells to the lymphatic endothelium, by utilising Stamper-Woodruff in vitro adhesion assay, Irjala et al. (2003) demonstrated the adhesion of both head and neck squamous cell

carcinoma (HNSCC) and lymphoma cells to lymphatic endothelium and HEVs. Although both tumours differ in their binding affinity to the endothelium, the incorporation of anti-CLEVER-1 antibody significantly reduced the lymphatic adhesion of these cancer cell lines suggesting the role of CLEVER-1 in tumour cells trafficking within the lymphatics. An immunohistochemistry (IHC) study with fresh frozen tumour materials demonstrated heterogeneous lymphatic expression of CLEVER-1 and that this expression was variable between tumours (Irjala et al., 2003). However, this study utilised just a small number of HNSCC samples (n=17) with limited follow-up information. Moreover, CLEVER-1 expression on lymphatic vessels was associated with lymph nodes metastasis and macrophage infiltrates suggesting that CLEVER-1 may be an important mediator of tumour cell metastasis to the lymph nodes which is regulated by inflammatory conditions, most likely by macrophage-associated cytokines (Ammar et al., 2011). Studies to date have shown very promising results regarding the potential of CLEVER-1 as a potential target in anticancer based therapy. However, the ligands of CLEVER-1 in adhesion and transmigration remains unidentified and how CLEVER-1 mediates these events is as yet unclear (Johnson and Jackson, 2008) The role of CLEVER-1 in tumour-endothelial adhesion will be discussed further in Chapter 3.

1.5.5 ICAM-1 and VCAM-1

Apart from lymphatic specific adhesion molecules described above, there are also a number of adhesion molecules which are typically expressed on endothelial cells such as ICAM-1 and VCAM-1. Intracellular adhesion molecule 1 (ICAM-1) or cluster of differentiation 54 (CD54) is a transmembrane glycoprotein in the immunoglobulin superfamily of adhesion molecules. ICAM-1 is expressed at low levels in fibroblasts, endothelial cells and some leukocytes. ICAM-1 has been intensively analysed as an important adhesive receptors on endothelial cells for the firm adhesion of leukocytes. This receptor binds to leukocytes β_2 integrin /LFA-1 in the leukocytes extravasation processes (Senger et al., 2002). ICAM-1 is also present on the surface of many cancer types and in cancer patients, a soluble form of ICAM-1 is present at an elevated level. ICAM-

1 plays an important role in the immune response and most importantly in the metastatic progression of various types of cancer. ICAM-1 expression in human lung cancer and melanoma was associated with advance stages of this cancer which has metastasised (Lin et al., 2006). In addition, ICAM-1 mediates leukocyte-endothelial cell adhesion and may play a role in the development of artherosclerosis and rheumatoid arthritis (Chen et al., 2011).

Vascular cell adhesion molecule 1 (VCAM-1) or cluster of differentiation 106 is a cell surface sialoglycoprotein, a type I membrane protein that is a member of the immunoglobulin superfamily. It is expressed at low concentrations in the membrane of leukocytes, vascular endothelial cells, macrophages and lymphocytes. VCAM-1 is a major ligand for β_1 integrin /very late antigen (VLA)-4 (Senger et al., 2002) and is mainly involved in the inflammation-related leukocyte extravasation. In addition, various inhibition studies have shown that the adhesion of cancer cells to endothelial cells is due to the interaction between the endothelial VCAM-1 and the $\alpha_4\beta_1$ integrins on the cancer cells (Saalbach et al., 2005; Zhu et al., 2002).

It was shown that the expression levels of ICAM-1 and VCAM-1 increases during inflammatory conditions (Hayes and Seigel, 2009) and their expression might be enhanced upon cytokine stimulation such as IL-1 and TNF-α. The expression level of ICAM-1 and VCAM-1 in normal and cytokines stimulated endothelial cells with a focus on lymphatic endothelial cells would be discussed further in Chapter 3.

1.6 Cancer and inflammation

The accumulation of inflammatory cells, i.e inflammatory infiltrate, in tumour microenvironment suggests a link between inflammation and cancer. Epidemiological studies have revealed an association between chronic inflammation and cancer development; for example, inflammatory bowel disease can lead to cancer in the large intestine, and gastric ulceration caused by Helicobacter pylori increases the risk of gastric cancer. Non-steroidal anti-inflammatory agents used as prevention and protection from various tumours

reflect the fact that inflammation is a risk factor for certain cancers. Tumour inflammatory microenvironment consists of (1) innate immune cells such as macrophages, neutrophils, dendritic cells, mast cells and natural killer cells and (2) adaptive immune cells which are the T and B lymphocytes. These cells together with the cancer cells and the surrounding stroma communicate with each other by direct contact or produce cytokine/chemokines to influence tumour growth and survival.

1.6.1 Macrophage

Macrophages are, besides the T cell lymphocytes, a major component of leukocyte infiltrate in the tumour microenvironment. These specialised phagocytic cells differentiate from monocytes and are recruited through the local expression of various chemo-attractants such as colony stimulating factor 1 (CSF-1), macrophage chemo-attractant protein 1 (MCP-1/CCL2), CCL5 and CXCL1 or through the action of other growth factors such as VEGF-A. In human tissue, macrophages are identified by the expression of specific proteins such as CD14, CD11b, EMR1 and CD68 (Mantovani et al., 2008).

There are two pathways of macrophage activation based on triggering signals; the classical M1 macrophage activation or the alternatively activated M2 macrophage also known as tumour associated macrophage (TAM). M1 macrophages are usually associated with an active defense mechanism against microorganisms and tumour cells response to interferon-γ and microbial products such as lipopolysaccharide (LPS) and produce high IL-12 (interleukin) and IL-23 production. By contrast, M2 forms which are activated by IL-4, IL-13, glucocorticoids, IL-10 and immunoglobulin complexes/TLR ligands can suppress immunity, are involved in matrix remodelling (Kzhyshkowska et al., 2008) and can promote both angiogenesis (Hussein, 2006; Joimel et al., 2010) and lymphangiogenesis (Coffelt et al., 2009; Jeon et al., 2008). In terms of lymphatic vessel formation, macrophages express lymphangiogenic growth factors, VEGF-C and VEGF-D which are able to stimulate the lymphatic vessel receptor, VEGFR-3 (Attout et al., 2009). Evidence also shows that macrophages promote metastasis via the production of various cytokines such

as TNF-α (Hahne et al., 1993) and IL1-β (Voronov et al., 2003). These authors suggest that both TNF-α and IL1-β enhance the expression of adhesion molecules on endothelial and malignant cells thus facilitating the invasion of malignant cells into the circulation and their dissemination to remote tissues - this topic will be discussed further in Chapters 3, 4 and 5.

1.6.2 IL-1β

Interleukin 1 beta (IL-1β) is a pro-inflammatory cytokine which belongs to the interleukin 1 gene family besides interleukin 1 alpha (IL-1α) and interleukin 1 receptor antagonist (IL-1Ra) (Song et al., 2003). This cytokine, which could evoke a wide variety of biological effects, is actively secreted by activated human monocytes and macrophages. IL-1β is first synthesised in the cytoplasm as biologically inactive 31kDa precursor, pro-inflammatory IL-1β (proIL-1β) which would remained cytosolic until it is cleaved by interleukin 1 converting enzyme (ICE)/caspase 1 to the active 17kDa form. Once released from the cells into the extracellular space or circulation, IL-1β can trigger interleukin 1 receptor 1 (IL-1R1) on both near and distant cells from the production site. This mature IL-1β released from the cells must compete with interleukin 1 receptor antagonist (IL-1Ra) which binds to IL-1R1 without triggering any signals. The cleavage of pro-IL-1β into its mature form by ICE is a physiologically controlled event; however, other enzymes such as trypsin, elastase and various other proteases can cleave and activate IL-1β under conditions of cell necrosis or mechanical injury (Dinarello, 1996; Voronov et al., 2003).

IL-1β is involved in the regulation of immune response and has been shown to promote inflammation, angiogenesis, tissue remodelling and metastatic spread in human cancer (Watanabe et al., 2012). This cytokine could upregulate major genes involved in inflammation such as nitric oxide synthase (iNOS), cyclooxygenas and phospholipase A2. IL-1β also exerts its effect through the regulation of other proliferative cytokines, growth factors, chemokines and adhesion molecules. A number of studies have reported the association between increased IL-1β expressions in tumour microenvironment with a more virulent tumour phenotype (Lewis, 2006; Saijo et al., 2002b). IL-1β was shown

to be linked with increased tumour invasiveness, angiogenesis and immune suppression of cancer cells. The involvement of IL-1β in the initial steps of metastasis will be discussed further in Chapters 3, 4 and 5.

1.6.3 TNF-α

Tumour necrosis factor alpha (TNF-α) is a multifunctional cytokine which plays diverse important roles in cell survival, proliferation, differentiation and cell death (Hahne et al., 1993; Wang and Lin, 2008). TNF-α is synthesised as a membrane-bound protein, pro-TNF-α which would then be cleaved by TNF-converting enzyme (TACE). TNF-α has been reported to play a role in various pathological responses such as rheumatoid arthritis, septic shock and transplant rejection (Wang and Lin, 2008).

In cancer biology, TNF-α was reported to play a dual role; with both tumour promotion and tumour regression effects. TNF-α expression was higher in various pre-neoplastic and tumour tissues which was associated with the progression of malignant diseases (Lin and Karin, 2007). TNF-α secretion by cancer cells could regulate expression of various cytokines, growth factors and adhesion molecules including expression of angiogenic factors such as vascular endothelial growth factor (VEGF) that could promote angiogenesis and cancer cell metastasis. TNF-α increased tumour cells invasiveness through upregulation of matrix metalloproteases and integrins (Sun et al., 2010; van Grevenstein et al., 2007). Anti-cancer effects of TNF-α may involve the activation of the immune cells such as cytotoxic T-lymphocytes or tumour infiltrating dendritic cells that could trigger a potent adaptive immune response leading to tumour recognition and preventing tumour promotion (Wang and Lin, 2008). The involvement of TNF-α in the initial steps of metastasis will be discussed further in Chapters 3, 4 and 5.

The aims and objectives of the present study were to;

1. Build upon previous work in breast cancer by examining the characteristics of lymphatic vessels (lymphatic vessel density and lymphatic vessel invasion), in cutaneous melanoma, in comparison to blood vessel

characteristics (microvessel density and blood vessel invasion) and to examine such results in light of the level of inflammatory infiltrate (macrophage) and their relationship to tumour clinicopathological characteristics and patient prognosis.

2. Conduct in-vitro investigations of tumour cell adhesion, migration and transmigration using lymphatic and vascular endothelial cell models to understand the regulation of lymphovascular invasion in the tumour and the influence of macrophage and/or macrophage associated factors in these processes.

These aims will form the basis of subsequent chapters.

CHAPTER 2: DISTRIBUTION AND CHARACTERISTICS OF LYMPHATIC AND BLOOD VESSELS, AND OF LYMPHOVASCULAR INVASION IN MELANOMA

2.1 Abstract

Background: Vascular invasion (both to blood and lymphatic) is an important step in the metastatic cascade. Unlike blood vessels, less is known about the role of lymphatic vessels in cancer progression. Progress of lymphatic research has been slow primarily due to the lack of tools which were able to specifically distinguish lymphatic from blood endothelial cells. This chapter aims to investigate the incidence of lymphatic vessel invasion and lymphatic vessel density in comparison with blood vessel invasion and blood vessel density as prognostic biomarkers in cutaneous melanoma patients. In addition, the topography and characteristics of lymphatic and blood vessels in cutaneous melanoma and the association of these characteristics with clinicopathologic variables and clinical outcome were studied. The role of tumour associated macrophages in these processes was also investigated.

Methods: A series of 202 primary invasive cutaneous melanoma samples with a mean follow up time of 38 months were used in this study. Three commercially available immunohistochemical (IHC) markers were used to stain the 202 sections; D2-40, CD34 and CD68 to stain lymphatic vessels, blood vessels and macrophages respectively. To access lymphatic vessel density, each tumour section was divided into two zones; intra-tumoural and peri-tumoural. Positive vessels were counted in each zone which was normalised to the tumour surface area. The sum of lymphatic vessel density in the two zones gave total lymphatic density. Microvessel density and the number of macrophages in each tissue section were counted using the Chalkley method in three hotspot areas with the average value used for analysis. Lymphatic vessel invasion was determined as the presence of tumour cells within D2-40 stained vessels while blood vessel invasion was determined as the presence of tumour cells within CD34 positive vessels but D2-40 negative. All characteristics of lymphatic and blood vessels were tested for association with clinicopathological variables and clinical outcome. The frequency of vascular invasion assessed by IHC was also compared with haematoxylin and eosin (H&E) staining.

Results: Using IHC, vascular invasion was detected in 29.9% of cases (55/184) compared to 8.0% of vascular invasion (16/199) detected by H&E. Of the vascular invasion detected by IHC, 85.5% (47/55) was via the lymphatic vessels opposed to 9.1% (5/55) having blood vessel invasion alone. 5.4% of tumours (3/55) had both lymphatic and blood vessel invasion. Lymphatic vessel invasion was significantly associated with tumour stage, Breslow thickness, Clarks level, ulceration and mitotic rate ($p<0.001$). No associations were observed between relapse-free survival or overall survival with any characteristics of lymphatic and blood vessels assessed. High macrophage counts were significantly associated with Breslow thickness, ulceration and mitotic rate ($p<0.001$, $p<0.001$ and $p=0.005$ respectively). High macrophage counts were also significantly associated with high microvessel density ($p=0.003$) and lymphatic vessel invasion ($p=0.002$).

Conclusions: Vascular invasion in melanoma is essentially invasion of lymphatic vessels with conventional assessment of vascular invasion, by H&E, underestimating its incidence. Detection is significantly improved by the use of IHC. The association of lymphatic vessel invasion with markers of aggressive disease suggests importance in melanoma. A high macrophage count associated with lymphatic vessel invasion suggesting a role for macrophage and/or their associated factors in mediating the process of metastatic spread via lymphatic vessels.

2.2 Introduction and aims

2.2.1 Melanoma

2.2.1.1 Epidemiology and risk factors

Melanoma, a malignant tumour of melanocytes, is the most lethal and most aggressive form of human skin cancer. This disease is mainly localised in the skin (cutaneous melanoma) but can also occur in other body parts such as the bowel and the eyes (uveal melanoma). Although the incidence of melanoma accounts for 5 to 7% of all skin cancers, the mortality data indicated that melanoma causes nearly 80% of all skin cancer related deaths (Gahl et al., 2009). In the USA, 60,000 new cases of melanoma were recorded annually with a mortality rate of 8,000 people and these figures are expected to rise over the next decade at a faster rate compared to other cancers (Lazovich et al., 2010). One reason for this is may be because of the depletion in stratospheric ozone layer leading to increases in solar UV-B radiation at the surface of the earth (Norval et al., 2011)

The risk factors of developing melanoma, as in most cancers, are both intrinsic and environmental with interaction between the two. The incidence of melanoma increases with age with a median age at diagnosis of 57 years (Rajer et al., 2005). Epidemiological studies have established the role of sunlight exposure as the most relevant causative factor of melanoma (Pfeifer and Besaratinia, 2011; Rass and Reichrath, 2008). The risk of melanoma increases with intermittent sun exposure and there is a strong association between early life exposure to UV-A and UV-B with the risk of melanoma in adulthood (Norval et al., 2011). Individuals with three or more atypical (dysplastic) naevi and multiple melanocytic naevi are also considered a significant risk factor for melanoma (Mackie et al., 2009).

5% of all cutaneous melanoma occurs in familial settings involving germline mutations in cell cycle regulatory genes; CDKN2A (cyclin dependent kinase inhibitor 2A) and CDK4 (cyclin dependent kinase 4) genes (Liang et al., 2011). Another melanoma susceptibility gene is the MC1R (melanocortin 1 receptor)

gene which encodes a protein located on the surface of melanocytes. Melanocytes produce melanin, a pigment that gives colour to skin, hair and eyes. Eumelanin and pheomelanin are two forms of melanin produced by melanocyes. Individuals with typical melanoma susceptible phenotypes such as pale Caucasian skin, red or blonde hair and blue eyes possess a higher proportion of MC1R variants which reduce the ability of MC1R to stimulate eumelanin production causing melanocytes to make more pheomelanin. Pheomelanin does not protect individuals from UV radiation; they are more prone to sunburn reactions and other UV-induced skin damaged making these individuals prone to a higher risk of melanoma (Fargnoli et al., 2010; Thody et al., 1991). Table 2-1 summarises the established and postulated risk factors for cutaneous melanoma.

Table 2-1: Established and postulated risk factors for cutaneous melanoma. Adapted from (Mackie et al., 2009)

Risk factors for cutaneous melanoma	
Personal history of melanoma	Invasive cutaneous melanoma in one/more first degree relatives
Multiple melanocytic naevi (>100)	Multiple clinically atypical (dysplastic) naevi (>3)
High/intermittent sun exposure	Higher socioeconomic status
Race/pale Caucasian skin	Use of sunbeds
Red/blonde hair	Occupation
Age	

Key genetic changes in melanoma involve the mitogen-activated protein kinase (MAPK) pathway which regulates crucial cellular activities such as gene expression, apoptosis, proliferation and differentiation of cells. Extensive mutation analysis has identified that BRAF and NRAS mutations occurr in two thirds and one third of melanomas respectively (Curtin et al., 2006). Both mutations cause increased expression of cyclin D1, a regulatory subunit involved in controlling a cell progression through G1 to S phase of the cell cycle. Phosphatidylinositol 3' kinase pathway (PI3K) is also mutated in melanoma. Loss of heterozygosity has been demonstrated in the PTEN (phosphatase and tensin homolog) locus which acts as a tumour suppressor and negative growth

regulator of PI3K activity. Subsequently, this activates the AKT pathway which phosphorylates a number of targets involved in the regulation of cell growth and survival. Figure 2-1 illustrates the MAPK and PI3K pathway in cutaneous melanoma. Besides these, the p53 and retinoblastoma pathway which are frequently inactivated in a number of human cancers are also inactivated in melanoma. Mutational analysis have estimated the incidence of p53 mutation to be 15 to 25% of melanomas (Bardeesy et al., 2001) while mutation of retinoblastoma pathway occurs in 10 to 40% of total melanoma incidence (Fecher et al., 2007a).

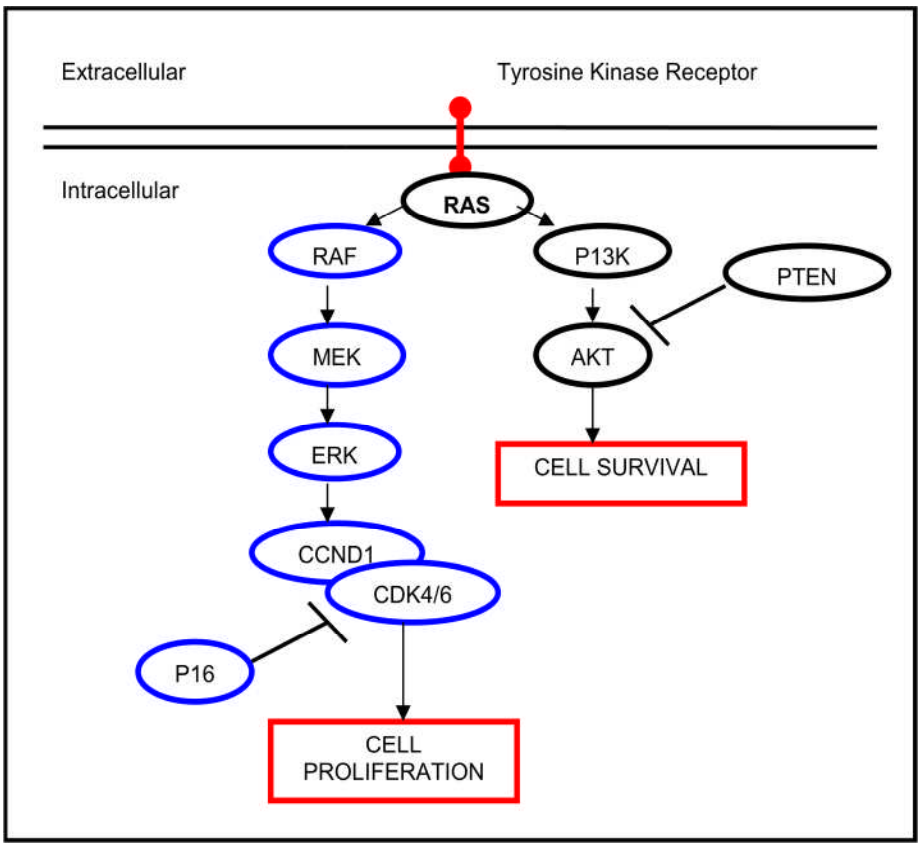


Figure 2-1: The MAPK and PIK3 pathway in melanoma. Receptor tyrosine kinases (RTK) are the key regulators for many cellular processes in human. Defect in any genes involved in this pathway may play critical role in the development of melanoma and its progression. MAPK signalling cascade will be activated upon signals from the RTK which eventually promote cell proliferation. Similarly, these signals will also promote cell survival via the PI3K pathway.

The primary treatment of melanoma is surgical excision with safety excision margins around the tumour site. Narrow excision margins of tumour site (1 to 2

cm) are appropriate compared to wide excision (>3cm) as studies have consistently found equivalent rate of local recurrence and disease free and overall survival between both tumour margins (Balch et al., 2001a; Lens et al., 2002). Interferon alpha-2b (Intron A) is usually used for adjuvant treatment of melanoma which was shown to increase relapse free and overall survival in the group that received high dose of this biologic therapy compared to control group (Kirkwood et al., 1996). However, the use of high dose interferon treatment is still controversial and inconclusive because this treatment was shown to have no effect in overall survival especially in patients with sentinel node micrometastasis (Wheatley et al., 2003).

2.2.2 Histological classification of melanoma

The histological classification of melanoma is based on the microscopic appearance of the tumour. For malignant melanoma, histological criteria of each subtype are related to the location and organisational pattern of melanocytes, cytological features and location of the malignant melanoma (Duncan, 2009). Four major subtypes of malignant melanomas are (1) superficial spreading malignant melanoma (SSMM), (2) nodular malignant melanoma (NMM), (3) acral lentiginous malignant melanoma (ALMM) and (4) lentigo maligna melanoma (LMM).

SSMM is the most frequent form of cutaneous melanoma characterised by the presence of a radial growth phase of epithelioid melanocytes in a pagetoid fashion along the epidermis. Pagetoid is a term used to describe upward spreading of melanocytes into the epidermis. It is common in sun exposed skin especially on the trunk of males and on the lower extremities of females. 25% of SSMM evolved from pre-existing congenital or dysplastic nevi (Forman et al., 2008).

NMM is the second most common types of malignant melanoma. This disease is characterised by the presence of a vertical growth phase and the absence of a radial growth phase. NMM is the most aggressive of all histological types as it grows rapidly in thickness. It can occur anywhere in the body, usually found on

the sun exposed areas of the skin without the need of a pre-existing mole (Gahl et al., 2009).

ALMM is the most frequent histological type of melanoma occurring in Asian and Black populations (Phan et al., 2007). It is observed on the palms, soles and nailbeds (subungual skin) and is characterised by lentiginous proliferation of melanocytes along the basal layer of the epidermis in the radial growth phase (Kuchelmeister et al., 2000).

LMM develop from lentigo maligna, a non invasive skin growth also known as melanoma in-situ or Hutchinson's melanotic freckle. This disease typically found on sun-exposed areas such as the face and neck of elderly people and histologically characterised by the presence of atypical melanocytes along the dermal-epidermal junction (McKenna et al., 2006).

Besides the four major subtypes, other unusual or rare variants of melanoma include desmosplastic melanoma, nevoid melanoma, small cell melanoma, mucosal melanoma and soft tissue melanoma. Histological subtypes of malignant melanoma and its incidence are summarised in Table 2-2.

Table 2-2: Histological subtypes of malignant melanoma and their incidence. Adapted from (Forman et al., 2008; McKenna et al., 2006).

Subtypes	***Incidence (%)***	***Subtypes***	***Incidence (%)***
Major subtypes		**Rare variants**	
Superficial spreading	50 – 75	Desmoplastic melanoma	2 – 4
Nodular	15 – 35	Nevoid melanoma	<1
Lentigo maligna	5 – 15	Small cell melanoma	<1
Acral lentiginous	5 – 10	Mucosal melanoma	<1
		Soft tissue melanoma	<1

2.2.3 Staging of melanoma

The TNM (Tumour Nodes Metastasis) system by the American Joint Committee on Cancer (AJCC) is the most commonly used system to describe the stages of melanoma. This system classifies melanoma based on their (1) tumour size (T),

(2) lymph node involvement (N) which includes in-transit metastasis and (3) distant metastasis (M) which includes plasma level of lactate dehydrogenase enzyme (reviewed in section 2.2.4.8). A summary of AJCC TNM staging system in melanoma is shown in Appendix A-Table A1. The stage groupings and the 5-year overall survival rates are shown in Appendix A-Table A2 (Balch et al., 2009).

2.2.4 Prognosis

2.2.4.1 Breslow depth

At present, the most important feature that influences the prognosis of melanoma patients is Breslow's depth; the thickness of tumour invasion in millimeters. Breslow's depth is measured vertically from the top of granular layer of the epidermis to the deepest point of tumour penetration. Table 2-3 shows the depth of melanoma invasion and approximate 10-year survival rates of patients (Marghoob et al., 2000).

Table 2-3: Breslow thickness (mm) and the approximate 10-year survival rates of patients with melanoma Adapted from (Marghoob et al., 2000).

Breslow thickness (mm)	*Approximate 10-year survival rates (%)*
<1.00	> 92
1.01 – 2.00	80
2.01 – 4.00	63
> 4.00	50

2.2.4.2 Mitotic rate

Mitotic rate, expressed as the number of cells exhibiting mitosis per square milimeter, is an indicator of tumour proliferative rate. According to a recent review by AJCC (Balch et al., 2009), it is identified as the second most powerful and independent predictive factor of survival after Breslow thickness in the T category of the TNM staging system. Many studies have also demonstrated a highly significant correlation between increasing mitotic rates and declining survival rates (Azzola et al., 2003; Murali et al., 2010). Based on the AJCC review, the most significant correlation with survival was identified at a threshold

of at least 1 mitotic rate per mm^2 determined histologically in H&E stained tissue (Balch et al., 2009).

IHC determination using a cell proliferation marker, Ki67, has been shown to correlate with worse prognosis in melanoma (Gimotty et al., 2005) but results have not been consistent (Ohsie et al., 2008). Although Ki-67 is associated with cell proliferation, only a subset of the cells expressing this marker will actually undergo mitosis (Scholzen and Gerdes, 2000).

2.2.4.3 Clark's level of invasion

Clark's level of invasion describes the level of melanoma invasion based on anatomical structure of the skin. This measurement provides a system to relate the degree of melanoma penetration into the skin to the 5-year survival rates after surgical removal of melanoma. Five anatomical skin levels are classified in this system with increase prognostic significant at higher level as follows;

1. Level I: in-situ melanoma which is confined to the epidermis.
2. Level II: invasion into papillary dermis
3. Level III: invasion into the junction of papillary dermis and reticular dermis but no extension to the reticular dermis.
4. Level IV: invasion into reticular dermis
5. Level V: invasion into deep subcutaneous tissue.

With the revised 6^{th} edition of melanoma staging system in 2001, Clark's level was only used to predict prognosis in patients with thin tumours (<1.0mm) as it was shown to be less predictive of outcome and less reproducible in patients with thicker tumour (>1.0mm). However, mitotic rate has replaced Clark's level as the primary criteria in defining T1 patients in the 7^{th} edition of melanoma staging system (Balch et al., 2009; Balch et al., 2001b).

2.2.4.4 Distant metastasis

Lung, central nervous system, liver and bone are the most frequently involved distant sites of metastasis. Metastasis is the term which refers to the spread of tumour cells from primary tumour site in which cancer develops to other site of

the body and this has been discussed in greater details in Section 1.2-Chapter 1. The site of metastatic dissemination can influence prognosis. Of note, superficial distant metastases, for example to the skin, subcutaneous tissue or lymph nodes has better prognostic value that visceral distant metastases such as to the lung, liver or bone (Chen et al., 2009). Table 2-4 shows site of distant metastases in melanoma and the one-year survival rates.

Table 2-4: Site of distant metastasis and 1 year survival rates in melanoma. Adapted from (Balch et al., 2001b).

Site of distant metastasis	***1-year survival rates (%)***
Superficial distant metastasis (distant skin, subcutaneous sites, distant lymph nodes) (M1a)	59
Metastases to the lung (M1b)	57
Any visceral sites	41

* M1a = melanoma patients with distant metastasis in the skin, subcutaneous tissue or distant lymph nodes and a normal LDH level.
*M1b = melanoma patients with metastasis to the lung (or with a combination of lung and skin or subcutaneous metastases) and a normal LDH level.

2.2.4.5 Ulceration

Histologically, ulceration is defined by the absence of an intact epidermis layer over any part of the tumour, which commonly occurs in 20 to 60% of melanoma patients. It can act as independent predictive factor of disease recurrence and worse overall survival (McCready et al., 2000).Survival rates of patients with ulcerated melanoma are lower compared to patients with a non-ulcerated melanoma of the same stage. For example, in stage IIB melanoma (i.e. tumour thickness of >2mm with no nodal involvement and no distant metastasis), patients with ulceration have a 5 year survival rates of 68% compared to 71% in patient without the presence of ulceration (Balch et al., 2009). Furthermore, although ulceration can occur in melanoma of any thickness, its presence is significantly linked to thicker tumours (Walters et al., 2007).

2.2.4.6 Histological type

From the four major histological types of melanoma (reviewed in section 2.2.2) lentigo maligna melanoma is associated with the worse prognosis, followed by

superficial spreading malignant melanoma, acral lentiginous malignant melanoma and nodular malignant melanoma. However, histological type is not a significant independent variable when other prognostic factors are included in multivariate analysis (Morales et al., 1992; Phan et al., 2007).

2.2.4.7 Age

Patient's age is an independent prognostic factor with respect to overall survival. There was a significant decline in the survival rate of patients with melanoma with increasing age at diagnosis (Rutkowski et al., 2010). Each 10 years increase in age was associated with a steady decrease in both 5 and 10 year survival rates as shown in table 2-5.

Table 2-5: 5- and 10-year survival rates by age group. Adapted and reproduced from (Balch et al., 2001b). Copyright© 2001 by American Society of Clinical Oncology with permission conveyed through Copyright Clearance Center Inc.

Age group (years)	***5 year survival rates (% ± SE)***	***10 year survival rates (% ± SE)***
10 – 19	87 ± 2.6	81 ± 3.5
20 – 29	87 ± 1.1	77 ± 1.6
30 – 39	86 ± 0.8	77 ± 1.2
40 – 49	85 ± 0.8	75 ± 1.2
50 – 59	82 ± 0.9	69 ± 1.3
60 – 69	78 ± 1.0	63 ± 1.6
70 – 79	71 ± 1.7	56 ± 2.7
$\geq$ 80	60 ± 5.0	43 ± 7.0

2.2.4.8 Prognostic biomarkers

The use of prognostic biomarkers in cancer is very important as this help to measure disease progression and the effect of treatments on individual patients. Due to the aggressive nature of cutaneous melanoma, there is a critical need to identify the subset of patients with localised lesions that would benefit from adjuvant treatment after surgery.

Lactate dehydrogenase (LDH), an enzyme involved in the conversion of pyruvate and lactate is routinely measured to check for tissue damage

especially to the heart, skeletal muscle, brain and lungs. In patients with cancer, elevated LDH level in the blood is used as an indicator of metastatic cancer. Although LDH is not specific for melanoma, its measurement is useful at time of diagnosis and for post surgery monitoring. The updated AJCC melanoma staging system demonstrates elevated serum LDH levels as an independent and significant predictor of survival especially in stage IV patients. LDH is the only serum biomarker that has been included in the current melanoma staging system (Balch et al., 2009).

S-100B is another serum biomarker that shows promising results in melanoma as an indicator of relapse. This protein is a member of S100 protein family that modulates a large number of biological activities via calcium binding. S100B protein is involved in the regulation of cell morphology, cell differentiation, cell motility, transcription and cell cycle progression. Overexpression of intratumoural S100B has been detected in malignant melanoma with the strength of expression was found to correlate with the degree of malignancy (Harpio and Einarsson, 2004). Comprehensive analysis in high-risk melanoma patients revealed that increased S-100B serum concentration could be used as an indicator of early distant metastasis which is highly specific and relatively sensitive compared to using LDH (Domingo-Domenech et al., 2007; Egberts et al., 2009).

IHC is a widely used technique to investigate protein expression in tumour tissue. High throughput analysis of proteins across large cohort of patients can be studied using tissue microarray where hundreds of samples can be spotted on a single glass slide. Many IHC based studies have been able to identify proteins which could be useful as prognostic biomarkers in melanoma. For example, increased expression of adhesion molecules such as MCAM/MUC18, L1-CAM and CAECAM-1 are significantly associated with worse disease free survival (Li et al., 2002; Xie et al., 1997). Ki-67, PCNA and metallothionine; proteins which contributed to replicative potential are also associated with disease free and overall survival (Fecher et al., 2007b).

Many other markers of malignant melanoma have been identified. Although some of them have been shown to be of diagnostic and prognostic value, the results need to be validated in adequately powered prospective studies for them to be used clinically.

2.2.4.9 Lymph node involvement

The extent of regional lymph node involvement is one of the most reliable prognostic factors available and has long been used as a criterion of tumour aggressiveness. Prognosis is less favourable with increasing numbers of involved lymph nodes (Koskivuo et al., 2007).

Sentinel lymph node (SLN) biopsy is a technique developed to surgically assess subclinical nodal metastasis without the invasiveness and morbidity associated with standard lymphadenectomy; surgical removal of one or more groups of lymph nodes (Nowecki et al., 2008). To determine the sentinel lymph node (first lymph node the tumour is draining into), a low activity radioactive substance and a blue dye is injected near the tumour. The path of the radioactive material towards the draining lymph nodes is recorded and the blue dye assists the surgeon visually in finding the SLN, which is then removed and checked for the presence of cancer cells by a pathologist (Essner, 2006). SLN biopsy is usually carried out in patients with a Breslow thickness of >1mm (Niakosari et al., 2005b). However, in some cases, patients with a tumour thickness less than 1mm are sometimes positive for SLN biopsy because of the aggressive nature of some thinner tumours. Therefore, in addition to these criteria, younger age, sex, mitotic rate, location of primary tumour, the presence and absence of ulceration and lymphovascular invasion are additional clinicopathological criteria suggested as important predictive factor of SLN biopsy in melanoma (Chao et al., 2004; Cuellar et al., 2004; Kesmodel et al., 2004; Sondak et al., 2004). However, although SLN biopsy is used routinely in melanoma patients, the false negative rate reported with this procedure is rather alarming. From the literature, the rate of false negative SLN was reported between 0.4 to 14% (Morton et al., 1999; Phan et al., 2009; Topar et al., 2006). In addition, it was reported that tumour related deaths were not significantly higher in positive SLN

patients compared to patients with negative SLN (Topar et al., 2006), suggesting the need of additional prognostic criteria in melanoma progression.

Lymphovascular invasion encompassing both lymphatic vessel invasion and blood vessel invasion is a commonly reported histological finding in primary tumour assessment. The detection of vascular invasion in primary tumour indicates tumours metastatic potential. In melanoma, it has been reported as a predictor of shorter overall survival and disease free survival (Kashani-Sabet et al., 2001). Currently, lymphovascular invasion is identified using conventional H&E staining as the presence of tumour emboli within vascular spaces lined by a single layer of endothelial cells. Unfortunately this technique cannot discern between blood vessel and lymphatic vessel invasion, and in addition it cannot distinguish tumour emboli that completely obliterate the lumen of vessels or distinguish retraction artefacts from true tumour emboli which eventually will contribute to the false negative and false positive interpretation of those samples.

With advances in immunohistochemical techniques and the discovery of selective markers, blood vessels can now be reliably differentiated from lymphatics. The use of lymphatic specific markers (reviewed in Section 1.4) has enabled lymphatic characteristic in neoplasms to be studied in a robust fashion and by using these markers, lymphatic vessel invasion has been shown to be an important characteristic in various settings and an independent prognostic factor in certain cancers such as breast carcinoma (Mohammed et al., 2008). Lymphatic vessel invasion was reported to not only correlate significantly with SLN positivity but also has strong correlations with distant metastasis, overall survival and disease free interval (Doeden et al., 2009b; Petersson et al., 2009). Questions exist as to whether the detection of tumour cells within lymphatic vessels using lymphatic specific markers should be routinely assessed clinically and whether lymphatic vessel invasion positivity should be use as a predictive factor of SLN biopsy in melanoma and if so, which of these markers give reliable and robust results.

With the discovery of these lymphatic specific markers, a number of studies have investigated lymphatics in solid tumours, however many questions remain unanswered. There is still debate concerning the topography and characteristics of lymphatic vessels in tumour specimens. Lymphangiogenesis, the formation of lymphatic vessels, is thought to play a role in the metastatic dissemination of cancer via lymph vessels. VEGF-C and VEGF-D are novel members of the VEGF family, which can regulate lymphatic vessel formation via VEGFR-3, a receptor predominantly expressed by the lymphatic vessel during development (Plate, 2001; Skobe et al., 2001; Stacker et al., 2001). Activated VEGFR-3 stimulates lymphatic endothelial cell proliferation, increasing the ability of cancer cells to metastasise to lymph vessels in addition to lymph nodes. However, whether actual lymphangiogenesis occurs inside the tumour is still debatable as various publications show variability over the presence of intratumoural lymphatics verses peritumoural lymphatics (Gao et al., 2008; Ji et al., 2007; Longatto-Filho et al., 2007; Williams et al., 2003). Studies using colorectal carcinoma (Omachi et al., 2007) and gastric carcinoma (Gao et al., 2008) have concluded that peritumoural lymphatic vessels, rather that intratumoural are responsible for metastatic spread via the lymphatics. They showed that increased peritumoural lymphatic vessel density (LVD) correlates with increased nodal metastasis and decreased overall survival. Opposing results have been reported which demonstrated that lymphatic proliferation does occur in the intratumoural area and based on the existence of tumour emboli within vessels in this area, it was concluded that these vessels do function as one of the pathways for metastatic spread (Ji, 2006; Mohammed et al., 2008).

Other than using lymphatic specific markers to identify lymphatic vessel density and lymphatic vessel invasion, the assessment of microvessel density and blood vessel invasion is also important in the study of tumour blood vascularity. Angiogenesis, the formation of new blood vessels has long been appreciated to play an important role in tumour progression by being the route for tumour cell dissemination as well as providing nutrients for the growing tumour mass. Both angiogenesis and lymphangiogenesis share similar regulatory mechanisms at the cellular level, occurring through sequential steps that involve the expression

of vascular endothelial growth factors (VEGF's) and matrix metalloproteinases (MMP's) followed by endothelial cell migration, proliferation and organisation into functional vessels. However, although both angiogenic and lymphangiogenic pathways share some common regulatory mechanisms, there are also a distinct regulatory pathways that are related to their specific physiological functions.

CD34 is a widely used endothelial marker to detect the presence of blood vessels. CD34 detects a 110-kDa transmembrane glycoprotein selectively expressed on majority of haematopoietic stem/progenitor cells, bone marrow stromal cells, endothelial cells, embryonic fibroblasts and some nervous tissue. In addition, it is also expressed on dermal dendritic cells, perifolicullar cells of normal skin and stromal spindle-shaped cells in Kaposi's sarcoma (Nickoloff, 1991).

CD31, also known as platelet endothelial cell adhesion molecule-1 (PECAM-1) has also been used as a panvascular marker to demonstrate the presence of endothelial cells in histological tissue sections. This 130-kDa transmembrane glycoprotein is found in large quantities on the surface of endothelial cells and is also expressed on most cells of hematopoietic lineage including monocytes, platelets, neutrophils and some T-cell subsets. The encoded CD31 protein is involved in leukocyte transendothelial migration as well as angiogenesis (Privratsky et al., 2010).

Factor VIII related antigen or von Willebrand factor (vWf) has been used to study angiogenesis in various human tumours. This factor is synthesised in endothelial cells and megakaryocytes and under pathological condition, it mediates the adhesion of platelets to the walls of injured vessels (Muller et al., 2002).

During invasion, tumour cells interact with their microenvironment and are influenced by signals from endothelial, stromal, immune cells and inflammatory cells present in tumour microenvironment. Inflammatory infiltrates such as neutrophils, monocytes, lymphocytes, natural killer cells and macrophages often

exist in this environment and are believed to help promote tumour growth and invasion into the circulation. Of these, macrophages (reviewed in Section 1.6.1-Chapter 1) represent a major inflammatory component of many tumours (Qian and Pollard, 2010) and have been associated with tumour progression due to their involvement in inducing angio- and lymphangiogenesis. Patients with high macrophage densities seem to have worse prognosis in many types of cancers. Therefore, it is of interest in the current study to examine the correlation of macrophage infiltrates in tumour specimens with characteristics of blood and lymphatic vessels as assessed by immunohistochemical markers.

CD68 is a heavily glycosylated transmembrane protein with a molecular weight of 110-kDa which is highly expressed by human monocytes and tissue macrophage. CD68, a homologue of mouse macrosialin, is closely related to the lysosomal associated, mucin-like, membrane protein family. A small fraction of CD68 is found on the cell surface however it is predominantly located in lysosomal membranes. CD68 is widely used as pan-macrophage marker in IHC and this antibody detects both subtypes of macrophages; protumoural M2 macrophages and classical immunostimulating macrophages. as well as various cells of macrophage lineage including monocytes, histiocytes, giant cells, Kupffer cells and osteoclasts (Holness and Simmons, 1993).

The aims and objectives of the current chapter were to;

1. Study the topography and characteristics of lymphatic vessels in cutaneous melanoma and to examine their associations with clinicopathological criteria.
2. Distinguish between lymphatic vessel invasion and blood vessel invasion in cutaneous melanoma by comparing the differential expression of blood vascular marker, CD34 and lymphatic marker, D2-40 in FFPE samples using IHC.
3. Compare between conventional assessment of vascular invasion using H&E with that determined using IHC.
4. Investigate the role of lymphatic and blood vessel invasion, and lymphatic and microvessel density as prognostic biomarkers in cutaneous melanoma patients.

5. Investigate the role of tumour associated macrophage in influencing lymphatic vessel invasion and lymphatic vessel density as well as blood vessel invasion and microvessel density in cutaneous melanoma patients by investigating the associations between macrophage counts and characteristics of the vessels.

The results of this chapter have been published in the journal of Modern Pathology.

2.3 Materials and Methods

2.3.1 Patients and specimens

This study was conducted on 202 consecutive FFPE archival specimens of cutaneous melanoma obtained from the Royal Surrey County Hospital. Ethical approval for this study was granted by Leeds (East) REC (06/Q1206/149). Clinical characteristics of the patients and tumours are summarised in Table 2-6.

Table 2-6: Clinicopathological characteristics of patients

Clinical feature	*Number (%)*	*Clinical feature*	*Number (%)*
Gender		**Sentinal node biopsy**	
Female	99 (49.0)	Negative	70 (34.7)
Male	103 (51.0)	Positive	132 (65.3)
Breslow thickness (mm)		**Histological subtype**	
<1.4	57 (28.2)	Superficial spreading	141 (74.2)
≥1.4 <2.1	50 (24.8)	Nodular	37 (19.5)
≥2.1 <3.4	46 (22.8)	Other	12 (6.3)
≥3.4	49 (24.3)	Not determined	12
Melanoma site		**Diameter (mm)**	
Trunk	73 (36.1)	<9	65 (33.9)
Leg	67 (33.2)	9-12	65 (33.9)
Arm	45 (22.3)	>12	62 (32.3)
Head/Neck	17 (8.4)	Not determined	10
Ulceration		**Number of positive nodes**	
No	145 (74.4)	0	70 (34.7)
Yes	50 (25.6)	1 or 2	123 (60.9)
Not determined	7	≥3	9 (4.5)
Clarks level		**Mitotic rate (per mm^2)**	
II/III	44 (22.0)	<3	68 (33.8)
IV	141 (70.5)	3-7	62 (30.8)
V	15 (7.5)	>7	71 (35.3)
Not determined	2	Not determined	1
Perineural infiltration		**Regression**	
No	186 (98.4)	No	171 (85.5)
Yes	3 (1.6)	Yes	29 (14.5)
Not determined	13	Not determined	2
Tumour infiltrating lymphocytes (TILs)		**Microsatellites**	
Absent	52 (26.4)	No	163 (86.7)
Non-brisk	126 (64.0)	Yes	25 (13.3)
Brisk	19 (9.6)	Not determined	14
Not determined	5		

The median age of patients at time of diagnosis was 53 years (range: 14 to 88 years). 65.3% (n=132) patients were positive for SLN biopsy with 28.2% (n=57) having Breslow thickness of <1.4mm. 61.6% (n=62) patients relapsed and 45 patients died from the disease. Mean follow up period for the cohort was 38 months. Relapse-free survival was calculated from the date of initial surgery to the first relapse or from the date of the initial surgery to the last date known to have not relapsed for those censored. Overall survival time was calculated from the date of initial surgery to death or from the date of initial surgery to the last date known to be alive for those censored.

2.3.2 Immunohistochemistry

Three consecutive FFPE melanoma sections from each patient were stained with commercially available markers; CD34, D2-40 and CD68 to stain blood vessels, lymphatic vessels and macrophages respectively. Staining optimisation was conducted on tonsil tissue and breast composite whole sections before using them in the main melanoma cohort. 4µm thick whole sections from each specimen were deparaffinised in two histolene baths for 5 minutes each then rehydrated in a series of descending ethanol concentrations (100%, 90%, 70%, 50% and 30% in water for 1 minute at each concentration). For CD34 and CD68, antigen retrieval was carried out in 0.01molL^{-1} sodium citrate buffer (pH6) in a microwave for 20 minutes; 10 minutes at full power (750W) followed by 10 minutes at low power (450W). For D2-40, antigen retrieval was not required (Mohammed et al., 2007). Endogenous hydrogen peroxidase activity was then blocked in 0.3% hydrogen peroxide in methanol for 10 minutes. Non-specific staining was blocked using normal swine serum (DAKO) (1:5 dilution in Tris buffered saline, TBS).

Following blocking, sections were incubated with the primary antibody diluted in blocking solution (CD34 1:500, D2-40 1:100, CD68 1:100) for 1 hour at room temperature. Details of antibodies used are summarised in Table 2-7. Unbound primary antibody was washed with TBS prior to the addition of biotinylated secondary antibody (1:100 dilution in blocking buffer) for 30 minutes followed by the addition of avidin-biotinylated horseradish peroxidase (1:100 dilution in

blocking buffer) for another hour at room temperature (StreptABComplex/HRP Duet, Mouse/Rabbit kit, DAKO, K0492). For CD34 and D2-40, immunohistochemical reactions were developed using 3, 3́ diaminobenzidine (Liquid DAB+ Substrate Chromogen System, DAKO, K3468) for 7 minutes. Sections were then counterstained with Gills formula Haematoxylin (Vector Laboratories) for 3 minutes and rinsed off under running tap water. Sections were dehydrated in a series of ascending ethanol concentrations (30%, 50%,70%, 90% and 100% in water for 1 minute at each concentration), fixed in histolene and mounted with DPX. For CD68, immunoreactions were visualised using permanent red solution (AEC+ Substrate Chromogen, DAKO) to distinguish macrophages from melanocytes, added for 20 mins prior to counterstaining with Gills formula Haematoxylin. Slides were mounted straight away, omitting the dehydration steps, using aqueous-based Glycergel mounting medium (Dako, 0365). Sections were left to dry overnight before viewing under the microscope.

Tonsil sections were used as both positive and negative controls each time staining was conducted. The procedure as above was applied for the positive controls. For the negative control, primary antibody was omitted. In addition, H&E staining was conducted on a consecutively cut sections and used to assess vascular invasion alongside CD34 and D2-40 staining.

Table 2-7: Antibodies used in IHC

Antibody	***Source***	***Type***	***Antigen Retrieval***	***Concentration***
CD34	AbD Serotec MCA5479 (Oxford, UK)	Rabbit polyclonal	Microwave	1:500
D2-40	Covance SIGNET 3730-1000 (Cambridge, UK)	Mouse monoclonal	Not required	1:100
CD68	Abcam AB955-500 (Cambridge, UK)	Mouse monoclonal	Microwave	1:100

2.3.3 Microscopic analysis

2.3.3.1 Assessment of microvessel density (MVD) and macrophage count

Sections stained with CD34 and CD68 were used for the evaluation of microvessel density and macrophages count respectively using the Chalkley 25-point counting method which has been reliably used to assess angiogenesis in malignant tumours (Fox et al., 1994; Fox et al., 1995). With this method, a 'hotspot,' i.e. the area with highest vessels/macrophages staining, was identified in each of the stained slides at 100x magnification. The Chalkley eyepiece graticule was adjusted so that there was a maximum overlap between graticule points and the positively stained vessels/macrophages (Figure 2-2). These points of overlap were counted at 200x magnification. The mean value from three hotspot areas for an individual tumour was used for analysis. 20% of slides were examined by two independent pathologists blinded to clinicopathologic data and scores. Rabab A. A. Mohammed assessed MVD and blood vessel invasion and Bernard Ho examined the macrophages count.

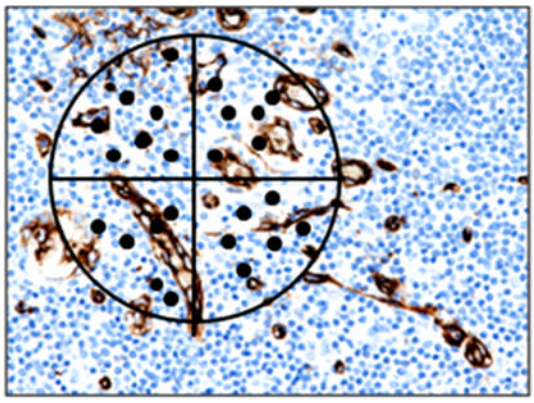


Figure 2-2: Representation of a hotspot of CD34 positive vessels superimposed with Chalkley graticule. The grid is adjusted so that there was a maximum overlap between positively stained vessels with the black dots of the graticule. Average value from 3 counts is the MVD for an individual tumour which was used for analysis (200x magnification).

2.3.3.2 Assessment of location/distribution of lymphatic vessels and lymphatic vessel density

Sections stained with D2-40 were used for the evaluation of lymphatic vessel density across the whole section. Two characteristics of lymphatic vessels were examined i.e. (1) the distribution of lymphatic within (intra-tumoural) and around (peri-tumoural) the tumour and (2) the lymphatic vessel density across the

whole section. With this method, each tumour section was examined by low power magnification (40x) and divided into two areas, intra-tumoural being the area of the tumour, and peritumoural area being the surrounding normal area within one high power microscopic field under the microscope (100x magnification) as shown in Figure 2-3. Positively stained vessels were counted across the total tumour area by counting fields of view using 100x magnification representing a surface area of 153.86mm^2. Lymphatic vessel density in each area was obtained by dividing the sum of lymphatic vessel density in all fields in that area, presented as vessels/mm^2. Total lymphatic vessel density for each specimen is the sum of both intra-tumoural lymphatic vessel density with peri-tumoural lymphatic vessel density. 30% of slides were examined by three independent assessors blinded to clinicopathological data and scores. Mark J Vasco assessed lymphatic vessel density and lymphatic vessel invasion and Martin Cook and Ian O. Ellis assessed lymphatic vessel invasion.

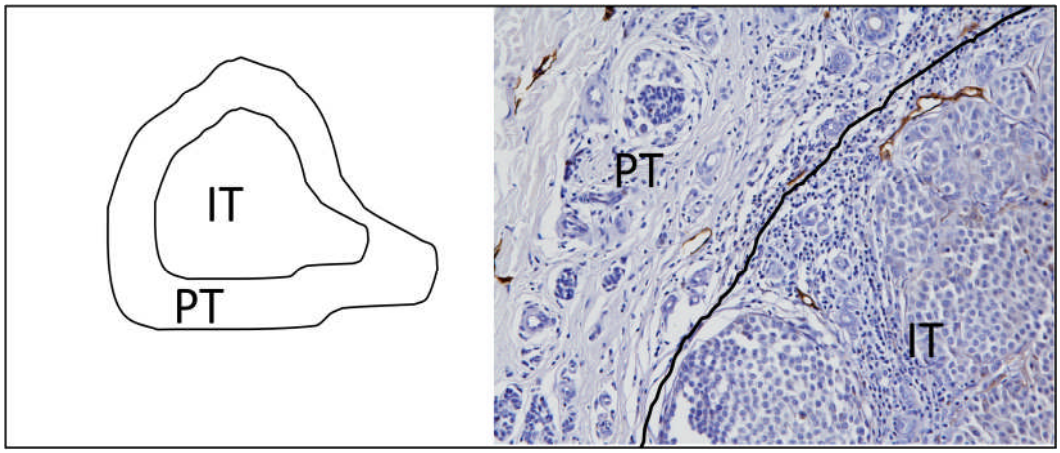


Figure 2-3 Representation of how tumour sections were divided into two areas to assess the distribution of lymphatic vessels. Left panel: schematic diagram of a tumour section divided into intratumoural-IT and peritumoural-PT area. Right panel: division of IT and PT area in an actual tumour section (200x magnification).

Most studies have used the hotspot methodology to assess lymphatic vessel density in tissue samples (Doeden et al., 2009b; Giorgadze et al., 2004). Although the method of examining lymphatic vessel density across the whole tumour section as adopted in this study is time consuming, it allows all

lymphatic vessels across the whole tumour section to be assessed enabling information of lymphatic distribution to be obtained (Mohammed et al., 2007).

2.3.3.3 Assessment of vascular invasion

Sections stained with D2-40 and CD34 were used for the evaluation of vascular invasion. Lymphatic vessel invasion was identified as the presence of tumour cells within a D2-40 stained vessel. As CD34 can also stain a subset of lymphatic vessels, blood vessel invasion was defined when tumour cells were detected in CD34 positive but D2-40 negative vessels. Sections with lymphatic vessel invasion or blood vessel invasion were categorised as vascular invasion positive. All probable lymphatic vessel invasions were reviewed by consultant pathologist (Prof Ian Ellis and/or Martin Cook). Vascular invasion was reported as negative, probable or positive. In term of analysis, probable vascular invasions were group with the positive cases. Vascular invasion in H&E stained sections were also examined by consultant pathologist (Mark Vasco and/or Martin Cook) and were reported as negative, probable or positive. The frequency of vascular invasion detected by H&E staining was compared with that detected by IHC. False positive cases were recorded when H&E sections were positive for vascular invasion but negative in IHC. False negatives were recorded when vascular invasion was negative in the H&E sections but positive in IHC. The location of lymphatic vessel invasion was also assessed in a similar way as was used in the lymphatic vessel density assessment, i.e. within the total tumoural area, intra-tumoural or within the area surrounding the tumour, peri-tumoural. Some tumours were not scored due to missing tissue during the staining and preparation techniques or due to a lack of peri-tumoural area.

2.3.4 Statistical analysis

Microvessel density, lymphatic vessel density and macrophage count were plotted in distribution histograms and classified into two groups according to the median value (high$\geq$median, low<median). Statistical analysis was performed using these groups with grouped clinicopathological parameters. Vessel invasion was divided into negative, probable or positive groups and used as a

basis to determine association between all parameters (microvessel density, lymphatic vessel density, blood vessel invasion, lymphatic vessel invasion and macrophage count). Using Pearson Chi Square test of association (χ^2) (or Fisher's Exact test if a cell count was <5), the relationship between vascular invasion, vessel density and clinicopathological data were assessed. Overall survival and relapse free survival analyses were performed using the Kaplan-Meier method and the statistical significance between groups assessed by the long rank test. Multivariate survival analysis was performed using Cox regression analysis. An arbitrary significance level of 1% was used. Concordance between results from individual observers was evaluated using intraclass correlation coefficient (ICC). Statistical analysis was carried out by Sabreena Safuan, Sarah Storr as well as collaborators from Leeds University; Angana Mitra and Julia Newton-Bishop using SPSS version 17.0 and STATA version 10 (StataCorp 2007, Stata statistical software: Release 10, College Station, TX: Stata CorpLP).

2.4 Results

2.4.1 Distribution of lymphatic and blood vessels in cutaneous melanoma

Figure 2-4 summarises the distribution of lymphatic vessels within the intra-tumoural and peri-tumoural area as well as the total lymphatic vessel density count in 202 cutaneous melanoma samples used in this study. The majority of lymphatic vessels were located in the peri-tumoural area and were observed in all cases where this area was assessable (181/181). Intra-tumoural lymphatic vessels were detected in 86.4% of tumours (165/191). Peri-tumoural lymphatic vessel density ranged from 0.009 to 0.198/mm^2 and intra-tumoural lymphatic vessel density ranged from 0.000 to 0.130/mm^2. Total lymphatic vessel density ranged from 0.004 to 0.266/mm^2. The medians of intra-tumoural, peri-tumoural and total lymphatic vessel density were 0.010/mm^{2,} 0.060/mm^2 and 0.077/mm^2 respectively.

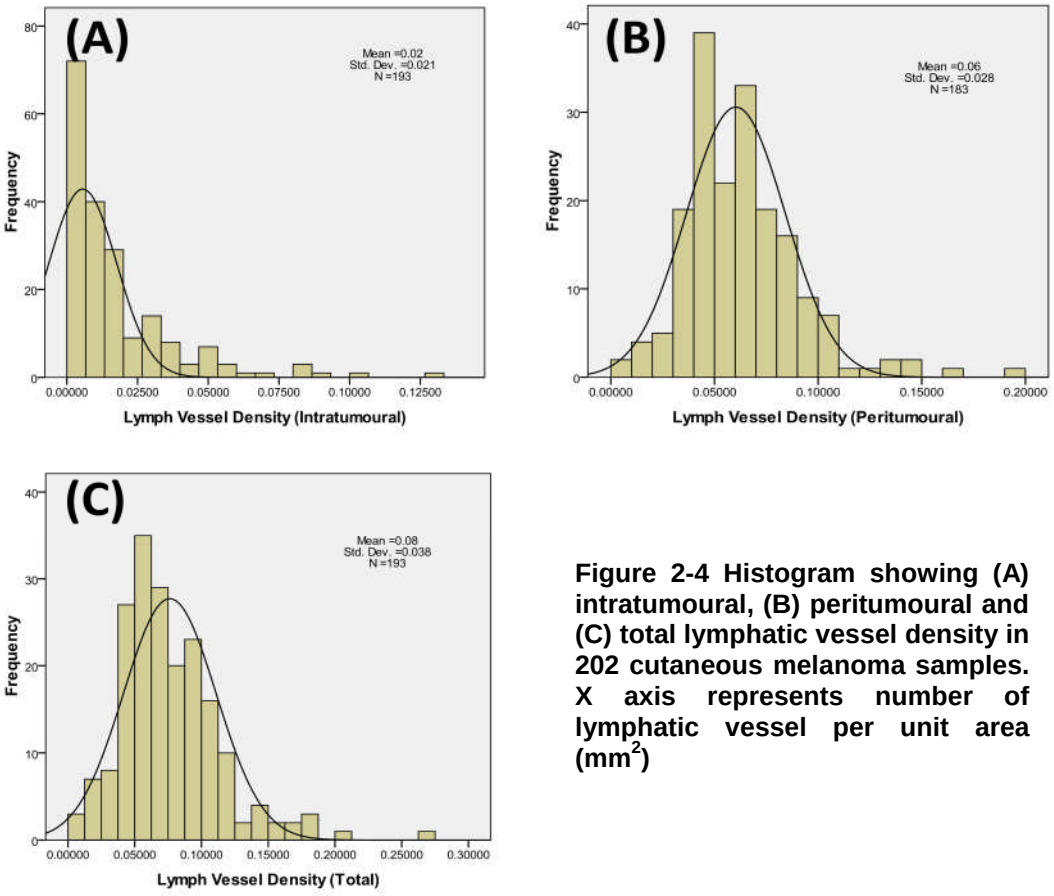


Figure 2-4 Histogram showing (A) intratumoural, (B) peritumoural and (C) total lymphatic vessel density in 202 cutaneous melanoma samples. X axis represents number of lymphatic vessel per unit area (mm^2)

Figure 2-5 shows the distribution of microvessel density in the total cohort of 202 cutaneous melanoma patients. Microvessel density ranged from 0.667 to 9.000 with a median of 4.883 vessels. Lymphatic vessel density and microvessel density were not directly compared due to the difference in their assessment methods; which were required due to the high frequency of blood vessels observed in all samples in comparison to the relatively low level of lymphatic vessel density in the same samples. However, it was observed that the density of blood vessels were much higher than that of lymphatic vessels under the same field view (Figure 2-6).

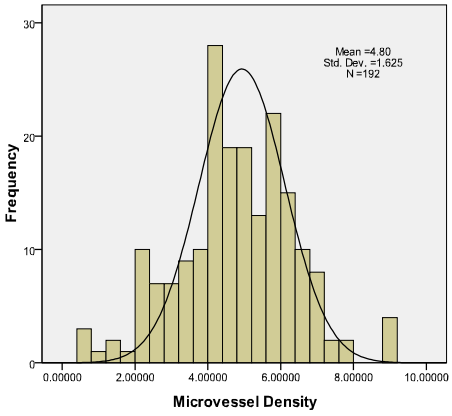


Figure 2-5 Histogram showing microvessel density in 202 cutaneous melanoma samples. X axis represents number of microvessel per hotspot count.

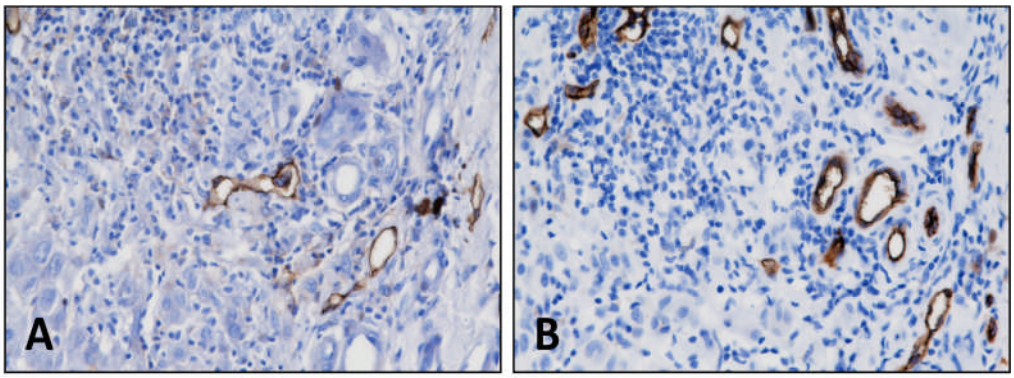


Figure 2-6 Morphology and distribution of (A) lymphatic vessels and (B) blood vessels in melanoma. It was observed that the density of blood vessels was much higher compared to lymphatic vessels under the same microscopic field view. 200x magnification.

2.4.2 Relationship between lymphatic and microvessel density with clinicopathological variables

Table 2-8 and 2-9 show the association between lymphatic vessel density and microvessel density respectively with clinicopathologic variables in melanoma. No significant association was observed between intra-tumoural lymphatic vessel density and clinicopathologic variables however, it was marginally associated with relapse status (χ^2=4.91, d.f.=1, p=0.03) and tumour infiltrating lymphocytes (TILs) (χ^2=6.08, d.f.=2 p=0.05). Peri-tumoural lymphatic vessel density and total lymphatic vessel density was significantly associated with Clarks level of invasion (Fisher's Exact test, p=0.01 and p=0.01 respectively). Total lymphatic vessel density was also significantly associated with the absence of tumour infiltrating lymphocytes (χ^2=11.21, d.f.=2, p=0.004). High microvessel density was associated with the presence of ulceration (χ^2=17.79, d.f.=1, p<0.001) and high mitotic rate (p=0.002). It was positively associated with stage (χ^2=12.91, d.f.=2, p<0.001) and was marginally associated with positive sentinel lymph nodes biopsy (χ^2=4.01, d.f.=1, p=0.05).

2.4.3 Distribution and frequency of blood and lymphatic vessel invasion in melanoma detected by IHC and H&E staining

Vascular invasion; both lymphatic and blood vessel invasion was assessed in consecutive melanoma sections. Intratumoural, peritumoural or any lymphatic vessel invasion (total invasion from combination of intratumoural and peritumoural invasion) was detected in 17.3% (33/191), 14.9% (27/181), 26.9% (50/186) of cases respectively. Blood vessel invasion occurred in 4.2% (8/190) cases, 3 of which also had lymphatic vessel invasion.

The presence of vascular invasion determined by IHC and H&E was also directly compared. Invasion of both blood and lymphatic; categorised as any vascular invasion was detected in 29.9% of cases (55/184) by IHC opposed to 8.0% of cases (16/199) detected by H&E. Of the vascular invasion detected by IHC 85.5% (47/55) were purely invasion of lymphatics, 9.1% (5/55) were solely via the blood vessels while 5.4% had both lymphatic and blood vessel invasion (3/55).

Table 2-8: Association between lymphatic vessel density and clinicopathological characteristics

Categories		***Lymphatic Density n(%)***								
		Intra-tumoural			*Peri-tumoural*			*Total*		
		Low	*High*	*p value*	*Low*	*High*	*p value*	*Low*	*High*	*p value*
Gender	Female	48 (50.5)	44 (45.8)	0.52	45 (50.6)	44 (47.8)	0.71	50 (52.1)	42 (44.2)	0.28
	Male	47 (49.5)	52 (54.2)		44 (49.4)	48 (52.2)		46 (47.9)	53 (55.8)	
Sentinal node biopsy (SNB)	Negative	38 (40.0)	28 (29.2)	0.12	35 (39.3)	31 (33.7)	0.90	36 (37.5)	30 (31.6)	0.39
	Positive	57 (60.0)	68 (70.8)		26 (29.9)	28 (30.8)		60 (62.5)	65 (68.4)	
Relapse Status	No	57 (61.3)	71 (76.3)	0.03	61 (70.1)	63 (69.2)	0.90	61 (65.6)	67 (72.0)	0.34
	Yes	36 (38.7)	22 (23.7)		26 (29.9)	91 (30.8)		32 (34.4)	26 (28.0)	
Staging	IA/IB	23 (25.6)	17 (18.1)	0.69	23 (26.7)	17 (19.3)	0.58	22 (24.2)	18 (19.4)	0.78
	IIA	6 (6.7)	5 (5.3)		6 (7.0)	5 (5.7)		6 (6.6)	5 (5.4)	
	IIB/IIC	6 (6.7)	5 (5.3)		5 (5.8)	6 (6.8)		5 (5.5)	6 (6.5)	
	IIIA	38 (42.2)	45 (47.9)		33 (38.4)	44 (50.0)		37 (40.7)	46 (49.5)	
	IIIB	17 (18.9)	22 (23.4)		19 (22.1)	16 (18.2)		21 (23.1)	18 (19.4)	
Number of positive nodes	0	38 (40.0)	28 (29.2)	0.31	35 (39.3)	31 (33.7)	0.35	36 (37.5)	30 (31.6)	0.37
	1 or 2	53 (55.8)	63 (65.6)		48 (53.9)	58 (63.0)		54 (56.3)	62 (65.3)	
	≥3	4 (4.2)	5 (5.2)		6 (6.7)	3 (3.3)		6 (6.3)	3 (3.2)	
Melanoma site	Trunk	40 (42.1)	32 (33.3)	0.27	35 (39.3)	32 (34.8)	0.44	35 (36.5)	37 (38.9)	0.12
	Leg	25 (26.3)	38 (39.6)		30 (33.7)	29 (31.5)		35 (36.5)	28 (29.5)	
	Arm	23 (24.2)	19 (19.8)		20 (22.5)	21 (22.8)		23 (24.0)	19 (20.0)	
	Head/neck	7 (7.4)	7 (7.3)		4 (4.5)	10 (10.9)		3 (3.1)	11 (11.6)	
Histological subtype	Superficial spreading	65 (74.7)	70 (75.3)	0.21	68 (79.1)	64 (74.4)	0.72	67 (75.3)	68 (74.7)	0.55
	Nodular	14 (16.1)	20 (21.5)		13 (15.1)	17 (19.8)		15 (16.9)	19 (20.9)	
	Other	8 (9.2)	3 (3.2)		5 (5.8)	5 (5.8)		7 (7.9)	4 (4.4)	

Table 2-8: Cont..

Categories		***Lymphatic Density***								
		Intra-tumoural			*Peri-tumoural*			*Total*		
		Low	*High*	*p value*	*Low*	*High*	*p value*	*Low*	*High*	*p value*
Diameter (mm)	<9	29 (32.2)	32 (34.8)	0.90	32 (37.6)	28 (31.8)	0.70	29 (32.2)	32 (34.8)	0.28
	9-12	31 (34.4)	29 (31.5)		26 (30.6)	31 (35.2)		26 (28.9)	34 (37.0)	
	>12	30 (33.3)	31 (33.7)		27 (31.8)	29 (33.0)		35 (38.9)	26 (28.3)	
Breslow thickness (mm)	<1.4	31 (32.6)	23 (24.0)	0.40	27 (30.3)	27 (29.3)	1.00	29 (30.2)	25 (26.3)	0.47
	≥1.4 <2.1	21 (22.1)	24 (25.0)		21 (23.6)	22 (23.9)		22 (22.9)	23 (24.2)	
	≥2.1 <3.4	18 (18.9)	26 (27.1)		21 (23.6)	22 (23.9)		18 (18.8)	26 (27.4)	
	≥3.4	25 (26.3)	23 (24.0)		20 (22.5)	21 (22.8)		27 (28.1)	21 (22.1)	
Ulceration	No	69 (76.7)	67 (71.3)	0.41	66 (75.9)	64 (73.6)	0.73	69 (75.0)	67 (72.8)	0.74
	Yes	21 (23.3)	27 (28.7)		21 (24.1)	23 (26.4)		23 (25.0)	25 (27.2)	
Clarks level	II/III	20 (21.5)	22 (22.9)	0.37	20 (22.7)	22 (23.9)	**0.01**	20 (21.3)	22 (23.2)	**0.01**
	IV	63 (67.7)	69 (71.9)		56 (63.6)	68 (73.9)		61 (64.9)	71 (74.7)	
	V	10 (10.8)	5 (5.2)		12 (13.6)	2 (2.2)		13 (13.8)	2 (2.1)	
Mitotic rate (per mm^2)	<3	38 (40.4)	26 (27.1)	0.15	36 (40.9)	26 (28.3)	0.20	39 (41.1)	25 (26.3)	0.08
	3-7	26 (27.7)	32 (33.3)		24 (27.3)	31 (33.7)		24 (25.3)	34 (35.8)	
	>7	30 (31.9)	38 (39.6)		28 (31.8)	35 (38.0)		32 (33.7)	36 (37.9)	
Perineural infiltration	No	85 (96.6)	90 (100)	0.12	81 (96.4)	85 (100.0)	0.12	86 (96.6)	89 (100.0)	0.25
	Yes	3 (3.4)	0 (0.0)		3 (3.9)	0 (0.0)		3 (3.4)	0 (0.0)	
Regression	No	78 (83.9)	82 (85.4)	0.77	75 (86.2)	75 (81.5)	0.40	81 (86.2)	79 (83.2)	0.57
	Yes	15 (16.1)	14 (14.6)		12 (13.8)	17 (18.5)		13 (13.8)	16 (16.8)	
Microsatellites	No	76 (86.4)	79 (88.8)	0.63	69 (83.1)	78 (91.8)	0.09	74 (84.1)	81 (91.0)	0.16
	Yes	12 (13.6)	10 (11.2)		14 (16.9)	7 (8.2)		14 (15.9)	8 (9.0)	
Tumour infiltrating lymphocytes (TILs)	Absent	32 (34.4)	18 (19.1)	0.05	28 (32.2)	19 (20.7)	0.21	35 (37.6)	15 (16.0)	**0.004**
	Non-brisk	54 (58.1)	64 (68.1)		51 (58.6)	62 (67.4)		50 (53.8)	68 (72.3)	
	Brisk	7 (7.5)	12 (12.8)		8 (9.2)	11 (12.0)		8 (8.6)	11 (11.7)	

Table 2-9: Association between microvessel density, macrophage count and blood vessel invasion with clinicopathological characteristics

Category		*Microvessel density*			*Macrophage count*			*Blood vessel invasion*			*Vascular invasion*		
											H&E		
		Low	*High*	*p value*	*Low*	*High*	*p value*	*Absent*	*Present*	*p value*	*Absent*	*Present*	*p value*
Gender	Female	44 (46.3)	45 (47.4)	0.88	51 (53.7)	44 (44.0)	0.18	86 (47.3)	3 (37.5)	0.73	90 (49.2)	6 (37.5)	0.37
	Male	51 (51.3)	50 (52.6)		44 (46.3)	56 (56.0)		96 (52.7)	5 (62.5)		93(50.8)	10 (62.5)	
Sentinal node biopsy (SNB)	Negative	38 (40.0)	25 (26.3)	0.05	32 (33.7)	36 (36.0)	0.73	61 (33.5)	2 (25.0)	1.00	68(37.2)	2(12.5)	0.06
	Positive	57 (60.0)	70 (73.7)		63 (66.3)	64 (64.0)		121 (66.5)	6 (75.0)		115(62.8)	14(87.5)	
Relapse Status	No	62 (68.1)	63 (67.0)	0.87	66 (72.5)	65 (65.7)	0.31	121 (68.4)	4 (50.0)	0.23	126(70.8)	8(50)	0.09
	Yes	29 (31.9)	31 (33.0)		25 (27.5)	34 (34.3)		56 (31.6)	4 (50.0)		52(29.2)	5(50)	
Staging	IA/IB	29 (31.2)	10 (11.1)	**<0.001**	22 (23.9)	19 (19.6)	0.05*	39 (22.2)	0 (0.0)	0.47	42(23.7)	1(6.7)	0.21
	IIA	6 (6.5)	3 (3.3)		5 (5.4)	6 (6.2)		9 (5.1)	0 (0.0)		10(5.6)	1(6.7)	
	IIB/IIC	2 (2.2)	9 (10.0)		2 (2.2)	10 (10.3)		10 (5.7)	1 (14.3)		12(6.8)	0(0.0)	
	IIIA	44 (47.3)	40 (44.4)		48 (52.2)	37 (38.1)		80 (45.5)	4 (57.1)		75(42.4)	11(73.3)	
	IIIB	12 (12.9)	28 (31.1)		15 (16.3)	25 (25.8)		38 (21.6)	2 (28.6)		38(21.5)	2(13.3)	
Number of positive nodes	0	38 (40.0)	25 (26.3)	0.06*	32 (33.7)	36 (36.0)	0.91	61 (33.5)	2 (25.0)	0.41	68 (37.2)	2(12.5)	0.09
	1 or 2	51 (53.7)	67 (70.5)		59 (62.1)	59 (59.0)		113 (62.1)	5 (62.5)		107(58.5)	13(81.3)	
	≥3	6 (6.3)	3 (3.2)		4 (4.2)	5 (5.0)		8 (4.4)	1 (12.5)		8(4.4)	1(6.3)	
Melanoma site	Trunk	34 (35.8)	37 (39.0)	0.82	32 (33.7)	39 (39.0)	0.19	67 (36.8)	4 (50.0)	0.38	65(35.5)	7(43.8)	0.62
	Leg	34 (35.8)	28 (29.5)		39 (41.1)	27 (27.0)		59 (32.4)	3 (37.5)		61(33.3)	5(31.3)	
	Arm	20 (21.1)	23 (24.2)		19 (20.0)	25 (25.0)		43 (23.6)	0 (0.0)		43(23.5)	2(12.5)	
	Head/neck	7 (7.4)	7 (7.4)		5 (5.3)	9 (9.0)		13 (7.1)	1 (12.5)		14(7.7)	2(12.5)	
Histological subtype	Superficial spreading	70 (77.8)	64 (71.9)	0.36	73 (81.1)	63 (67.0)	0.03	130 (75.1)	4 (66.7)	0.37	127 (73.8)	12 (80.0)	0.80
	Nodular	14 (15.6)	21 (23.6)		11 (12.2)	26 (27.7)		34 (19.7)	1 (16.7)		35(20.3)	2(13.3)	
	Other	6 (6.7)	4 (4.5)		6 (6.7)	5 (5.3)		9 (5.2)	1 (16.7)		10(5.8)	1(6.7)	

Table 2-9: Cont…

		Microvessel density			***Macrophage count***			***Blood vessel invasion***			***Vascular invasion (H&E)***		
		Low	*High*	*p value*	*Low*	*High*	*p value*	*Absent*	*Present*	*p value*	*Absent*	*Present*	*p value*
Diameter (mm)	<9	36 (38.7)	22 (25.0)	0.14	36 (40.0)	27 (28.1)	0.23	55 (31.4)	3 (50.0)	0.45	63(36.0)	2(13.3)	0.02
	9-12	28 (30.1)	34 (38.6)		27 (30.0)	26 (37.5)		61 (34.9)	1 (16.7)		54(30.9)	10(66.7)	
	>12	29 (31.2)	32 (36.4)		27 (30.0)	33 (34.4)		59 (33.7)	2 (33.3)		58(33.1)	3(20.0)	
Breslow thickness (mm)	<1.4	35 (36.8)	18 (18.9)	0.02	34 (35.8)	20 (20.0)	**<0.001**	53 (29.1)	0 (0.0)	0.24	55 (30.1)	1 (6.3)	0.14
	≥1.4 <2.1	21 (22.1)	24 (25.3)		31 (32.6)	17 (17.0)		42 (23.1)	3 (37.5)		45 (24.6)	4 (25.0)	
	≥2.1 <3.4	22 (23.2)	21 (22.1)		20 (21.1)	25 (25.0)		41 (22.5)	2 (25.0)		40 (21.9)	5 (31.3)	
	≥3.4	17 (17.9)	32 (33.7)		10 (10.5)	38 (38.0)		46 (25.3)	3 (37.5)		43 (23.9)	6 (37.5)	
Ulceration	No	80 (87.0)	54 (59.3)	**<0.001**	79 (85.0)	60 (62.5)	**<0.001**	130 (73.9)	4 (57.1)	0.39	129 (72.9)	13 (86.7)	0.36
	Yes	12 (13.0)	37 (40.7)		14 (15.0)	36 (37.5)		46 (26.1)	3 (42.9)		48 (27.1)	2 (13.3)	
Clarks level	II/III	23 (24.2)	19 (20.4)	0.62	20 (21.3)	22 (22.2)	0.75	42 (23.2)	0 (0.0)	0.33	43 (23.6)	0 (0.0)	0.04
	IV	66 (69.5)	65 (69.9)		68 (72.3)	68 (68.7)		124 (68.5)	7 (100.0)		126 (69.2)	13 (86.7)	
	V	6 (6.3)	9 (9.7)		6 (6.4)	9 (9.1)		15 (8.3)	0 (0.0)		13 (7.1)	2 (13.3)	
Mitotic rate (per mm^2)	<3	41 (43.2)	21 (22.3)	**0.002**	36 (38.3)	27 (27.0)	**0.005**	61 (33.7)	1 (12.5)	0.29	64 (35.2)	3 (18.8)	0.03
	3-7	30 (31.6)	28 (29.8)		35 (37.2)	26 (26.0)		56 (30.9)	2 (25.0)		51 (28.0)	10 (62.5)	
	>7	24 (25.3)	25 (47.9)		23 (24.5)	47 (47.0)		64 (35.4)	5 (62.5)		67 (36.8)	3 (18.8)	
Perineural infiltration	No	87 (96.7)	87 (100.0)	0.25	86 (96.6)	94 (100.0)	0.11*	168 (98.2)	6 (100.0)	1.00	170 (99.4)	13 (86.7)	0.02
	Yes	3 (3.3)	0 (0.0)		3 (3.4)	0 (0.0)		3 (1.8)	0 (0.0)		1 (0.6)	2 (13.3)	
Regression	No	84 (88.4)	75 (80.7)	0.14	83 (88.3)	81 (81.8)	0.21	151 (83.9)	8 (100.0)	0.61	155 (85.2)	13(86.7)	1.00
	Yes	11 (11.6)	18 (19.3)		11 (11.7)	18 (18.2)		29 (16.1)	0 (0.0)		27 (14.8)	2(13.3)	
Microsatellites	No	79 (87.8)	74 (86.0)	0.73	78 (87.6)	80 (86.0)	0.75	149 (87.6)	4 (66.7)	0.18	149 (87.6)	11(73.3)	0.13
	Yes	11 (12.2)	12 (13.0)		11 (12.4)	13 (14.0)		21 (12.4)	2 (33.3)		21(12.4)	4(26.7)	
Tumour infiltrating lymphocytes (TILs)	Absent	28 (30.4)	21 (22.3)	0.45	35 (37.2)	16 (16.5)	**0.005**	44 (24.7)	5 (62.5)	0.07	48(26.7)	4(26.7)	0.59
	Non-brisk	56(60.9)	63(67.0)		51 (54.3)	71 (73.2)		116 (65.2)	3 (37.5)		113(62.8)	11(73.3)	
	Brisk	8(8.7)	10 (10.6)		8 (8.5)	10 (10.3)		18 (10.1)	0 (0.0)		19(10.6)	0(0.0)	

184 samples had both H&E and IHC results available and vessel invasion in these cases were compared. In this group of cases, 14 patients had vascular invasion evident in sections stained by H&E, 9 of which were shown to be false positives by IHC (64.33%). Of the 170 cases negative for vascular invasion determined by H&E, 50 were false negative (29.4%). Examples of H&E and IHC staining are shown in Figure 2-7, 2-8 and 2-9.

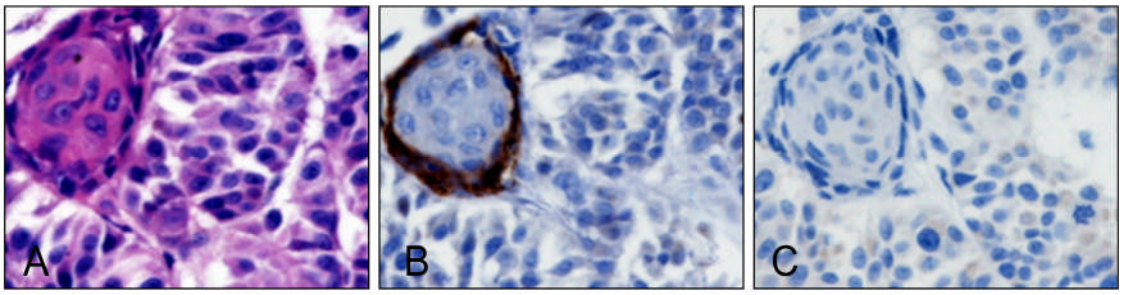


Figure 2-7 Example of false negative determinant in H&E stained tissue. In this tumour, vascular invasion in H&E stained tissue (A) was scored as negative. However, using IHC, vascular invasion was scored as positive. This specimen will be scored as lymphatic vessel invasion positive because tumour emboli were detected within D2-40 vessels (B) but CD34 negative (C). 200x magnification

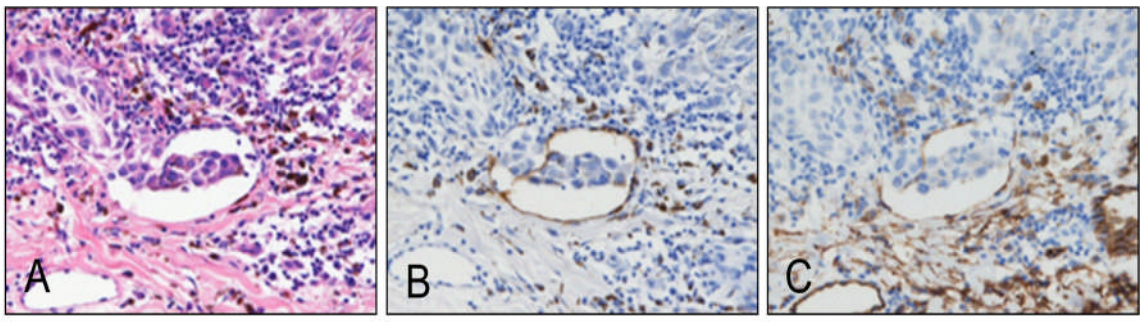


Figure 2-8 Example of true positive lymphatic vessel invasion determination in H&E stained tissue. In this tumour, vascular invasion in H&E stained tissue (A) was scored as positive. The presence of vascular invasion is clearly observed in D2-40 stained tissue (B) and was scored as lymphatic vessel invasion positive. Consecutive sections stained with blood marker, CD34 (C) was also shown. 200x magnification

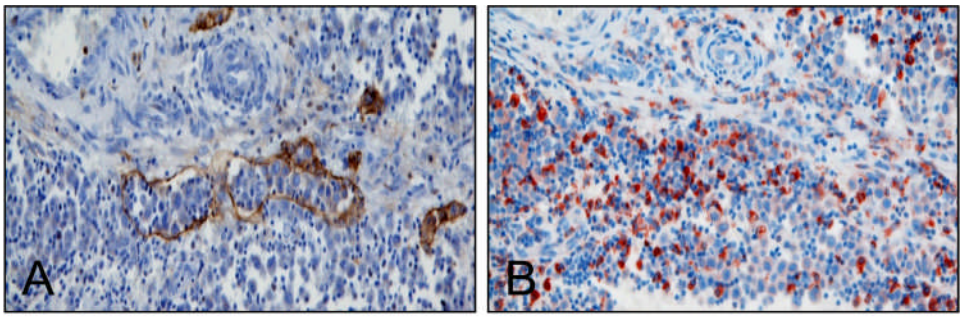


Figure 2-9 Examples of positive lymphatic vessel invasion and macrophage infiltrates. In this tumour, the presence of vascular invasion is clearly observed in D2-40 stained tissue (A) and was scored as lymphatic vessel invasion positive. Consecutive section showed macrophage infiltrates surrounding the lymphatic vessels (B).

2.4.4 Relationship between vascular invasion and macrophage count with clinicopathological characteristics

Intra-tumoural lymphatic vessel invasion was significantly associated with increased stage (Fisher's Exact test, p=0.004), increased Breslow thickness (Fisher's Exact test, p<0.001), the presence of ulceration (χ^2=14.69, d.f.=1, p<0.001), increased Clark's level (Fisher's Exact test, p<0.001), high mitotic rate (Fisher's Exact test, p<0.001), the presence of microsatellites (χ^2=6.18, d.f.=1, p=0.01) and nodular histological subtype (χ^2=18.94, d.f.=2, p<0.001) (Table 2-10). Significant association of peri-tumoural lymphatic vessel invasion was observed with increased stage (Fisher's Exact test, p<0.001), increased Breslow thickness (Fisher's Exact test, p=0.002) the presence of ulceration (χ^2=16.99, d.f.=1, p<0.001), and nodular histological subtype (Fisher's Exact test, p<0.001) (Table 2-10). However, peri-tumoural lymphatic vessel invasion was not associated with Clark's level, mitotic rate or the presence of microsatellites. Any lymphatic vessel invasion was significantly associated with increased stage (Fisher's Exact test p<0.001), increased Breslow thickness (χ^2=31.56, d.f.=3, p<0.001), the presence of ulceration (χ^2=22.59, d.f.=1, p<0.001), increased Clark's level (χ^2=16.83, d.f.=2, p<0.001), high mitotic rate (χ^2=18.90, d.f.=2, p<0.001), the presence of microsatellites (χ^2=6.64, d.f.=1, p=0.01), and nodular histological subtype (χ^2=28.47, d.f.=2, p<0.001). Blood vessel invasion was not significantly associated with any clinicopathological variable. Table 2-10 summarises the association of these parameters with clinicopathological criteria of cutaneous melanoma.

Table 2-10: Association between lymphatic vessel invasion with clinicopathological characteristics

Category		***Lymphovascular invasion***									***Any vessel invasion***		
		Intra-tumoural			*Peri-tumoural*			*Any lymphovascular invasion*					
		Absent	*Present*	*p value*	*Absent*	*Present*	*p value*	*Absent*	*Present*	*p value*	*Absent*	*Present*	*p value*
Gender	Female	78 (49.4)	14 (42.4)	0.47	76 (49.4)	13 (48.1)	0.91	68 (50.0)	23 (46.0)	0.63	64 (49.6)	25 (45.5)	0.61
	Male	80 (50.6)	19 (57.6)		78 (50.6)	14 (51.9)		68 (50.0)	27 (54.0)		65 (50.4)	30 (54.5)	
Sentinal node biopsy (SNB)	Negative	58 (36.7)	8 (24.2)	0.17	58 (37.7)	8 (29.6)	0.42	53 (39.0)	13 (26.0)	0.10	49 (38.0)	14 (25.5)	0.10
	Positive	100 (63.3)	25 (75.8)		96 (62.3)	19 (70.4)		83 (61.0)	37 (74.0)		80 (62.0)	41 (74.5)	
Relapse Status	No	110 (71.0)	18 (58.1)	0.16	109 (72.2)	15 (55.6)	0.08	98 (73.1)	29 (60.4)	0.10	93 (73.2)	31 (58.5)	0.05
	Yes	45 (29.0)	13 (41.9)		42 (27.8)	12 (44.4)		36 (26.9)	19 (39.6)		34 (26.8)	22 (41.5)	
Staging	IA/IB	38 (25.0)	2 (6.3)	**0.004**	39 (26.4)	1 (3.8)	**<0.001**	37 (28.2)	3 (6.3)	**<0.001**	35 (28.0)	3 (5.8)	**<0.001**
	IIA	10 (6.6)	1 (3.1)		10 (6.8)	1 (3.8)		9 (6.9)	2 (4.2)		8 (6.4)	2 (3.8)	
	IIB/IIC	7 (4.6)	4 (12.5)		6 (4.1)	5 (19.2)		5 (3.8)	6 (12.5)		5 (4.0)	6 (11.5)	
	IIIA	71 (46.7)	13 (37.5)		69 (46.6)	8 (30.8)		62 (47.3)	17 (35.4)		59 (47.2)	20 (38.5)	
	IIIB	26 (17.1)	13 (40.6)		24 (16.2)	11 (42.3)		18 (13.7)	20 (41.7)		18 (14.4)	21 (40.4)	
Number of positive nodes	0	58 (36.7)	8 (24.2)	0.34	58 (37.7)	8 (29.6)	0.05	53 (39.0)	13 (26.0)	0.16	49 (38.0)	14 (25.5)	0.09
	1 or 2	93 (58.9)	23 (69.7)		91 (59.1)	15 (55.6)		78 (57.4)	33 (66.0)		76 (58.9)	36 (65.5)	
	≥3	7 (4.4)	2 (6.1)		5 (3.2)	4 (14.8)		5 (3.7)	4 (8.0)		4 (3.1)	5 (9.1)	
Melanoma site	Trunk	63 (39.9)	9 (27.3)	0.43	59 (38.3)	8 (29.6)	0.77	56 (41.2)	13 (26.0)	0.26	53 (41.1)	16 (29.1)	0.46
	Leg	52 (32.9)	11 (33.3)		48 (31.2)	11 (40.7)		42 (30.9)	19 (38.0)		39 (30.2)	21 (38.2)	
	Arm	32 (20.3)	10 (30.3)		35 (22.7)	6 (22.2)		28 (20.6)	14 (28.0)		28 (21.7)	14 (25.5)	
	Head/neck	11 (7.0)	3 (9.1)		12 (7.8)	2 (7.4)		10 (7.4)	4 (8.0)		9 (7.0)	4(7.3)	
Histological subtype	Superficial spreading	120 (81.6)	15 (45.5)	**<0.001**	121 (81.8)	11 (45.8)	**<0.001**	111 (85.4)	22 (46.8)	**<0.001**	106 (85.5)	24 (48.0)	**<0.001**
	Nodular	20 (13.6)	14 (42.4)		19 (12.8)	11 (45.8)		13 (10)	20 (42.6)		13 (10.5)	20 (40.0)	
	Other	7 (4.8)	4 (12.1)		8 (5.4)	2 (8.3)		6 (4.6)	5 (10.6)		5 (4.0)	6 (12.0)	

Table 2-10		***Lymphovascular invasion***									***Any vessel invasion***		
Category		*Intra-tumoural*			*Peri-tumoural*			*Any lymphovascular invasion*					
		Absent	*Present*	*p value*	*Absent*	*Present*	*p value*	*Absent*	*Present*	*p value*	*Absent*	*Present*	*p value*
Diameter (mm)	<9	53 (34.9)	8 (26.7)	0.09	49 (33.6)	11 (40.7)	0.66	46 (35.1)	14 (29.8)	0.52	42 (33.6)	15 (30.0)	0.60
	9-12	45 (29.6)	15 (50.0)		50 (34.2)	7 (25.9)		41 (31.3)	19 (40.4)		40 (32.0)	20 (40.0)	
	>12	54 (35.5)	7 (23.3)		47 (32.2)	9 (33.3)		44 (33.6)	14 (29.8)		43 (34.4)	15 (30.0)	
Breslow thickness (mm)	<1.4	51 (32.3)	3 (9.1)	**<0.001**	52 (33.8)	2 (7.4)	**0.002**	49 (36.0)	5 (10.0)	**<0.001**	47 (36.4)	5 (9.1)	**<0.001**
	≥1.4 <2.1	42 (26.6)	3 (9.1)		38 (24.7)	5 (18.5)		37 (27.2)	6 (12.0)		34 (26.4)	8 (14.5)	
	≥2.1 <3.4	35 (22.2)	9 (27.3)		36 (23.4)	7 (25.9)		30 (22.1)	14 (28.0)		28 (21.7)	16 (29.1)	
	≥3.4	30 (19.0)	18 (54.5)		28 (18.2)	13 (48.1)		20 (14.7)	25 (50.0)		20 (15.5)	26 (47.3)	
Ulceration	No	121 (79.6)	15(46.9)	**<0.001**	119 (80.4)	11 (42.3)	**<0.001**	109 (83.2)	23 (47.9)	**<0.001**	103 (82.4)	26 (50.0)	**<0.001**
	Yes	31 (20.4)	17 (53.1)		29 (19.6)	15 (57.7)		22 (16.8)	25 (52.1)		22 (17.6)	26 (50.0)	
Clarks level	II/III	39 (25.0)	3 (9.1)	**<0.001**	40 (26.1)	2 (7.4)	0.04	37 (27.4)	5 (10.0)	**<0.001**	36 (28.1)	5 (9.3)	**<0.001**
	IV	111 (71.2)	21 (63.6)		103 (67.3)	21 (77.8)		93 (68.9)	35 (70.0)		87 (68.0)	39 (72.2)	
	V	6 (3.8)	9 (27.3)		10 (6.5)	4 (14.8)		5 (3.7)	10 (20.0)		5 (3.9)	10 (18.5)	
Mitotic rate (per mm^2)	<3	62 (39.5)	2 (6.1)	**<0.001**	57 (37.3)	5 (18.5)	0.09	55 (40.7)	7 (14.0)	**<0.001**	52 (40.6)	8 (14.5)	**<0.001**
	3-7	52 (33.1)	6 (18.2)		47 (30.7)	8 (29.6)		43 (31.9)	13 (26.0)		41 (32.0)	14 (25.5)	
	>7	43 (37.4)	25 (75.8)		49 (32.0)	14 (51.9)		37 (27.4)	30 (60.0)		35 (27.3)	33 (60.0)	
Perineural infiltration	No	143 (97.9)	32 (100.0)	1.00	141 (98.6)	25 (96.2)	0.40	124 (98.4)	47 (97.9)	1.00	118 (98.3)	50 (98.0)	1.00
	Yes	3 (2.1)	0 (0.0)		2 (1.4)	1 (3.8)		2 (1.6)	1 (2.1)		2 (1.7)	1 (2.0)	
Regression	No	128 (82.1)	32 (97.0)	0.03	131 (86.2)	19 (70.4)	0.04	114 (85.1)	41 (82.0)	0.61	107 (89.3)	46 (83.6)	0.917
	Yes	28 (17.9)	1 (3.0)		21 (13.8)	8 (29.6)		20 (14.9)	9(18.0)		20 (15.7)	9 (16.4)	
Microsatellites	No	132 (90.4)	23 (74.2)	**0.01**	126 (88.7)	21 (80.8)	0.26	115 (91.3)	36 (76.6)	**0.01**	110 (91.7)	38 (76.0)	**0.006**
	Yes	14 (9.6)	8 (25.8)		16 (11.3)	5 (19.2)		11 (8.7)	11 (23.4)		10 (8.3)	12 (24.0)	
Tumour infiltrating lymphocytes (TILs)	Absent	41 (26.5)	9 (28.1)	0.41	42 (27.6)	5 (18.5)	0.23	36 (26.9)	12 (24.5)	0.19	32 (25.2)	16 (29.6)	0.19
	Non-brisk	96 (61.9)	22 (68.8)		92 (60.5)	21 (77.8)		81 (60.4)	35 (71.4)		79 (62.2)	36 (66.7)	
	Brisk	18 (11.6)	1 (3.1)		18 (11.8)	1 (3.7)		17 (12.7)	2 (4.1)		16 (12.6)	2 (3.7)	

Vascular invasion determined by IHC (intra-, peri-tumoural lymphatic vessel or blood vessel invasion), was positively associated with increased stage (Fisher's Exact test, $p<0.001$), increased Breslow thickness ($\chi^2=29.00$, d.f.=3, $p<0.001$), the presence of ulceration ($\chi^2=19.51$, d.f.=1, $p<0.001$), increased Clark's level ($\chi^2=15.94$, d.f.=2, $p<0.001$) and the presence of microsatellites ($\chi^2=7.69$, d.f.=1, $p=0.006$). In addition, vascular invasion was also significantly associated with nodular histological subtype ($\chi^2=26.65$, d.f.=2, $p<0.001$) and these data are summarised in table 2-9.

High macrophage count was associated with the presence of ulceration ($\chi^2=12.23$, d.f.=1, $p<0.001$), Breslow thickness ($\chi^2=24.49$, d.f.=3, $p<0.001$), mitotic rate ($\chi^2=10.67$, d.f.=2, $p=0.005$) and the presence of non brisk or brisk TILs ($\chi^2=10.53$, d.f.=2, $p=0.005$). Table 2-9 summarises the association between macrophage count and the clinicopathological data.

2.4.5 Relationship between vessel density, vessel invasion and macrophage count

Intra-tumoural lymphatic density was significantly associated with intra-tumoural lymphatic vessel invasion ($\chi^2=8.05$, d.f.=1, $p=0.005$) but no significant association was observed with peri-tumoural invasion. Blood microvessel density was associated with intra-tumoural, peri-tumoural and any lymphatic invasion ($\chi^2=8.08$, d.f.=1, $p=0.004$; $\chi^2=6.65$, d.f.=1, $p=0.010$; $\chi^2=14.77$, d.f.=,1 $p<0.001$ respectively).

High macrophage count was significantly associated with the presence of any lymphatic vessel invasion ($\chi^2=9.15$, d.f.=1, $p=0.002$), blood microvessel density ($\chi^2=8.94$, d.f.=1, $p=0.003$) and IHC determined vascular invasion ($\chi^2=8.66$, d.f.=1, $p=0.003$), indicating the likely importance of macrophage in the process of tumour vascularisation and lymphatic dissemination. Table 2-11 shows the associations between these variables.

Table 2-11: Association between variables used in the study (LVD, LVI, MVD, BVI and macrophage count)

		lymphatic density			Lymphatic invasion			Blood vessel density	Blood vessel invasion	Macrophage	Any vessel invasion
		IT	PT	Total	IT	PT	Total				
lymphatic density	Intra-tumoural		**0.004**	**<0.001**	0.005	0.67	0.016	0.093	0.280	0.070	0.016
	Peri-tumoural	**0.004**		**<0.001**	0.752	0.028	0.156	0.060	0.120	0.603	0.117
	Total	**<0.001**	**<0.001**		0.079	0.074	0.071	0.125	0.721	0.048	0.138
Lymphatic invasion	Intra-tumoural	**0.005**	0.752	0.079		0.001	<0.001	**0.010**	0.150	0.012	<0.001
	Peri-tumoural	0.670	0.028	0.074	0.001		<0.001	**0.004**	0.066	0.015	<0.001
	Total	0.016	0.156	0.071	<0.001	<0.001		**<0.001**	0.389	**0.002**	<0.001
blood vessel density		0.093	0.060	0.125	0.01	0.004	<0.001		0.279	**0.003**	<0.001
Blood vessel invasion		0.280	0.120	0.721	0.150*	0.066	0.389	0.279		0.726	<0.001
Macrophage		0.070	0.603	0.048	0.012	0.015	0.002	**0.003**	0.726		0.003
Any vessel invasion		0.016	0.117	0.138	<0.001	<0.001	<0.001	**<0.001**	<0.001	**0.003**	

2.4.6 Clinical outcome and vessel density, invasion and macrophage involvement

In univariate analysis, none of the variables assessed in this study were statistically associated with relapse-free or overall survival and were therefore not assessed in multivariate analysis (Figure 2-10 and 2-11).

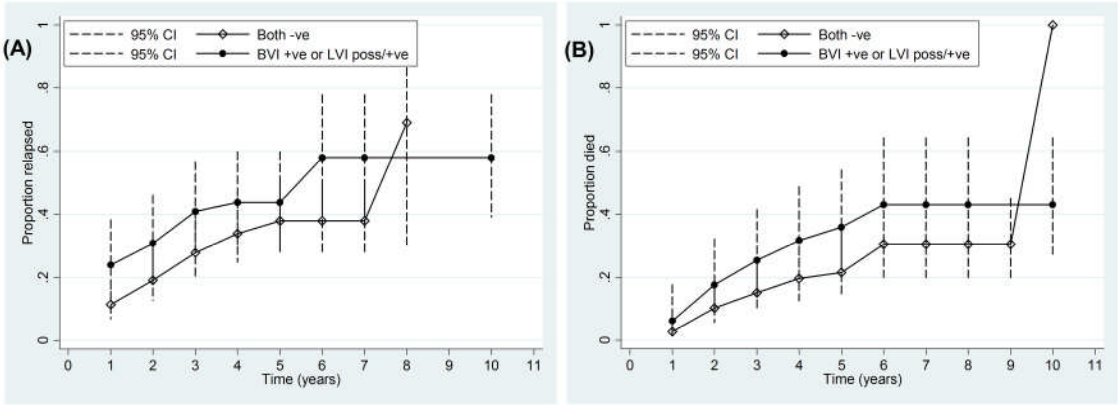


Figure 2-10 Kaplan-Meier analysis of vessels invasion (assessed by IHC) with relapsed free (A) and overall survival (B). No association were observed between IHC determinant vessels invasion with either relapsed free or overall survival in the 202 cohorts of melanoma samples used in this study.

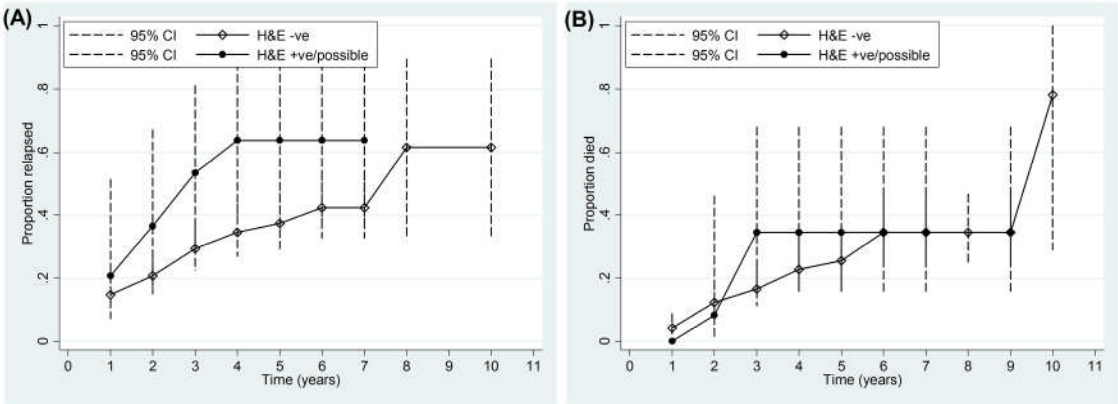


Figure 2-11 Kaplan-Meier analysis of vessels invasion (assessed by H&E) with relapsed free (A) and overall survival (B). No association were observed between H&E determinant vessels invasion with either relapsed free or overall survival in the 202 cohorts of melanoma samples used in this study.

2.5 Discussion

The aims of the current chapter were to study the topography and characteristics of lymphatic vessels in cutaneous melanoma and to examine their associations with clinicopathological criteria. In addition we aimed to distinguish between lymphatic vessel invasion and blood vessel invasion in cutaneous melanoma by comparing the differential expression of blood vascular marker, CD34 and lymphatic marker, D2-40 in FFPE samples using IHC. Furthermore, comparison between conventional assessments of vascular invasion using H&E with that determined using IHC marker was made. Finally, we aimed to investigate the role of lymphatic and blood vessel invasion, and lymphatic and blood vessel density as prognostic biomarkers in cutaneous melanoma patients. We sought to access the role of tumour associated macrophage in influencing lymphatic vessel invasion and lymphatic vessel density as well as blood vessel invasion and blood vessel density in cutaneous melanoma patients by investigating the associations between macrophage counts and characteristics of the vessels.

Questions exist regarding the distribution of lymphatic vessels in solid tumours. Initial studies in colorectal carcinomas had reported the absence of intra-tumoural lymphatics and concluded that peri-tumoural lymphatics are responsible for metastatic dissemination of cancer (Omachi et al., 2007). Functional intra-tumoural lymphatics were not detected in mouse tumours expressing elevated levels of VEGF-C (Weidner et al., 1991). Such findings suggest that functional lymphatics in the tumour margin alone are sufficient for lymphatic metastasis. The existence of intra-tumoural lymphatic vessels has been in doubt as it was thought that lymphatics could not penetrate into expanding primary tumours because of the high physical pressure existed inside the tumour mass (Stacker et al., 2002). However, with the discovery of new molecular markers that are lymphatic specific and with the emergence of better imaging techniques, intra-tumoural lymphatics have been observed (Mochizuki et al., 2004; Ribatti et al., 2006). In fact, the presence of intra-tumoural lymphatics in melanoma has been demonstrated by others (Cueni and

Detmar, 2009; Maddaluno et al., 2009). One study showed that intra-tumoural lymphatic vessel density is significantly higher in malignant melanomas compared to both common acquired nevi and dysplastic nevi (Baluk et al., 2007). However, this study did not report any associations with clinicopathological criteria. Whether increased intra-tumoural lymphatics in their study have any significant relationship to poor prognosis cannot be determined. In the present study which is the largest and most comprehensive of its type, intra-tumoural lymphatic vessels were detected in 86.4% of tumours and their significant association with a number of clinicopathological variables linked with a more aggressive disease may suggest their biological importance and vessel functionality. Previous studies using limited sample size, have also demonstrated the association between intra-tumoural lymphatic vessel density and positive SNB which was not observed in this study (Doeden et al., 2009b; Niakosari et al., 2008). Association of intra-tumoural lymphatic vessel density with distant metastasis was also reported suggesting an important role of these lymphatics in melanoma dissemination (Petersson et al., 2009).

In this cohort of melanoma patients, total lymphatic vessel density was associated with Clark's level and tumour infiltrating lymphocytes. Studies that have previously investigated lymphatic vessel density in melanoma have reported various associations with clinicopathological criteria. Lymphatic vessel density has been associated with age (Massi et al., 2006; Straume et al., 2003), the presence of inflammatory infiltrate (Dadras et al., 2003a; Massi et al., 2006), positive sentinel lymph node (Massi et al., 2006), tumour stage (Valencak et al., 2004) and tumour thickness (Straume et al., 2003). In the present study, no associations were observed between lymphatic vessel density with clinical outcome (relapse free or overall survival).

Microvessel density was associated with the presence of ulceration, high mitotic rate and stage. Associations between microvessel density with Clark's level and tumour stage have also been previously reported (Valencak et al., 2004). We also tested for correlations between blood and lymphatic vessel density. However, no correlations were observed between microvessel density and

lymphatic vessel density in melanoma and this observation was in accordance with other studies (Bono et al., 2004; Schoppmann et al., 2006). Current results, as with those found in breast cancer, suggest that although both angiogenic and lymphangiogenic pathways share some common regulatory mechanisms, there may also be distinct regulatory pathways (Mohammed et al., 2007).

The presence of vascular invasion is an important prognostic factor in most cancer types. In breast cancer, vascular invasion is associated with local recurrence and metastasis (Gonzalez-Vela et al., 2001; Parveen and Shahid, 1997; Rakha et al., 2011). However, the role of vascular invasion in melanoma is not as established. The assessment of vascular invasion in the primary tumour using conventional staining is problematic due to difficulties in identifying vessel endothelium surrounding tumour emboli, coupled with the inability to differentiate between capillaries and artefactual spaces caused by shrinking in routine histological sections (Fallowfield and Cook, 1989; Kashani-Sabet et al., 2001). Conventional staining is also unable to distinguish between lymphatic vessel invasion and blood vessel invasion.

In this study the detection rate of vascular invasion increases from 8% to 29.9% when using H&E compared to when IHC markers were used. As reported from different cohort of patients, the detection rate of vascular invasion using either H&E or IHC markers varies. The detection rates of vascular invasion determined in H&E stained tissues in comparison to lymphatic vessel invasion determined by IHC in previously published studies have ranged from 0% to 16%; n=44 (Niakosari et al., 2005a), 0% to 17%; n=36 (Sahni et al., 2005), 0% to 23%; n=74 (Petersson et al., 2009), 3.1% to 21.9%; n=64 (Fohn et al., 2011) and 4% to 37%; n=27 (Petitt et al., 2009). Clearly, the use of IHC markers in this study and other studies showed that this technique is able to overcome the limitations of using conventional H&E staining. D2-40, which is a selective lymphatic marker, was commonly used in studies examining the biological role of lymphatic in tumours while CD34 is used as selective blood vascular marker in angiogenesis studies. As reported in other study, CD34 is sometimes co-expressed with D2-40 on lymphatic vessels (Fidler, 1997).

Interestingly, this large series of patients shows that vascular invasion in melanoma is essentially invasion of lymphatic vessels (26.9% vs 4.2%). Reported detection rates for lymphatic vessel invasion are 16% (Doeden et al., 2009a; Doeden et al., 2009b; Niakosari et al., 2005a), 17% (Sahni et al., 2005), 21.9% (Fohn et al., 2011), 23% (Petersson et al., 2009), 33% (Niakosari et al., 2008; Xu et al., 2008), 37% (Petitt et al., 2009), 47% (Shields et al., 2004), and 63% (Emmett et al., 2010) in melanoma. A preference of lymphatic over blood vessel invasion has been reported previously with varying frequencies of blood vessel to lymphatic vessel invasion of 3% to 16% (Doeden et al., 2009a), 4.7% to 33% (Xu et al., 2008) and 43% to 47% (Shields et al., 2004) respectively. In fact, Shields and colleagues have developed a prognostic index based on their cohort of metastatic melanoma samples which combined the information on thickness, lymphatic density and lymphatic invasion, to generate a more efficient predictor of survival (Shields et al., 2004). However, only 21 samples were used, limiting the clinical value of this index. Further work is required to assess the robustness of the Shields index to consider the use of it in clinical settings. It would have been interesting to have assessed the prognostic value of the Shields index in the current cohort – this could be carried out through further work/investigations by others continuing the study.

In the present study, the presence of lymphatic vascular invasion was associated with markers of tumour aggressiveness such as increased stage, increased Breslow thickness, the presence of ulceration, increased Clark's level, high mitotic rate, the presence of microsatellites and nodular histological subtype. When analysis was repeated using lymphatic vessel invasion detected by IHC, significant association was observed with increased stage, increased Breslow thickness, the presence of ulceration, increased Clark's level and the presence of microsatellites. This is in agreement with previous work that reported significant associations of lymphatic vessel invasion with the presence of ulceration and younger age (Niakosari et al., 2008). However, no significant difference was observed with sentinel node biopsy or clinical outcome which also has been reported by previous studies (Petitt et al., 2009; Sahni et al., 2005). Lymphatic vessel invasion has been previously shown to be significantly

associated with sentinel lymph node status, distant metastasis, overall survival and disease free interval (Petersson et al., 2009). However, the Petersson study utilised dual markers, LYVE-1 and S-100 and on a small cohort (n=36). Whether the differences in these results are attributed to the small sample size or related to the use of a different marker of lymphatic endothelium, is unclear. In the current study, conducted in a robust fashion and in a large number of patients, no association of vessel characteristic with relapse-free or overall survival was observed. These results were unexpected due to their strong association with poor prognostic histological features of melanoma which may attest to the value of existing biomarkers. The mean follow up period of this cohort is 38 months; if followed up long enough, positive relationship with relapse-free or overall survival might be observed given the strong correlation of vessels characteristics with poor prognostic features. In addition, more samples could be added to the current cohort or using a different cohort altogether to confirm the association of lymphatic and blood vessels characteristic with clinical criterias.

The association of lymphatic vessel invasion with markers of tumour aggressiveness and sentinel node biopsy in this study, and others, shows that lymphovascular invasion by IHC should be routinely assessed clinically to complement the determination of SLN biopsy in melanoma patients. However, more studies need to be carried out in order to demonstrate a conclusive relation between the prognostic values of lymphatic vessel invasion with patient outcome.

It is unclear what factors drive lymphatic vessel invasion in a tumour rich in blood vessels. However, it was observed that lymphatic vessel invasion by tumour cells was associated with inflammatory cell infiltration around the vessels. In this study, the presence of high macrophage count was significantly associated with the presence of lymphatic vessel invasion, high microvessel density and total IHC determined vascular invasion. This result is supported by studies investigating the role of macrophages and inflammatory cells in inducing lymphangiogenesis in tumours and other tissues (Ammar et al., 2011;

Kerjaschki, 2005; Yano et al., 2006) and could be due to macrophages being a source of angio- and lymphangiogenic growth factors (Schoppmann et al., 2006). Tumour associated macrophage often accumulate in hypoxic regions of tumours and trigger the production of pro-angiogenic growth factors, such as VEGF, platelet derived endothelial cell growth factor, bFGF and TNF-α (Skobe et al., 2001). Tumour associated macrophage also produce VEGF-C and increased expression of this growth factor has been shown to increase in-vivo lymphangiogenesis in murine models (Padera et al., 2002).

In conclusion, results from the current study show that, even though there is a rich blood vessel network, vascular invasion in melanoma is essentially via lymphatic vessels and that conventional assessment of vascular invasion underestimates its incidence, which is significantly improved using IHC. The presence of vascular invasion (both blood and lymphatic) was associated with factors that indicate poor prognosis including increased stage, increased Breslow thickness and the presence of ulceration. Similarly, lymphatic vessel density was associated with Clark's level whilest microvessel density and macrophage count was associated with the presence of ulceration and increased mitotic rate. However, these parameters; microvessel density, lymphatic vessel density, lymphatic and blood vessel invasion were not associated with clinical outcome of relapse-free survival or overall survival.

Interestingly, the association of macrophage count with microvessel density and lymphatic vessel invasion, markers of neovascularisation and primary tumour growth, suggest a role of macrophage and/or their associated factors in metastatic cell dissemination via the lymphatic vessels. As will be described in the next chapter (Chapter 3), TNF-α and IL-1β are two major cytokines produced by tumour associated macrophages that have been shown to facilitate the invasion of tumour cells into the blood circulation. Subsequent chapters described experiments that were conducted to examine the function of TNF-α and IL-1β in influencing tumour cell (melanoma and breast) adhesion to blood and lymphatic endothelium and to investigate if this may account for the preferential invasion of lymphatics observed in patient tumours.

CHAPTER 3: ADHESION OF TUMOUR CELLS TO LYMPHATIC AND BLOOD ENDOTHELIUM

3.1 Abstract

Background and Aims: Vascular invasion is an important step in the metastatic cascade; tumour cell adhesion to blood and lymphatic vessels is followed by penetration through the vessel wall and adaptation into the local environment. Results from Chapter 2 show that, as we have also shown in breast cancer, even though melanomas have rich vascular networks, vascular invasion in melanoma is preferentially invasion of the lymphatics and that the association between macrophage infiltrate with lymphatic vessel invasion suggest a role for macrophage and/or their associated factors in cancer cell dissemination via lymphatic vessels. The primary aims of the current chapter were to compare the adhesion pattern of both melanoma and breast cancer cell lines to blood and lymphatic endothelial cell models (large vessel versus microvessel and primary versus immortalised cells) and to examine the effect of macrophage associated cytokines (TNF-α and IL-1β), macrophage conditioned media and tumour conditioned media on tumour-endothelial interactions. The role of an adhesion molecule, preferentially expressed on the lymphatics (i.e CLEVER-1 – common lymphatic endothelial and vascular endothelial receptor 1), might play in regulating lymphatic vessel invasion was also examined in addition to ICAM-1 and VCAM-1 adhesion molecules (intracellular adhesion molecule 1 and vascular cell adhesion molecule 1 respectively).

Methods: Melanoma (MeWo and SKMEL-30) and breast cancer (MDA-MB-231, MCF7) cell adhesion to lymphatic (hTERT-LEC – human telomerase reverse transcriptase lymphatic endothelial cells and hMVEC dLy Neo – human neonatal dermal lymphatic microvascular endothelial cells) and blood (HUVEC – human umbilical vein endothelial cells and hMEC-1 – human microvascular endothelial cells) endothelial models was assessed using static adhesion assays. The effect of TNF-α and IL-1β stimulation on endothelial cells and tumour cells alone or both together on the adhesive process was examined. In addition, the stimulatory effect on endothelial cells by tumour conditioned media and macrophage conditioned media on tumour cell adhesion pattern were also studied. ELISA (enzyme-linked immunosorbent assay) was used to examine the

concentration of IL-1β in both tumour conditioned media and macrophage conditioned media. Surface and intracellular expression of CLEVER-1 on the endothelial cells were examined by FACS (fluorescence activated cell sorting) in addition to the surface expression of ICAM-1 and VCAM-1.

Results: Melanoma and breast cancer cells exhibited a higher level of adhesion to blood compared to lymphatic endothelial cells ($p<0.001$). TNF-α stimulation of endothelial cells alone, or of tumour cells alone, did not significantly alter tumour-endothelial cell adhesion or patterns. However, when both tumour and endothelial cells were stimulated with TNF-α, a significant increased in adhesion was observed ($p<0.01$). This increased tumour cell adhesion was most notably observed in the lymphatic cell models ($p<0.001$). IL-1β stimulation of endothelial cells, tumour cells or both together showed a significant increase in the percentage of adhered tumour cells to the endothelial cell models with a higher increased adhesion to the lymphatic endothelial cells ($p<0.001$). In addition, no significant changes in tumour-endothelial cell adhesion observed when the endothelial cells were stimulated with tumour conditioned media. However, there was a significant increased in the percentage of adhered tumour cells (MDA-MB-231 and MeWo) to hMEC-1 and hTERT-LEC when both endothelial models were stimulated with macrophage conditioned media ($p<0.01$) which was significantly higher to the lymphatic endothelial cells. The effect of increased adhesion to lymphatics by macrophage conditioned media seemed to be associated with the amount of IL-1β present. When interleukin-1 converting enzyme (ICE) inhibitor was used, with macrophage conditioned media, the increased adhesion effect was removed.

CLEVER-1 was expressed intracellularly in both blood and lymphatic endothelial cell lines, however significant surface expression was only observed in the immortalised lymphatics, hTERT-LEC. Upon stimulation with TNF-α, only the primary lymphatic cells showed significant increased in surface expression of CLEVER-1 to match that of hTERT-LEC. No significant surface expression was ever observed with blood endothelium, even under stimulated conditions.

Interestingly, stimulation of HUVEC, hMEC-1, hTERT-LEC and hMVEC-dLy Neo with IL1-β did not show any significant altered CLEVER-1 expression.

ICAM-1 and VCAM-1 was expressed in HUVEC, hMEC-1 and hTERT-LEC with the highest surface expression observed in HUVEC. Upon stimulation with TNF-α and IL-1β, both ICAM-1 and VCAM-1 expression increased significantly in all cell models.

Conclusions: Tumour-endothelial cell adhesion is modulated by cytokine stimulation with both TNF-α and notably IL1-β, presumably secreted by macrophage, having strong influences in regulating adhesion particularly to the lymphatics thus modulating lymphatic vessels invasion. Although CLEVER-1 is an important lymphatic specific adhesion molecule, results from the current study suggest that it may not be the principal regulator of macrophage, or associated cytokine, up regulation of tumour cell lymphatic adhesion. This process may be regulated by ICAM-1 and VCAM-1, which increased upon stimulation with the macrophage associated cytokines; TNF-α and IL-1β.

3.2 Introduction

3.2.1 Breast Cancer

Breast cancer is the most frequent cancer diagnosed in women around the world which comprises 22.9% of all cancers (excluding non-melanoma skin cancers because registrations for this cancer type is likely to be less complete and less accurate compared to other cancers) with a 13.7% mortality rate worldwide. There is a 0.5% overall increase in incidence annually. In spite of such increasing incidence, mortality rates from breast cancer show a steady decline, especially in developed countries, that is attributed to better screening programmes, improved education and more effective treatments (Rosso et al., 2010).

There are a number of risk factors that predispose to breast cancer such as age, reproductive history (age of first birth, breast feeding and age of menopause), hormonal status (including age of menarche and endo-/exo-genous hormones), diet (high fat diet and alcohol consumption), environmental influences (exposure to ionizing radiation), body weight, physical activity and socioeconomic status. Familial breast cancer accounts 5-10% of total cases; and involves germline mutations in penetrant breast cancer susceptibility genes BRCA1 and BRCA2, amongst others such as p53, PTEN, ATM and RAD51 (Flanagan et al., 2010; Lose et al., 2006).

Histologically, breast cancer can be classified into (1) non-invasive carcinoma in-situ, (2) invasive carcinoma and (3) rare and uncommon tumours (inflammatory carcinoma and Paget's disease). Histological types of breast cancer, the frequency and the approximate 5-year survival rates are summarised in Table 3-1.

As in melanoma, breast cancer staging uses the TNM classification system. TNM staging categories for breast cancer are as shown in Appendix B-Table B1. Based on the combination of tumour size, lymph node status and distant metastasis, breast cancer is classified into four different stages as described in Appendix B-Table B2.

Table 3-1: Histological types of breast cancer, their frequency and the approximate 5-year survival rates. Reproduced and adapted from (Li et al., 2005). Copyright© 2005 by Cancer Research UK with permission conveyed through Copyright Clearance Center Inc.

Histological type	*Frequency (%)*	*Approximate 5-year survival (%)*
Infiltrating ductal carcinoma	63.6	79
Infiltrating lobular carcinoma	5.9	84
Infiltrating ductal and lobular carcinoma	1.6	85
Medullary carcinoma	2.8	82
Mucinous carcinoma	2.1	95
Paget's disease	1.0	79
Papillary carcinoma	0.8	96
Tubular carcinoma	0.6	96
Ductal carcinoma in-situ	3.6	>99
Lobular carcinoma in-situ	1.6	>99
Ductal and lobular carcinoma in-situ	0.2	>99

The prognosis of breast cancer is influenced by a number of factors such as tumour size, age and menopausal status, histological types and grade, lymph node involvement, lymphovascular invasion and hormone and growth factor receptors. These factors will be discussed below.

3.2.1.1 Tumour size

The size of the primary tumour is an independent poor prognostic factor in breast cancer. It has been established that the rate of distant recurrence increases in patients with large tumours as opposed to smaller tumours (Koscielny et al., 2009). Table 3-2 shows the 5-year overall survival of patients with different tumour size.

Table 3-2: Tumour size and the 5-year overall survival rates. Adapted from (McCready et al., 2000).

Tumour size (cm)	*5-year overall survival (%)*
<1.00	99
1.01-3.00	89
3.01-5.00	86

3.2.1.2 Age and menopausal status

Only 5 to 7% of breast cancers are diagnosed in women below 40 years of age. However, women in this group have a more aggressive cancer with higher mortality and recurrence rates compared with older women. The poor prognosis of younger patients is indication for adjuvant therapy which is offered to approximately 80% of such patients (van der Sangen et al., 2008). The poor prognosis breast cancers in younger women are more likely to be hormone receptor negative, higher grade, poorly differentiated and have higher HER-2/EGFR expression (Dobi et al., 2011).

3.2.1.3 Histological types and grade

A few histologic types of invasive carcinoma such as mucinous, tubular, medullary and papillary carcinoma have better prognostic value than invasive ductal carcinoma. Ductal carcinoma not otherwise specified (NOS) is the most invasive histological types of breast carcinoma which accounts for 70% of all breast carcinoma (Ellis et al., 1992).

Recently, gene expression profiling has been used to define breast cancer subtypes based on the molecular characteristics and its association with clinical outcome (van Veer et al., 2002; Weigelt et al., 2008). Based on the expression profiles, invasive breast cancer has been categorised into five groups; (a) luminal A (breast cancer with the highest expression of oestrogen receptor and expression of keratin 18 and 19 in luminal mammary cells), (b) luminal B (similar to luminal A but has moderate expression of oestrogen receptor), (c) basal like (oestrogen receptor negative and expression of cytokeratin 5, cytokeratin 17 and laminin), (d) normal breast like (expression of genes known

to be expressed in normal mammary tissue) and (e) ERRB2+ (oestrogen receptor negative and high expression of genes in the ERBB2 amplicons).

The grading system of breast cancer is based on the histological features of the tumour. The Scarff-Bloom-Richardson (SBR) is a widely used system for breast cancer which combined three histological characteristics of tumour to assign an overall grade; tubule formation, nuclear pleomorphism and mitotic count (Genestie et al., 1998; Zhang et al., 2010). In general, each factor is given the score of 1 to 3 with 3 being the worst and the score of each factor are added together to give the grade as shown in Table 3-3. In this grading system, score of 3 to 5 is assign overall tumour grade of 1, score of 6 to 7 is assign grade 2 and score of 8 or 9 is assign overall tumour grade of 3 with 5 years survival rate of 95, 75 and 50% in grade 1, 2 and 3 respectively.

Table 3-3: The Scarff-Bloom-Richardson grading system in breast cancer. Adapted from (Genestie et al., 1998),

Tubule formation	***Score***
>75%	1
10-75%	2
<10%	3
Nuclear pleomorphism	***Score***
Uniform cells, small	1
Variation in cells, moderate size	2
Marked variation	3
Mitotic count (per 10 high power field)	***Score***
≤7	1
8 to 14	2
>15	3

In breast cancer, prognostic indices which combine different prognostic criteria have been shown to be more powerful compared to using each of the factors alone. The Nottingham Prognostic Index (NPI), first developed in 1982, is an

established and widely used index (developed by breast cancer specialists at Nottingham University Hospital) which combines three prognostic factors of breast carcinoma, the lymph node stage, histological grade and tumour size in the following manner (Galea et al., 1992; Haybittle et al., 1982):

NPI = 0.2 x tumour diameter (cm) + lymph node stage (1=no nodes affected, 2=≤3 glands affected, 3=>3 glands affected) + tumour grade (1=grade I, 2=grade II, 3=grade III).

The higher the NPI value, the worse the prognosis (Galea et al., 1992). By applying this formula, scores fall into three bands; (a) a score of <3.4 suggests a good prognosis group with the highest chance of a cure, with the same short term survival of age-matched controls that have not had breast cancer but 15 to 20% of whom will experience metastasis and die of the disease over a 20-year period. (b) a score between 3.4 to 5.4 suggests intermediate level of prognosis with intermediate chance of a cure and (c) a score of >5.4 suggests the worse prognosis with a small chance of a cure. NPI is an excellent index for stratifying the prognosis of patients and in combination with oestrogen receptor status, HER-2 status, menopausal status, vascular invasion and patient's age, the NPI is used as a tool in decision making about the choice of adjuvant systemic therapy for early stage breast cancer patients after surgery. The absolute survival benefit of adjuvant systemic treatment in each NPI group is summarised in Table 3-4.

Table 3-4: The prognostic table. Adjuvant systemic treatment and 10-year survival. Adapted from (Feldman et al., 2002).

NPI	***Percentage survival, no drug***	***Polychemotherapy***			***Tamoxifen (for 5 years) all age***
		Age <50	**50-59**	**60-69**	
Risk reduction		27%	14%	8%	26%
<2.41	95%*	-	-	-	-
2.41-3.4	85%	89%	87%	86%	89%
3.41-4.4	70%	78%	74%	72%	78%
4.41-5.4	50%	64%	57%	54%	63%
>5.4	20%	42%	31%	26%	41%

*same as age-metched population without breat cancer.

3.2.1.4 Hormone and growth factor receptor

Oestrogen receptors (ER) and progesterone receptor (PR) located within the cell nucleus has prognostic value in breast cancer patients. Patients with ER-positive tumours have more favourable prognostic characteristics than patients with ER-negative tumours which relapse earlier (Garicochea et al., 2009). Patients with ER positive breast cancer will usually receive hormonal therapy. Tamoxifen is widely used for the treatment of early and advanced ER positive breast cancer in pre- and post-menopausal women. In addition, aromatase inhibitors are also given to post-menopausal women to lower the amount of oestrogen in their body. These treatments are usually given after surgery, chemotherapy and radiation therapy to block/reduce the effect of oestrogen in the body thus preventing disease recurrence.

3.2.1.5 Molecular marker

HER-2/neu also known as ErbB2 is a cell membrane surface receptor tyrosine kinase normally involved in signal transduction pathway causing cell growth and differentiation. This proto-oncogene is overexpressed in 25 to 30% of breast cancer patients with a significant decrease in 5-year survival rates (Weigelt et al., 2008). Besides HER-2/neu, mutation in tumour suppressor proteins such as p53 and bcl-2 which regulates the cell cycle and apoptotic pathway also influences the prognosis of breast cancer patients (Langerod et al., 2007). HER-2 positive breast cancer patients are usually given the drug Herceptin^{TM} (trastuzumab) that interfere with HER-2/neu receptors causing cell cycle arrest during the G1 phase.

3.2.1.6 Lymph node involvement

Involvement of axillary lymph nodes has been shown to be associated with disease free survival and overall survival in breast cancer patients and has been appreciated as the most reliable prognostic indicator. 50 to 70% of patients with axillary node involvement will develop local recurrence compared to 20 to 30% in node-negative patients (Fisher et al., 1983). The risk of recurrence is related to the number of positive lymph nodes. Patients with four

or more involved nodes have worse prognosis compared with patients with fewer involved nodes (Veronesi et al., 2006). 5 years survival with lymph node negative patients is 78% compared to 62 and 32% 5 years survival in patients with 1 to 3 lymph nodes and ≥4 lymph nodes positivity respectively.

However, approximately one-third or 15 to 20% of lymph node negative patients will ultimately develop distant metastasis if followed up long enough (Weigelt et al., 2005). There is an urgent need, as conventional marker (such as NPI) cannot stratify such patients, to identify new prognostic methods and markers in such patients. Recent works suggest that assessment of lymphovascular invasion may be able to stratify such lymph node patients (Abi-Raad et al., 2011; Mohammed et al., 2011; Rakha et al., 2011).

3.2.1.7 Lymphovascular invasion

As in melanoma, the presence of tumour cells within blood or lymphatic vessels detected microscopically is associated with poorer prognosis. Studies have found that the presence of vascular invasion is associated with the presence of axillary lymph nodes involvement, high risk of systemic relapse and increase local recurrence after wide local excision or mastectomy (Gonzalez-Vela et al., 2001). The identification of vascular invasion in the primary tumour has been associated with the ability to metastasise; however standardisation and adoption into clinical practice has not been achieved. As stated previously, the conventional assessment of vascular invasion is based upon detection of tumour cells within a vascular space lined with endothelial cells in tumour sections stained with H&E; however the assessment of such vascular invasion may be falsely reported due to retraction artifacts and may be missed if tumour cells are packed within a vessel. In one study, there was a low level of concordance (kappa=0.3) in the detection of vascular invasion by conventional means between two observers (Ellis et al., 2006) and the use of immunohistochemical marker such as D2-40 (reviewed in Section 1.4 -Chapter 1) demonstrated a very high level of concordance (kappa=0.9) between observers (Mohammed et al., 2007).

In a study from our group (Mohammed et al., 2008), using 177 well characterised primary invasive breast cancer specimens, 96.4% of specimens showed the presence of lymphatic vessel invasion while only 3.5% were blood vessel invasion. The presence of lymphatic vessel invasion in this study was significantly associated with the presence of lymph node metastasis, development of distant metastasis, regional recurrence, worse disease free interval and decreased overall survival. This is in accordance with other studies reporting the association of lymphatic vessel invasion with larger tumour size, lymph node metastasis and distant metastasis and also the independent role of lymphatic vessel invasion as a poor prognostic factor in breast cancer (Bono et al., 2004; Marinho et al., 2008; Viale et al., 2010).

Results from Chapter 1 showed that in melanoma, as also observed in breast cancer, although a rich blood vascular network is present, vascular invasion occurs predominantly via lymphatic vessels (85.5%) as opposed to blood vessels (4.2%). Biologically, this process requires the tumour cells to migrate toward the blood or lymphatic vessel; which is followed by tumour cell adhesion and migration through the vessel. This process differs dependent upon the vessel type, due to the presence or absence of basement membrane, supporting structure such as pericytes and the thickness of the vessel wall (Johnson and Jackson, 2008; Oliver and Detmar, 2002).

Although it was suggested that the metastatic spread of tumour cells via the lymphatics follows the routes of natural lymph drainage (Fidler, 1997; Mandriota et al., 2001), the exact mechanism of how tumour cells enter the lymphatic capillaries is still uncertain. Chemokines was shown to aid the movement of tumour cells towards lymphatic capillaries (Pepper and Skobe, 2003). Chemokine receptor-ligand relationships are important for regulating leukocyte trafficking and this relationship has been hypothesised to be exploited by cancer cells to modulate entry into the lymphatics. An example is the CCR7-CCL21 interaction. Transduction of B16 murine melanoma cell lines with a retroviral vector encoding the chemokine receptor CCR7 has shown an increased affinity towards lymphatic endothelium (Muller and Luscinskas, 2008). This interaction

could be blocked using neutralising antibodies against CCL21, which is produced by lymphatic endothelial cells and that is specific towards CCR7; suggesting that lymphatic vessels, through the action of chemokines, can attract tumour cells and eventually promote lymph nodes metastasis.

The role of lymphatic specific adhesion molecules may also be important in mediating tumour cells entry into and exit out of the lymphatics (Nathanson, 2003). Adhesion molecules are important because in order for tumour cells to migrate and transmigrate into the lymphatic vessels, they first require an adhesive interaction with lymphatic endothelial cells. To date, many adhesion molecules preferentially expressed on the lymphatics have been discovered and these adhesion molecules were reviewed in details in Section 1.5 -Chapter 1. The adhesive interaction between tumour cells and endothelial cells i.e tumour-endothelial interaction will be the focus of this chapter.

3.2.2 In vitro models of tumour-endothelial interactions

In vitro, endothelial cell monolayers have been used as a model system to investigate tumour-endothelial interactions to mimic interactions that occur in vivo. Current knowledge of tumour-endothelial interaction is largely derived from studies using blood endothelial cells. These studies use a range of endothelial cells to investigate tumour cell adhesion; however the most widely used cell type to investigate adhesion are large vein endothelial cells isolated from human umbilical cords (HUVEC). Studies investigating tumour cell adhesion to blood endothelium have identified a role for thrombospondin (TSP) in potentiating tumour cell metastasis. Adhesion of the breast cancer cell line MCF7 to HUVEC could be reduced by treatment with anti-TSP antibody (Incardona et al., 1995). HUVEC have also been used to investigate the role of intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) on the adhesion of breast cancer cells (MDA-MB-231) to blood endothelium (Nizamutdinova et al., 2008). Pre-treatment with tanshinone-1 significantly reduced adhesion of MDA-MB-231 to HUVEC through inhibition of ICAM-1 and VCAM-1 expression. Changes in cell surface glycoproteins on the breast cancer cell line, MDA-MB-231 can also change their adhesion to

HUVEC by down-regulating the expression of α-L-fucose, a monosaccharide overexpressed in many malignant tumours (Yuan et al., 2008). HUVEC have been used to study tumour-blood endothelial adhesion of many tumour types such as colon carcinoma (Gout et al., 2006); lung adenocarcinoma (Santoso et al., 2005) and melanoma (Kim et al., 1993).

In addition to large vein HUVEC, human microvascular endothelial cells, hMEC have also been used to study tumour-endothelial interactions. Increased adhesion of melanoma cell lines, MeWo, SKMEL-28, JPC 298 and HT144, occurs in Thy-1 activated human dermal microvascular endothelial cells (hDMEC); which suggests an important role of Thy-1 in tumour-blood endothelial interactions (Saalbach et al., 2005).

In contrast to blood endothelium, the mechanisms underlying the adhesion of tumour cells to the lymphatic endothelium is still poorly understood. Very few studies have utilised lymphatic endothelial cells, primarily due to the lack of molecular tools which were able to specifically distinguish lymphatic from blood endothelial cells. Studies investigating the interaction between tumour and lymphatic endothelium interaction have, however, become possible over the past 10 years following the discovery of lymphatic specific markers such as those mentioned in section 1.4 i.e. VEGFR-3 (Laakkonen et al., 2007), LYVE-1 (Banerji et al., 1999a), Prox-1 (Wigle et al., 2002), podoplanin (Schacht et al., 2005a) and D2-40 (Kahn and Alexander, 2002). Studies utilising primary lymphatic endothelial cells have shown an increase in the attachment of breast cancer cells (MCF7 and MDA-MB-231) following stimulation with anti-CCL-2 antibody which increases ICAM-1 expression on lymphatic endothelial cells (Kawai et al., 2009). In addition, Danussi and colleagues (2009) showed that the adhesion of breast cancer cells (MCF7) to human microvascular lung lymphatic endothelial cells (HMVEC-LLy) was reduced following treatment with an anti-Tn antibody, a common tumour associated carbohydrate antigen present in most human carcinomas (Danussi et al., 2009).

Studies conducted so far utilised either blood or lymphatic endothelial cells to study tumour-endothelial adhesion. No studies have conducted a direct side to

side comparison of blood versus lymphatic adhesion. In light of the importance of lymphatic invasion in the initial metastatic process, it is important to be able to compare tumour-endothelial adhesion pattern in both blood and lymphatic endothelial cell models. This knowledge is crucial towards understanding the regulation of metastasis via lymphatic vessels.

The aims and objectives of the current chapter were to;

1. Compare the adhesion pattern of melanoma and breast cancer cell lines to blood and lymphatic endothelial cell models. Different endothelial cell models were used; vascular versus lymphatic models and primary cells versus immortalised cell lines.

2. Investigate the stimulatory effect of macrophage associated cytokines; TNF-α and IL-1β, tumour conditioned media and macrophage conditioned media on tumour-endothelial interactions and if these may account for the preferential invasion of lymphatic vessels observed in tumours.

3. Study the surface and intracellular endothelial cell expression of CLEVER-1, ICAM-1 and VCAM-1 in vascular versus lymphatic models and primary cells versus immortalized cell lines; under control and stimulated conditions i.e. with TNF-α and IL-1β to examine the role that these adhesion molecules might play in regulating lymphatic vessel invasion.

3.3 Materials and Methods

3.3.1 Cell lines and culture

3.3.1.1 Blood endothelial cells

HUVEC: Human umbilical vein endothelial cells were isolated from human umbilical cords obtained from the Department of Obstetrics, City Hospital, Nottingham using a collagenase digestion technique (Jaffe et al., 1973). Before cell isolation, umbilical cords were stored in a sterile plastic container at 4^{o}C overnight or up to a maximum of one week. Trigene solution was used to wipe the outer surface of the cord to remove excess blood and debris. The cord was squeezed slightly to remove any clots forming inside the vein. To ensure maximum sterility, 2cm of the cord from both ends and all areas with clamp marks were cut off. The umbilical vein was cannulated using a sterile venflon® and washed with RPMI medium to remove blood and debris using a 20ml syringe inserted into the inlet pipe of the venflon®. 5ml of type I collagenase were added into the vein where the free end of the cord was clamped and then incubated at 37^{o}C for 40 minutes. The vein was washed with 20ml of HUVEC media (as described below) into a universal to stop the proteolytic activity of the collagenase solution and centrifuged at 1500rpm for 5 minutes. The resultant pellet was resuspended in 5ml of HUVEC medium (as described below) and plated in a T25 tissue culture flask pre-coated with 0.2% gelatin (Sigma, UK) for 20 minutes at 37^{o}C. Cells were incubated overnight and the media was changed the next day to remove cells debris, such as erythrocytes and leukocytes that remain floating, and these cells were grown to confluence. Cells from three cords were pooled into a T175 flask to give a single batch of experimental HUVEC. HUVEC were maintained in 37% nutrient mixture F-12 HAM media (Sigma, UK) in sterile water containing 3.7% 199 media (Sigma, UK), 20% iron supplemented donor calf serum (PAA Laboratories, Austria), 1% sodium bicarbonate (Sigma, UK), 14mM HEPES (Sigma, UK), 2mM L-Glutamine (Sigma, UK), 1% penicillin/streptomycin (Sigma, UK), 7.5U/ml heparin (CP Pharmaceuticals, UK), 25ng/ml EGF (Peprotech, UK) and 12.5ng/ml bFGF (Peprotech, UK). HUVEC were used between passage 2 and

6. HUVEC, being a primary culture were used at short passage windows because of the decrease expression of many proteins (for example angiotensin I converting enzyme and prostacyclin) at each passage due to accelerated senescence and spontaneous apoptosis. Upon recovery from liquid nitrogen, HUVEC were characterized with endothelial cell marker, CD34 (AbD Serotec, UK) using FACS to ensure specificity of the endothelial cells used in this study. Subsequently, all experiments involving HUVEC were carried out on 0.2% pre-gelatinised tissue culture flasks/plates.

hMEC-1: Human microvascular endothelial cells, obtained from ATCC, was the first immortalised cell line derived from human dermal microvascular endothelial cells (hDMEC), immortalised using SV40 large T-Antigen (Matsuo et al., 2006). Cells were grown in endothelial basal medium (Lonza, USA) with 10% iron supplemented donor calf serum, 1µg/ml hydrocortisone (Sigma, UK), 10ng/ml EGF and 1% penicillin/streptomycin. Cells were used between passage 4 and 17 and were characterized using CD34 and CD31 (AbD Serotec, UK) to ensure pure working blood endothelial cells population.

3.3.1.2 Lymphatic endothelial cells

hTERT-LEC: Human telomerase reverse transcriptase lymphatic endothelial cells (hTERT-LEC) were a kind gift from Professor M Pepper (Nisato et al., 2004). These cells are an immortalised lymphatic endothelial cell line established from human dermal microvascular endothelial cells (hDMEC) following transfection with a retrovirus containing the coding region of human telomerase reverse transcriptase. Cells were used between passage 27 and 34. hTERT-LEC were characterised by FACS using commercially available lymphatic marker, D2-40 (Covance, SIGNET, UK) to ensure pure working lymphatic endothelial cells population. hTERT-LEC were cultured in endothelial basal media (EBM) supplemented with the EGM-2 bullet kit (Lonza, USA).

hMVEC dLy Neo: Neonatal dermal lymphatic microvascular endothelial cells (hMVEC-dLy Neo), obtained from Lonza, are a primary lymphatic cell line. The medium for this cell line was prepared as hTERT-LEC. Cells were used

between passage 4 and 6 because of accelerated senescence and spontaneous apoptosis after passage 6.

3.3.1.3 Tumour cells

Two breast cancer cell lines; MCF7 (used between passage 23 and 35) and MDA-MB-231 (used between passage 15 and 28) and two melanoma cell lines; MeWo (used between passage 20 and 30) and SKMEL-30 (used between passage 18 and 30) were used in this study. These cells were obtained from ATCC. MCF7 and SKMEL-30 were maintained in RPMI-1640 (Sigma, UK), 10% iron supplemented donor calf serum with 1% penicillin/streptomycin. MDA-MB-231 were maintained in minimal essential medium EAGLE (Sigma, UK), 0.1mM non-essential amino acids solution (Sigma, UK), 2mM L-glutamine, 1% penicillin/ streptomycin and 1% iron supplemented donor calf serum . MeWo were maintained in minimum essential medium EAGLE, 10% iron supplemented donor calf serum, 2mM L-glutamine, 1% sodium bicarbonate, 0.1mM non-essential amino acids and 1mM sodium pyruvate (Sigma, UK).

3.3.2 Subculture of adherent cell lines

Adherent cells were cultured in tissue culture flasks with routine culture media (section 3.3.1) until 80 to 90% confluent. To subculture, complete medium was removed and cells washed with PBS (without Ca_{2+} and Mg_{2+}). Cells were detached from the flask by adding 5ml of 0.5mg/ml trypsin-EDTA (Sigma, UK) and incubated for 5 minutes at 37^{o}C. Complete media was used to deactivate the trypsin prior to centrifugation at 170g for 5 minutes to remove residual trypsin. The pellet was resuspended in complete media, mixed well and split into a new tissue culture flask.

3.3.3 Cryopreservation of cell lines

Cells were frozen to keep them at low passage number. Adherent cells lines were brought into suspension using trypsin-EDTA (section 3.3.2) and counted using haemocytometer or cell countess/automatic cell counter system (Life Technologies, NY). Cells were then centrifuged at 170g for 5 minutes. 1x10^{6}

were resuspended in 1ml of freezing medium (heat inactivated fetal calf serum or complete medium + 10% DMSO) and stored in a cryovial. Cells were placed at -80^{o}C overnight before transferring to liquid nitrogen until further use.

3.3.4 Peripheral blood mononuclear cells (PBMC) isolation

PBMC were isolated from whole blood using a density-gradient centrifugation method (Sheikh et al., 2004). Blood was collected, under informed consent from healthy donors, in a vacutainer tube (BD, UK) containing 170 IU heparin as anticoagulant. 2.5ml histopaque 1119 (Sigma, UK) was added to a 15ml tube to separate the granulocytes, followed by histopaque 1077 (Sigma, UK) to separate the mononuclear cells. 5ml of heparinised blood was layered on top of the histopaque 1119 and histopaque 1077 and centrifuged for 30 minutes at 670g followed by the removal of the PBMC at the second layer (Figure 3-1). Cells were washed twice in washing buffer (0.1% bovine serum albumin in iron supplemented donor calf serum) and counted by haemocytometer or cell countess prior to usage.

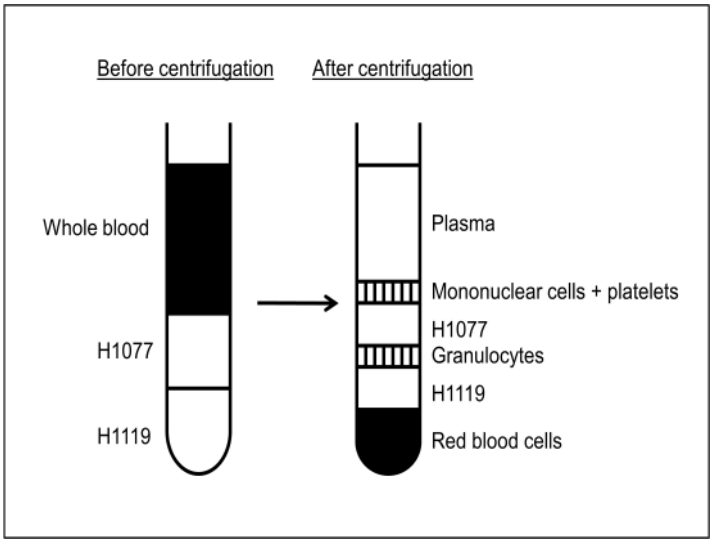


Figure 3-1 PBMC separation using density-gradient centrifugation method. Left tube represents 5ml of blood layered on top of histopaque 1077 and 1119. Right tube represents the layer of cells and liquid formed after centrifugation.

3.3.5 Generation of tumour conditioned media

In the tumour environment, tumour cells produce many cytokines and growth factors that are used for tumour expansion and tumour growth. Hewett and Murray (1996) developed a method to generate tumour conditioned media

which was used to mimic tumour microenvironment in vitro. In the current study, breast cancer (MDA-MB-231 and MCF7) and melanoma (MeWo and SKMEL-30) cell lines were used to generate tumour conditioned media. These cells were grown in T175 flasks until 90 to 100% confluent. Routine culture media was renewed overnight. The following day, media was removed, cells washed twice with PBS and 20ml of tumour conditioning media was added to each flask. Tumour conditioning media consists of HUVEC media or LEC basal media without serum or growth factors. Serum was not added to avoid possible loss of serum nutrients by tumour cells and to prevent a probable effect of conditioned serum metabolites on endothelial cells (Hewett and Murray, 1996). After 24 hours, media was collected and centrifuged at 670g for 10 minutes to remove cell debris prior to freezing at -80^{o}C. Tumour cells was washed with PBS, re-fed with their routine culture medium overnight before another batch of tumour conditioned media was harvested. Tumour conditioned media was harvested three times from each flask.

Prior to experimental use, the tumour conditioned media was supplemented with heat inactivated fetal calf serum to fulfill endothelial cells growth requirements (20%, 10%, 5% and 5% serum added prior to use on HUVEC, hMEC-1, hTERT-LEC and hMVEC dLy Neo respectively). 50U/ml of sterile polymyxinB-sulphate (Sigma, UK) was also added to neutralise any contaminating endotoxin present in the conditioned media. Proliferation assays were carried out by culturing tumour cells with normal cell culture media and with generated tumour conditioned media to ensure that such media was bioactive - results are shown in Appendix C-Figure C1.

3.3.6 Generation of macrophage conditioned media

Human monocytes were isolated from PBMC using a CD14+ selection method. This procedure was conducted by Mohamad El-Refaee (PhD student, University of Nottingham). PBMC were isolated from buffy coats by density gradient centrifugation as in Section 3.3.4. First, 90ml of PBS was added to 50ml of blood in a T75 flask. 35ml of the blood/PBS mixture was gently layered on top of a 15ml histopaque 1077 in a 50ml centrifuge tube which was

centrifuged at 670g for 30 minutes with no brakes applied. PBMC were harvested from the interphase of red blood cell layer and the histopaque layer. For washing, 10ml of PBS was added to the PBMC which were centrifuged at 170g for 10 minutes. Supernatant was discarded and the cell pellet was resuspended in 10ml PBS. Washing steps were repeated 3 times. After the third wash, a small sample was removed and counts using haemocytometer.

Monocytes were purified from PBMC using magnetic microbeads conjugated with anti CD14 (Miltenyi Biotec). PBMC were resuspended in 800µl MACS buffer (magnetic cell sorting buffer; PBS + 1% FCS + 2µM EDTA) and the magnetic microbeads were added at 20µl per $1x10^7$ PBMC. The mixture was incubated at 4^oC in the dark for 15 mins. Cells were centrifuged at 170g for 10 minutes, the pellet resuspended in 3ml MACS buffer and added to a negative depletion column. The column was washed 3 times with MACS buffer in which the CD14 negative cells were discarded. The column was removed from the magnet and placed over a fresh collection tube. 5ml of MACS buffer was added to the column and a plunger applied to elute CD14+ monocytes.

For macrophage preparation, $1x10^7$ $CD14^+$ monocytes were cultured for six days in RPMI-1640 + 5% FCS in the presence of 50ng/ml of macrophage-colony stimulating factor (M-CSF) (Peprotech, UK) in a Teflon flask (Thermo Scientific, UK). At day 7, this macrophage co-cultured conditioned media was harvested and stored at -20^oC until further use.

To study the production of IL-1β in different stimulatory conditions, $1x10^6$ macrophages per well were cultured (RPMI-1640 + 5% FCS) in a 12-well plate for 48 hours under following conditions;

(1) resting macrophage – unstimulated,

(2) tumour derived lysate (TDL) stimulation (MDA-MB-231 lysate). Cell lysates were prepared in RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA and 0.1% SDS) containing protease inhibitors (2 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 130 µm Bestatin, 14 µm E-64, 1 µm Leupeptin, 0.3 µm Aprotinin, Sigma) and

phosphatase inhibitor cocktails 1 and 2 (Sigma, UK). Cell lysates were stored at -80^{o}C until further used.

(3) Lipopolysaccharide gamma (LPS-γ) (Peprotech, UK) stimulation (500ng/ml). LPS acts as an endotoxin, which could promote the secretion of pro-inflammatory cytokines. In this study, LPS-γ was used to induce the production of IL-1β and the resulting supernatant was used to stimulate the endothelial and tumour cells in the in-vitro assays.

(4) TDL + interleukin converting enzyme (ICE) inhibitor (R&D Systems, UK) (250 mM).

(5) LPS-γ + ICE inhibitor.

ICE is a cysteine protease responsible for proteolytic activation of the biologically inactive IL-1β precursor to the proinflammatory active cytokine (Livingston, 1997). In this study, ICE inhibitor was used to inhibit the production of mature IL-1β to study the effect of IL-1β inhibition in the in-vitro assay. Optimisation of ICE inhibitor concentration needed to inhibit the production of IL-1β by LPS-γ stimulated macrophages was carried out before being used in the actual experiment. Optimisation result was shown in Appendix C –Figure C2.

These 5 different conditions of supernatants were harvested after 48 hours and stored at -20^{o}C until further used in the in-vitro assays.

3.3.7 Static adhesion assay

Endothelial cells were grown to confluence in a 24 well plate (optimised seeding density; 0.75x10^{5} cells/ml, 1.3x10^{5} cells/ml, 1.3x10^{5} cells/ml and 1.5x10^{5} cell/ml for HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLY cells respectively). Complete media was removed and replaced with growth factor free media in control wells or growth factor free media supplemented with (1) TNF-α (2.5ng/ml, 5ng/ml, 10ng/ml) (Peprotech, UK), (2) IL-1β (5ng/ml, 10ng/ml, 15ng/ml) (R&D Systems, UK), (3) TNF-α + IL-1β (5ng/ml + 1-ng/ml), (4) tumour

conditioned media or (4) macrophage conditioned media and incubated for 24 hours.

PBMC adhesion was determined by adding $4x10^5$ cells/well for 5 minutes. Non-adherent cells were washed from the endothelial layer with washed buffer (0.1% bovine serum albumin in PBS) and assessed visually by counting adherent cells in the central area of the well with a phase contrast microscopy at 10x magnification. PBMC adhesion to the endothelial cells with and without TNF-α and IL-1β stimulation was carried out immediately prior to commencing tumour cell adhesion experiments to assess immune cell-endothelial cell interactions, in addition to acting as a control to demonstrate that the endothelial cells and the cytokines were responding appropriately.

For tumour cell adhesion assays, tumour cells were trypsinised to prepare single cell suspensions. The effect of trypsin on tumour cell adhesion was further investigated as described in section 3.3.8 and results are shown in section 3.4.3. The cells were labeled with 1μM of Cell Tracker Green CMFDA (Invitrogen, USA) at $1x10^6$ per ml for 30 minutes at 37^oC. This concentration was shown not to affect cell viability and proliferation up to 24 hours (section 3.4.2). After labelling, tumour cells were resuspended in RPMI-1640 containing 10% iron supplemented donor calf serum. $0.5x10^5$ cells per well were incubated for 35 minutes at 37^oC on the different endothelial cell monolayers. Tumour cell adhesion time was optimised between 5 to 60 minutes and the results shown in section 3.4.2)

Non adherent cells were washed with RPMI-1640. Adherent tumour cells were counted using a fluorescence microscope (Nikon, Japan), in the central area of the well which was marked manually with grid lines on the bottom of the plate. 2 fields of view were counted in each well at 20x magnification. Experiments were conducted twice, both in triplicate. Results were expressed as the absolute number of cells adhered to the endothelial layer and as the percentage of cells adhered relative to control. Static adhesion assays were also performed under the following conditions: (1) stimulating tumour cells with TNF-α (2.5, 5 and 10ng/ml) or IL-1β (5ng/ml, 10ng/ml and 15ng/ml), (2) stimulating both

endothelial and tumour cells simultaneously with TNF-α (5ng/ml) or IL-1β (10ng/ml), (3) stimulating the endothelial cells with TNF-α (5ng/ml) + IL-1β (10ng/ml), (4) stimulating the endothelial cells with tumour conditioned media and (5) stimulating the endothelial cells with macrophage conditioned media.

3.3.8 Effect of trypsin dissociation on tumour cell adhesion

Trypsin-EDTA was used to dissociate adherent cells from tissue culture flasks to prepare cell suspension. However, this type of enzymatic treatment may affect tumour cell adhesion to the endothelium. To investigate the effect of the trypsin dissociation method on these cells, a direct comparison between trypsinised tumour cells or cells treated with EDTA was carried out. A confluent T75 flask of MCF7 cells was split 1:2 and cells were allowed to adhere overnight. Cells from one flask were trypsinised as in Section 3.3.2 and cells from the other flask were detached using 5mM of EDTA for 15 minutes and resuspended in RPMI-1640 + 10% serum. These two cells populations were labeled with cell tracker green and the adhesion pattern to the endothelial cells were compared as in Section 3.3.7.

3.3.9 Effect of gelatin on tumour cell adhesion

HUVEC cells were cultured on gelatin coated flasks to facilitate cell attachment. To investigate the effect of gelatin on tumour cell adhesion assay, a direct comparison of MDA-MB-231 adhesion to HUVEC and hTERT-LEC, both cultured with and without gelatin was carried out. The adhesion assay protocol was carried out as in Section 3.3.7.

3.3.10 ELISA

Enzyme-linked immunosorbent assay (ELISA) is a biochemical method used to detect the presence of an antibody or an antigen in a sample. In the current study this technique was used to detect the presence of IL-1β in tumour conditioned media and macrophage conditioned media using human IL-1β/IL-1F2 Duoset ELISA Development kit (R&D System, UK). A 96 well microplate was coated with capture antibody overnight at room temperature. Next day,

each well was aspirated and washed with wash buffer (0.05% Tween® 20 in PBS). Washing was carried out three times. After the last wash, any remaining wash buffer was removed by inverting the plate and blotting it against clean paper towel. Non specific binding was blocked by adding 300µl of reagent diluents (1% BSA in PBS, pH 7.2-7.4, 0.2µm filtered) in each well. The plate was covered with an adhesive strip and incubated at room temperature for one hour. 100µl of samples and standards, diluted in reagent diluents, were added to the test wells before incubation at room temperature for 2 hours. 100µl of detection antibody was added to the wells and incubated for 2 hours at room temperature before addition of 100µl of strepavidin-HRP (1:50 dilution in reagent diluents) for 20 minutes. Washing was conducted three times after each incubation. 100µl of substrate solution (1:1 of substrate reagent A and substrate reagent B; BDBioSciences, CA) was added to each well to allow colour change in response to HRP levels for 15 minutes. 50µl of sulphuric acid (2M) was then added to stop this reaction. The plate was lightly tapped to ensure thorough mixing. The optical density of each well was determined using a plate reader (Fluorostar Optima, BMG LabTech) at 450nm using Fluorostar Optima software version 1.30 R3.

3.3.11 Western blot

The specificity of the ICAM-1 and VCAM-1 antibodies was determined by Western blotting as described below (specificity of CLEVER-1 had been completed previously (Ammar et al., 2011).

(A) Cell lysate preparation

Endothelial cells and PBMC cell lysates were used to determine ICAM-1 and VCAM-1 antibody specificity. Endothelial cells (HUVEC and hMEC-1) were grown to confluence in 3 x T75 tissue culture flasks. Upon confluence, one flask of endothelial cells was stimulated with TNF-α (5ng/ml), one with LPS-γ (500ng/ml) diluted in growth factor free media and one flask acted as a control with only growth factor free media used. PBMC were isolated from fresh blood using the density gradient centrifugation method as described in Section 3.3.1.

The PBMC were incubated in T75 flasks at 37^{o}C for 24 hours in RPMI medium + 10% heat inactivated fetal calf serum with/out LPS-γ (500ng/ml) (Sigma, UK).

These cells (endothelial cells and PBMC) were trypsinised as described in Section 3.3.2. Cells were washed with 1ml PBS to remove any residue of serum proteins and centrifuged at 690g for 10 minutes. The supernatant was discarded and 1ml lysis buffer (2mM Tris-HCl, 135mM NaCl, 1.5mM $MgCl_2$, 1% Triton X-100 and 10% glycerol) added to the cell pellet with 10µl of protease inhibitor cocktail (Sigma, UK) to maintain and preserve protein functionality following cell lysis. Cell lysates were stored at -20^{o}C.

(B) Protein measurement by Bio-Rad protein assay kit (Bio-Rad Laboratories, USA)

The Bio-Rad protein assay is a colourimetric method to measure the concentration of solubilised protein in cell lysates. It involves the addition of an acidic dye to the protein solution and a differential color change occurs in response to the protein concentration. The absorbance value was determined by a microplate reader. A protein standard curve was generated using bovine serum albumin diluted in lysis buffer by plotting the absorbance values against corresponding protein concentrations. The absorbance readings of unknown protein lysates at 650nm were plotted on the standard curve to determine the protein concentration in each cell lysate - this was carried out in triplicate. The determination of the protein concentration, in respective cell lysates, was carried out to ensure equal loading of protein in the wells of the SDS-PAGE Western blot gel.

(C) Buffers used in Western Blot

- **4X SDS/sample buffer**: To prepare a non-reducing sample buffer, 1.52g Tris base (Sigma, UK), 60% glycerol (Sigma, UK), 8% SDS (Sigma, UK) and 10mg bromophenol blue (Sigma, UK) were diluted in 100ml ultrapure water. The pH was adjusted to 6.8 with 1M hydrochloric acid (HCl). To prepare a reducing sample buffer, 200µl/ml of β-mercaptoethanol (Sigma, UK) was added to the above mixture.

- **0.5M Tris-HCl/SDS pH 6.8**: 6.05g Tris base and 0.4% SDS were mixed with 50ml ultrapure water. The pH was adjusted to 6.8 with 1M HCl and ultrapure water was added to 100ml total volume.
- **1.5M Tris-HCl/SDS pH 8.8**: 18.2g Tris base and 0.4% SDS were mixed with 50ml ultrapure water. The pH was adjusted to 8.8 with 1M HCl and ultrapure water was added to 100ml total volume.
- **Electrode buffer/10X Tris-Glycine**: 30.3g Tris-base and 144g glycine (Sigma, UK) were mixed in 1L distilled water.
- **1X running buffer**: 100ml 10X Tris-Glycine, 10ml 10% SDS and 900ml distilled water.
- **Transfer buffer**: 50ml 10X Tris-Glycine, 100ml methanol and 350ml distilled water.

(D) Gel preparation and electrophoresis

12% SDS-PAGE resolving gels and 5% stacking gels were prepared using the recipes described in Table 3-5. Gels were left to set for 30 minutes each at room temperature. 25µg of protein from each sample was loaded into the wells of the Western blot gel. Before loading, cell lysates were mixed with 4X SDS sample buffer (1 volume of sample to 3 volume of sample buffer) and incubated at 100^{o}C for 5 minutes to denature the proteins. These protein samples were loaded at equal amounts in the wells of the SDS-PAGE gel (total volume 20µl). 1.5µl of Western blot molecular weight marker (rainbow marker) (GE Healthcare, UK) with 1.5µl of Western blot magic marker (Invitrogen, NY, USA) were also loaded and the gel was run on 125V for 90 minutes. Following electrophoresis, the stacking gel was removed and the gel blotted on a nitrocellulose membrane (Millipore, MA, USA) at 25V for 90 minutes. The membrane was then blocked overnight in blocking buffer (5% milk (SMA First Infant Milk, Ireland) in 0.1% PBS-Tween) at 4^{o}C. The blocking buffer was removed and the membrane incubated with either ICAM-1 or VCAM-1 anti-antibody (1:100 in blocking buffer) (Thermo Scientific, UK) for 1 hour at room temperature. The membrane was washed 3 x 5 minutes with 0.1% PBS/Tween before incubation with secondary α-mouse HRP-antibodies (1:1000) (R&D

System, UK) for 1 hour at room temperature. Washing was carried out 3 x 5 minutes and Amersham ECL reagent (GE Healthcare, UK) was added to the membrane for 1 minute. An Amersham hyperfilm ECL (GE Healthcare, UK) was exposed on top of the membrane for 3 minutes and developed to view bands. β-actin antibody was used to ensure equal loading of sample in the gel. The nitrocellulose membrane was incubated with β-actin-HRP conjugated antibody (1:1000 in blocking buffer) (Invitrogen, UK) for 1 hour at room temperature and the membrane was developed as above.

Table 3-5: Recipe for 12% resolving gel and 5% stacking gel used in western blot

Reagents	***12% resolving gel (ml)***	***Reagents***	***5% stacking gel (ml)***
H_2O	6.6	H_2O	3
30% acrylamide-bis	8.0	30% acrylamide-bis	0.65
1.5M Tris-HCl/SDS pH 8.8	5.2	0.5M Tris-HCl/SDS pH 6.8	1.25
10% APS	0.2	10% APS	0.05
TEMED	0.008	TEMED	0.0005

3.3.12 Flow cytometry of endothelial cell CLEVER-1, ICAM-1 and VCAM-1 expression

Flow cytometry was carried out to detect surface or intracellular expression of cellular proteins. Indirect flow cytometry was carried out using mouse α-CLEVER-1 antibody (obtained via collaboration with Dr Marco Salmi, Finland), anti-ICAM-1 and anti-VCAM-1 antibody (R&D System. UK) and a FITC-labelled rabbit anti-mouse antibody (DAKO, Denmark). Both surface and intracellular expression of CLEVER-1 was assessed on four endothelial cell models; HUVEC, hMEC-1, hTERT-LEC and hMVEC dLy Neo. In addition, the influence of TNF-α and IL1-β on CLEVER-1 expression in these cell lines was investigated. Surface expression of ICAM-1 and VCAM-1 were assessed on three endothelial cell models; HUVEC, hMEC-1 and hTERT-LEC. WinMDI 2.8 (Purdue University Cytometry Laboratories, Indiana) and FlowJo software version 7.6.1 was used to analyse flow cytometry results.

(A) Surface staining

Endothelial cells were trypsinised and counted using a haemocytometer. 2 x 10^5 cells were aliquoted into a 10ml FACS tube and centrifuged at 170g for 5 minutes. The supernatant was removed and cells resuspended in 100µl of CLEVER-1 antibody diluted in FACS buffer (PBS + 0.1% bovine serum albumin-BSA) bringing the total concentration of anti-CLEVER-1 to 20 µg/ml. 5µg/ml of ICAM-1 and VCAM-1 antibody were also used to asses expression of these adhesion molecules using this procedure. For controls, cells were either incubated in FACS buffer alone or with IgG isocontrol (20µg/ml). These cells were incubated on ice for one hour in the dark. 1ml FACS buffer was added to wash the cells (to remove any unbound antibody) and then centrifuged (170g, 5 minutes). Supernatant was discarded and the cell pellet was resuspended in 100µl of secondary antibody (rabbit anti-mouse FITC; 1:50 dilution in FACS buffer) prior to incubation on ice for 1 hour in the dark. Washing steps followed and finally, cells were resuspended in 400µl of FACS buffer and fluorescence readings were measured using FACS scan (Becton Dickinson, Sunnyvale Ca, USA).

(B) Intracellular staining

Endothelial cells were trypsinised and fixed with 1ml 4% formaldehyde (diluted in PBS) for 30 minutes at room temperature. The cells were centrifuged at 1000rpm for 5 minutes and supernatant discarded. 1ml FACS buffer then added to wash the cells, centrifuged at 170g for 5 minutes and supernatant discarded. The cells were permeabilised in 70% methanol for 10 minutes and centrifuged (170g, 10 minutes). Supernatant was removed and cells resuspended in 1.5ml FACS buffer. The cells were evenly distributed into three FACS tubes; unlabelled cells, cells with IgG isocontrol (DAKO, Denmark, 20µg/ml) and cells with CLEVER-1 antibody and centrifuged at 170g for 10 minutes. The cells were then treated as in the surface staining procedures

3.3.13 Statistical analysis

For flow cytometry, results were reported as geometric mean ± standard deviation. The student t-test was used to calculate the significance value of CLEVER-1, ICAM-1 and VCAM-1 expression between endothelial cell models and between unstimulated controls with TNF-α/IL1-β stimulated endothelial cells.

For adhesion assays, the student t-test was used to evaluate variation between endothelial cell models and individual treatments (control and TNF-α/IL-1β stimulated conditions). A p value <0.05 defined significant relationships. All statistical analysis was carried out using GraphPad Prism version 3.02 and Microsoft Excel 2007.

3.4 Results

3.4.1 The effect of cell tracker green labeling on tumour cell viability and proliferation

To assess the effect of fluorescent labelling on cell viability and proliferation, the trypan blue exclusion viability assay was used. Trypan blue is a vital stain used to selectively colour dead tissues or cells blue without killing living cells. No difference in MDA-MB-231 cells viability count between the labelled and unlabelled cells was observed (Figure 3-2). There was also no difference in the proliferative rate between the labeled and unlabelled cells up to 72 hours. The same effect was also observed with MeWo cell line (Figure 3-3). Therefore, cell tracker green was used for tumour cell labeling in subsequent adhesion assays.

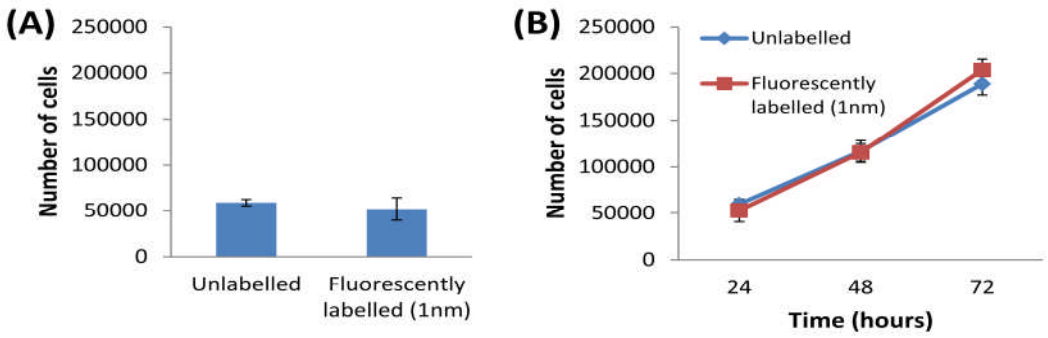


Figure 3-2 The effect of cell tracker green labelling on MDA-MB-231 cell viability (A) and cell proliferation (B). There was no significant different in MDA-MB-231 cell viability (at 24 hours) and proliferation (up to 72 hours) between the fluorescently labeled and unlabelled cells. Pooled data from 2 independent experiments, each carried out in triplicate (n=6).

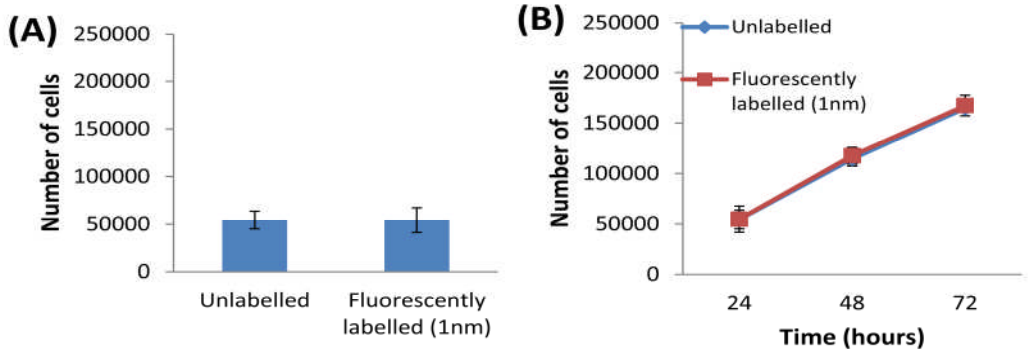


Figure 3-3 The effect of cell tracker green labelling on MeWo cell viability (A) and cell proliferation (B). There was no significant different in MeWo cell viability (at 24 hours) and proliferation (up to 72 hours) between the fluorescently labelled and unlabelled cells. Pooled data from 2 independent experiments, each carried out in triplicate (n=6).

3.4.2 Optimisation of tumour cell adhesion assay

MDA-MB-231 and MCF7 cell lines were used to determine the optimal adhesion time of tumour cell adhesion. Each cell line was left to adhere to HUVEC, hMEC-1 and hTERT-LEC for 5, 15, 35, 45 and 60 minutes and the number of adhered cells compared at the end of the assay. Results show that the number of adhered MDA-MB-231 cells increased significantly up to 35 minutes and then plateaud in all endothelial cell models used. If there was an increased in the number of adhered tumour cells after 35 minutes, this increased is non-significant. Similar adhesion patterns were also observed with MCF7 cells in which no significant increase in the number of adhered cells was observed after 35 minutes for HUVEC and hMEC-1 cells. However, the adhesion of MCF-7 cells to hTERT-LEC increased significantly up to 45 minutes and plateaud at 60 minutes. Yet, based on MDA-MB-231 and MCF-7 adhesion results, 35 minutes was used as the adhesion time for subsequent tumour cell assays (Figure 3-4).

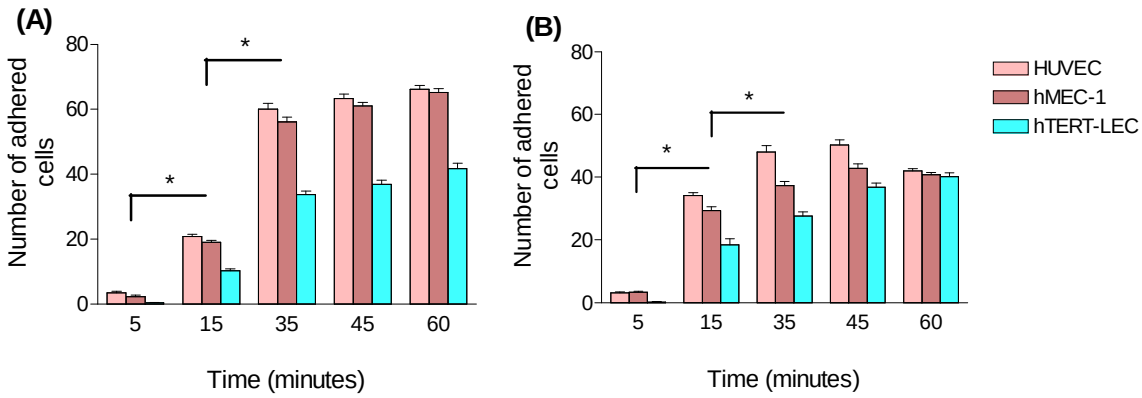


Figure 3-4 Optimisation of MDA-MB-231 (A) and MCF7 (B) adhesion time to HUVEC, hMEC-1 and hTERT-LEC. The number of adhered tumour cells to the endothelial cells was assessed at different incubation time. Both tumour cells showed significant increased in the number of cells adhered between 5 to 35 minutes and plateau thereafter. Pooled data from 2 independent experiments, each carried out in triplicate (n=6).
***represents significant difference (p<0.05) between two time points.**

3.4.3 The effect of trypsin dissociation method on tumour cell adhesion

To assess the effect of trypsin on the adhesive property of tumour cells, adhesion assays were conducted using cells detached using either trypsin or EDTA. There was no difference in MCF7 cell adhesion to HUVEC between the

two dissociation methods as shown in Figure 3-5. Therefore, tyrpsin was used to detach adherent tumour cells in this study.

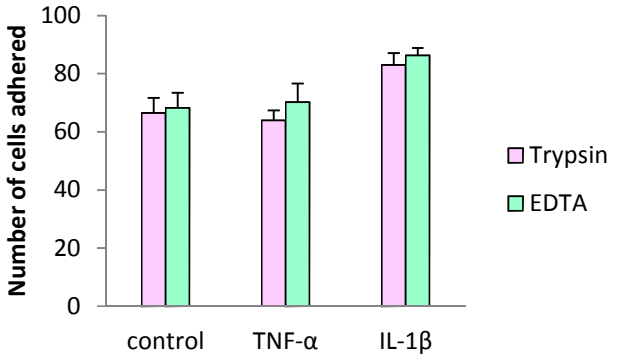


Figure 3-5 Effect of trypsin dissociation method on MCF7 adhesion to HUVEC under control, TNF-α and IL-1β stimulated conditions. Pooled results from two independent experiments each carried out in triplicate (n=6).

3.4.4 The effect of gelatin on tumour cell adhesion

To assess the effect of gelatin coating (used to culture HUVEC cells) on tumour cell adhesion, adhesion assays were carried out with MDA-MB-231 cells on HUVEC or hTERT-LEC cells cultured on tissue culture plates coated with and without gelatin. It was observed that coating tissue culture flask/well with gelatin has no significant effects on tumour cell adhesion as shown in Figure 3-6 and that, as will be discussed in greater detail later (Section 3.4.6), tumour cells exhibited a preference for blood endothelial cells adherence as opposed to lymphatic endothelial cells adherence.

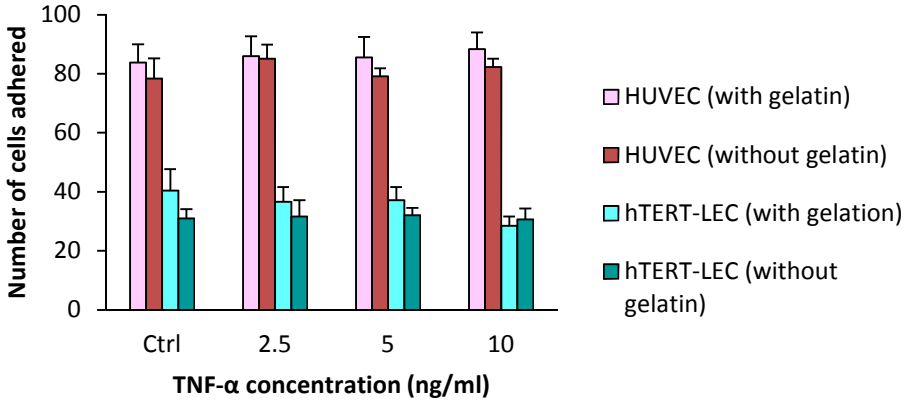


Figure 3-6 The effect of gelatin coating on MDA-MB-231 adhesion to HUVEC under control and TNF-α stimulated conditions. There was no significant different in MDA-MB-231 adhesion to HUVEC or hTERT-LEC; with or without gelatin. Pooled results from two independent experiments each carried out in triplicate (n=6).

3.4.5 PBMC adhesion to HUVEC, hMEC-1, hTERT-LEC and hMVEC dLy Neo.

PBMC adhesion to unstimulated and TNF-α /IL-1β -stimulated HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo was conducted prior to conducting any tumour cell adhesion experiments. Endothelial cells pre-stimulation with TNF-α and IL-1β for 24 hours caused a significant increased in PBMC adhesion compared to the unstimulated controls ($p<0.0001$ and <0.01 respectively) (Figure 3-7). PBMC adhesion to all endothelial cell models increased with increasing TNF-α concentration and a plateau was observed at 10ng/ml. For IL-1β, a plateau was observed at 15ng/ml. PBMC adhesion to TNF-α/IL-1β -stimulated HUVEC was more than that to hMEC-1 but was not significantly different ($p>0.05$). There was no significant difference in the number of cells adhered to either of the lymphatic endothelial models, hTERT-LEC and HMVEC-dLY Neo. However, adhesion of PBMC was significantly higher to stimulated blood endothelial cells (HUVEC and hMEC-1) than to the lymphatic endothelial cells (hTERT-LEC and HMVEC-dLy Neo), ($p<0.05$) for both cytokines, except when the endothelial cells were stimulated with 15ng/ml of IL-1β where the differences were not statistically significant.

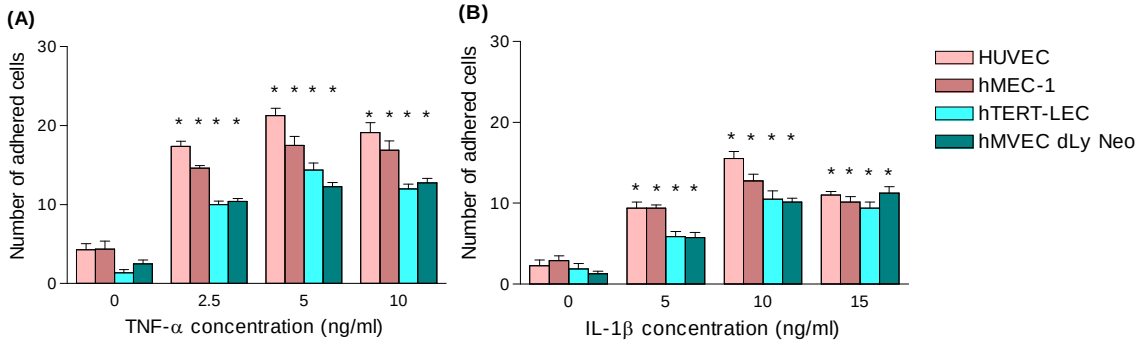
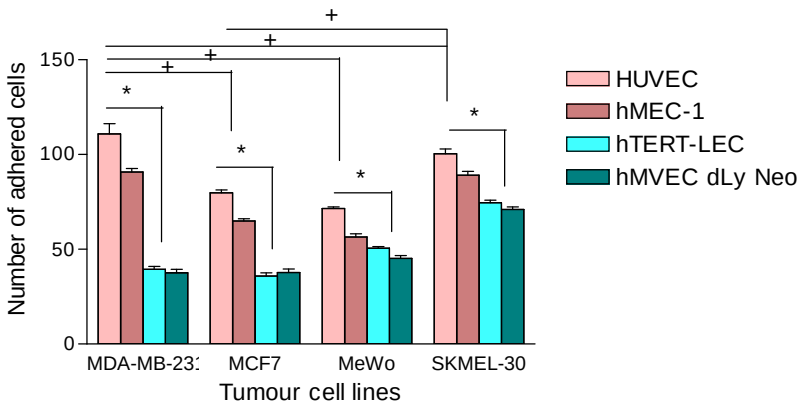


Figure 3-7 PBMC adhesion to HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo under (A) TNF-α and (B) IL-1β stimulation. Stimulation of endothelial cells with TNF-α or IL-1β caused a significant increase in the number of PBMC adhered to all endothelial cell models compared to the unstimulated controls ($p<0.0001$ and $p<0.01$ respectively). PBMC adhesion to all endothelial cell models increased with increasing concentrations of both cytokines which plateau at 10 and 15ng/ml for TNF-α and IL-1β respectively. Error bars represent standard deviation in 28 independent experiments, each in duplicate (n=56). P value was assessed by paired T-test, * represent statistical significant compared to control groups ($p<0.05$).

3.4.6 Tumour cell adhesion to HUVEC, hMEC-1, hTERT-LEC and hMVEC dLy Neo under control conditions

MCF7, MDA-MB-231, MeWo and SKMEL-30 showed similar overall adhesion patterns to all four endothelial cell models, under control conditions, however with different affinity as shown in Figure 3-8. Of the four tumour cell lines, MDA-MB-231 cells had the highest affinity towards the blood endothelium (HUVEC 110.88±14.95 and hMEC-1 90.63±6.00) while SKMEL-30 had the highest affinity towards lymphatic endothelial cells (hTERT-LEC 74.67±5.76 and HMVEC-dLy Neo 72.00±4.84). Both the breast cancer cell lines showed relatively less adherence to the lymphatic rather than the blood endothelial cells in comparison to the melanomas. Although MDA-MB-231, MCF7, MeWo and SKMEL-30 showed preferential adhesion towards the blood endothelial cells as opposed to lymphatic endothelial cells, only the breast cancer cell lines shows significant difference ($p<0.001$). There was no significant difference in the tumour cell adhesion patterns between the primary and the immortalised cell lines of the same origin; blood and lymphatics.



	MDA-MB-231	MCF7	MeWo	SKMEL-30
HUVEC	110.88 ±14.95	79.75 ±4.50	71.75 ±3.86	98.92 ±6.68
hMEC-1	90.63 ±6.00	64.88 ±3.27	56.25 ±5.19	90.67 ±6.68
hTERT-LEC	39.38 ±4.37	35.88 ±5.00	50.42 ±3.06	74.67 ±5.76
hMVEC dLy Neo	37.50 ±5.45	37.75 ±5.15	44.08 ±4.94	72.00 ±4.84

Figure 3-8 Tumour cell adhesion to HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo under control conditions. All endothelial cell models show the same adhesion pattern with the 4 tumour cells used, MDA-MB-231, MCF7, MeWo and SKMEL-30; in which higher adherence to the blood endothelial models, HUVEC is observed. Pooled results from 28 independents experiments, each in triplicate (n=84). * significant difference within group, + significant difference between groups)

3.4.7 The effect of TNF-α and IL1-β on the adhesion of tumour cells to the different endothelial models

The percentage adhesion of each of the tumour lines; MDA-MB-231, MCF7, MeWo and SKMEL-30 to TNF-α-stimulated HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo was not significantly increased when compared to the unstimulated endothelial models as shown in Figure 3-9.

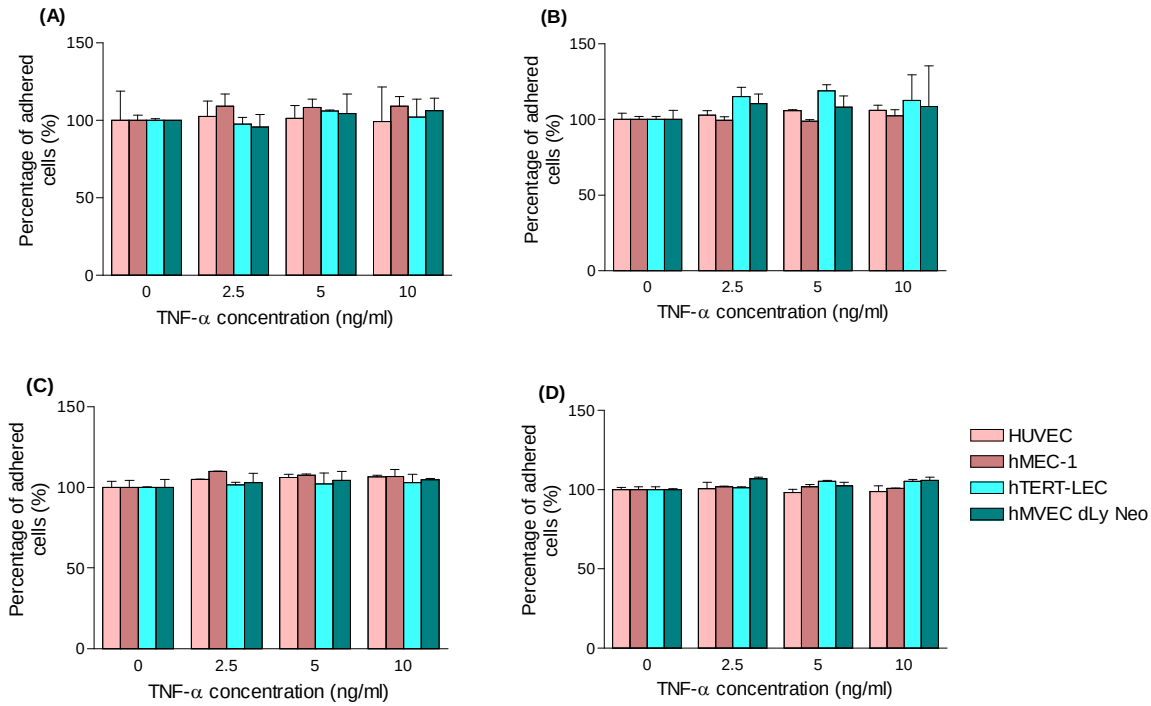


Figure 3-9 MDA-MB-231 (A), MCF7 (B), MeWo (C) and SKMEL-30 (D) adhesion to TNF-α stimulated HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo at different TNF-α concentrations relative to the unstimulated controls. Pre-stimulation of the endothelial cells with TNF-α for 24 hours did not have any significant effect on the adhesion of tumour cells to either blood (HUVEC and hMEC-1) and lymphatic (hTERT-LEC and HMVEC-dLy Neo) endothelial cell models. Pooled results from two independent experiments, each carried out in triplicate (n=6).

Pre-stimulation of the tumour cell lines, MDA-MB-231, MCF7, MeWo and SKMEL-30 with TNF-α did not significantly increased their adhesion to unstimulated blood endothelial cell models; except for the adhesion of TNF-α stimulated MDA-MB-231 to HUVEC ($p<0.001$). However, pre-stimulation of the tumour cell lines with TNF-α shows significant increased of MDA-MB-231,

MCF7 and MeWo cells adhesion to the unstimlated lymphatic endothelial cells, hTERT-LEC ($p<0.05$) as shown in Figure 3-10.

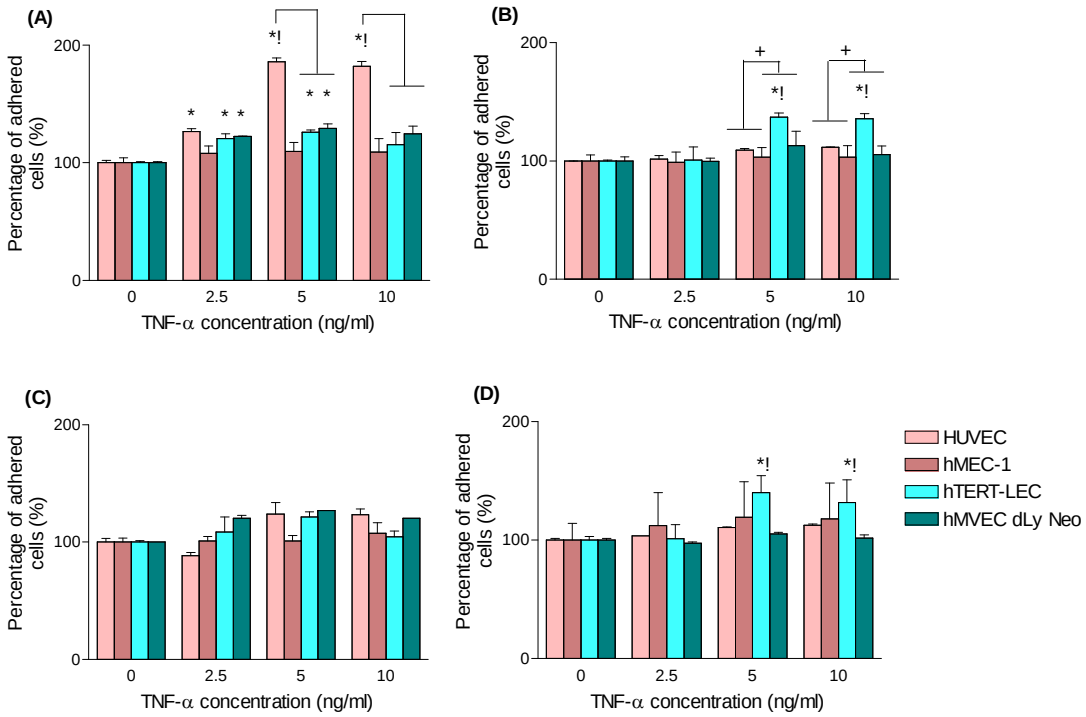


Figure 3-10 Adhesion of TNF-α stimulated tumour cells; MDA-MB-231 (A), MCF7 (B), MeWo (C) and SKMEL-30 (D) to unstimulated HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo. Percentage of tumour cell adhesion to all endothelial cell lines did not vary significantly under control and stimulated conditions with the exception of MDA-MB-231 adhesion to HUVEC ($p<0.001$) and the adhesion of MDA-MB-231, MCF7 and MeWo to hTERT-LEC ($p<0.05$). Pooled results from two independent experiments, each carried out in triplicate (n=6). P value was assessed by paired T-test, * represent statistical significant compared to control groups ($p<0.05$). ! represent statistical significant compared to 2.5ng/ml of TNF-α and + represent statistical significant between blood and lymphatic endothelial cell models.

Pre-stimulation of the endothelial cells with IL-1β shows significant increased in MDA-MD-231, MCF7, MeWo and SKMEL-30 cells adhesion towards HUVEC, hMEC-1, hTERT-LEC and hMVEC dLy Neo, with a more pronounced percentage increased observed in both breast cancer cell lines compared to the melanomas. In MDA-MB-231 and MCF7, the percentage increased was higher towards the lymphatic endothelial cells compared to the blood endothelial cells ($p<0.001$ and $p<0.0001$ respectively) as shown in Figure 3-11.

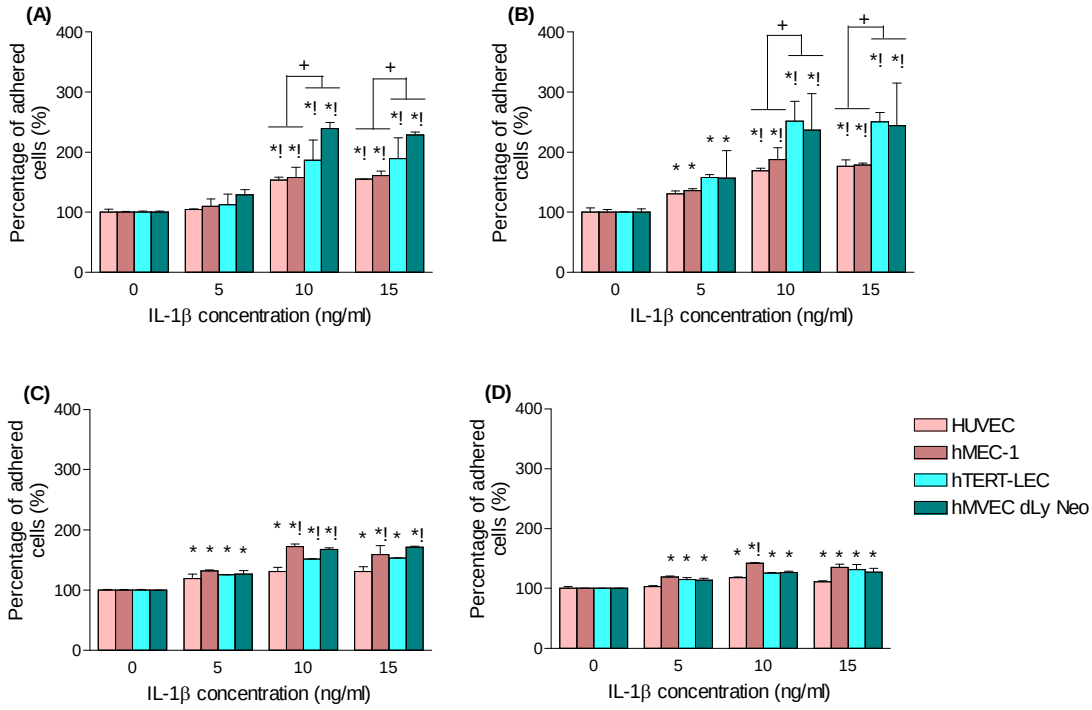


Figure 3-11 MDA-MB-231 (A), MCF7 (B), MeWo (C) and SKMEL-30 (D) adhesion to IL-1β stimulated HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo relative to the unstimulated controls. Pre-stimulation of the endothelial cells with IL-1β for 24 hours significantly affect the adhesion of tumour cells to both blood (HUVEC and hMEC-1) and lymphatic (hTERT-LECand hMVEC dLy Neo) endothelial cell models. Increased was more significant in breast cancer cell lines compared to the melanoma cell lines. In breast cancer cell lines, percentage increased was more to the lymphatic cell models compared to the blood endothelial models ($p<0.001$ and $p<0.0001$ respectively). Pooled results from two independent experiments, each carried out in triplicate (n=6). * represent statistical significant compared to control groups ($p<0.05$). ! represent statistical significant compared to 5ng/ml of IL-1β and + represent statistical significant between blood and lymphatic endothelial cell models.

When the tumour cells: MDA-MB-231, MCF7, MeWo and SKMEL-30 were stimulated with IL-1β, tumour-endothelial adhesion increased significantly across the four unstimulated endothelial cell models; HUVEC, hMEC-1, hTERT-LEC and hMVEC dLy Neo. In MDA-MB-231 and SKMEL-30 adhesion, the percentage adhesion was higher towards the lymphatic endothelial cells opposed to the blood endothelial cells as shown in Figure 3-12.

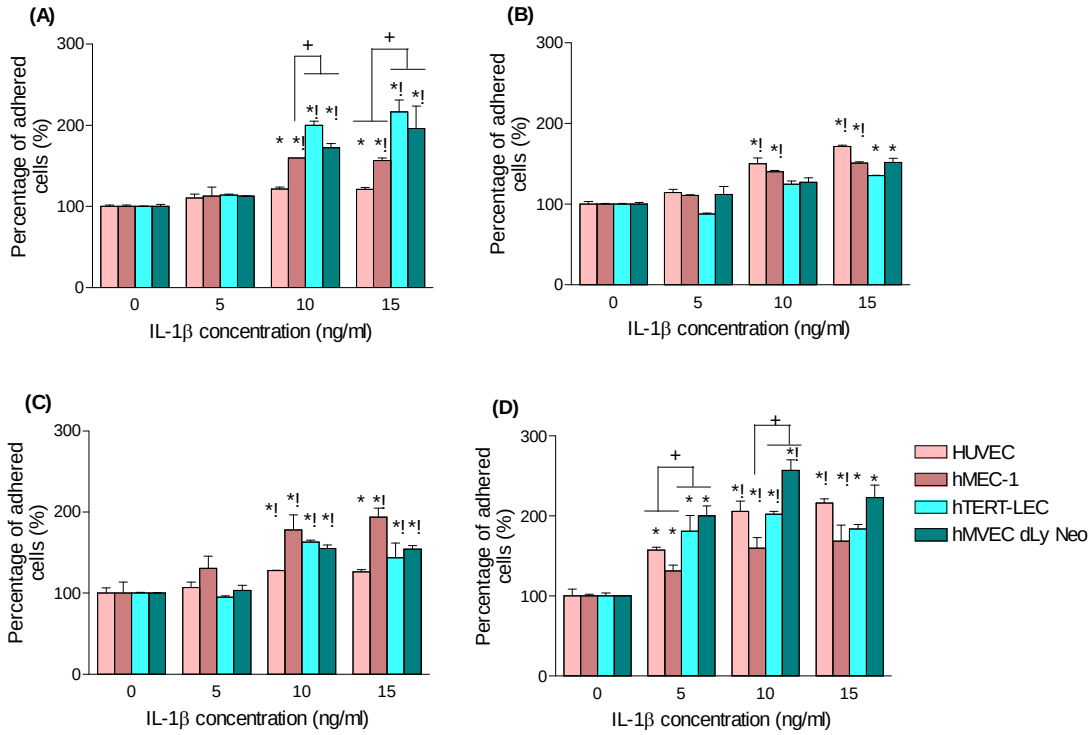


Figure 3-12 Adhesion of IL-1β stimulated tumour cells; MDA-MB-231 (A), MCF7 (B), MeWo (C) and SKMEL-30 (D) to unstimulated HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo relative to the unstimulated controls. Pre-stimulation of the tumour cells with IL-1β for 24 hours significantly affect the adhesion of tumour cells to blood (HUVEC and hMEC-1) and lymphatic (hTERT-LECand hMVEC dLy Neo) endothelial cell models. Increased was more significant to the lymphatic endothelial cells compared to the blood endothelial cells in MDA-MB-231 and SKMEL-30. Pooled results from two independent experiments, each carried out in triplicate (n=6). * represent statistical significance compared to control groups (p<0.05). ! represent statistical significant compared to 5ng/ml of IL-1β and + represent statistical significant between blood and lymphatic endothelial cell models.

As both breast cancer and both melanoma cell lines showed a similar adhesion pattern with similar affinity to the endothelial cell models of the same origin, double stimulation of TNF-α (both tumour and endothelial cells were stimulated simultaneously) was conducted using one breast cancer (MDA-MB-231) and one melanoma cell line (MeWo) to study the adhesion patterns towards the immortalised endothelial cells; hMEC-1 and hTERT-LEC. When MDA-MB-231 and the endothelial cells were both simultaneously stimulated with TNF-α, there was a significant increased in tumour cell adhesion to hMEC-1 ($p=0.02$) and hTERT-LEC ($p<0.001$). The relative percentage of adhered tumour cells was significantly higher to the lymphatic compared to the blood endothelial cell models ($p<0.05$). Similar results were also observed with melanoma where

there was a significant increased in adhesion of MeWo to hMEC-1 ($p<0.001$) and hTERT-LEC ($p<0.0001$); as with the breast line the percentage adhesion of MeWo cells were also higher in the lymphatic compared to the blood endothelial cell model (Figure 3-13).

When MDA-MB-231 and the endothelial cells (hMEC-1 and hTERT-LEC) were both simultaneously stimulated with IL-1β, there was a significant increased in tumour cell adhesion to hMEC-1 and hTERT-LEC ($p<0.001$ and $p<0.0001$ respectively). Interestingly, the relative percentage of adhered tumour cells was significantly higher to the lymphatic compared to the blood endothelial cell models ($p<0.0001$). With the melanoma cell line (MeWo), although significant increased in adhesion to hMEC-1 ($p<0.001$) and hTERT-LEC ($p<0.001$) was observed, the percentage adhesion was of similar affinity towards both lymphatic and blood endothelial cells (Figure 3-13).

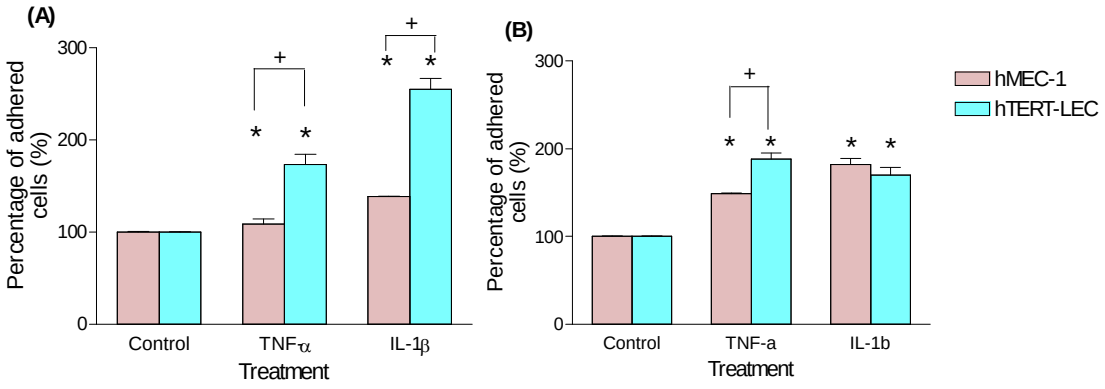


Figure 3-13 MDA-MB-231 (A) and MeWo (B) cell adhesion to hMEC-1 and hTERT-LEC when both the tumour cells and the endothelial cells were stimulated with TNF-α /IL-1β. Significantly increased tumour-endothelial adhesion was observed in both tumour cell lines with higher percentage adhesion to the lymphatic compared to the blood endothelial cells. Pooled results from two independent experiments, each carried out in triplicate (n=6). P value was assessed by paired T-test, * represent statistical significance compared to control groups ($p<0.05$). + represent statistical significant between blood and lymphatic endothelial cell models.

Figure 3-14 shows the adhesion pattern of MDA-MB-231 and MeWo to TNF-α, IL-1β and TNF-α+IL-1β stimulated hMEC-1 and hTERT-LEC. As seen previously and in this assay, TNF-α and IL-1β stimulation of endothelial cells, tumour cells or both together increased MDA-MB-231 adhesion to both blood

and lymphatic endothelial cell models with a preferential increased to the lymphatic endothelial cells; with IL-1β inducing greater adhesion than TNF-α. Combining TNF-α and IL-1β stimulation on hMEC-1 and hTERT-LEC did not significantly affect the adhesion pattern of MDA-MB-231 cells to both endothelial cell models compared to stimulation with IL-1β alone.

A different pattern was observed with MeWo in which no significant increased was observed with TNF-α stimulation. However, with IL-1β stimulation, although a significant increased was observed, there was no significant different between blood and lymphatic endothelial cell models i.e between hMEC-1 and hTERT-LEC. As observed with MDA-MB-231 cells, combining TNF-α and IL-1β stimulation of endothelial cells did not significantly increased MeWo cell adhesion to blood or lymphatic endothelial cells in comparison to IL-1β stimulation alone.

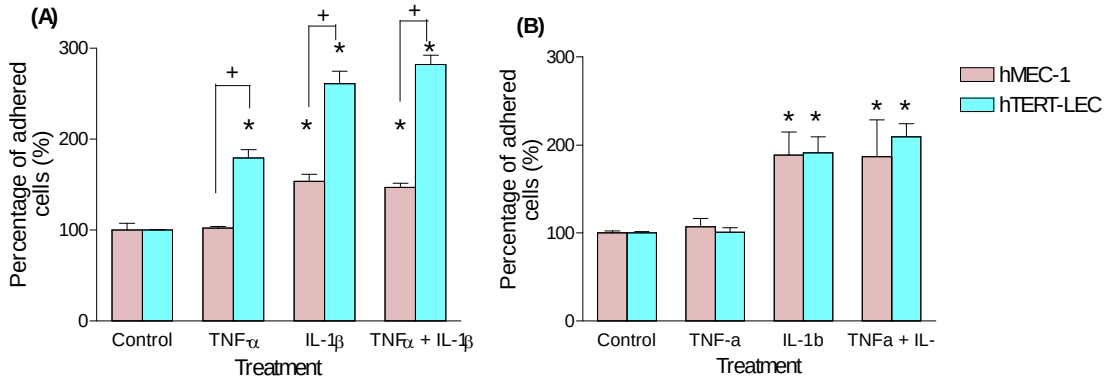


Figure 3-14 MDA-MB-231 (A) and MeWo (B) cell adhesion to TNF-α , IL-1β and TNF-α + IL-1β stimulated hMEC-1 and hTERT-LEC relative to the unstimulated controls. Pre-stimulation of the endothelial cells with TNF-α for 24 hours cause significant increased in MDA-MB-231 adhesion to hTERT-LEC ($p<0.001$) while IL-1β stimulation for 24 hours significantly affect the adhesion of both tumour cells to hMEC-1 and hTERT-LEC, which were higher to the lymphatic endothelial cells. Upon stimulation with TNF-α + IL-1β, no significant changes observed compared to when the cells were stimulated with IL-1β alone. Pooled results from two independent experiments, each carried out in triplicate (n=6). P value was assessed by paired T-test, * represent statistical significance compared to control groups ($p<0.05$). + represent statistical significant between blood and lymphatic endothelial cell models.

3.4.8 The effect of tumour conditioned media on the adhesion of tumour cells to the endothelium

To study the effect of tumour conditioned media on the adhesion of tumour cells to lymphatic and blood endothelium, hMEC-1 and hTERT-LEC were incubated for 24 hours with tumour conditioned media. As observed in Figure 3-15, there was no significant increased in the adhesion of MDA-MB-231 when the endothelial cells were pre-incubated with MDA-MB-231 tumour conditioned media. Similarly, there was no effect in the percentage adhesion of MCF7, MeWo and SKMEL-30 cells towards the pre-incubated endothelial cells with tumour conditioned media.

To ensure that the adhesive effect observed in this assay is a true effect, proliferation assays were carried out by culturing tumour cells with normal cell culture media and with the tumour conditioned media used to in this assay to ensure that these media ware bioactive - results are shown in Appendix C-Figure C1.

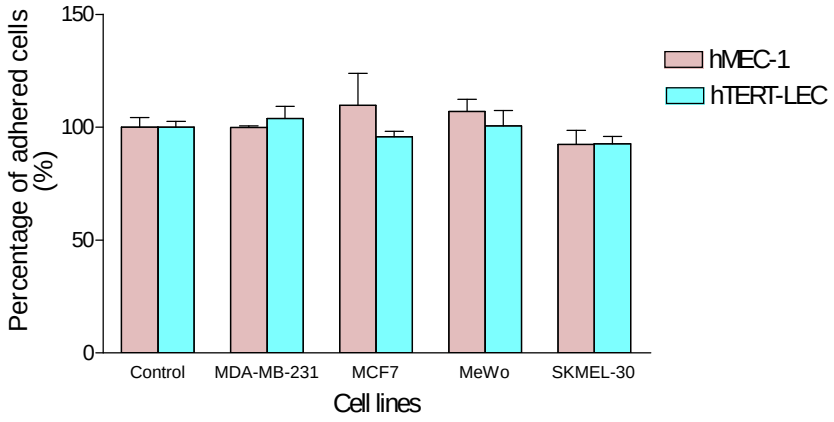


Figure 3-15 MDA-MB-231, MCF7, MeWo and SKMEL-30 adhesion to hMEC-1 and hTERT-LEC stimulated with tumour conditioned media relative to the unstimulated control. Tumour conditioned media stimulation for 24 hours did not have any significant effect on the four tumour cell adhesion to the endothelial cells. Pooled results from two independent experiments each carried out in triplicate (n=6).

3.4.9 The effect of macrophage conditioned media on the adhesion of tumour cells to the endothelium

Macrophage conditioned media from day 7 monocyte-macrophage cultured was obtained from three different donors. Pre-incubation of the endothelial cells (hMEC-1 and hTERT-LEC) with this conditioned media for 24 hours caused a significant increased in the adhesion of MDA-MB-231 and MeWo cells to both hTERT-LEC and hMEC-1 cells relative to the controls ($p<0.05$). The percentage increased in the adhesion of MDA-MB-231 cells was higher to the tumour conditioned media stimulated hTERT-LEC compared to the tumour conditioned media stimulated hMEC-1 across the three donors ($p<0.001$, $p=0.008$, $p<0.001$ for donor 1, donor 2 and donor 3 respectively) (Figure 3.16A). Similar results were also observed in MeWo-endothelial adhesion in which the percentage increased were higher to the tumour conditioned media stimulated lymphatic endothelial cells opposed to the tumour conditioned media stimulated blood endothelial cells in the three donors ($p<0.01$, $p<0.01$ and $p=0.001$ for donor 1, donor 2 and donor 3 respectively)(Figure 3.16B).

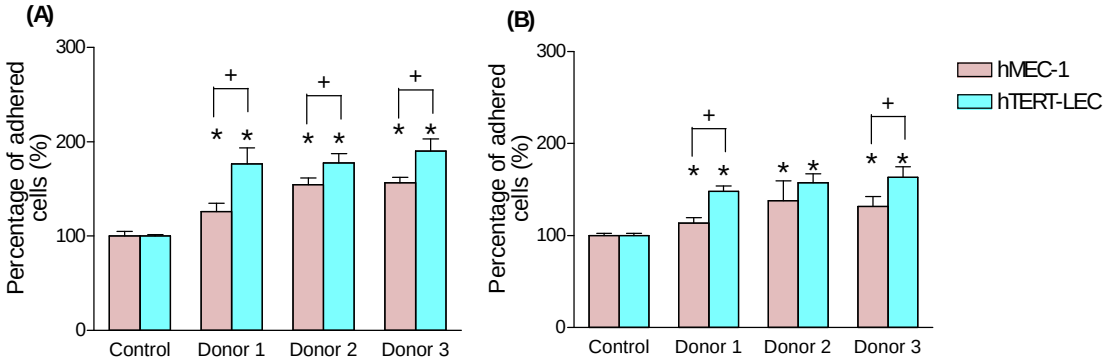


Figure 3-16 MDA-MB-231 (A) and MeWo (B) cells adhesion to tumour conditioned media stimulated hMEC-1 and hTERT-LEC relative to the unstimulated controls. Pre-stimulation of the endothelial cells for 24 hours caused significant increase in both tumour cells adhesion to hMEC-1 and hTERT-LEC which was notably higher to the lymphatic endothelial cells. Pooled results from two independent experiments each carried out in triplicate (n=6). P value was assessed by paired T-test, * represent statistical significance compared to control groups ($p<0.05$). + represent statistical significant between blood and lymphatic endothelial cell models.

3.4.10 IL1-β concentration in tumour conditioned media and macrophage conditioned media

An ELISA was conducted (as in Section 3.3.10) to determine the concentration of IL-1β in macrophage and tumour conditioned media used in the static adhesion assays above (Section 3.4.8 and 3.4.9). No IL-1β was detected in any of the tumour conditioned media used. However, the macrophage conditioned media from the three different donors showed 69.7, 131.8 and 88.8 pg/ml of IL-1β in donor 1, donor 2 and donor 3 respectively. These results were shown in Figure 3-17.

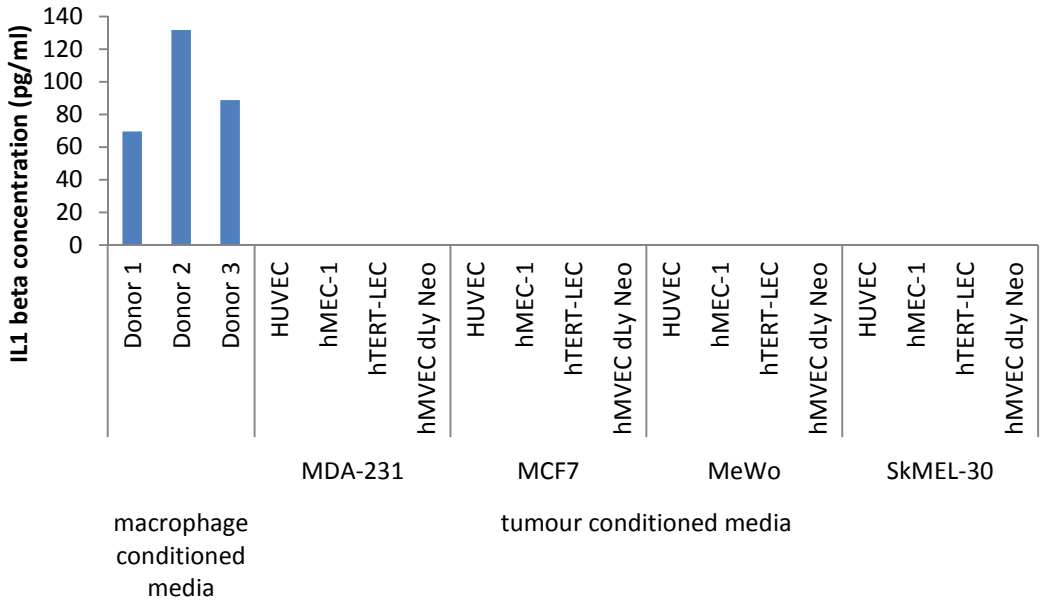


Figure 3-17 IL-1β concentration in macrophage conditioned media (from three different donors) and tumour conditioned media used to stimulate endothelial cells in static adhesion assay. IL-1β was detected in macrophage conditioned media but not tumour conditioned media.

3.4.11 The effect of macrophage conditioned media (5 different conditions with/out ICE inhibitors on the adhesion of tumour cells to the endothelium.

In unstimulated conditions, MDA-MB-231 cells adhered with similar affinity to hMEC-1 and hTERT-LEC across all three donors. These donors are different than the one used in previous adhesion assay and were designated donor 4, 5

and 6. Upon stimulation with macrophage conditioned media generated from LPS-γ stimulated macrophages, there was an increased in the number of MDA-MB-231 cells adhered to both hMEC-1 ($p<0.001$, $p<0.0001$ and $p<0.001$ in donor 4, donor 5 and donor 6 respectively) and hTERT-LEC ($p<0.001$, $p<0.0001$ and $p<0.001$ in donor 4, donor 5 and donor 6 respectively) with no preferential adhesion to either hMEC-1 or hTERT-LEC. The adhesion of MDA-MB-231 cells to hMEC-1 and hTERT-LEC stimulated with macrophage conditioned media generated from TDL stimulated macrophages was not statistically significant compared to the control condition. However, there was a significant decreased in the adhesion of MDA-MB-231 cell when the endothelial cell models were stimulated with macrophage conditioned media generated from LPS-γ+ICE inhibitor and LPS-γ+TDL+ICE inhibitor stimulated macrophages across the three donors (Figure 3-18).

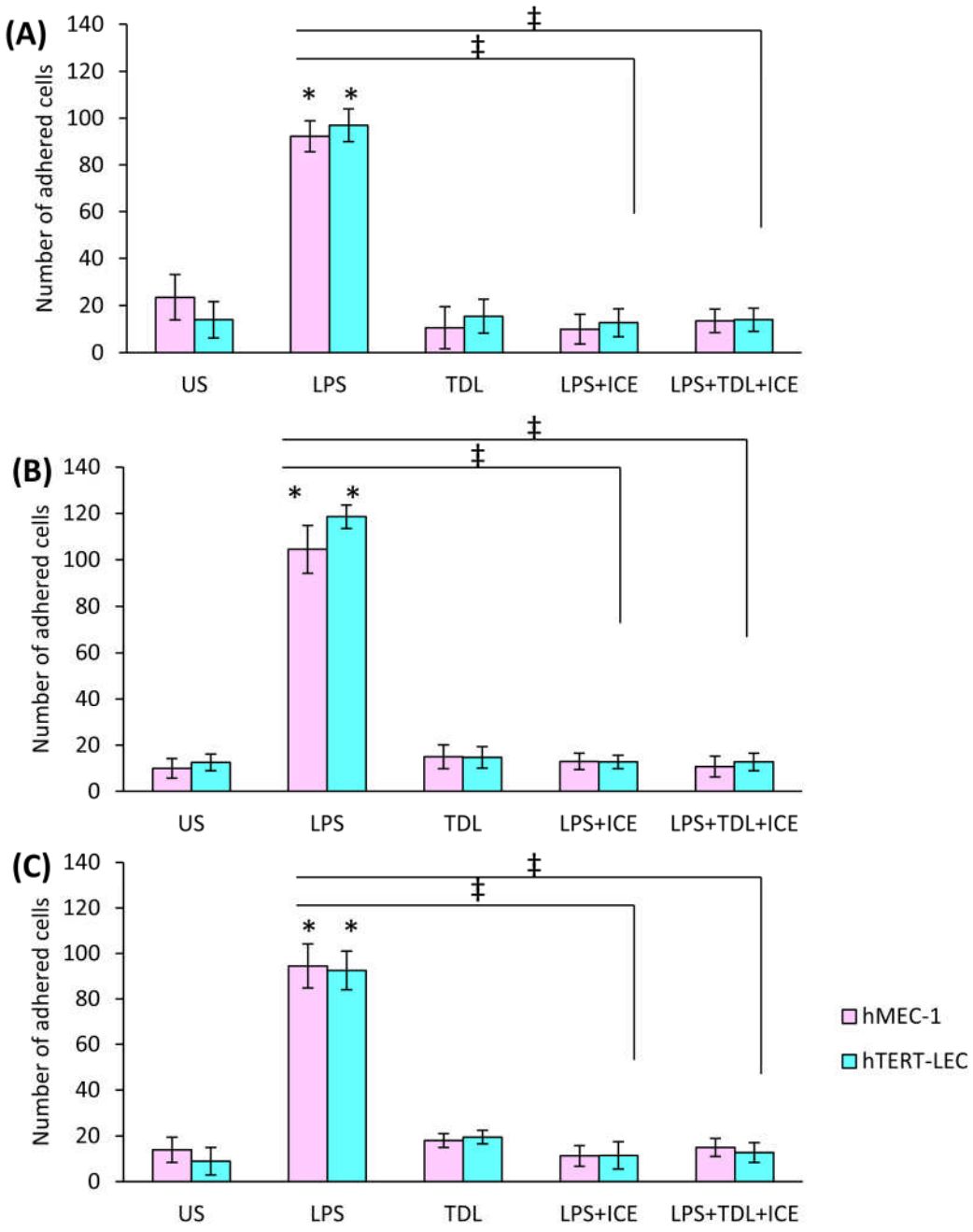


Figure 3-18 Adhesion of MDA-MB-231 cells to hMEC-1 and hTERT-LEC stimulated with macrophage conditioned media from 3 different donor; (A) Donor 4, (B) Donor 5 and (C) Donor 6. Under control conditions, MDA-MB-231 cells shows similar adhesive ability and affinity across both endothelial cells. Adhesion of MDA-MB-231 cells increased significantly across hMEC-1 and hTERT-LEC when they were stimulated with macrophage conditioned media generated from LPS-γ stimulated macrophages. MDA-MB-231 cells adhesion decreased significantly across hMEC-1 and hTERT-LEC in macrophage conditioned media generated from LPS-γ+ICE and LPS-γ+TD+ICE stimulated macrophages in all 3 donors. Pooled data from two independent experiments, each carried out in duplicate (n=4). (*significant difference compared to unstimulated controls) (‡ significant different compared to LPS-γ stimulated group).

3.4.12 IL-1β concentration in macrophage conditioned media

As`previously, an ELISA was conducted to determine the concentration of IL-1β in macrophage conditioned media used in the adhesion assay as described above (Section 3.4.11). No IL-1β was detected in the unstimulated macrophage supernatant across the three donors used. Upon stimulation with LPS-γ, there was a significant increased in the concentration of IL-1β detected in all donors; 166.29, 270.36 and 118.24 pg/ml for donor 4, donor 5 and donor 6 respectively in which the highest increased was observed in donor 5. When ICE inhibitor was added to the LPS-γ stimulated macrophage, the percentage reduction of IL-1β concentration was 82.9, 74.3, 62.5% respectively (Figure 3-19).

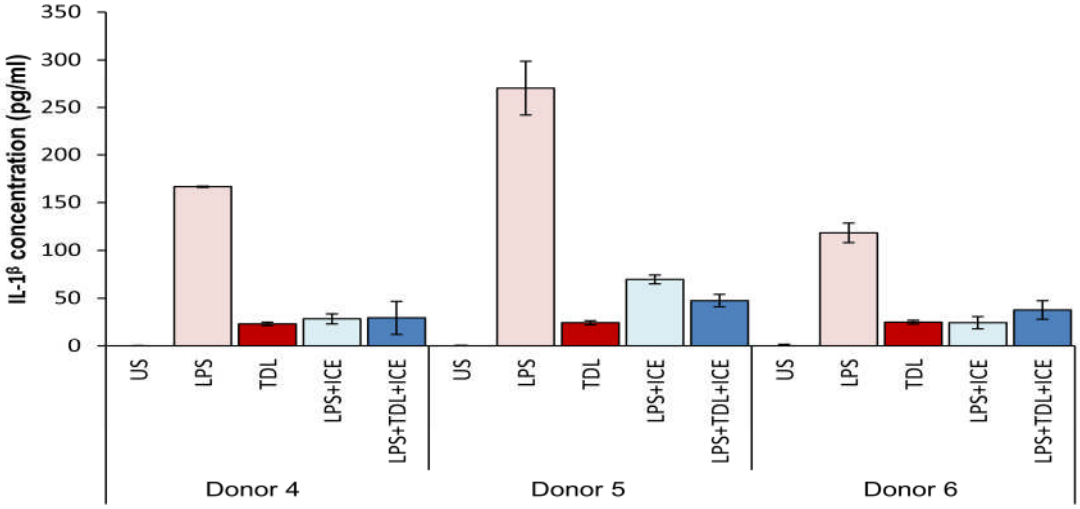


Figure 3-19 IL-1β concentration in macrophage conditioned media (5 conditions) generated from three donors used to stimulate hMEC-1 and hTERT-LEC cells in transendothelial migration assay.

3.4.13 Western blot result for ICAM-1 and VCAM-1 expresion

Figure 3-20 shows ICAM-1 and VCAM-1 protein expression by Western blot. Anti ICAM-1 and anti VCAM-1 bands were detected at 90 and 80 kDa respectively. HUVEC, hMEC-1 and PBMC lysates expressed low level of ICAM-1 under control condition. ICAM-1 expression level was higher in TNF-α stimulated HUVEC and hMEC-1 cells as well as LPS stimulated PBMC. Similarly, VCAM-1 was expressed in lower level in HUVEC and hMEC-1 lysates and the expression was higher in TNF-α stimulated cells. However, under

control condition, HUVEC expressed lower level of ICAM-1 compared to hMEC-1 lysate. No non-specific bands were detected indicating that the anti-ICAM-1 and anti-VCAM-1 antibodies used were specific and detected the correct protein bands. These antibodies were therefore used to assess ICAM-1 and VCAM-1 protein expression via FACS analysis.

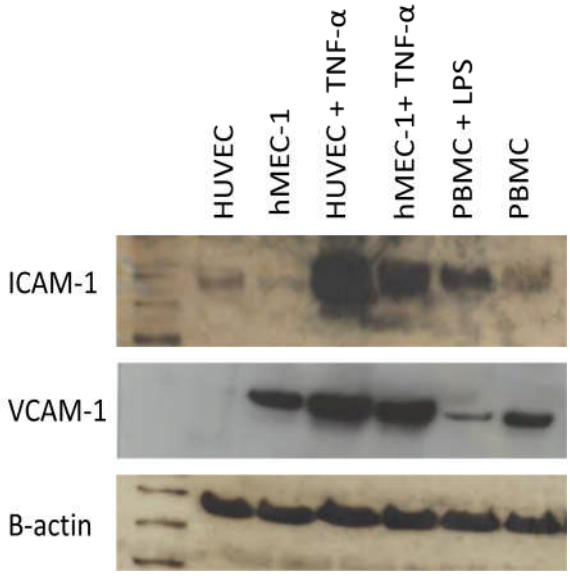


Figure 3-20 ICAM-1 and VCAM-1 expression in HUVEC, hMEC-1 and PBMC cell lysate in control and cytokine stimulated conditions. 25µg of protein were loaded into each well. Native ICAM-1 and VCAM-1 expression was detected in PBMC lysate with stronger expression in TNF-α.LPS stimulated lysates. B-actin bands show equal protein loading into each well.

3.4.14 Endothelial cell expression of CLEVER-1, ICAM-1 and VCAM-1

FACS was used to examine both surface and intracellular expression on unstimulated and TNF-α/IL-1β stimulated endothelial cells. Results show that CLEVER-1 was expressed intracellularly in both blood and lymphatic endothelium with the highest expression in hTERT-LEC (geometric mean (GM) ±SD 36.46±9.33). The GM of HUVEC, hMEC-1 and HMVEC-dLy Neo were 18.65±1.49, 14.67±1.03 and 13.87±0.56 respectively (Figure 3-21). Interestingly, as seen in previous studies (Ammar et al., 2011) CLEVER-1 surface expression was only detected on hTERT-LEC in comparison with low expression on HUVEC, hMEC-1 and HMVEC-dLy Neo (12.11±3.86, 1.43±1.15, 0.80±1.29 and 0.85±0.70 respectively). Upon stimulation with TNF-α, only HMVEC-dLy Neo showed a significant increase of CLEVER-1 expression on both surface (17.91±0.59) and intracellular staining (26.35±1.59). No significant increase in CLEVER-1 expression was observed in any of the endothelial cell lines stimulated with IL-1β (Figure 3-21).

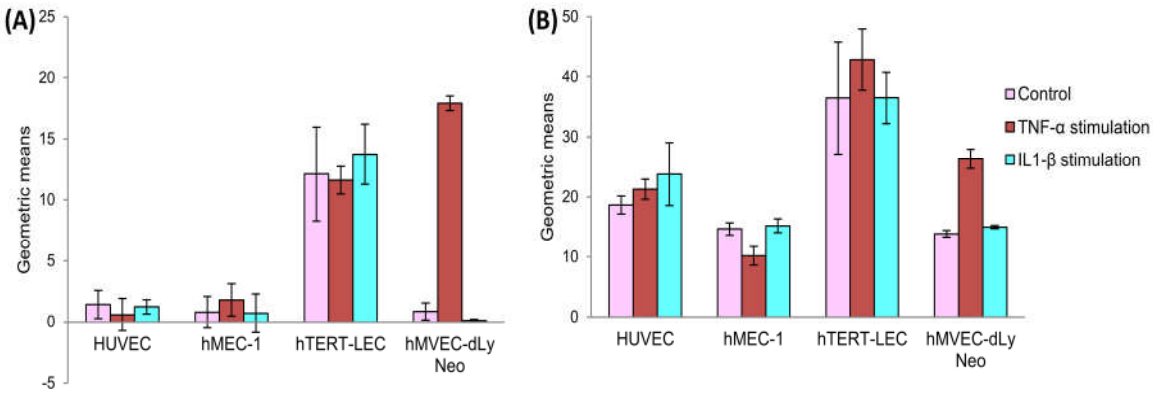


Figure 3-21 CLEVER-1 surface (A) and intracellular (B) expression on HUVEC, hMEC-1, hTERT-LEC and hMVEC dLy Neo under control and TNF-α/IL-1β stimulation. CLEVER-1 surface expression was only detected on hTERT-LEC in comparison with low expression on HUVEC, hMEC-1 and HMVEC-dLy. Only HMVEC-dLy Neo showed significant increased of CLEVER-1 expression on both surface and intracellular staining upon stimulation with TNF-α. No significant increase in CLEVER-1 expression observed in all endothelial cell lines stimulated with IL1-β. Pooled results from three independent experiments each carried out in duplicate (n=6).

Surface expression of ICAM-1 and VCAM-1 was also examined by FACS analysis. Under control conditions, the expression of ICAM-1 was the highest in HUVEC (14.55±1.12) compared to hMEC-1 (2.44±0.25) and hTERT-LEC (3.91±0.74). Upon stimulation with TNF-α/IL-1β, there was an increase in ICAM-1 expression observed across blood and lymphatic endothelial cells (Figure 3-22). VCAM-1 expression was also increased in TNF-α/IL-1β stimulated HUVEC, hMEC-1 and hTERT-LEC (Figure 3-22).

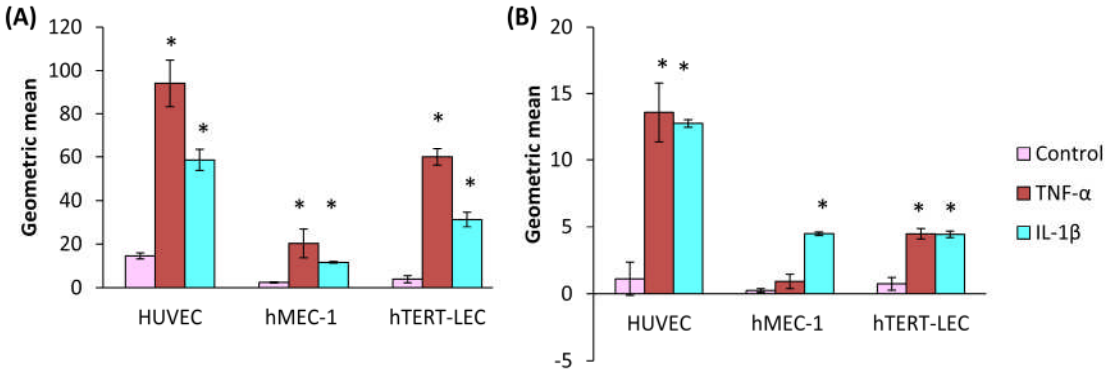


Figure 3-22 Surface expression of (A) ICAM-1 and (B) VCAM-1 on HUVEC, hMEC-1 and hTERT-LEC under control and TNF-α/ IL-1β stimulation. VCAM-1 surface expression was detected in all endothelial cell lines. Upon stimulation with TNF-α and IL-1β, there was an increase in ICAM-1 surface expression. VCAM-1 expression of endothelial cells also increased when HUVEC, hMEC-1 and hTERT-LEC were stimulated with TNF-α/IL-1β. Pooled results from two independent experiments each carried out in duplicate (n=4).(*significant different compared to unstimulated controls).

3.5 Discussion

In the present chapter, the adhesion pattern of breast cancer and melanoma cell lines to blood and lymphatic endothelial models (large vessel versus microvessel and primary versus immortalised cells) was compared. The stimulatory effect of macrophage associated cytokines (TNF-α and IL-1β), macrophage conditioned media and tumour conditioned media on tumour-endothelial adhesion was also compared to study whether these may account for the preferential invasion of lymphatic observed in both melanoma (Chapter 2) and breast cancer (Mohammed et al., 2008) patients. The role of CLEVER-1, which is an adhesion molecule preferentially expressed on lymphatic endothelial cells, in terms of regulating lymphatic vessel invasion was studied, in addition to the stimulatory effect of TNF-α and IL-1β on CLEVER-1 expression on the blood and lymphatic endothelial cell models. ICAM-1 and VCAM-1 expression on the endothelial cell models under control and cytokines stimulated conditions were also investigated.

PBMC adhesion to the unstimulated and TNF-α/IL-1β stimulated HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo was assessed. Adhesion of PBMC was greater to vascular than lymphatic endothelial cells under control conditions. As PBMC were of blood origin, it may be that they are programmed to interact more with blood endothelial barrier compared to the lymphatic barrier. PBMC adhesion to TNF-α stimulated endothelial cells was significantly higher than the unstimulated controls (Salmi et al., 2004). Similarly, IL-1β stimulated HUVEC have been shown to increase PBMC adhesion compared to the unstimulated controls (de Oca et al., 2005). This may be due to increased expression of adhesion molecules such as ICAM-1 and VCAM-1 in inflamed HUVEC and human dermal lymphatic endothelial cells in order to recruit immune cells to the site of inflammation (Louise et al., 2006; Sun et al., 2010; van Grevenstein et al., 2007); which was also shown in this study showing that the expression level of ICAM-1 and VCAM-1 on HUVEC, hMEC-1 and hTERT-LEC was upregulated upon stimulation with TNF-α/IL-1β. The number of PBMC adhered to all TNF-α stimulated endothelial cells was significantly higher

compared to IL-1β stimulated endothelial cells which is in accordance with previous studies comparing PBMC adhesion to TNF-α, IL-1β and interferon-γ stimulated human skin microvascular endothelial cells from adults (HMVEC-Ad) and human coronary arterial endothelial cells (HCAEC) (Watabe et al., 2007).

The adhesion of PBMC to the endothelial models was used as an internal experimental control prior to the assessment of tumour cell adhesion to ensure that both cytokines and endothelial cell models were behaving as expected. When PBMC adhesion was compared against tumour cell adhesion to HUVEC, hMEC-1, hTERT-LEC and hMVEC dLy Neo, results shows that tumour cells have different adhesion kinetics than leukocytes. This may partially attributed to size differences between tumour cells and leukocytes (Wu et al., 2001). The optimal adhesion time for tumour cells was 35 minutes which is in accordance with previous tumour cell adhesion studies where 30 minutes and 1 hour adhesion times were used (Carrire et al., 2005; Jurisic et al., 2010).

In tumour cell adhesion, the breast cancer cell lines, MDA-MB-231 and MCF7, and the melanoma cell lines, MeWo and SKMEL-30, showed different adhesive affinity towards the endothelial cell models, which demonstrated the heterogenous nature of tumour cell adhesion (Eccles and Welch, 2007). Of the four tumour cell lines, MDA-MB-231 had the highest affinity towards all endothelial cell models, which may be related to the highly invasive and aggressive nature of this tumour (Kim et al., 2010; Walter-Yohrling et al., 2003), which expressed high level of vimentin, a marker of mesenchymal phenotype. MCF7 on the other hand expresses markers of luminal epithelial phenotype; estrogen and progesterone receptors positive and HER2/Neu negative, and are less aggressive compared to MDA-MB-231 (Mladkova et al., 2010). Although the four tumour cell lines showed differences in terms of adhesive potential, they all showed preferential adhesion to unstimulated blood endothelial cells as opposed to the lymphatic endothelial cells (Figure 3-8). This is interesting in light of current observations and others (Doeden et al., 2009b; Kuroda et al., 2010; Mohammed et al., 2007) in breast cancer and melanoma that show vascular invasion in these tumour types is principally of lymphatic vessels.

Experiments comparing the adhesion of a panel of breast cancer and melanoma cells on blood and lymphatic endothelial cells have not previously been reported. However, the preferential adhesion of colon carcinoma cells, HT29, to lung microvascular endothelial cells (HMVEC) as opposed to large vein endothelial cells (HUVEC) has been reported (ten Kate et al., 2006). This is in contrast to our current observations made using breast cancer and melanoma cells, in which preferential adhesion of all four cell lines, MDA-MB-231, MCF7, MeWo and SKMEL-30 was observed on HUVEC, reflecting diverse nature of endothelial cells arising from different vascular beds or different adhesion patterns of various tumour types (Murakami et al., 2001; Swerlick et al., 1992). Although the Kate et al (2006) study used fluorescently labeled tumour cells to differentiate them from the endothelial cells, as in the current study, the end measurement to assess adhesion differs as their study used total fluorescence, assessed using a plate reader, to measure cell adhesion rather than counting total number of fluorescent cells.

There was no difference in tumour cell adhesion to primary lymphatic cells (HMVEC-dLy Neo) and immortalised lymphatic cells (hTERT-LEC), suggesting that despite the immortalisation procedure, hTERT-LEC retain a phenotype similar to the primary cells from which they were derived (Nisato et al., 2004). Therefore, hTERT-LEC is a useful *in-vitro* model to study lymphatic-endothelial interactions, which can overcome the problem of a short passage window when using primary lymphatics.

The preferential adhesion of tumour to blood rather than lymphatic endothelial cells was unexpected, considering our previous histopathology based findings in tumour specimens. This may be due to cells being exposed to inflammatory conditions and associated cytokines in the tumour milieu. TNF-α and IL-1β, both are multifunctional cytokines have been known to stimulate angiogenesis, growth, proliferation and invasion of tumour cells via various signaling pathways such as the NF-ĸB and MAPK pathway (Chen and Goeddel, 2002; Wajant et al., 2003). Tumour cell adhesion to TNF-α-stimulated HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo did not vary significantly when compared to

the unstimulated endothelium. Similar results have been described, in which no significant changes are observed in the adhesion of melanoma cell lines; WM9, WM239 and WM119 to unstimulated or TNF-α-stimulated HUVEC (Voura et al., 1998). However, others have shown that tumour cell adhesion increases with TNF-α-stimulation. The colon cancer cell line, HT29 adhered more to TNF-α stimulated HUVEC and lung microvascular endothelial cells than the unstimulated controls. The adhesion of breast MDA-MB-231 cancer cells to HUVEC was shown to increase approximately six fold following TNF-α-stimulation compared to the unstimulated counterpart (Nizamutdinova et al., 2008). No such increased was observed in the current study, using similar cell models; however both the TNF-α concentrations and the incubation time differ. In their study, HUVEC cells were stimulated for 4 hours in which significant increase in the adhesion molecules, ICAM-1 and VCAM-1 was observed compared to the unstimulated control. In this study, the endothelial cells were incubated for 24 hours with TNF-α, at which time, the maximal increase in adhesion molecules such as E-selectin, ICAM-1 and VCAM-1 was reported (Hagi-Pavli et al., 2004).

However, upon IL-1β stimulation, a significant increased of tumour cell adhesion was observed which was notably higher to the lymphatic endothelial cells, suggesting a novel and important role for IL1-β in cancer cell adhesion to lymphatic vessels. Pre-stimulation of either endothelial cells or tumour cells with IL1-β increased the percentage adhesion of MDA-MB-231, MCF7, MeWo and SKMEL-30 towards HUVEC, hMEC-1, hTERT-LEC and hMVEC dLy Neo with similar adhesive affinity towards the endothelial of the same origin i.e. blood and lymphatics. Dejana and colleague (1998) have also reported similar results. Using three human colorectal carcinoma (HT-29, HCC-P2988, and HCC-M1410) and one human melanoma (A-375) cell line, a significant increase in the adhesion pattern of these cells towards IL-1β stimulated HUVEC could also be observed (Dejana et al., 1998).

As the two breast (MDA-MB-231 and MCF7) and the two melanoma (MeWo and SKMEL-30) cell lines showed a similar adhesion pattern towards blood and

lymphatic endothelial cells, experiments were therefore conducted to examine the effect of TNF-α and IL-1β activated MDA-MB-231 and MeWo cells in influencing the adhesion towards TNF-α and IL-1β activated hMEC-1 and hTERT-LEC. A significant increased was observed when both the tumour and the endothelial cells were simultaneously stimulated with TNF-α suggesting that dual activation with TNF-α might be necessary to promote tumour-endothelial cell adhesion. In tumour cells, TNF-α and IL-1β acts through NF-ĸB induction of chemokine receptor CXCR4 and up-regulation of molecules such as monocyte chemoattractant protein-1 (MCP-1), IL-8 and ICAM-1. In endothelial cells, both cytokines up-regulate various receptors such as lectin-like oxidized low-density lipoprotein (oxLDL) receptor-1 (LOX-1) (Choo et al., 2006; Katerinaki et al., 2003; Liang et al., 2007; Wang and Lin, 2008). Interestingly, the percentage adhesion of both MDA-MB-231 and MeWo was higher to the lymphatic endothelial model (hTERT-LEC), as opposed to the blood endothelial cells (hMEC-1). Such results suggest that although in control conditions, tumour cells adhered more to the blood endothelium, TNF-α and IL-1β activation increases the percentage of adherent cells, which was higher to lymphatic endothelial cells. Such results support the hypothesis that in melanoma and breast cancer, lymphatic adhesion, and subsequently metastasis by this route, can be modulated by cytokine stimulation.

Results from current experiments suggested that macrophage secreted cytokines i.e. TNF-α, and more importantly IL-1β, have strong influences in regulating adhesion of tumour cells to the lymphatic endothelial cells and thus may modulate lymphatic vessel invasion. Involvement of TNF-α in malignancy comes from the observation that mice which lack the gene for TNF-α are resistant to skin cancer (Moore et al., 1999). Similarly, in melanoma cell models as well as breast and prostate cancer, the absence of metastasis was observed in mice models lacking IL-1β gene (Pollard, 2008). In addition, interleukin-1 receptor antagonist (IL-1ra) inhibits the increased adhesion of A375M melanoma cells on IL-1β activated HUVEC, supporting the fact that IL-1β mediated tumour-endothelial interaction is involved in the adhesion and thus metastatic process (Chirivi et al., 1993).

When both blood (HUVEC and hMEC-1) and lymphatic models (hTERT-LEC and HMVEC-dLy Neo) were stimulated with tumour conditioned media or macrophage conditioned media, only the macrophage conditioned media stimulated endothelial cells exhibited increased tumour cell adhesion. These results suggest that cytokines secreted by macrophages, rather than tumour cells, may contribute to the increased invasiveness of tumour cells - this is supported by the ELISA results where IL-1β was detected in macrophage conditioned media but not tumour conditioned media. The fact that IL-1β is not detected in tumour conditioned media used in this study is interesting because tumour cells, besides macrophages could also produced IL-1β in both normal settings and in the tumour microenvironment. This may be due to the effect of IL-6 or IL-10, which could suppress IL-1β production in the tumour (Bogdan et al., 1992; Jensen et al., 2009). IL-6 and IL-10 ELISA's could be carried out on tumour conditioned media to confirm the expression of these cytokines, and determine how IL-1β is regulated in these cells. However, IL-1β concentration, from the macrophage media, seemed to correlate with the number of cells adhered to the endothelial cell models. IL-1β has been shown to increase the expression of a number of adhesion molecules which eventually promotes tumour-endothelial cell adhesion (Pollard, 2008) as observed in this study. When ICE inhibitor was used with the LPS-γ stimulated macrophages, the increased adhesion effect was removed and may be due to the fact that there was a decreased in the expression of cell adhesion molecules on both blood and lymphatic endothelial cells.

Therefore, the role that adhesion molecules preferentially expressed on the lymphatic endothelial cells play in regulating lymphatic vessel invasion was also examined. Adhesion molecules are of particular interest as they regulate tumour cell entry into, and exit out of, the lymphatics. Based on our historical, and current, data, CLEVER-1 is preferentially expressed on the surface of lymphatic endothelial cells (hTERT-LEC). Interestingly, CLEVER-1 expression in lymphatic vessels was associated with lymph node metastasis (Ammar et al., 2011). In this chapter, we compared CLEVER-1 expression between blood and lymphatic endothelial cells, and between primary and immortalised cell lines,

showing that although all endothelial cell models expressed CLEVER-1 intracellularly, only the immortalised lymphatics, hTERT-LEC showed significant surface expression of CLEVER-1. Similar results have been published showing that CLEVER-1 is differentially expressed in lymphatic, but not vascular endothelial cells in normal skin in-vivo (Salmi et al., 2004). Upon stimulation with TNF-α, only the primary lymphatics, HMVEC-dLy Neo showed a significant increase in CLEVER-1 expression; both total and surface, suggesting that hTERT-LEC represent an activated LEC model, at least in terms of CLEVER-1 expression. Stimulation of HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo with IL1-β did not show any significant altered CLEVER-1 expression. Such results, in light of the adhesion assay results, suggest that although CLEVER-1 may be an important lymphatic specific adhesion molecule, it may not be a principal regulator of lymphatic vessel invasion. In tumour sections CLEVER-1 expression was observed in blood vessels, lymphatic vessels and macrophages, however, only CLEVER-1 expression in lymphatic vessels was associated with lymph nodes metastasis (Ammar et al., 2011). Based on these results, CLEVER-1 knockdown in lymphatic cells would be required to further examine the role that this molecule plays in mediating tumour cell trafficking via lymphatic vessels. However, at this stage, studying CLEVER-1 was discontinued from the current study.

Surface expression of adhesion molecules, ICAM-1 and VCAM-1 were also examined. Upon stimulation with TNF-α/IL-1β, increased expression of these adhesion molecules could be observed in HUVEC, hMEC-1 and hTERT-LEC cells which is in accordance with other studies (Brigati et al., 2002; Hayes and Seigel, 2009), suggesting that these adhesion molecules might play important role in regulating tumour-endothelial adhesion processes. As in CLEVER-1, ICAM-1 and VCAM1 knockdown would be required to examine the role of these molecules in mediating tumour cell trafficking into the circulation.

In summary, our previous translational investigations as discussed in Chapter 2, and by others (Mohammed et al., 2008), have demonstrated that lymphatic vessels play an important role in metastatic disease. We have shown in the

current chapter that TNF-α, and more importantly IL-1β stimulation, of both tumour and endothelial cells causes a significant increase in adhesion, of which the highest increase is observed on lymphatic endothelial cell models. A suitable *in vitro* lymphatic endothelial cell system is important to understand the complex mechanisms governing tumour cell adhesion and migration towards the lymphatics, which in some tumour types, occurs more frequently than invasion of blood vessels. Current results suggest that metastasis resultant from lymphatic vessel-tumour cell adhesion is modulated by cytokine stimulation, which may represent an important therapeutic target in breast cancer and melanoma.

In addition to tumour-endothelial adhesion, tumour cells migration through the extracellular matrix and tumour cell transendothelial migration is a pre-requisite for metastatic dissemination into the circulation. Therefore, we next sought to study whether TNF-α and IL-1β have any effect upon tumour cell migration, and endothelial transmigration, which is the focus of Chapter 4.

CHAPTER 4: TUMOUR CELL MIGRATION AND BLOOD VERSUS LYMPHATIC ENDOTHELIAL TRANSMIGRATION

4.1 Abstract

Background and Aims: In the initial metastatic process, the capability of cancer cells to metastasise depends on their ability to detach from the tumour mass, migrate through the extracellular matrix, adhere to the endothelium and transmigrate between blood and/or lymphatic endothelial cells into the circulation. Results in Chapter 3 demonstrated that tumour-endothelial cell adhesion can be modulated by cytokine stimulation and that TNF-α, and most importantly IL1-β secreted by macrophage, strongly influenced tumour cell adhesion to lymphatic endothelial cells; thus potentially modulating lymphatic endothelial transmigration. The aim of the current chapter was to investigate the effect of TNF-α, IL-1β, tumour conditioned media and macrophage conditioned media stimulation on breast cancer and melanoma cell migration. In addition, the transmigration pattern of both melanoma and breast cancer cell lines across blood and lymphatic endothelial cell models were compared following stimulation with either IL-1β or macrophage conditioned media.

Methods: The effect of TNF-α, IL-1β, tumour conditioned media and macrophage conditioned media stimulation on melanoma (MeWo and SKMEL-30) and breast cancer (MDA-MB-231 and MCF7) cell migration was investigated using scratch wound assays. Melanoma (MeWo) and breast cancer (MDA-MB-231) transmigration across the lymphatic (hTERT-LEC) and blood (hMEC-1) endothelial cell models was assessed using Boyden chamber transmigration assays. The effect of IL-1β and macrophage conditioned media stimulation of endothelial cells on the transmigratory process was also examined.

Results: The percentage reduction of scratch area at different time points represented tumour cell migration in the scratch wound migration assay. MDA-MB-231 showed the highest percentage of wound closure followed by MCF7, SKMEL-30 and MeWo under control conditions. Following exposure to TNF-α and IL-1β, the migration of all four tumour cell lines was increased. Increased migration was higher in IL-1β stimulated cells compared to TNF-α stimulated cells for all of the tumour cell lines assessed. There was no significant

difference in the migration ability of MDA-MB-231, MCF7, MeWo and SKMEL-30 when the cells were stimulated with tumour conditioned media compared to the unstimulated controls. A significant increase in the percentage of wound closure was observed at 24 hours post-wounding when the cells were stimulated with macrophage conditioned media from donor 2 ($p<0.0001$) and donor 3 ($p=0.023$).

Results from the static transendothelial migration assay, show that MDA-MB-231 cells exhibit similar transmigratory abilities across both hMEC-1 and hTERT-LEC which increased significantly upon stimulation with IL-1β (p-values <0.001 and <0.001 for hMEC-1 and hTERT-LEC respectively). MeWo cell transmigration under control conditions showed similar affinity across both hMEC-1 and hTERT-LEC, however with a lower number of cells migrating across the endothelium compared to MDA-MB-231 cells. However, upon stimulation with IL-1β, there was an increased in the transmigratory ability of MeWo cells across hMEC-1 and hTERT-LEC (p-values <0.00001 and <0.0001 respectively) which was notably observed across hMEC-1 cells. When hMEC-1 and hTERT-LEC were stimulated with LPS-γ stimulated macrophage conditioned media, there was an increased in the number of cells transmigrated across the endothelial cell monolayer; hMEC-1 and, particularly, hTERT-LEC. However, when hMEC-1 and hTERT-LEC were stimulated with macrophage conditioned media generated with LPS-γ+ICE and LPS-γ+TDL+ICE stimulation, the number of transmigrated MDA-MB-231 cells decreased significantly compared to both control and LPS stimulated macrophages suggesting that it was IL1β in the macrophage lysate that was responsibe for the increased transmigration.

Conclusions: Tumour cell migration and transmigration across the lymphatic and blood endothelium can be modulated by cytokine stimulation. TNF-α and especially IL-1β, are inflammatory cytokines secreted by macrophage that have strong influences in regulating tumour cell migration and transmigration across endothelial cells, particularly across lymphatic endothelium.

4.2 Introduction

Tumour cell adhesion to blood or lymphatic vessels is an important step in the process of vascular invasion and entry into the circulation. Results presented in Chapter 2 showed that in melanoma, vascular invasion is principally of lymphatic vessels opposed to blood vessels even though a rich vascular network was present. Similar patterns were also observed in breast cancer patients, where 85.5% vascular invasion occurs via lymphatic vessels (Mohammed et al., 2008). A highly significant association between high macrophage counts and the presence of lymphatic vessel invasion was also observed, which may suggest that macrophage, or macrophage associated factors, play a role in promoting invasion through lymphatic vessels. In vitro results, from Chapter 3, show that TNF-α and IL-1β, which are cytokines secreted by macrophage, can influence tumour cell adhesion to lymphatic and blood endothelial cell models, but with a preferential increase observed to lymphatic endothelial cells.

In addition to tumour cell adhesion, the migration of tumour cells through the extracellular matrix is also a pre-requisite in the initial metastatic progression. Cell migration is a cyclic process involving continuous formation and disassembly of matrix adhesions (Etienne-Manneville, 2008). This multistep model of cell migration has been investigated in non-neoplastic cells such as fibroblasts and keratinocytes during normal physiological processes including wound healing and immune cell trafficking. However, studies on tumour cells show that these cells use similar migratory mechanisms to those that occur in normal cells. In response to migratory and chemotactic stimuli, cells form a membrane protrusion which is driven by actin polymerization and assembly of cell filaments. Cell protrusions attach to the surrounding tissue structure or extracellular matrix, contract and subsequently generate a traction force that leads to forward gliding of the cells. The molecular mechanisms of cell migration are summarised in Figure 4-1.

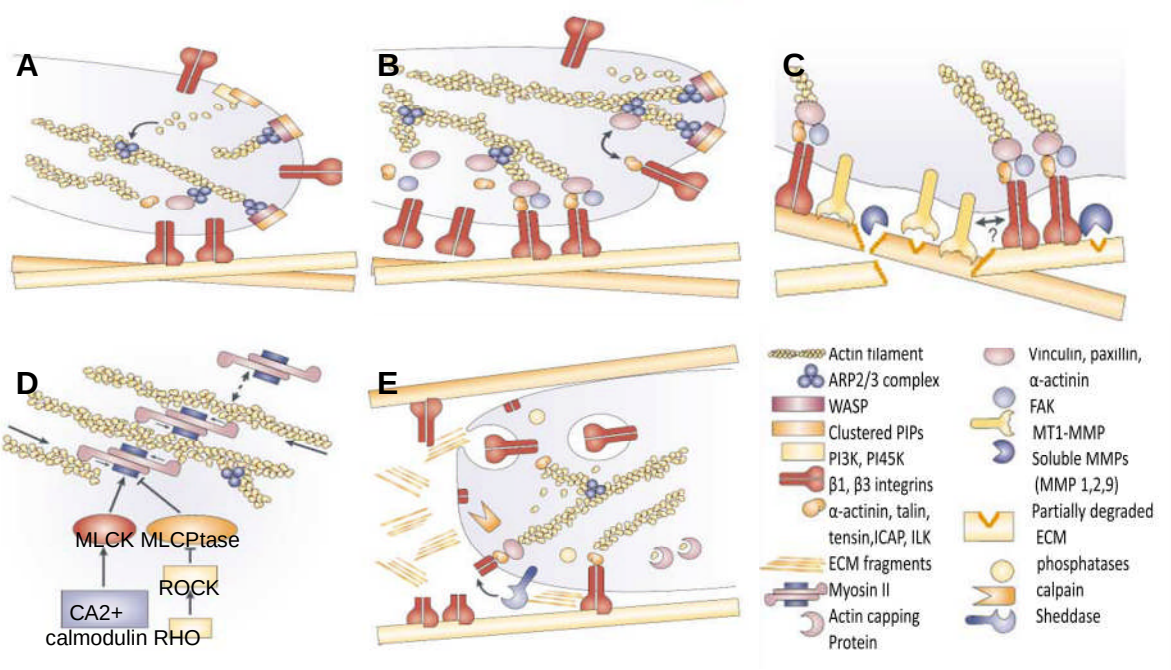


Figure 4-1 A five step model of cell migration. (A) Formation of cell protusions is driven by actin polymerization which binds to ARP2/3 and Wiscott-Aldrich protein (WASP). ARP2/3/WASP complex binds to inner leaflet of the plasma membrane via phosphoinosites (PIPs) and CDC42 inducing pseudopod extension. (B) Formation of focal contact. Integrins cluster in the cell membrane and come into contact with ECM inducing phosphorylation and dephosphorylation signals into the cell. (C) Surface proteases cleave ECM components (collagen, fibronectin, laminins, pro-MMPs). (D) Active myosin II binds to actin filaments generating actomyosin contraction. (E) Detachment of the trailing edge. Focal contact disassembly occurs at the trailing edge leading to integrin detachment from the substrate. Integrins detached from the substrate become internalised by endocytotic vesicles to be recycled towards the leading edge or deposited onto the substrate. Reproduced and adapted from (Friedl and Wolf, 2003). Copyright© by Nature Publishing Group with permission conveyed through Copyright Clearance Center Inc.

The process of migration can be studied using a wound healing assay which is a convenient and inexpensive method for the analysis of cell migration in vitro. This assay involves creating a wound or a scratch in a cell monolayer and capturing an image of wound closure at regular intervals before quantifying the rate of migration. Wound healing assays have been used to study the ability of cells to polarise and migrate and to study the effect of cell-matrix and cell-cell interactions in the migratory process (Etienne-Manneville and Hall, 2001; Nobes and Hall, 1999).

Transmigration of cancer cells across the endothelium follows migration and adhesion steps in the initial metastatic cascade. This process, termed

intravasation i.e the entry of cells into the vasculature is less well characterised than extravasation i.e the movement of cells from the circulation into the tissue which mimics leukocytes extravasation following inflammation. Controversy exists in relation to whether tumour cells actively transmigrate across blood or lymphatic vessels in response to chemokines/growth factors or whether they passively enter the circulation without active migratory mechanisms (van Zijl et al., 2011). In term of lymphatic capillaries, the suggestion has been put forward that transmigration may be predominately a passive process due to the structure of the lymphatic vessels which lack basement membrane and inter-endothelial tight junctions. In addition, tumour lymph/angiogenesis generates immature and poorly organised vessels that might allow shedding cancer cells to pass. Yet, many studies have supported the hypothesis that intravasation is an active process involving the interactions between tumour cells, endothelial cells and the microenvironment with expression of growth factors and adhesion molecules and secretion of various chemokines playing an important role.

The tumour microenvironment consists of many cell types, the population of which is determine by the types of tumour (Pollard, 2008). Examples of cells present in the tumour environment include fibroblasts, adipocytes and haematopoietic and immune cells such as leukocytes, lymphocytes, mast cells and macrophages.

4.2.1 The involvement of macrophage in tumour cell invasion

Tumour associated macrophage (reviewed in Section 1.6.1 -Chapter 1) which populate the microenvironment of most tumours have been shown to promote and enhance tumour cell invasion. Studies using human and mouse mammary carcinoma have shown that tumour associated macrophage are present in at least three separate populations in the tumour microenvironment; (1) in the stroma surrounding tumour cells, (2) accumulating around the edge of dying cells in necrotic areas and (3) aligned along the abluminal side of vessels (Sahai, 2007). Such tumour associated macrophages produce epidermal growth factor (EGF) and those that are clustered around vessels may establish EGF gradients that attract tumour cells towards the vessels thus promoting

intravasation. A paracrine signaling loop was identified between carcinoma cells and macrophages, where tumour cell EGF receptors and macrophage CSF-1 receptors are activated by macrophage-produced EGF and tumour cell-produced CSF-1 respectively, leading to increased migration and invasion (Wyckoff et al., 2000). In separate experiments, the production of TNF-α, IL-1β, MMP-2, -3, -7 and -9 have been shown to be upregulated in macrophages when co-cultured with breast cancer cells (Pollard, 2008). However, the authors did not discuss on how breast cancer could promote such an effect. Therefore, questions exist on how breast cancer could promote such an effect and whether the increase in these cytokines in macrophages could increase tumour cells metastatic capacity.

Although not widely studied, similar to the tumour-endothelial adhesion (discussed in Chapter 3), reports thus far have utilised either blood endothelial cells, mostly HUVEC, or very occassionaly lymphatic endothelial cells to examine the transmigration of tumour cells across the endothelium. The focus of the current chapter was to conduct a side to side comparison of tumour cell transendothelial migration across blood versus lymphatic endothelial cells and determine what factors might influence this process.

The aims and objective of the current chapter were to;

1. Investigate the effect of macrophage associated cytokines; TNF-α and IL-1β and the effect of tumour conditioned media and macrophage conditioned media on breast cancer (MDA-MB-231 and MCF7) and melanoma (SKMEL-30 and MeWo) cell migration.

2. Compare the transmigration pattern of melanoma (MeWo) and breast cancer (MDA-MB-231) cell lines across blood and lymphatic endothelial cell models; hMEC-1 and hTERT-LEC respectively, under control and IL-1β stimulated conditions in addition to the effect of macrophage conditioned media in this process.

4.3 Materials and Methods

4.3.1 Optimisation of seeding density for scratch wound migration assay

Optimisation of tumour cells seeding density was carried out before carrying out scratch wound migration assay to ensure 100% confluent monolayer prior to wounding. MDA-MB-231, MCF7, MeWo and SKMEL-30 was added at $1x10^5$, $2x10^5$, $3x10^5$, $4x10^5$ and $5x10^5$ per well in duplicate wells. The tumour cells were incubated at 37^oC for 24 hours. After 24 hours, the tumour cells were observed under the microscope to determine their confluency. Photomicrographs of the cells were also taken at 10x microscopic magnification. Optimisation was carried out twice for each tumour cell line, each in duplicate.

4.3.2 Scratch wound migration assay

Scratch wound migration assays were conducted by plating $4x10^5$ MDA-MB-231cells/well in a 12 well tissue culture plate and incubating for 24 hours prior to wounding to ensure a confluent monolayer (as optimised in Section 4.3.1). 24 hours post-plating, the standard culture media was removed and replaced with growth factor and serum free media with or without TNF-α (2, 5 and 10ng/ml) or IL-1β (5, 10 and 15ng/ml) for a further 24 hours. Confluent cell monolayers were scratched with a p20 pipette tip done at a 90 degree angle to create a wound, devoid of adherent cells. Wound scratch at 90 degree angle to the tissue culture plate was shown to produce a more consistent and reproducible width as shown by Yimin Zhang in a separate PhD project.

Cell debris was washed away twice with PBS and cells were cultured in growth factor and serum free media with 10µg/ml of mytomycin-c (Sigma, UK) for the duration of this assay to inhibit cellular proliferation. Wound closure was monitored by photomicrographs taken at predetermined time points (0, 2, 4, 6 and 24 hours) after the scratch was made. Figure 4-2 shows representative photomicrograph of MDA-MB-231 scratch wound migration assay taken at 0, 4, 6 and 24 hours after the scratch was made on a confluent cell monolayer.

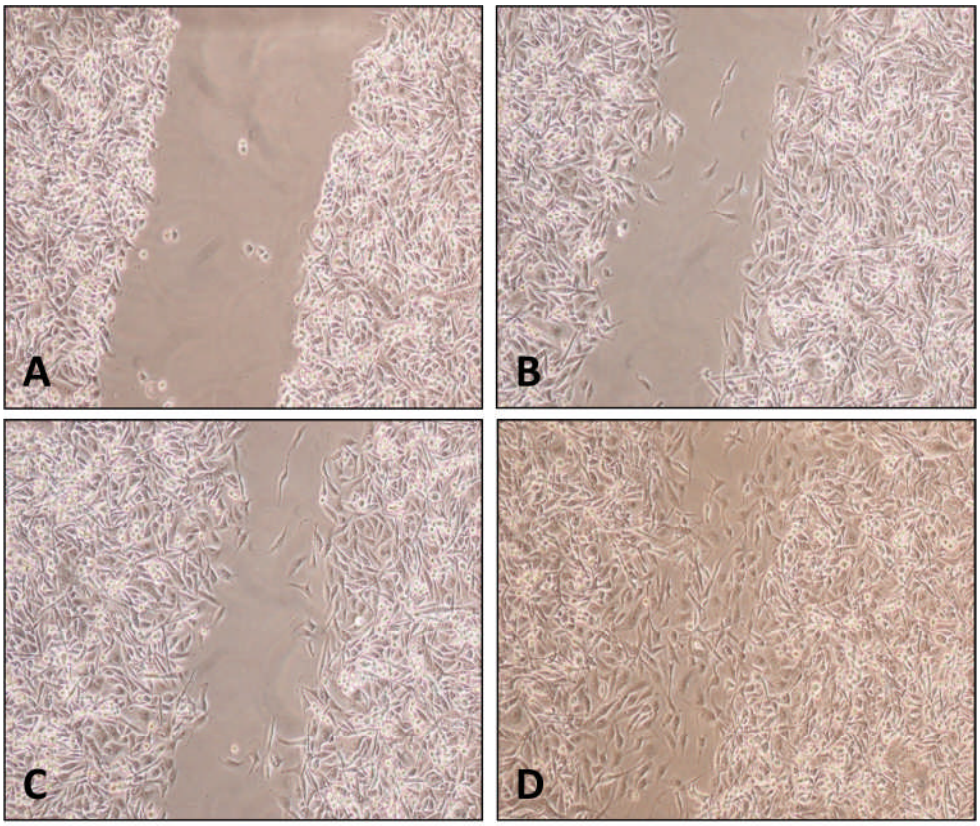


Figure 4-2 MDA-MB-231 scratch wound migration assay. Confluent cell monolayers were scratch with a pipette tip to create a wound, devoid of adherent cells. Photomicrographs were then taken at (A) 0, (B) 4, (C) 6 and (D) 24 hours. Percentage reduction of the scratch area at different time points represented the rate of tumour cell migration. (100x magnification).

The percentage reduction of the scratch area at different time points represented the tumour cells migratory ability and was measured using ImageJ 1.43u software (National Institute of Health, USA). Experiments were conducted twice, each in triplicate.

As 5ng/ml TNF-α and 10ng/ml IL-1β was determined to be an optimal concentration used to stimulate MDA-MB-231 cells in initial adhesion assays (mentioned above), these concentrations were also used to examine MCF7, MeWo and SKMEL-30 cell migration. Scratch wound migration assays were also conducted to examine the effect of tumour conditioned media (generated as described in Section 3.3.5) on MDA-MB-231, MCF7, MeWo and SKMEL-30 and the effect of macrophage conditioned media (generated as described in section 3.3.6) on MDA-MB-231 cells migration. These macrophage conditioned

media were from three new donors and were designated donor 7, 8 and 9. IL-1β levels in these media; the macrophage and tumour conditioned media, was determine by ELISA as described in Section 3.3.10.

4.3.3 Optimisation of coating agents and endothelial cell seeding density for transmigration assay

The transmigration assay was carried out using Transwell chambers (Costar, CA, USA) with 6.5mm diameter polycarbonate membrane filters of 8µm pore size which were used to form dual compartments in a 24 well tissue culture plate. Prior to transmigration assay experiments, the total number of endothelial cells (hMEC-1) to be added per transwell was optimised so that a confluent intact cell monolayer was obtained. A confluent cell monolayer is essential for transendothelial migration assay. In addition to the seeding density, the concentrations and the types of coating on which to grow the endothelial cells was also optimised. The Transwell inserts are made of polycarbonate membrane on which it is difficult to culture the endothelial cells on, to obtain a cell monolayer. Cell attachment can be improved by coating the culture surface with matrix constituents. Coating agents used in this optimisation procedure were gelatin (0.2%) (Sigma, UK) and rat tail collagen type I (GIBCO, Paisley, UK) in 3 different concentrations; 5, 10 and 15µg/cm^3.

To coat the polycarbonate membrane with gelatin, 0.2% gelatin diluted in PBS was added to the inserts and incubated at 37^{o}C for 30 minutes. The media was then removed and washed twice with PBS prior to the addition of endothelial cells. To coat the polycarbonate membrane with collagen, 5, 10 and 15µg/cm^3 rat tail collagen type I diluted in 0.02M acetic acid was added to the inserts and incubated at 37^{o}C for 1 hour. The remaining solution was aspirated from the inserts, washed twice with PBS to remove all the acid and air dried for 30 minutes in the fume hood prior to usage.

hMEC-1 cells at different seeding densities (5x10^4, 7.5x10^4, 1x10^5 and 2x10^5) were then added to the coated inserts and incubated at 37^{o}C for 24 hours after which time the media was removed and cells washed twice with PBS. To check

for an intact cell monolayer, 100µg/ml of Lucifer yellow was added to the inserts or to Transwell inserts without cells as control. 600µl of PBS was added to the lower wells. The plate was incubated at 37^{o}C for 30 minutes. Fluorescence leakage of each well was determined using a plate reader (Fluorostar Optima, BMG LabTech) (excitation 458nm, emission 520nm) using Fluorostar Optima software version 1.30 R3. The lucifer yellow leakage range for an intact cell monolayer is typically 0.3% to 2% reduction of control. A leakage value of >2% of control suggests that cell monolayer was likely compromised during the assay (Costar, USA).

4.3.4 Optimisation of tumour cell labeling for transmigration assay

Tumour cells used in the transmigration assay were fluorescently labeled to differentiate them from the endothelial cells. Optimisation was carried out using MDA-MB-231 and MeWo cells labeled with different concentrations of Cell Tracker Green CMFDA (Invitrogen, USA). MDA-MB-231 and MeWo cells in suspension were labeled with 1nm, 2.5nm, 5nm, 10nm and 15nm of cell tracker green diluted in RPMI serum free media at 1x10^{6} cells per ml for 30 minutes at 37^{o}C. Labeled cells were centrifuged at 170g for 10 minutes. Supernatant discarded and cells resuspended in RPMI media and cultured in a 12 well plate at 37^{o}C. After 24 hours, the fluorescently labeled tumour cells were viewed under the fluorescence microscope to access their fluorescence signal. The labeled cells were also trypsinised and counted to assess their viability and proliferation. The labeled cells also were monitored at 48 and 72 hours post labeling.

4.3.5 Transmigration assay

In the transmigration assay optimisation (Section 4.3.3), coating the upper compartment (insert) of the Boyden chamber Transwell system with rat tail collagen type I gave more reproducible and consistent results in generating endothelial cell monolayers. So, this coating agent was used in all subsequent transmigration assay experiments.

Endothelial cells (seeding density: $2x10^5$ for both hMEC-1 and hTERT-LEC cells in 100µl cultured media) were added to the coated insert and grown to confluence by incubating them at 37^oC for 24 hours with 600µl of complete media in the lower compartment. After 24 hours, complete media was removed and replaced with growth factor free media in control wells or growth factor free media supplemented with IL-1β (10ng/ml) and incubated at 37^oC for a further 24 hours.

Prior to conducting the transmigration assay, lucifer yellow leakage was used to check for intact endothelial cells monolayers (Section 4.3.3). If an intact endothelial cell monolayer was obtained, the transmigration assay would proceed by first labelling tumour cells. The tumour cells were trypsinised to produce a single cell suspension and then counted using the cell countess system. Cells at $1x10^6$ per ml were then labeled with 5nM of Cell Tracker Green CMFDA (Invitrogen, USA) for 30 minutes at 37^oC. This concentration was shown not to affect cell viability and proliferation up to 72 hours (section 4.4.7). After labelling, tumour cells were resuspended in RPMI-1640. $1x10^5$ cells per well (100µl volume) were seeded on top of the endothelial cell monolayer. 600µl of RPMI-1640 was added to the lower compartment. As a positive control, 600µl of heat inactivated fetal calf serum was added to the lower chamber and these cells were left to transmigrate for 16 hours at 37^oC (Tumour cell transmigration time was optimized between 4 to 24 hours and the results shown in Section 4.4.8)

At the end of the assay, the endothelial cells and the non-migrated tumour cells in the upper chamber were cleansed with a cotton swab. Tumour cells that had transmigrated to the lower face of the filter were manually counted using a fluorescence microscope (Nikon, Japan). 2 fields of view were counted in each well at 10x magnification. Experiments were conducted twice, both in duplicate. Results were expressed as the absolute number of cells transmigrated and as the percentage of cells transmigrated relative to control. Transmigration assays were also performed by stimulating the endothelial cells with macrophage conditioned media.

4.3.6 Statistical analysis

All data are represented as mean ± standard deviation (SD) of two independent experiments each performed in duplicate/triplicate. In both migration and transmigration assays, the student t-test was used to evaluate variation between control and individual treatments (TNF-α /IL-1β stimulated conditions). A p-value less than 0.05 was used to define significant relationships. All statistical analysis was carried out using GraphPad Prism software version 3.02 (GraphPad Software Inc.).

4.4 Results

4.4.1 Optimisation of seeding density for scratch wound migration assay

In a scratch wound migration assay, cultured cells need to be 100% confluent prior to wounding to ensure accurate results. Results from the optimisation experiments show that $4x10^5$ MDA-MB-231 cells per well in a 12 well plate is the appropriate seeding density for the cells to be 100% confluent after 24 hours incubation at 37°C (Figure 4-3). At $5x10^5$ cells/well, MDA-MB-231 was too confluent and the cells started to lift off from the tissue culture plate, and therefore failed to develop an intact monolayer. For MCF7, MeWo and SKMEL-30, $5x10^5$ cells per well was the appropriate seeding density to ensure confluent monolayers after 24 hours incubation at 37°C (Figure 4-4).

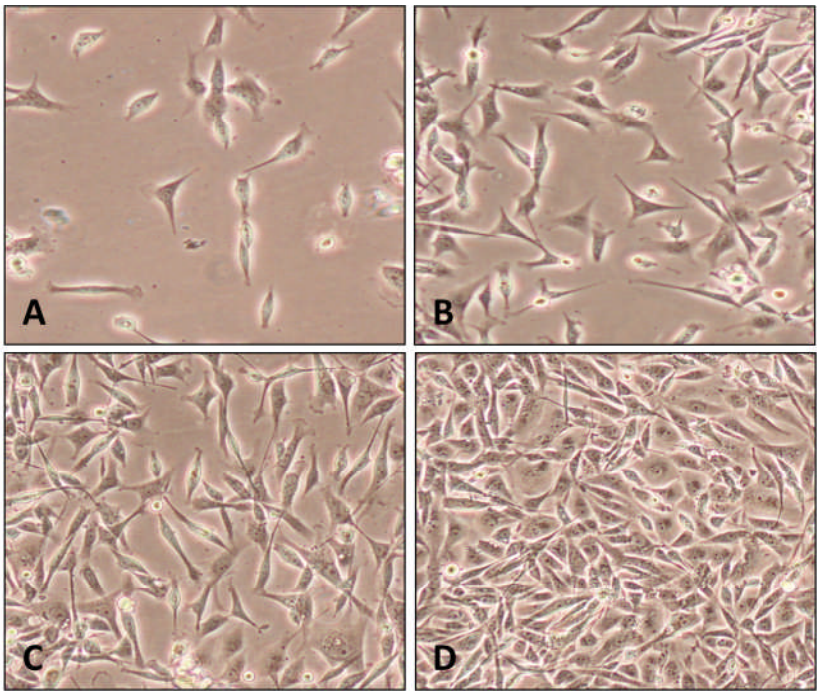


Figure 4-3 Optimisation of MDA-MB-231 cells seeding density for scratch wound migration assay. MDA-MB-231 were seeded at (A)$1x10^5$, (B)$2x10^5$, (C)$3x10^5$ and (D)$4x10^5$ cells per well in a 12 well plate. Cells were incubated at 37°C for 24 hours after which time, photomicrographs of the cells were taken to assess confluency. Cells need to be 100% confluent prior to wounding. Optimisation was carried out twice, each in duplicate (200x magnification).

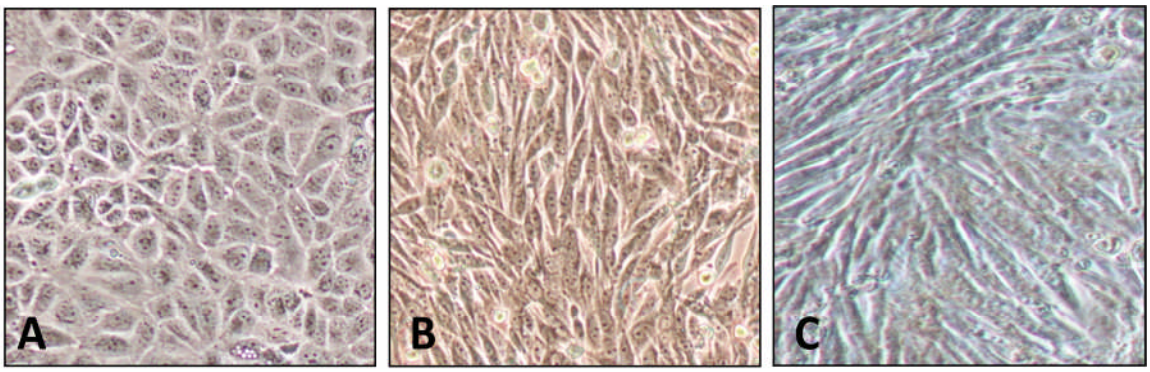


Figure 4-4 Confluent cell monolayer of (A) MCF7, (B) MeWo and (C) SKMEL-30 for scratch wound migration assay. Cells were seeded at $5x10^5$ per well to ensure confluent monolayer after 24 hours incubation at 37^oC (200x magnification)

4.4.2 Optimisation of TNF-α and IL-1β concentration for scratch wound migration assay

The MDA-MB-231 cell line was used to determine the optimal TNF-α and IL-1β concentration to stimulate tumour cells in the scratch wound migration assay. Confluent MDA-MB-231 cells were stimulated with 3 different concentrations of TNF-α (2.5, 5 and 10ng/ml) and the scratch wound migration assay was carried out as in Section 4.3.2.

Results show that there was no significant increased in the percentage of wound closure when MDA-MB-231 cells were stimulated with 2.5ng/ml TNF-α at any time point accessed. The percentage of wound closure increased significantly when MDA-MB-231 cells were stimulated with 5 and 10ng/ml TNF-α at 4, 6 and 24 hours post wounding compared to the unstimulated control cells (Figure 4-5). No significant increased was observed in the percentage of wound closure between 5 and 10ng/ml TNF-α stimulation. Therefore, 5ng/ml TNF-α was used to stimulate tumour cells in subsequent migration assays.

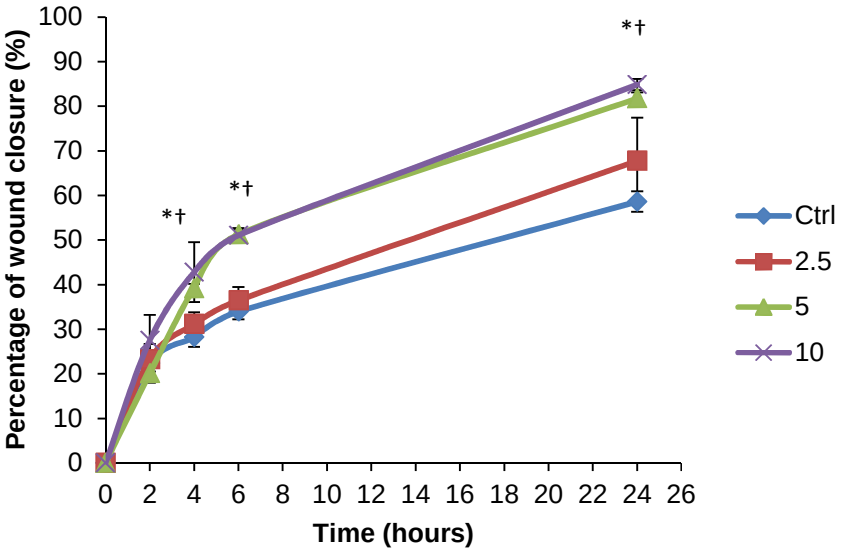


Figure 4-5 The effect of TNF-α on MDA-MB-231 cell migration. The percentage of wound closure increased significantly when cells are stimulated with 5 and 10ng/ml TNF-α compared to the unstimulated control. No significant increased in the percentage of wound closure was observed between 5 and 10ng/ml TNF-α. Pooled data from 2 independent experiments each carried out in triplicate (n=6).(* significant difference of 5ng/ml TNF-α stimulation compared to control group, † significant difference of 10ng/ml TNF-α stimulation compared to control group).

Three different concentrations of IL-1β i.e 5, 10 and 15ng/ml were used to stimulate confluent MDA-MB-231 cells for 24 hours to determine the optimal IL-1β concentration to stimulate tumour cells in scratch wound migration assays. The scratch wound migration assay was carried out as described as in Section 4.3.2.

Results show that the percentage of wound closure increased significantly when MDA-MB-231 cells are stimulated with 5, 10 and 15ng/ml IL-1β at all time points assessed, as shown in Figure 4-6. A significant increased of wound closure was observed when comparing between 5 and 10ng/ml of MDA-MB-231 stimulation at the 24 hours time point. However, no significant increased in the percentage of wound closure was observed between 10 and 15ng/ml IL-1β at all time points assessed. Therefore, 10ng/ml IL-1β was used to stimulate tumour cells in subsequent migration assays.

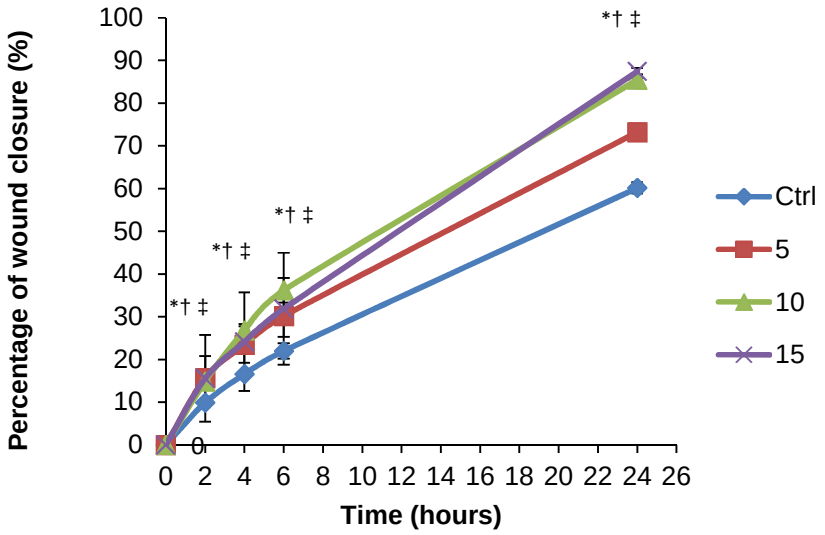


Figure 4-6 The effect of IL1-β on MDA-MB-231 cell migration. The percentage of wound closure increased significantly when cells were stimulated with 5, 10 and 15ng/ml IL-1β. No significant increased in the percentage of wound closure was observed between 10 and 15ng/ml IL-1β. Pooled data from 2 independent experiments each carried out in triplicate (n=6).(* significant difference of 5ng/ml IL-1β stimulation compared to control group, † significant difference of 10ng/ml IL-1β stimulation compared to control group, ‡ significant difference of 15ng/ml IL-1β stimulation compared to control group)

4.4.4 The effect of TNF-α and IL-1β on tumour cell migration

The percentage reduction of the scratch area at different time points represents the rate of tumour cell migration. Under control conditions, MDA-MB-231 shows the highest percentage of wound closure (67.43 ±9.85%) followed by MCF7 (55.67 ±6.91%), SKMEL-30 (27.68 ±6.91%) and MeWo (18.42 ±5.72%).

Following exposure to TNF-α, the migration of all four tumour cell lines increased which was particularly noticeable with MDA-MB-231. MDA-MB-231 stimulated with TNF-α had a significantly higher migration rate compared to the unstimulated control at all time points assessed (2 to 24 hours post wounding). At 24 hours, the wound closure of TNF-α-stimulated MDA-MB-231 cells was 89.42 ±16.70% compared to the unstimulated control i.e, 67.43 ±9.85%. MCF7 (control 55.67 ±6.91% and TNF-α 66.02 ±8.05%), MeWo (control 18.42 ±5.72% and TNF-α 29.45 ±10.13%) and SKMEL-30 (control 27.68 ±6.91% and TNF-α 35.56 ±6.23%) cells also showed significantly higher migratory ability at 24 hours post wounding; p-value <0.01, <0.01 and 0.014 respectively.

Following exposure to IL-1β, the migration of all four tumour cell lines increased which was significant at 2 hours post wounding in MDA-MB-231 and MeWo cell lines compared to control cells. Similar to TNF-α stimulation, IL-1β stimulation of MDA-MB-231 cells had significantly higher migratory ability compared to the other tumour cell lines used in this study. The percentage cell migration of these tumour cell lines was 89.20 ±1.57%, 71.57 ±6.04%, 33.82 ±5.75% and 39.34 ±5.03% for MDA-MB-231, MCF7, MeWo and SKMEL-30 respectively

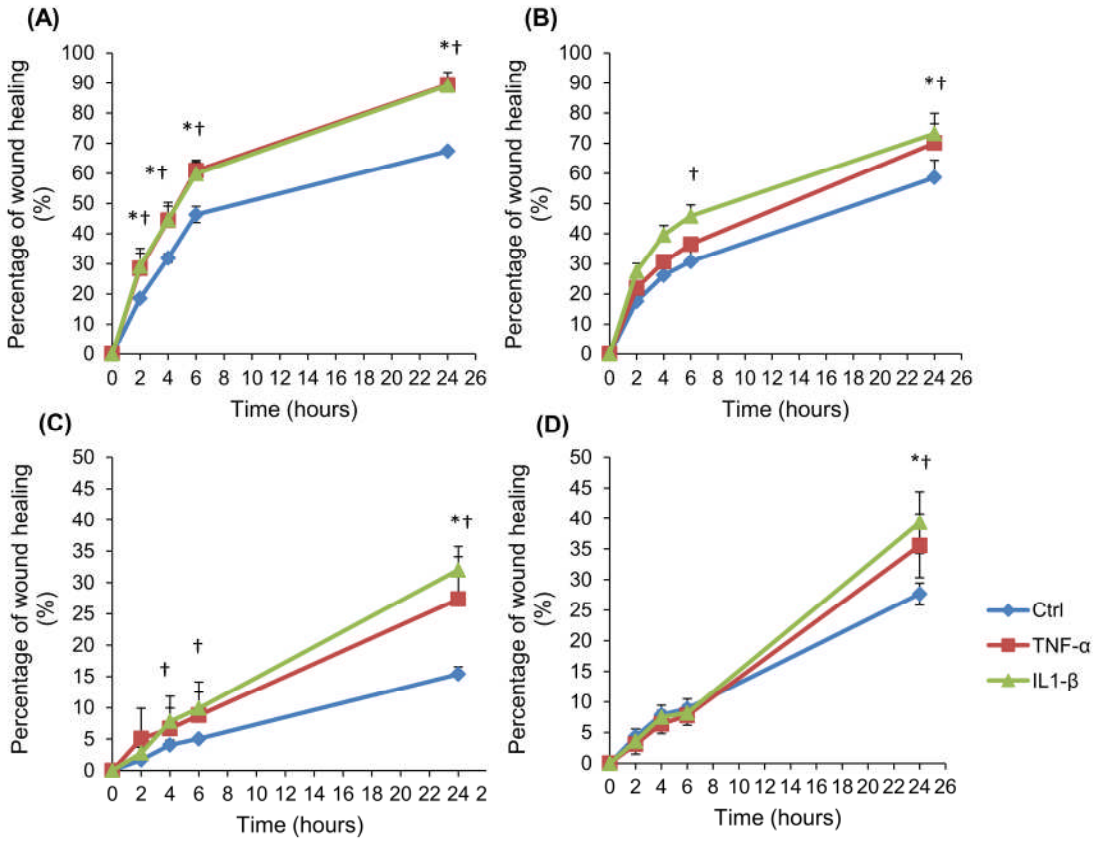


Figure 4-7 The effect of TNF-α and IL-1β stimulation on (A) MDA-MB-231, (B) MCF7, (C) MeWo and (D) SKMEL-30 cell migration. Stimulation of all tumour cells with TNF-α and IL-1β caused significant increased in the migratory ability of all tumour cells at 24 hours which was notably higher with IL-1β stimulation. MDA-MB-231 stimulated with TNF-α and IL-1β had a significantly higher migration rate compared to the unstimulated control at all time points assessed (p<0.001). MCF7, MeWo and SKMEL-30 showed significantly higher migratory ability at 24 hours with TNF-α (p value =0.01, =0.0054 and 0.014 respectively) and IL-1β stimulation (p value <0.0001, p<0.0001 and p=0.0002 respectively). Pooled data from two independent experiments, each carried out in triplicate (n=6). (*significant difference of TNF-α stimulation compared to control group; †significant difference of IL-1β stimulation compared to control group).

4.4.5 The effect of tumour conditioned media stimulation on tumour cell migration

To study the effect of tumour conditioned media on tumour cell migration, MDA-MB-231, MCF7, MeWo and SKMEL-30 were incubated with the cells' own tumour conditioned media generated as described in Chapter 3. There was no significant different in the migration rate of MDA-MB-231 cells compared to the unstimulated controls and this effect was observed across all cell lines used in this study (Figure 4-8).

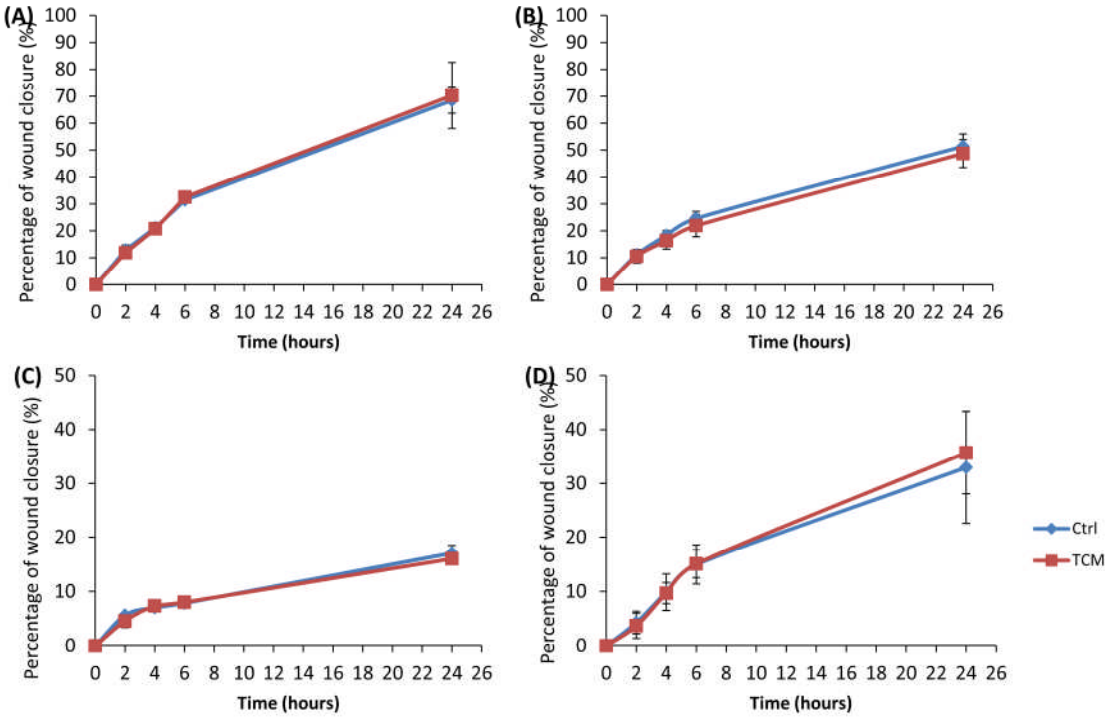


Figure 4-8 The effect of tumour conditioned media stimulation on (A) MDA-MB-231, (B) MCF7, (C) MeWo and (D) SKMEL-30 cell migration. Stimulation of tumour cells with tumour conditioned media caused no significant effect in the migratory ability across the cell lines used compared to the unstimulated controls. Pooled data from two independent experiments, each carried out in triplicate (n=6).

4.4.6 The effect of macrophage conditioned media stimulation on tumour cell migration

To study the effect of macrophage conditioned media on tumour cell migration, MDA-MB-231 cells were incubated with macrophage conditioned media

generated from three different donors as described in Chapter 3 (donor 7, donor 8 and donor 9). Under control condition, the percentage cell migration of MDA-MB-231 was 67.63 ±4.82 which is in accordance with previous migration assay experiments.

There was no significant difference in the migration rate of MDA-MB-231 cells compared to the unstimulated controls when cells were stimulated with macrophage conditioned media generated from donor 1. A significant difference in the percentage of wound closure was, however, observed when cells were stimulated with macrophage conditioned media generated from donor 3 but only at the 24 hour time point ($p=0.023$) whereas media generated from donor 2 showed a significant difference at 4, 6 and 24 hours post wounding ($p=0.04$, $p=0.01$, $p<0.001$ respectively) (Figure 4.9).

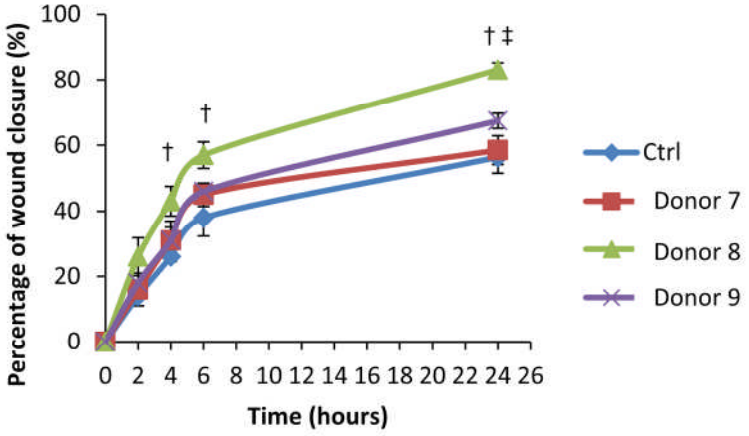


Figure 4-9 The effect of macrophage conditioned media stimulation on MDA-MB-231 cell migration. MDA-MB-231 cells were stimulated with macrophage conditioned media generated from three different donors for 24 hours post wounding. Macrophage conditioned media generated from donor 7 caused no significant effect in the migratory ability of MDA-MB-231 cells compared to control. Significant different in the percentage of wound closure was observed when cells were stimulated with macrophage conditioned media from donor 8 (at 4, 6 and 24 hours) and donor 9 (at 24 hours). Pooled results from 2 independent experiments each carried out in duplicate (n=4). † and ‡ represent statistical significant of macrophage conditioned media generated from donor 8 and donor 9 respectively.

An ELISA was conducted to determine the concentration of IL-1β in macrophage conditioned media used in the haplotaxis migration assay described above. 20.13, 270.36 and 137.71 pg/ml of IL-1β was detected in

macrophage conditioned media generated from donor 7, donor 8 and donor 9 respectively (Figure 4-10).

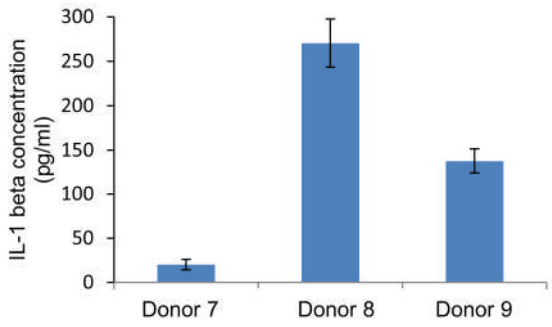


Figure 4-10 IL-1β concentration in macrophage conditioned media generated from three donors (donor 7, donor 8 and donor 9) used to stimulate MDA-MB-231 cells in scratch wound migration assay.

4.4.7 Optimisation of coating agents and endothelial cells seeding density for transmigration assay

Optimisation of coating agents and endothelial cell seeding density was carried out prior to transmigration assay experiments so that a confluent cell monolayer could be obtained (Section 4.3.3). Results show that the most suitable coating agent, agent concentration and cell seeding density to obtain a cell monolayer is 10µg/cm^3 of rat tail collagen I using 2x10^5 cells per well because at this concentration, there was <4% reduction in Lucifer yellow flux to the bottom well (Figure 4-11). Although from the manufecturer's recommendation that Lucifer yellow leakage for an intact cell monolayer is typically 2% reduction of control, 4% reduction is the lowest value that could be optained from the optimisation experiments in this study. Therefore, it was decided that all subsequent transmigration assay experiments would use 10µg/cm^3 of rat tail collagen I as coating agent with 2x10^5 endothelial cells per well.

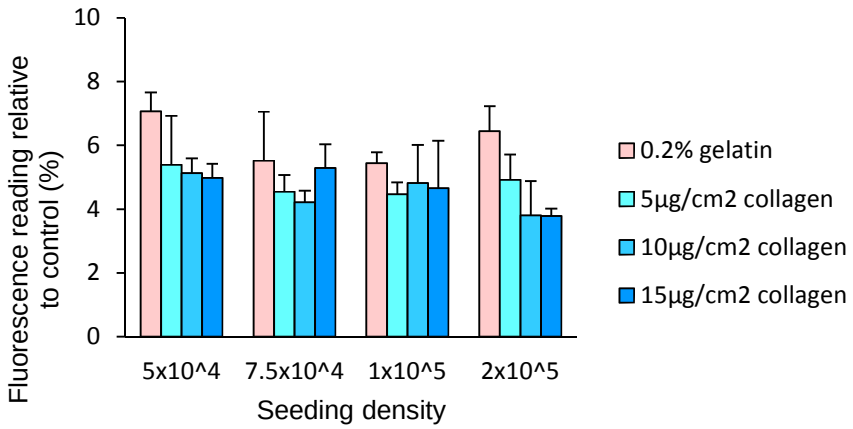


Figure 4-11 Optimisation of coating agents and endothelial cell seeding density on tranwell inserts for transmigration assay in order to obtain a cell monolayer. 10µg/cm^3 of collagen at 2x10^5 cells per well were used in the subsequent transmigration assay as there was <4% reduction in Lucifer yellow flux to the bottom well at this concentration and density opposed to Lucifer yellow flux in control wells. Error bars represents standard deviation from two independent experiment, each carried out in duplicate (n=4).

4.4.8 The effect of cell tracker green labeling on tumour cell viability and proliferation

In the static transmigration assay, tumour cells usually take up to 24 hours to transmigrate across endothelial monolayers. Therefore, fluorescently labeled tumour cells need to be viable for the duration of the assay. To assess the effect of fluorescent dye on cell viability, the trypan blue exclusion method was used. This method is based on the principle that viable cells possess an intact cell membrane which does not take up the blue dye. Dead cells will take up the dye as their membrane can no longer control the passage of macromolecules. Cell counting was used to assess the effects on cell proliferation (all cells were counted to give a total cell count, including viable and non-viable cells).

Results show that there was no difference in MDA-MB-231 cell viability between the labelled and unlabelled cells across the range of concentrations i.e 1, 2.5, 5, 10 and 15nm up to 48 hours (Figure 4-12). However, at 72 hours post labeling, there was a reduction in MDA-MB-231 cell viability at 5nm to 15nm concentrations. There was also no difference between the total cell count i.e cell proliferation between the labelled and unlabelled cells at 1, 2.5, 5 and 10nm

concentrations up to 72 hours time point. The total cell count of MDA-MB-231 decreased significantly at 72 hours post labeling with 15nm dye concentrations.

Similar effects were also observed with MeWo cells in which there was no significant different in MeWo cell viability up to 24 hours post labeling at all concentrations used compared to the unlabelled cells. However, at 48 and 72 hours post labeling, there was a reduction in MeWo cell viability at 10 to 15 nm concentrations. There was no significant difference in MeWo proliferation rate at 1nm to 5nm concentrations. However, at 48 and 72 hours post labeling with 10 and 15nm concentrations, the total cell count of MeWo significantly decrease as compared to the unlabelled cells (Figure 4-13).

Therefore, based on the results from these two cell lines i.e. MDA-MB-231 and MeWo, 5nm cell tracker green was used for tumour cell labeling in subsequent transmigration assays.

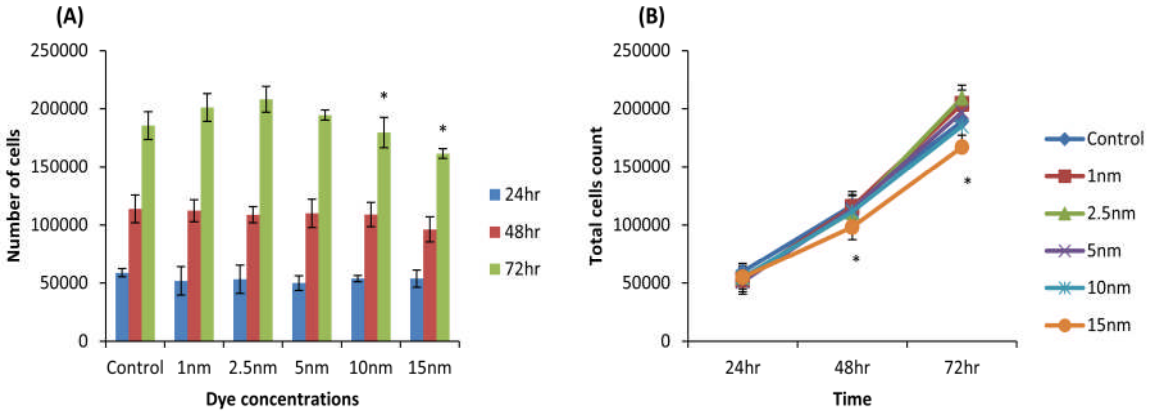


Figure 4-12 The effect of cell tracker green labelling on MDA-MB-231 cell viability (A) and cell proliferation (B). There was no significant different in MDA-MB-231 cell viability up to 48 hours across the range of concentration compared to the unlabelled cells. At 72 hours post labeling, there was a reduction in MDA-MB-231 cell viability at 5nm to 15nm concentrations. There was no significant different in MDA-MB-231 proliferation rate at 1nm to 10nm concentrations. The proliferation rate decrease at 15nm concentration at 48 and 72 hours opposed to unlabelled cells. *represent significant difference compared to control group. Pooled data from 2 independent experiments, each carried out in triplicate (n=6).

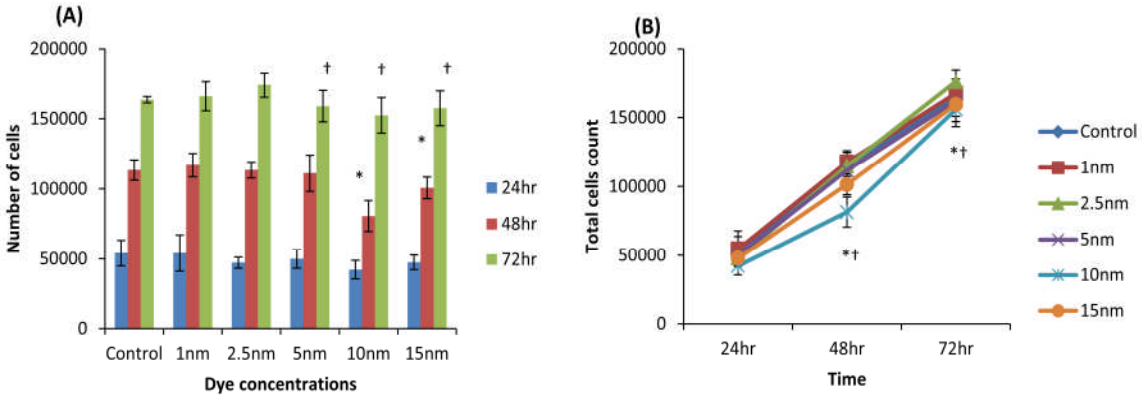


Figure 4-13 The effect of cell tracker green labelling on MeWo cell viability (A) and cell proliferation (B). There was no significant different in MeWo cell viability up to 24 hours across the range of concentration used compared to the unlabelled cells. However, at 48 and 72 hours post labeling, there was a reduction in MeWo cell viability at 10nm to 15 nm concentrations. There was no significant different in MeWo proliferation rate at 1nm to 5nm concentrations. However, the proliferation rate decrease at 10nm and 15nm dyes concentrations at 48 and 72 hours opposed to unlabelled cells. * represent significant difference compared to control group. Pooled data from 2 independent experiments, each carried out in triplicate (n=6).

4.4.9 Time point optimisation for tumour cell migration across endothelial cell monolayers

The MDA-MB-231 breast cancer cell line was used to optimise the adhesion time required for tumour cell transmigration. This tumour cell line was left to transmigrate across hMEC-1 cells for 4, 8, 16 and 24 hours and the number of transmigrated tumour cells attached to the lower face of the polycarbonate membrane filters between the negative control and the positive control wells were compared at the end of the assay (Figure 4-14 and 4-15).

The number of MDA-MB-231 cells that has transmigrated across hMEC-1 cells increased significantly from 4 to 24 hours in both negative and positive control wells. At 4 and 8 hours, the number of tumour cells that has transmigrated across hMEC-1 cells was less than 50 cells and if this time point was chosen, the accuracy of the statistical analysis could be debated. At 24 hours time point, more than 200 MDA-MB-231 cells had transmigrated, which would affect the counting accuracy because the cells were very close together (Figure 4-14). Therefore, the 16 hour time point was chosen as the incubation time for subsequent tumour cell transendothelial migration assays.

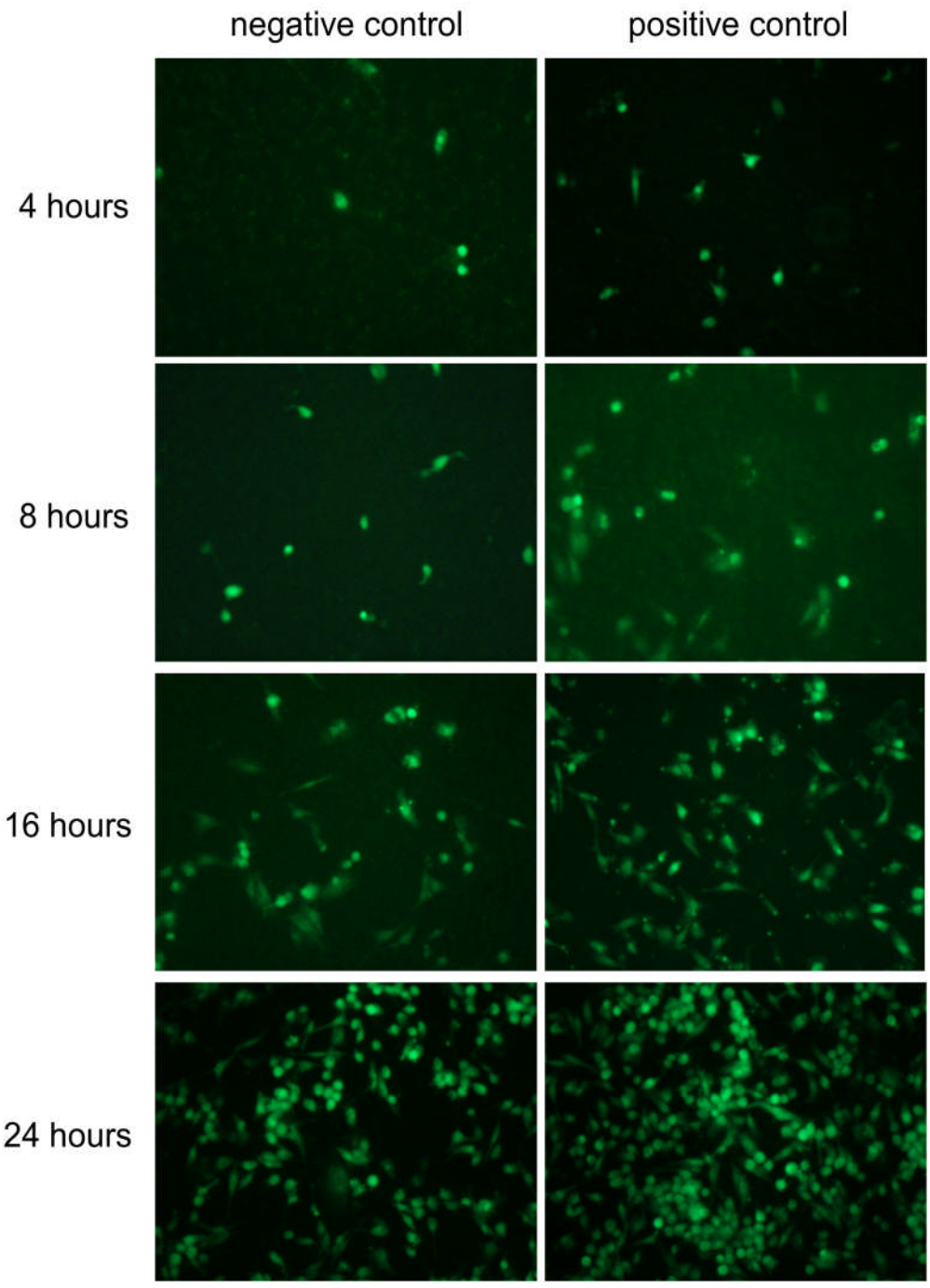


Figure 4-14 The number of transmigrated MDA-MB-231 cells attached to the lower face of the polycarbonate membrane filters between negative (wells without serum) and positive (wells with 100% heat inactivated fetal calf serum) control wells at 4, 8, 16 and 24 hours.

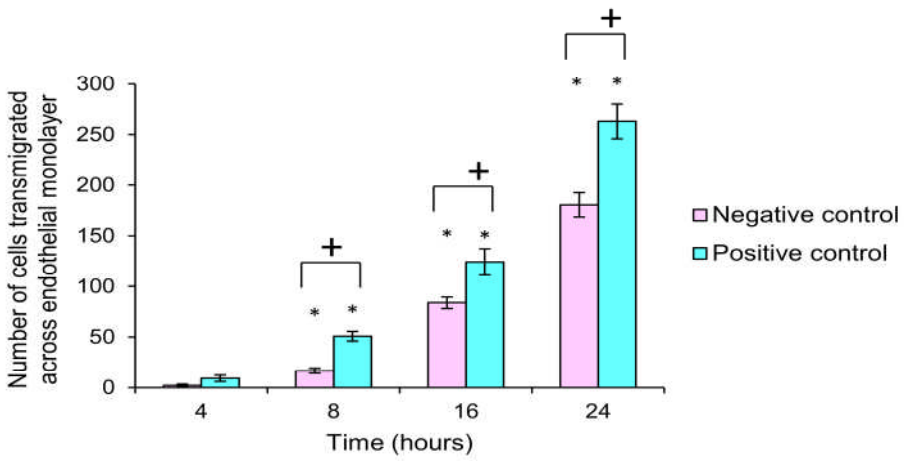


Figure 4-15 Optimisation of MDA-MB-231 transmigration time across hMEC-1 cells. The number of MDA-MB-231 cells migration across hMEC-1 cells was assessed at different incubation time. Both negative (wells without serum) and positive (well with 100% heat inactivated fetal calf serum) controls showed significant increase in the number of tumour cells migrated across hMEC-1 cells between 4 to 24 hours. Pooled data from two independent experiments, each carried out in duplicate (n=4). * represent significant difference compared to 4 hours time point and + represent significant difference between negative and positive control group.

4.4.10 Tumour cell migration across hMEC-1 and hTERT-LEC under control and IL-1β stimulation

Under unstimulated conditions, MDA-MB-231 show similar transmigratory abilities across both hMEC-1 and hTERT-LEC, in that there was no significant difference in the number of MDA-MB-231 cells that transmigrated between hMEC-1 and hTERT-LEC. The transmigratory ability of MDA-MB-231 increased significantly in response to IL-1β stimulation of the endothelial cells across both hMEC-1 and hTERT-LEC monolayers (p-value <0.001 and <0.001 respectively) with similar affinity across both endothelial models (139.5± 6.1 cells vs 149.5±5.3 cells across hMEC-1 and hTERT-LEC respectively). MeWo cell transmigration under control conditions showed similar transmigratory ability across both hMEC-1 (11.3± 0.7 cells) and hTERT-LEC (11.8±1.4 cells), however with a lower number of cells migrating across the endothelium compared to MDA-MB-231 cells. However, upon stimulation with IL-1β, there was an increased in the transmigratory ability of MeWo cells across hMEC-1 and hTERT-LEC (p value <0.00001 and <0.0001 respectively) which was more notable across hMEC-1 (Figure 4-16).

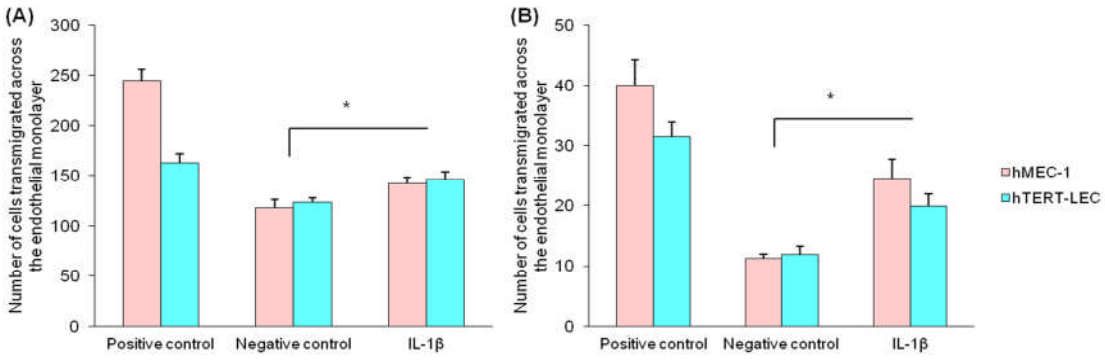


Figure 4-16 The number of MDA-MB-231 (A) and MeWo (B) cells migration across hMEC-1 and hTERT-LEC under control and IL-1β stimulated conditions. Positive controls (wells with 100% heat inactivated fetal calf serum) shows that the assays were in working conditioned as expected. MDA-MB-231 cells shows an increased migration across both hMEC-1 and hTERT-LEC cells with similar affinity to both endothelial cells (p value <0.001 and <0.001 respectively). Transmigration of MeWo cells were also significantly increased across IL-1β stimulated hMEC-1 and hTERT-LEC (p value <0.00001 and p<0.0001). Pooled data from two independent experiments, each carried out in duplicate (n=4). (* significant different compared to negative controls).

4.4.11 The effect of macrophage conditioned media stimulation on MDA-MB-231 transmigration across hMEC-1 and hTERT-LEC

The 5 conditions of macrophage conditioned media used in this assay were generated from 3 donors as described in Chapter 3. Under control conditions, MDA-MB-231 showed similar transmigratory ability across hMEC-1 and hTERT-LEC across all 3 donors. When hMEC-1 and hTERT-LEC were stimulated with macrophage conditioned media generated from LPS-γ stimulated macrophages, there was an increased in the number of cells transmigrated across the endothelial cell monolayer. It should be remembered (Section 3.4.12) that the IL-1β concentrations (in conditioned media from LPS stimulated macrophages) for these donors were as follows; 166.29 pg/ml for donor 4, 270.36 pg/ml for donor 5 and 118.24 pg/ml for donor 6. In donor 5 and donor 6, the increased was significantly noted with the lymphatic endothelial cells, hTERT-LEC opposed to hMEC-1 cells ($p=0.03$ and $p<0.001$ in donor 5 and donor 6 respectively). Supernatants generated with TDL stimulated macrophages showed no significant difference in term of the number of transmigrated cells across both hMEC-1 and hTERT-LEC in all donors (IL-1β concentrations were 23.23, 24.31 and 24.96pg/ml for donor 4, donor 5 and donor 6 respectively). However, when hMEC-1 and hTERT-LEC were

stimulated with macrophage conditioned media generated with LPS-γ+ICE inhibitor and LPS-γ+TDL+ICE inhibitor stimulation, the number of transmigrated MDA-MB-231 cells decreased significantly compared to both control and LPS-γ stimulated macrophages in all the 3 donors (Figure 4-17). ELISA results showed that the IL-1β concentration in these macrophage conditioned media were 28.44, 69.52 and 24.39pg/ml from LPS-γ+ICE stimulated macrophages and 29.41, 47.43 and 37.71pg/ml from LPS-γ+TDL+ICE stimulated macrophages (donor 4, 5 and 6 respectively)

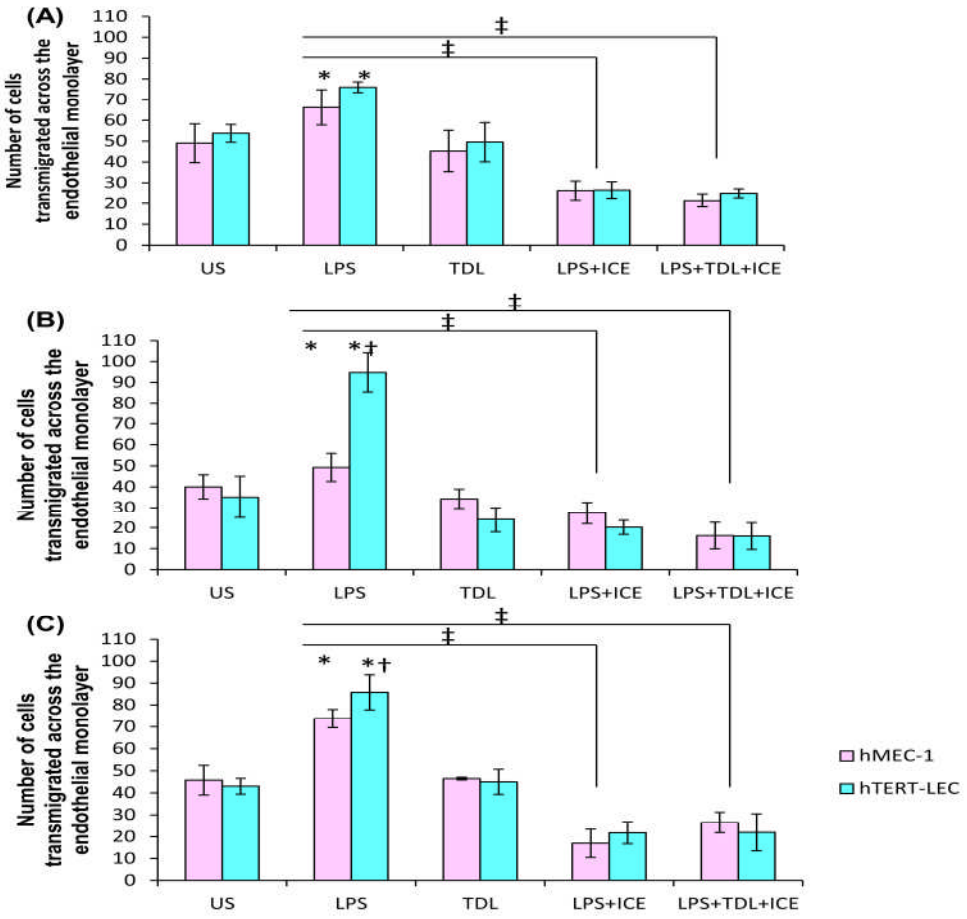


Figure 4-17 MDA-MB-231 cells migration across hMEC-1 and hTERT-LEC stimulated with macrophage conditioned media from; (A)donor 4, (B)donor 5 and (C) donor 6. Under control conditions, MDA-MB-231 cells shows similar transmigratory ability across the endothelial cells. Transmigration of MDA-MB-231 cells increased significantly across hMEC-1 and hTERT-LEC with macrophage conditioned media stimulation from LPS-γ stimulated macrophages, which decreased significantly in macrophage conditioned media from LPS-γ+ICE and LPS-γ+TDL+ICE stimulated macrophages. Pooled data from two independent experiments, each in duplicate (n=4). *represent significant difference compared to controls. †represent significant difference between hMEC-1 and hTERT-LEC of the same group and ‡represent significant difference compared to LPS stimulated hMEC-1 and hTERT-LEC.

4.5 Discussion

In the initial metastatic process, cancer cells must break from the primary tumour mass, migrate through the extracellular matrix, adhere and transmigrate across blood or lymphatic endothelial cells before entering the circulation and extravasating into the tissue to establish a secondary tumour site. Each of these steps is multi-component processes with regulation dependent upon different molecules and proteins that co-operate to ensure cancer cells survival and spread. Results from Chapter 3 demonstrated that tumour-endothelial cell adhesion can be modulated by cytokine stimulation and that TNF-α and, especially, IL1-β secreted by macrophages strongly influence adhesion to lymphatic endothelial cells; thus potentially modulating lymphatic vessel invasion. The aim of the current chapter was to study the migratory ability of four tumour cell lines; MDA-MB-231, MCF7, MeWo and SKMEL-30 under control conditions and examine the effect of TNF-α, IL-1β, tumour conditioned media and macrophage conditioned media stimulation. The transmigration pattern of both melanoma (MeWo) and breast cancer (MDA-MB-231) cell lines across blood (hMEC-1) and lymphatic (hTERT-LEC) endothelial cell models were also studied and compared. The effect of IL-1β and macrophage conditioned media upon tumour cell migration across blood and lymphatic endothelial cells were also examined.

Results from the scratch wound migration assays showed that TNF-α and IL-1β-stimulation altered the migratory ability of all tumour cell lines used in this study (MDA-MB-231, MCF7, MeWo and SKMEL-30), which was observed at numerous time points. These observations are in agreement with other studies showing that migration of human cutaneous melanoma and cervical cancer cell lines (HBL and HW756) is increased by these pro-inflammatory cytokines. Both TNF-α and IL-1β have been shown to upregulate integrin expression in human melanoma cells thus increasing the ability of cells to bind to extracellular substrate and increase invasion via fibronectin binding (Katerinaki et al., 2003). A maximal effect on cell migration, with the cell lines used in this study, was observed at 20-24 hours which in accordance with other studies (Hidalgo et al.,

2005; Katerinaki et al., 2003), during which an increase in ICAM-1, VCAM-1 (Morandini et al., 1998) and integrin (Zhu et al., 2002) expression on the tumour cells was observed. Of the four tumour cell lines used in this study, MDA-MB-231 had significantly higher migratory rate compared to the other tumour cell lines both in control and TNF-α/IL-1β stimulated conditions followed by MCF7, SKMEL-30 and MeWo. The differences in migratory ability of these tumour lines demonstrated the heterogenous nature of tumour cell migration. MDA-MB-231 cells originated from a highly invasive and aggressive tumour which might explain the high motility rate (Swamydas et al., 2011).

When the tumour cells were stimulated with tumour conditioned media, there were no significant changes in the migratory ability of MDA-MB-231, MCF7, MeWo and SKMEL-30 observed across all time points assessed. Tumour conditioned media consists of various proteins, cytokines, chemicals and growth factors; all of which may influence the migration rate of tumour cell lines but no effect was observed in this current study. It could be argued that the tumour conditioned media used is not biologically active, thus the observed results. However, as described in Chapter 3, when tumour cells were cultured with the same tumour conditioned media, they still proliferated at the same rate as those cultured with normal media.

When MDA-MB-231 cells were stimulated with macrophage conditioned media, there was an increased in the percentage of wound closure and the migratory ability observed compared to the unstimulated control. The fact that IL-1β was detected in the macrophage conditioned media and no IL-1β was detected in the tumour conditioned media may suggest that IL-1β could play a role in such tumour cell migration and the initial metastatic process. In a previous study, IL-1β stimulated MCF7 cells were shown to express S100A4, which is a metastasis promoting protein, as well as increase secretion of MMP-9 and MMP-2, which are capable of degrading extracellular matrix proteins during cell migration (Franco-Barraza et al., 2010).

It may be argued that results from the scratch wound migration assays are not based on cell migration alone but could be affected by cell proliferation. The use

of mitomycin-C for the duration of the assay from 0 hour post wounding ensures that the effect observed is a true migratory effect. Mitomycin-C is an antibiotic that can exerts anti-proliferative effect in living cells by arresting cells in the G2/M phase of the cell cycle (Granada et al., 2005) up to 48 hours following exposure to a single dose of mitomycin-C (Lama and Fechtner, 2003).

Transmigration of tumour cells across the endothelial cells follows on from migration and adhesion in the metastatic cascade. Therefore, tumour transendothelial migration was also off interest in this study. The Boyden chamber system is a dual compartment system assay and the most commonly used model to study tumour cell transmigration in-vitro (Okada et al., 1994). Both hMEC-1 and hTERT-LEC endothelial cells were cultured on collagen coated surfaces as they did not form monolayers when cultured directly on the polycarbonate membrane surface of the transwell inserts. The use of extracellular constituents such as collagen, laminin, gelatin, fibronectins and others help to facilitate endothelial cell attachment to the membrane surface. Most studies involving culture of endothelial cells have utilised rat tail collagen type I as the coating agent which not only helps the endothelial cells to attach to the cultured surface but also maintain their native differentiation characteristics and functions (Ghislin et al., 2011; Voura et al., 1998).

In the assay used in the current study, tumour cells were labeled with a fluorescent dye; cell tracker green CMFDA, which is a fluorescent chloromethyl derivative that can diffuse through live cell membranes. This dye reacts with the intracellular components inside the cells to emit fluorescent signals which, reportedly, do not affect cell viability up to 72 hours. The use of fluorescent dye in this assay helps to differentiate between tumour and endothelial cells, and in doing so, improves the accuracy of quantifying the number of transmigrated cells across the endothelium (Wu et al., 2010).

Experiments comparing transendothelial migration of tumour cell lines; MDA-MB-231 and MeWo across blood and lymphatic endothelial cells in a single experiment have not been reported previously. As in the adhesion assay described in Chapter 3, most transendothelial migration assays have used large

vein endothelial cells (HUVEC) to study tumour cell transmigration. Under control conditions, MDA-MB-231 and MeWo show similar transmigratory abilities across both blood and lymphatic endothelial cells however with different affinity. MDA-MB-231, which is a more aggressive tumour type, transmigrated in higher numbers compared to MeWo cells. One previous study has shown that human fibrosarcoma HT-1080 and lung carcinoma Calu-1 cells exhibit significantly higher transmigration across bovine aortic endothelial cells (BAEC) monolayers compared to the colon carcinoma cell lines (SW620, SW480, HT29 and Colo 205) (Li and Zhu, 1999). They concluded that the HT-1080 and Calu-1 cells may associate with a higher capacity to spread in-vivo.

Upon stimulation with IL-1β, there was a significant increased in the number of MDA-MB-231 cells that transmigrated across both hMEC-1 and hTERT-LEC with similar affinity to both endothelial cell types. Similarly, upon stimulation with IL-1β, there was also an increased in the transmigratory ability of MeWo cells across hMEC-1 and hTERT-LEC which was more notably observed across hMEC-1 cells. Such results, with IL-1 altering transmigration across endothelium, may be due to enhanced contractile force generation and cytoskeletal remodeling which can increase transmigration and invasion efficiency across the endothelial barrier, as has been observed with chemokines such as Gro-β and IL-8 (Claudia et al., 2008).

Upon stimulation with macrophage supernatant generated from LPS-γ stimulated macrophages, an increased transmigratory effect was observed with hTERT-LEC cells compared to hMEC-1 cells and was statistically significant in donor 2, the donor with the highest level of IL-1β. The differences in these results may be due to the differences in IL-1β; i.e. one is recombinant IL-1β whereas that from macrophage is naturally occurring. Many studies have also reported contrasting results in relation to IL-1β in tumour microenvironment; tumour promoting and tumour regression and they conclude that the biological effect of IL-1β may depend on the doses of this cytokine and the condition of IL-1β administration (Saijo et al., 2002a).

When both hMEC-1 and hTERT-LEC cells were stimulated with macrophage conditioned media generated from LPS-γ+ICE inhibitors stimulated macrophages, the number of cells transmigrated across both endothelial cells decreased significantly across all three donors compared to using LPS-γ alone. IL-1β ELISA results, from the macrophage media, seemed to correlate with the number of cells transmigrated. IL-1β has been shown, in separate studies, to decrease the amount of $p125^{FAK}$ molecules in HUVEC which eventually blocked the formation of focal contacts, resulting in decreased strength of endothelial cell attachment with the extracellular matrix. The decreased adhesiveness might enable tumour cells to migrate beneath the endothelial cells more easily (Iwaki et al., 1997). It remains to be seen if this explanation applies to current results. IL-1β stimulation on endothelial cells increased the expression of adhesion molecules such as ICAM-1 and VCAM-1 resulting in the increased of tumour-endothelial cell adhesion eventually leading to increased tumour transendothelial migration.

In addition, when both hMEC-1 and hTERT-LEC were stimulated with macrophage conditioned media generated from LPS-γ+ICE inhibitors stimulated macrophages,it was also observed that the number of MDA-MB-231 cells transmigrated was lower than the controls (endothelial cells without any stimulation). As stated earlier, ICE is a cysteine protease with its predominant role in cleaving immature IL-1β into its active mature form (review in Chapter 1-Section 1.6.2). However, ICE could also cleaved IL-18, a pro-inflammatory cytokine produce by cells of haematopoietic lineage including monocyes/macrophage hence, the effect observed in this transwell migration assay might be due to this fact. IL-18 ELISA should be carried out if this assay was to be repeated to confirm on this information.

It would also be good however, to carried out Lucifer yellow test on the endothelial cells monolayer after 24 hours stimulation with IL-1β and macrophage conditioned media to show that the integrity of the monolayers are not affected with both stimulation. However, in the adhesion assay (described in Chapter 3), when the endothelial cell monolayers were stimulated with either IL-

1β or macrophage conditioned media, the integrity of cells monolayer was not compromised as observed under the light microscope. Such result reflected that the effect observed with IL-1β and macrophage media stimulation in the traswell migration assay is a true effect and not as a result of non-intact cells.

In summary, results in the current chapter show that pro-inflammatory cytokines, TNF-α and IL-1β may play important roles in regulating melanoma and breast cancer cell migration and transmigration in-vitro. IL-1β stimulation of endothelial cells increased both melanoma and breast cancer cell transmigration across hMEC-1 and hTERT-LEC. In the case of IL-1β generated from macrophage supernatant, the transmigration of MDA-MB-231 cells was more notably observed across hTERT-LEC. This study is important as it optimised various factors involved in the transmigration protocol to create a fully working transendothelial transmigration assay procedures to be used in the near future. In terms of the tumour transendothelial migration, additional experiments need to be carried out to validate the results observed using MDA-MB-231 by using more tumour cell lines. Transendothelial migration under flow conditions would also be useful as it more closely replicates in vivo processes. Due to the findings, as discussed in Chapter 3 and the present chapter, that IL-1β, particularly macrophage derived, modulates lymphatic vessel adhesion, migration and transmigration, it was interesting to investigate the expression of IL-1β expression in tumour specimens - this is the focus of the following chapter.

CHAPTER 5: IL-1B EXPRESSION IN BREAST TISSUE

5.1 Abstract

Background and Aims: The initial metastatic process depends on the ability of cancer cells to separate from the primary tumour, migrate through the extracellular matrix protein, adhere and transmigrate between blood or lymphatic vessels to enter the circulation. Results from previous chapters have demonstrated that IL-1β, secreted by macrophages, modulates lymphatic endothelial cell adhesion, migration and transmigration. The aim of the current chapter was to investigate the expression of IL-1β in tumour specimens and test for association with lymphatic vessel invasion. Furthermore, this chapter sought to test for association between IL-1β expression and clinicopathologic variables and clinical outcome.

Methods: IL-1β expression was investigated using a tissue microarray (TMA) approach. TMAs were supplied preprepared using 0.6mm cores from 1511 early stage invasive breast cancer patients. A commercially available anti-IL-1β antibody was used to stain the TMA using a Novolink Novocastra Polymer detection kit. A histochemical score (H-score) which includes the assessment of staining intensity and the percentage of positive cells was used to assess the stained slides after scanning the slides with a Nanozoomer Digital Pathology Scanner.

Results: Results show that high IL-1β expression in the tumour cells was significantly associated with older patients (p=0.001), low tumour stage (p=0.011), PgR positive tumours (p=0.007), non-basal status (p=<0.001), the absence of lymphatic vessel invasion (p=0.01 and p=<0.001 respectively) as well as no distant metastasis (p=0.012). In Kaplan-Meier analyses, low IL-1β expression in the tumour cells was significantly associated with breast cancer specific survival (p=0.017) and disease free interval (p=0.008) which lost significance in multivariate Cox regression analysis.

Conclusions: Results from the current chapter show that high IL-1β expression was associated with the absence of lymphatic vessel invasion. However, results from previous in-vitro chapters show that IL-1β exerts a positive effect on

tumour cell adhesion, migration and transmigration and that macrophages are the main producer of IL-1β. A more specific IL-1β staining on macrophages, using a double IHC methodology, is recommended to evaluate the correlation of this parameter with lymphatic invasion and other clinical criteria.

5.2 Introduction

Tumour cell migration, adhesion and transmigration across blood or lymphatic vessels are important in the initial vascular invasion process. In-vitro results from previous chapters have demonstrated that IL-1β can influence tumour cell migration, in addition to tumour cell adhesion and transmigration to/across lymphatic and blood endothelial cell models, with a preferential increase observed to/across lymphatic endothelial cells. This IL-1β is likely, as suggested by results in Chapters 3 and 4, produced by tumour associated macrophages present in abundance in the tumour microenvironment with, from in vitro experiments, relatively less produced by tumor cells (from tumour conditioned media experiments mentioned earlier).

The interaction between cancer cells and tumour associated macrophages (reviewed in Chapter 1) are pivotal in the metastatic dissemination of cancer. There exists a complex paracrine signaling loop between tumour associated macrophages and tumour cells to activate each other. Upon activation by cancer cells, tumour associated macrophages can release various growth factors, cytokines, inflammatory mediators and proteolytic enzymes, many of these factors are key agents in cancer metastasis (Yunping Luo et al., 2006).

Table 5-1 shows various cytokines secreted by macrophages and their role in the immune response. IL-1β (reviewed in Chapter 1) is one of the many cytokines secreted by macrophages as a native 31kDa precursor, which is proteolytically processed to its active 17kDa form by IL-1β converting enzyme (ICE) or caspase 1. IL-1β production by cell types other than monocytes and macrophages has been reported in the literature. These include melanoma, sarcoma, hepatoblastoma as well as ovarian carcinoma cells (Burger et al., 1994; Dinarello, 1996). In animal models, IL-1β production by melanoma cells correlates with the ability to undergo metastasis to the liver. In many cases, the production of IL-1β by tumour cells caused increased expression of adhesion molecules and cell surface proteins which in turn contributed to increased cancer cell dissemination (Pollard, 2008; Wyckoff et al., 2000).

Table 5-1 Common cytokines secreted by macrophages.Adapted from (Baay et al., 2011; Pollard, 2008; Qian and Pollard, 2010).

Cytokines	*Primary functions*
TNF-α	• Increases the expression and cellular responsiveness to growth factor • Induces signaling pathway leading to cell proliferation
IL-1β	• Co-stimulatory cytokines with antigen presenting cells and T cells • Involved in inflammation, fever and the acute phase response
IFN α/β	• Activates natural killer cells and macrophages • Induction of Class I MHC on all somatic cells
IL-6	• Involved in the acute phase response of the immune response • Produce synergistic effect with IL-1β and TNF-α in the tissue and tumour microenvironment
IL-10	• Inhibits other cytokine production such as IL-6 and IL-1 • Promotes B cell proliferation and antibody production
IL-12	• Promotes natural killer cells proliferation and IFN- γ production
IL-13	• Stimulates growth and proliferation of B cells • Inhibits the production of macrophage inflammatory cytokines
IL-18	• Promotes natural killer cell activity and induces the production of IFN-γ

In contrast, IL-1β has been reported to exert a growth inhibitory effect on tumour cells (melanoma, breast, cervical and ovarian carcinoma cells). In these studies, IL-1β was proposed to cause arrest in cell cycle regulatory pathway, increased oxygen radicals release and nitric oxide regeneration (Hanauske et al., 1992; Olle et al., 2000). In fact, low doses of IL-1β are postulated to increase anti-tumour defense mechanisms or could boost the effect of antitumour adjuvants. IL-1β was reported to produce a synergistic effect in reducing tumour growth in-vitro when combined with doxorubicin, cisplatin and other cytokines such as TNF-α and IL-6 (Olle et al., 2000).

Based upon such literature findings, and results generated in Chapters 3 and 4, it was of interest to study IL-1β expression in tumour tissue. The aim of this chapter was to examine expression of IL-1β in breast tumour tissue, to test for associations with lymphatic vessel invasion and assess the association of this expression with clinicopathologic variables and clinical outcome.

5.3 Materials and Methods

5.3.1 Western blot

The specificity of the anti-IL-1β antibody was determined prior to immunohistochemical staining of patients' specimen using western blot technique as described in Section 3.3.11 using tumour (MDA-MB-231, MCF7, MeWo and SKML-30) and PBMC cell lysates.

Numerous antibodies from different suppliers were examined by Western blotting to determine specificity before deciding on the best ones to be taken forward for assessment in immunohistochemical staining of patient tissues i.e anti-IL-1β antibody from Abcam, UK, R&D, UK and Thermo Scientific, US.

5.3.2 Optimisation of IL-1β antibody

Optimisation of IL-1β antibody concentration was carried out using 3 different immunohistochemical staining kits; DAKO kit (DAKO, Denmark), Vectastain Universal Elite ABC kit (Vector Laboratories, San Francisco, USA) and Novolink Novocastra polymer detection kit (Leica, Denmark), each using 4 different IL-1β antibody concentrations; 1:50, 1:70; 1:100 and 1:150. Liver and breast composite tissue sections were used in the optimisation experiments. Liver was used to optimise the IL-1β staining as per manufacturers recommendation as it has been reported as being a high IL-1β producer.

Staining was also carried out using IL-1β antibody which was pre-incubated with IL-1β recombinant protein overnight to ensure that the IL-1β staining observed was specific. Such incubation with excess protein, corresponding to the epitope recognised by the antibody, was carried out to neutralise the antibody thereby making it unable to bind to the epitope present in the tissue sections. By comparing the staining from the blocked antibody with the antibody alone, the staining specificity can be determined.

5.3.3 Patients and specimens

This study was conducted on 1511 early stage invasive breast cancer patients treated at Nottingham University Hospitals between 1987 and 1998. Ethical approval for this study was granted by the Nottingham Research Ethics committee 2 under the title 'development of a molecular genetic classification of breast cancer.' The median age of patients at time of diagnosis was 54 years (range: 18 to 72 years). 39.5% (n=752) patients relapsed and 505 patients died from the disease. Relapse-free survival was calculated from the start of primary treatment to the first relapse. Breast cancer specific survival was defined as the time interval between the start of primary surgery to death from breast cancer. Clinical characteristics of patients are summarised in Table 5-2.

5.3.4 Immunohistochemistry

IL-1β expression was investigated using prepared (by others within supervisory research groups) tissue microarrays (TMAs) which were constructed using 0.6mm cores from 1511 tissue samples (single cores) as described above. The tissue sections were stained with commercially available marker of IL-1β (Thermo Scientific, UK). This marker was tested and optimised on liver and breast composite sections before using them in the main cohort. The TMA slides were placed on a 60^{o}C hotplate for 10 minutes and were allowed to cool before placing them in a Leica autostainer slide rack. The rack was loaded in the Leica Autostainer XL Staining System ST5010 (Minnesota, USA) for dewaxing and rehydrating. Briefly, the slides were deparaffinised in two xylene baths for 5 minutes each then rehydrated in a series of descending industrial methylated spirit concentrations followed by water for 5 minutes. The slides were then loaded onto a sequenza slide rack (ThermoFisher Scientific, NY, USA). A Novolink Novocastra polymer detection kit (Leica, Germany) was used to stain the tissue sections. 100µl peroxidase block was added onto each slide for 5 minutes and then washed 2x5 minutes with TBS. 100µl of protein block was then added for another 5 minutes and washing was carried out as above before the addition of IL-1β antibody (1:70 dilution in Leica antibody diluent) for 1 hour at room temperature. The slides were then washed 2x5 minutes.

Table 5-2 Clinicopathological characteristics of patients

Clinical feature	*Number (%)*	*Clinical feature*	*Number (%)*
Age		**Size**	
≤40	129 (8.50)	≤ 2cm	904 (59.8)
>40	1382 (91.5)	> 2cm	598 (39.6)
		not determined	9 (0.60)
Tumour stage		**Tumour grade**	
1	921 (61.0)	1	253 (16.7)
2	448 (29.6)	2	499 (33.0)
3	135 (8.90)	3	750 (49.6)
not determined	7 (0.50)	not determined	9 (0.60)
LVI		**BVI**	
negative	1309 (86.6)	negative	1508 (99.8)
positive	202 (13.4)	positive	3 (0.20)
NPI		**Distant metastasis**	
<3.4	454 (30.0)	No	1025 (67.8)
3.4-5.4	768 (50.8)	Yes	478 (31.6)
>5.4	279 (18.5)	Not determined	8 (0.50)
Not determined	10 (0.70)		
Basal status		**ER status**	
Non basal	1118 (74.0)	negative	387 (25.6)
Basal	291 (19.3)	positive	1085 (71.8)
Not determined	102 (6.80)	Not determined	39 (2.60)
PgR status		**Her2 status**	
negative	599 (39.6)	negative	1278 (84.6)
positive	827 (54.7)	positive	201 (13.3)
Not determined	25 (6.70)	Not determined	32 (2.10)
Triple negative status			
Non triple negative	1207 (79.9)		
Triple negative	261 (17.3)		
Not determined	43 (2.80)		

100µl of post primary block was added for 30 minutes, washed and 100µl of Novolink polymer was added for another 30 minutes. DAB solution (1:20 DAB chromogen in DAB substrate buffer) was applied to the slides for 5 minutes prior to 2x5 minutes washing. The slides were stained with Novolink haematoxylin for another 6 minutes. Slides were removed from the sequenza trays and place in a Leica autostainer for dehydration steps in a series of ascending industrial methylated spirit concentrations followed by 2x5 minutes in xylene. Sections were mounted with DPX mounting medium (Leica, Germany) and were left to dry overnight in a fume cupboard.

Breast composite sections were used as both positive and negative controls when the IL-1β staining was conducted. The procedure as above was applied for the positive control. For the negative control, primary antibody was omitted.

5.3.5 TMA scoring

A histochemical score (H-score) was used to assess the stained slides after scanning the slides with a Nanozoomer Digital Pathology Scanner (Hamamatsu Photonics). H-score includes the assessment of the staining intensity as well as the percentage of positive cells. For the intensity assessment, tissue sections were scored 0 to 3 which correspond to negative, weak, moderate and strong staining intensity. The percentage of positive cells at each intensity level was estimated. This score was then calculated as (0 X negatively stained %) + (1 X weakly stained %) + (2 X moderately stained %) and (3 X strongly stained %). Therefore, the range of possible score for each slide is between 0 to 300. Figure 5-1 shows photomicrograph of representative staining intensity from the breast tissue microarray. 30% of the cores were double assessed by an independent assessor (Sarah Storr) blinded to clinicopathologic data and scores. Single measure intraclass correlation coefficients between scores were 0.839 showing excellent concordance between scorers.

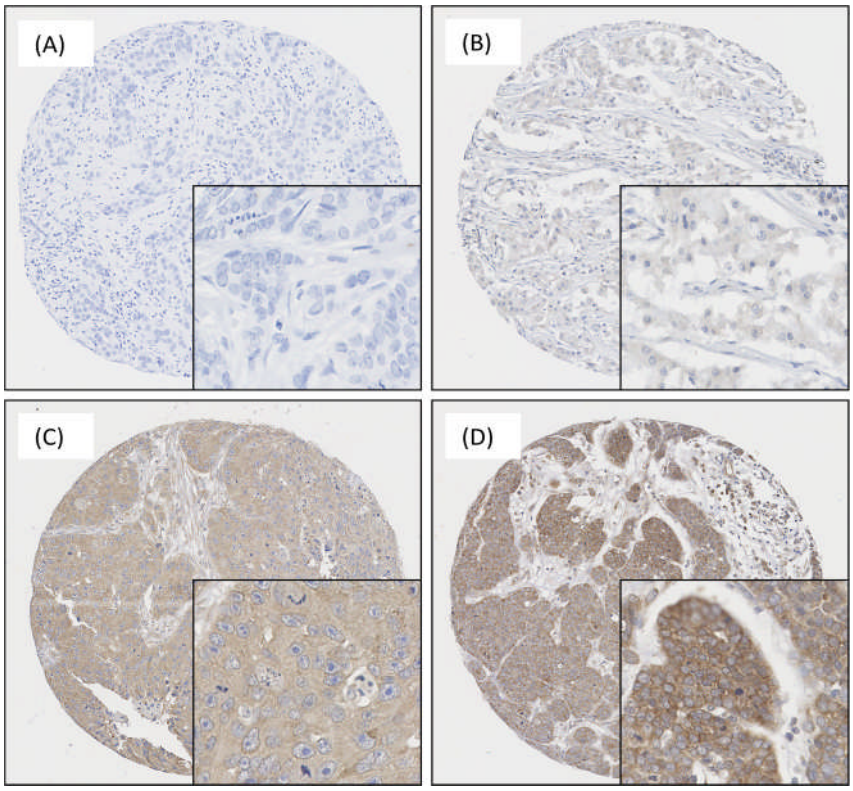


Figure 5-1 Photomicrograph showing representative IL-1β staining intensity in breast tissue microarray used in this study which corresponds to (A) 0 –negatively stained, (B) 1 –weakly stained, (C) 2 –moderately stained and (D) 3 –strongly stained of the H-score assessment. 100x magnification. Inset box 200x magnification.

5.3.6 Statistical analysis

Cross table and Pearson Chi Square test of association (χ^2) (or Fisher's Exact test if a cell count was <5) were used to measure the relationship between categorised protein expression with clinicopathological data. Overall survival and relapse-free survival analyses were performed using the Kaplan-Meier method and the statistical significance between groups assessed by the log rank test. Multivariate survival analysis was performed using Cox regression analysis. P value of <0.05 was considered statistically significant. Concordance between results from individual observers was evaluated using intraclass correlation coefficient (ICC). Statistical analysis was carried out using SPSS version 17.0. Stratification cut-points were determined using X-Tile software and were determined prior to statistical analyses (Camp et al., 2004).

5.4 Results

5.4.1 Western blotting for IL-1β expression

As mentioned previously, three anti-IL-1β antibodies from three different suppliers were tested to ensure that the best possible antibody was used in the immunohistochemical staining of patients' specimens. IL-1β protein expression and binding specificity was investigated by Western blot. Antibodies were directed against mature human IL-1β with the expected molecular weight of 17kDa. Such antibodies also recognise native IL-1β of 31kDa.

Figure 5-2 shows IL-1β expression using the anti-IL-1β antibody supplied from Abcam, UK. As mentioned above, this antibody also recognises native IL-1β of 31kDa as detected in LPS-γ stimulated PBMC lysate. However, the specificity of IL-1β antibody was questionable as it also gave non-specific bands in the tumour lysates used i.e MDA-MB-231, MCF-7, MeWo and SKMEL-30.

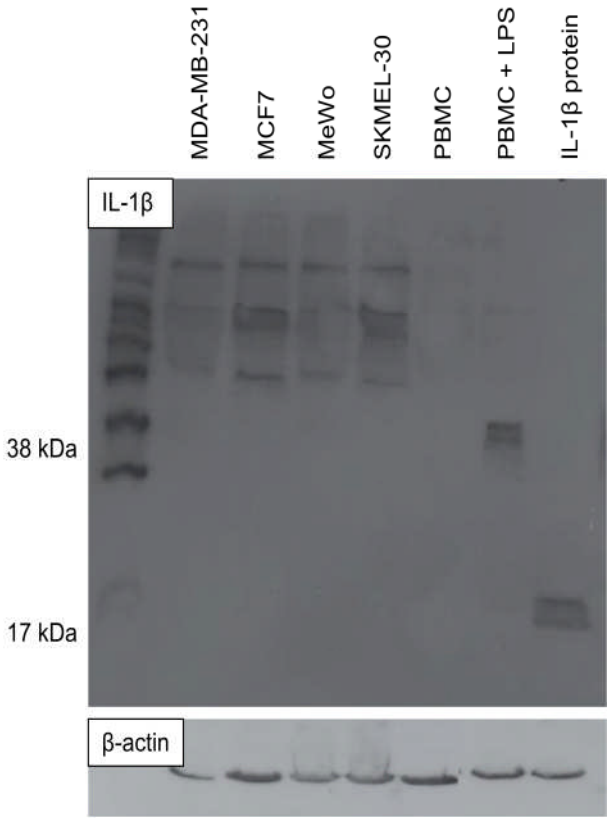


Figure 5-2 IL-1β (Abcam, UK) expression in MDA-MB-231, MCF7, MeWo, SKMEL-30, PBMC and PBMC+LPS-γ by Western blot. 25µg of protein were loaded into each well. Native IL-1β expression was detected in LPS-γ stimulated PBMC. Mature IL-1β was detected in IL-1β recombinant protein sample. However, non-specific bands were observed in the tumour lysates and therefore the use of this antibody in immunohistochemistry was omitted. B-actin bands show equal protein loading into each well.

Figure 5-3 shows IL-1β protein expression assessed using an anti-IL1β antibody supplied by Thermo Scientific, UK. A band of the expected size was observed in the PBMC lysate and was expressed at a higher level in LPS-γ stimulated PBMC lysate compared to the unstimulated PBMC control. In the IL-1β beta recombinant protein sample, an IL-1β band was observed at 17kDa. No expression of IL-1β could be observed in the tumour lysates i.e. MDA-MB-231, MCF7, MeWo and SKMEL-30 lysates. This Western blot result suggested that the anti-IL-1β antibody used was specific and detects the correct IL-1β protein band – it was therefore used to assess IL-1β expression in controls (liver and breast composite blocks) and breast cancer tissue samples via immunohistochemistry.

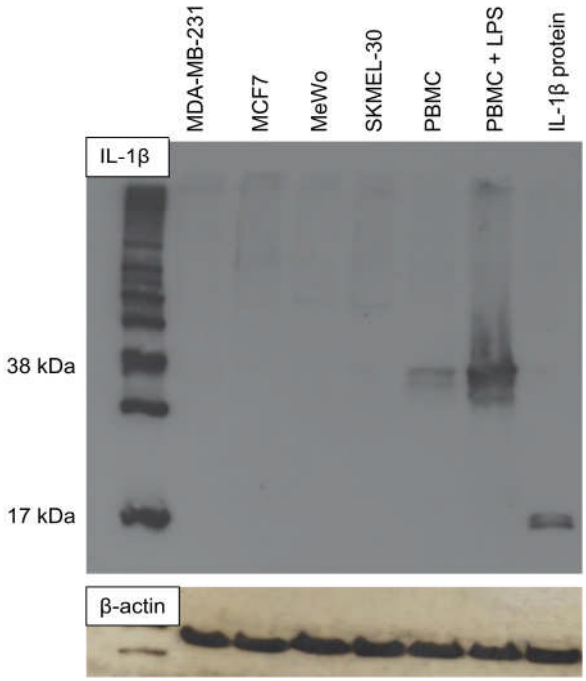


Figure 5-3 : IL-1β (Thermo Scientific, UK) expression in MDA-MB-231, MCF7, MeWo, SKMEL-30, PBMC and PBMC+LPS by western blot. 25µg of protein were loaded into each well. Native IL-1β expression was detected in PBMC lysate with stronger expression in LPS-γ stimulated PBMC. Mature IL-1β was detected in IL-1β recombinant protein sample. No IL-1β band was observed in tumour lysates. B-actin bands show equal protein loading into each well.

5.4.2 Optimisation of IL-1 antibody concentration for IHC

As mentioned above, optimisation of anti-IL-1β antibody (Thermo Scientific, US) in tumour sections was carried out using 3 different immunohistochemical staining kits i.e DAKO kit (DAKO, Denmark), Vectastain Universal Elite ABC kit (Vector Laboratories, San Francisco, USA) and Novolink Novocastra polymer

detection kit (Leica, Denmark), each using 4 different IL-1β antibody concentrations; 1:50, 1:70; 1:100 and 1:150. 1:70 dilution of this antibody using Novolink Novocastra polymer detection kit was determined to give the optimal staining on the liver and breast tissue sections.

When breast composite sections were stained with IL-1β, staining could be observed in the cytoplasm of the cells. Using the blocking recombinant protein, there was no staining observed on the cell cytoplasm indicating that the staining observed with this antibody is a specific for IL-1β (Figure 5-4).

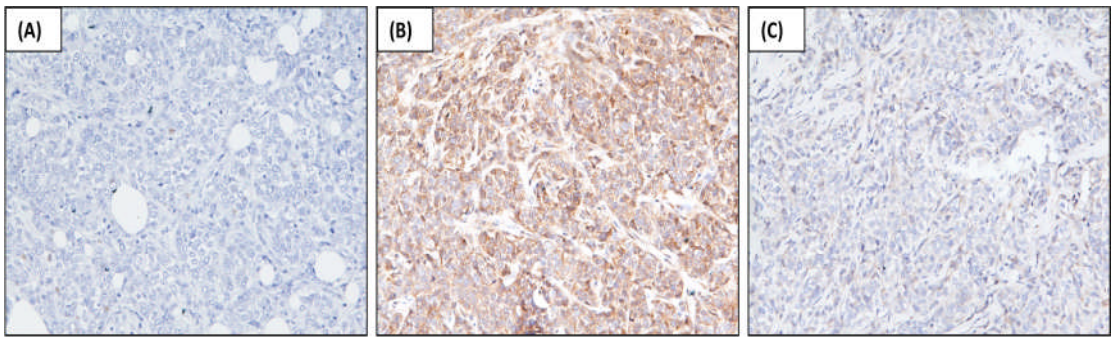


Figure 5-4 Representative of IL-1β staining on breast tissue composite sections; No staining was observed in the negative control section (A). Cytoplasmic staining was observed with IL-1β antibody (B) which was not observed when recombinant protein was used (C). 200x magnification

5.4.3 IL-1β staining in the breast tissue microarray

Cytoplasmic staining was observed with the IL-1β antibody displaying staining heterogeneity between tumour cells (Figure 5-5). This anti-IL-1β antibody also stained a subset of inflammatory cells. The median H-score for cytoplasmic IL-1β expression was 125 with a range between 0 and 230. X-tile software was used to determine the cut off point separating the score into low and high staining intensity. This cut off point was used to determine correlation with clinicopathologic variables. In this study, IL-1β had an H-score cut off point of 35 with 259 (17.1%) cases with low score and 1252 (82.9%) cases having high score. The H-score histogram is shown in Appendix D-Table D2.

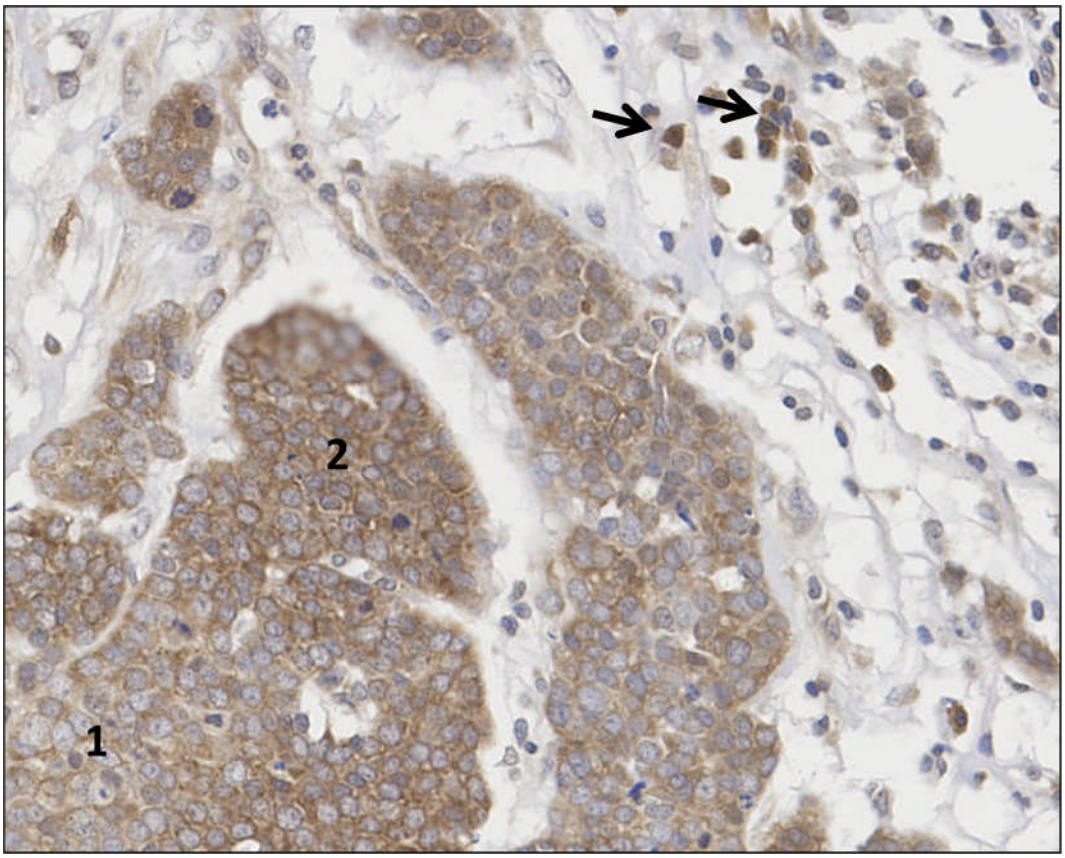


Figure 5-5 The staining pattern of anti-IL-1β antibody on breast tissue microarray. There was staining heterogeneity observed between tumour cells in the same section - for example, in this section, low (1) and moderate (2) staining intensity could be observed. IL-1β also stained a subset of inflammatory cells as shown by the arrow. The staining of IL-1β is specific with no staining observed on stromal and fat cells.

5.4.4 Relationship between IL-1β expression with clinicopathological variables

Table 5-3 shows the association of IL-1β expression with clinicopathologic variables. High IL-1β expression was significantly associated with older patients (χ^2=10.26; d.f.=1; p=0.001), low tumour stage (χ^2=9.01; d.f.=2; p=0.011), PgR positive tumours (χ^2=7.72; d.f.=1; p=0.007), non-basal status (χ^2=51.23; d.f.=1; p=<0.001), the absence of lymphatic vessel invasion (χ^2=7.41; d.f.=1; p=0.01 and χ^2=21.34; d.f.=1; p=<0.001 respectively) as well as no distant metastasis (χ^2=6.45; d.f.=1; p=0.012).

Table 5-3 The association between IL-1β expression and clinicopathologic variables.

Variable		*IL-1β*		
		Low	*high*	*P value*
Age	≤40	9 (3.50)	120 (9.60)	**0.001**
	>40	250 (96.5)	1132 (90.4)	
Size	≤ 2cm	156 (61.4)	748 (59.9)	0.674
	> 2cm	98 (38.6)	500 (40.1)	
Tumour stage	1	174 (68.2)	747 (59.8)	
	2	56 (22.0)	392 (31.4)	**0.011**
	3	25 (9.80)	110 (8.80)	
Tumour grade	1	59 (23.2)	194 (15.5)	
	2	79 (31.1)	420 (33.7)	**0.012**
	3	116 (45.7)	634 (50.8)	
LVI	negative	206 (79.5)	1103 (88.1)	**<0.001**
	positive	53 (20.5)	149 (11.9)	
BVI	negative	259 (100)	1249 (99.8)	1.000
	positive	0 (0.00)	3 (0.20)	
Any vessel invasion	negative	206 (79.5)	1100 (87.9)	0.001
	positive	53 (20.5)	152 (12.1)	
NPI	<3.4	86 (34.0)	368 (29.5)	
	3.4-5.4	130 (51.4)	638 (51.1)	0.136
	>5.4	37 (14.6)	242 (19.4)	
Distant metastasis	No	158 (61.5)	867 (69.6)	**0.012**
	Yes	99 (38.5)	379 (30.4)	
Basal status	Non basal	151 (62.4)	967 (82.9)	**<0.001**
	Basal	91 (37.6)	200 (17.1)	
ER status	negative	69 (28.2)	318 (25.9)	0.475
	positive	176 (71.8)	909 (74.1)	
PgR status	negative	122 (50.0)	477 (40.4)	**0.007**
	positive	122 (50.0)	705 (59.6)	
Her2 status	negative	210 (83.3)	1068 (87)	0.130
	positive	42 (16.7)	159 (13)	
Triple negative status	non triple negative	206 (84.1)	1001 (81.8)	0.464
	triple negative	29 (15.9)	222 (18.2)	

5.4.5 Relationship between IL-1β expression with clinical outcome

From Kaplan-Meier analyses, low IL-1β expression was significantly associated with breast cancer specific survival (p=0.017) and disease free interval (p=0.008) (Figure 5-6). However, in multivariate Cox regression analysis, IL-1β expression was not an independent indicator of breast cancer specific survival (HR=1.084; 95% CI=0.846-1.390; p=0.523) and disease free interval (HR=0.874; 95% CI=0.711-1.075; p=0.202). In these multivariate analyses, the potential confounding factors included were patient age, tumour stage, PgR status, intratumoural and peritumoural lymphatic vessel invasion as well as distant metastasis.

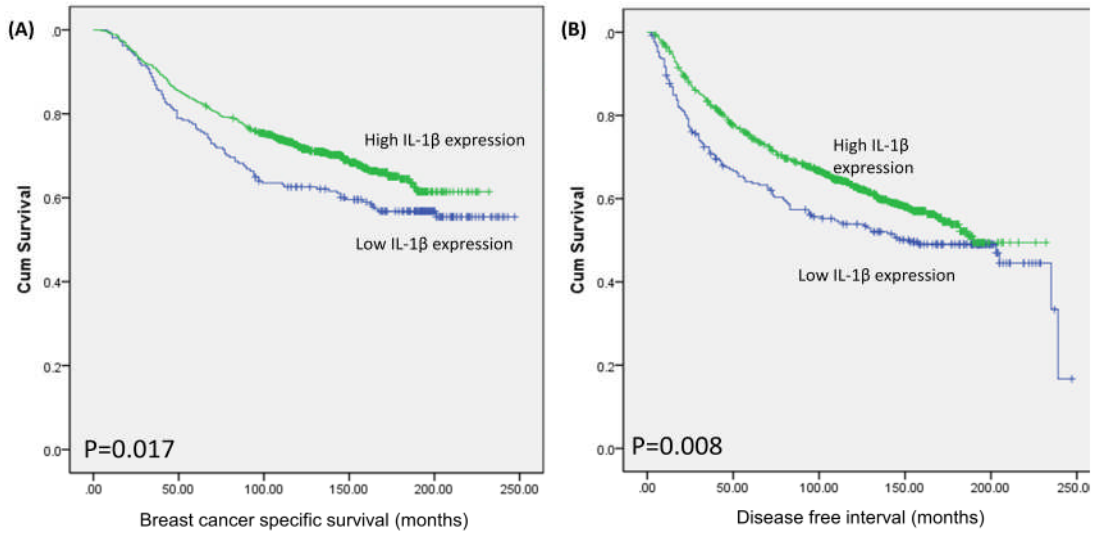


Figure 5-6 Kaplan-Meier analysis of breast cancer specific survival and disease free interval of IL-1β expression. Low IL-1β expression was significantly associated with breast cancer specific survival (p=0.017) and disease free interval (p=0.008).

5.5 Discussion

In previous chapters (Chapter 3 and Chapter 4), it was found that IL-1β may play an important role in regulating the adhesion, migration and transmigration of cancer cells, especially to/across lymphatic endothelial cells. The main aim of this chapter was to investigate the expression of IL-1β and any association it may have with lymphatic vessel invasion in tumour specimens. Furthermore we sought to investigate any apparent associations between this protein expression and clinicopathologic variables and clinical outcome.

To achieve this, 1511 early stage breast cancer specimens with known standard clinical variables were used to demonstrate associations with IL-1β expression. High IL-1β expression was significantly associated with older patients i.e more than 40 years old and this result is in agreement with another study which showed that the basal level of IL-1β and other pro-inflammatory cytokines could be elevated as a result of aging (Barbieri et al., 2003). This might be due to the fact that in older population, the baseline level of IL-10 was reduced compared to younger population (Hacham et al., 2004). IL-10 is a pleiotropic cytokine that can suppress the production of other inflammatory cytokines such as IL-1β, TNF-α, IL-2, IL-3 and IFN-γ.

There are surprisingly few studies that have examined the expression of IL-1β in breast cancers. IL-1β expression in human breast cancer was shown to correlated inversely with estrogen receptor levels (Miller et al., 2000). In one study, IL-1β expression was detected in the cytoplasm of invasive ductal breast carcinoma cells and correlated with poor differentiation and decreasing estrogen receptor expression (Singer et al., 2003). However, no such association between IL-1β expressions with oestrogen receptor status was observed in this study. In this current study, high IL-1β expression was significantly associated with the absence of lymphatic vessel invasion as well as the absence of distant metastasis. In survival analyses, low IL-1β expression in the cytosolic compartment was significantly associated with breast cancer specific survival and disease free interval; which lost their significance in multivariate analyses. A diverse effect of IL-1β on tumour development has

been described in the literature. IL-1β exerts its anti-tumour effect through the ability to (1) co-stimulate T cell activation, generating a sustained immune response (2) to induce the secretion of other cytokines such as IL-2, granulocyte-macrophage colony-stimulating factor and IFN-γ and (3) to potentiate the differentiation of immune surveillance cells. (Song et al., 2003). In addition, IL-1β has been reported to reduce tumour growth and proliferation by cytocidal or cytotoxic effects (Bhakdi et al., 1990). The expression of several genes associated with growth arrest such as gro-α and gro-β has been described with the addition of IL-1β to melanoma cell line (Rangnekar et al., 1992). Administration of exogenous IL-1β in-vivo could lead to tumour regression and vascular collapsed especially when this cytokine was administered in a large amount (100 to 400μg/kg). In this experiment, IL-1β was proposed to act with IFN-γ in a synergistic manner to increase tumour killing (Rice and Merchant, 1992).

However, in light of the results discussed in Chapter 3 and Chapter 4 that IL-1β could influence the adhesion, migration and transmigration of tumour cells to the lymphatic endothelium, immunohistochemistry results in this chapter are somewhat unexpected. IL-1β is initially synthesised as an immature proIL-1β precursor molecule which lacks a signal peptide and remains cytosolic until it is cleaved by IL-1β converting enzyme into an active and mature IL-1β. Mature IL-1β is secreted into the cellular compartment through a membrane channel. In the in-vitro adhesion, migration and transmigration experiments, the recombinant IL-1β used to stimulate the endothelial and tumour cells was the purified 17kDa IL-1β in its active form. Yet, the anti-IL-1β antibody used to stain tumour specimens in this chapter detects both the 31kDa immature IL-1β precursor (proIL-1β) and the mature IL-1β. Such differences might account for the differences in the in-vitro observations and in the patient's tumours.

In a study by Song and colleagues (2003), the mode of presentation and the compartmentalisation of the active form of IL-1β within the tumour cells and its microenvironment determined its effect in the malignant process. Their study showed that the secreted form of IL-1β, and not the mature IL-1β in the

intracellular compartment, could increase tumour growth, invasion and metastasis through the up-regulation of growth factors, angiogenesis-promoting factors, matrix metalloproteinase and the expression of adhesion molecules. Following cleavage of proIL-1β by ICE, most mature IL-1β remains cytosolic because the secretion of IL-1β is an inefficient process that requires additional signals such as exogenous ATP to promote secretion. Song and colleagues (2003) demonstrated that active IL-1β secretion by fibrosarcoma cells increases their invasive potential and the degree of invasiveness depends on the concentration of secreted IL-1β. Other studies have also reported similar results in which increased invasiveness of lung carcinoma cells was observed when the cells were transfected with signal sequence IL-1β (ssIL-1β) construct, to allow IL-1β secretion through the ER-Golgi pathway (Saijo et al., 2002a). In the previous in-vitro chapters (Chapter 3 and 4), the IL-1β used is the secreted form of IL-1β in the macrophage supernatant generated from LPS-γ activated macrophages. However, in the current chapter, the IL-1β detected was the IL-1β in the cytoplasm of the cells and this difference might account for the results observed.

Initial results, in Chapter 2 and previous studies (Mohammed et al., 2007; Storr et al., 2011), examining how lymphatic vessel invasion might be regulated have identified that lymphatic vessel invasion was particularly evident when macrophage infiltrates were immediately adjacent to the lymphatic vessels, it may be that in addition to the type of IL-1β being produced (immature and mature IL-1β) that concentration gradients within the tumour milieu exist. It would be interesting, as mentioned earlier, to assess the level of IL-1β production from macrophage via double immmuno techniques or to assess IL-1β levels adjacent to lymphatic vessel invasion. If IL-1β activity cannot be directly assessed, it would also be of interest to assess the levels of proteins involved in the maturation process i.e ICE/caspase and examine their expression and localisation.

In summary, results in the current chapter show that high expression of IL-1β in tumour cells correlates with the absence of lymphatic vessel invasion, both

intratumoural and peritumoural invasion, and that low IL-1β expression correlates with breast cancer specific survival and disease free interval. Further work is required to fully elucidate the role that macrophage play in regulating lymphovascular invasion, with increased attention being focused on the role that IL-1β plays in this process.

CHAPTER 6: GENERAL DISCUSSION AND SUGGESTIONS FOR FUTURE WORK

As mentioned in previous chapters, tumour progression to invasive metastatic disease often begins with lymphatic and/or blood vessel invasion. In contrast to blood vessels, research on lymphatic vessels has lagged behind, primarily due to the lack of molecular tools capable of specifically distinguishing lymphatic endothelial cells from blood endothelial cells. It was not until recent years that the study of the characteristics of lymphatic vessels in tissues and cell culture were possible, following the discoveries of lymphatic specific markers. The aims of the current research project were to (1) examine the characteristics of lymphatic vessels in cutaneous melanoma and their relationship to tumour clinicopathological characteristics and patient prognosis, (2) to conduct in-vitro investigations of adhesion, migration and transmigration, conducting comparisons between lymphatic and blood endothelial cells and (3) to investigate the influence of macrophage and macrophage associated cytokines in the initial metastatic processes of lymphovascular invasion.

In the first component of the project (Chapter 2), lymphatic vessels in cutaneous melanoma samples were examined using the lymphatic marker, D2-40. They were found to be mostly located in the peritumoural area of the tumour with fewer vessels in the intratumoural area. Intratumoural lymphatic vessel invasion was, however, present and was significantly associated with increased stage, increased Breslow thickness, the presence of ulceration, increased Clark's level, high mitotic rate, the presence of microsatellites and nodular histological subtype. The majority of studies examining lymphatic topography in cutaneous melanoma have reported the presence of intratumoural lymphatics (Giorgadze et al., 2004; Niakosari et al., 2005b), some of which were shown to be correlated with poor disease free and overall survival (Dadras et al., 2003b) and distant metastasis (Doeden et al., 2009b). Intratumoural lymphatics were also detected in other human tumours such as breast cancer (Bono et al., 2004; Mohammed et al., 2011), gastric cancer (Wang et al., 2010), hepatocellular carcinoma and non small cell lung cancer (Renyi-Vamos et al., 2005). In contrast, in breast and cervical cancer (Thelen et al., 2009; Williams et al., 2003), functional intratumoural lymphatics were not reported suggesting that the distribution of tumoural lymphatics varies between tumour types and also

anatomical site. In our group's previous work with breast cancer we found that peritumoural lymphatics were detected in all 177 specimens whilest intratumoural lymphatics were detected in 41% of the specimens. Such vessels are functional in that tumour emboli are observed within them. Such work was verified in separate expanded cohorts (Mohammed et al., 2011; Rakha et al., 2011).

In the current cohort of melanoma tumour specimens, although microvessel density was much higher compared to lymphatic vessel density in the same tissue section, vascular invasion in melanoma was essentially invasion of lymphatic vessels (26.9% vs 4.2%). A preference of such lymphatic invasion over blood vessel invasion was reported previously with varying frequencies of blood vessel to lymphatic vessel invasion of 3% to 16% (Doeden et al., 2009a), 4.7% to 33% (Xu et al., 2008) and 43% to 47% (Shields et al., 2004) respectively.

As mentioned above, in the current study, the presence of lymphatic vessel invasion was associated with increased stage, increased Breslow thickness, the presence of ulceration, increased Clark's level, high mitotic rate and the presence of microsatellites; all of these factors are markers of tumour aggressiveness. In other studies, lymphatic vessel invasion was also associated with younger age, the presence of ulceration, sentinel lymph nodes positivity and distant metastasis (Niakosari et al., 2005b). As a result, lymphatic invasion has been suggested as a prognostic indicator for the progression and spread of malignant melanoma and other cancers. In fact, Shields and colleagues have developed a prognostic index based on their cohort of metastatic melanoma samples which combined the information on thickness, lymphatic density and lymphatic invasion, to generate a more efficient predictor of survival (Shields et al., 2004). However, only 21 samples were used, limiting the clinical value of this index. Further work is required to assess the robustness of the Shields index to consider the use of it in clinical settings. It would have been interesting to have assessed the prognostic value of the Shields index in the current cohort

– this could be carried out through further work/investigations by others continuing the study.

It is unclear what factors influence lymphatic vessel invasion in tumours rich in blood vessels. However, it was observed that lymphatic vessels in tumours, where lymphatic vessel invasion was noted, were associated with a dense inflammatory infiltrate. There are a number of reports in the literature, showing the presence of inflammatory infiltrates in the tumour microenvironment and suggesting a link between inflammation and cancer (Hagemann et al., 2005; Johnson et al., 2006). Macrophages were usually detected in abundance surrounding tumoural vessels. It was therefore off interest in this study to examine if there was any correlation between macrophage infiltrates and the presence of lymphatic invasion. The presence of high macrophage count was associated with the presence of lymphatic vessel invasion which was supported by other studies investigating the role of macrophages and inflammatory cells in inducing lymphangiogenesis in tumours and other tissues (Kerjaschki, 2005; Yano et al., 2006). Such inflammatory cells have been shown to produce lymphangiogenic growth factors and cytokines thereby contributing to lymphangiogenesis (Kerjaschki, 2005).

The conclusion of this part of the project was that, as previously found with breast cancer, lymphatics play an important role in melanoma cell progression by being important routes for tumour cell dissemination and that this process might be driven by macrophages and their secreted factors.

In-vitro investigation of tumour cell adhesion, migration and transmigration

In the initial metastatic process, tumour cells must first be able to detach from the primary tumour mass, migrate through the extracellular matrix, and adhere to the blood or lymphatic endothelial cells before transmigrating across the endothelial cells to enter the circulation or lymph. Many phenotypical and biochemical changes occur to/in the tumour cells during these processes which involve alterations of growth factor signalling, cell-cell adhesion, gene

expression, motility and cell shape (Leber and Efferth, 2009). Furthermore, stromal cells in the tumour microenvironment may also affect the aforementioned changes via secretion of various cytokines. Our histopathology studies suggest that inflammatory cells might play an important role in regulating at least some of these processes. Based on our findings that macrophage might influence lymphatic vessel invasion in tumour specimens, the second part of the study dealt with in-vitro investigations of adhesion, migration and transmigration between lymphatic and blood endothelial cells in control conditions and the influence of macrophage and macrophage associated cytokines in these processes.

Tumour cell adhesion to blood (HUVEC and hMEC-1) and lymphatic endothelial cells (hTERT-LEC and hMVEC-dLy Neo) was compared. The side-side comparison between tumour cell adhesion to lymphatic and blood endothelial cells was novel and has not been examined in previous studies. Previous in vitro studies of tumour cell adhesion usually included only one endothelial cell model, mostly HUVEC or microvascular endothelial cells. Tumour cell adhesion to lymphatic endothelium has, however, been studied before using fresh frozen lymph node whole sections (Irjala et al., 2003) and non small cell lung carcinoma cell adhesion to lymphatic endothelial cells (Irigoyen et al., 2007), however with no direct comparison with a vascular comparator. In the current study, MDA-MB-231, MCF7, MeWo and SKMEL-30 cell lines were used to demonstrate the adhesion pattern of these cells towards large vein and microvessel endothelial cells, HUVEC and hMEC-1 respectively as well as primary and immortalised lymphatic endothelial cells, hMVEC-dLy Neo and hTERT-LEC respectively. Under unstimulated conditions, these cell lines showed preferential adhesion to blood endothelial cells as opposed to the lymphatic endothelial cells. The preferential adhesion of tumour to blood and lymphatic endothelial cells was unexpected, considering the findings in patients specimens showing that vascular invasion in these tumour types is principally lymphatic vessels. Such conflicting findings raised important questions about the physiological mechanisms of lymphatic vascular invasion; are there molecular mechanisms that could initiate and control the tumour-endothelial

interaction which eventually drive the tumour cells to lymphatic endothelial cells or was the preferential lymphatic vessel invasion simply a reflection of differences in vessel structure (i.e. lymphatic vessels being ‘easier’ to transmigrate)?

The correlation between high macrophage count and the presence of lymphatic vessel invasion as observed in patients’ samples could indicate that macrophages, inflammatory cells present in abundance in the tumour microenvironment, might influence lymphatic vessel invasion. Therefore, TNF-α and IL-1β, both of which are macrophage secreted cytokines, were used to stimulate the endothelial cells (HUVEC, hMEC-1, hTERT-LEC and hMVEC-dLy Neo) and tumour cells (MDA-MB-231, MCF7, MeWo and SKMEL-30) to determine whether these factors alter the adhesion, migration and transmigration patterns of these tumour cells.

Tumour cell adhesion to TNF-α-stimulated HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo did not vary significantly when compared to the unstimulated endothelium. However, a significant increased was observed when both the tumour and the endothelial cells were simultaneously stimulated with TNF-α suggesting that dual activation with TNF-α might be necessary to promote tumour-endothelial cell adhesion. Upon IL-1β stimulation, a significant increased of tumour cell adhesion was observed which was notably higher to the lymphatic endothelial cells, suggesting a novel and important role for IL1-β in cancer cell adhesion to lymphatic vessels. This was supported by the fact that in mice models lacking the IL-1β gene, the absence of metastasis was observed (Moore et al., 1999). Pre-incubation of human microvascular endothelial cells with IL-1β were also shown to increase the adhesion of human pancreatic cancer cell lines.

Questions then arise as to whether the increased tumour cell binding is dependent upon the up-regulation of adhesion molecules on the endothelial cells and if so, which adhesion molecules are responsible? Therefore, in addition to the in-vitro adhesion assays, the role of adhesion molecules expressed on the endothelial cell models in regulating lymphatic vessel invasion

was also examined. CLEVER-1, common lymphatic endothelial and vascular endothelial receptor 1, is differentially expressed in lymphatic, but not vascular endothelial cells (Salmi et al., 2004). In this study, CLEVER-1 expression between blood and lymphatic endothelial cells, and between primary and immortalised cell lines was compared. Results showed that although all endothelial cell models expressed CLEVER-1 intracellularly, only the immortalised lymphatics, hTERT-LEC, showed significant surface expression of CLEVER-1. Upon stimulation with TNF-α, only the primary lymphatic cells model, hMVEC-dLy Neo showed significant increase of CLEVER-1 expression on both surface and intracellular staining suggesting that hTERT-LEC may be a model of activated lymphendothelium.However, stimulation of HUVEC, hMEC-1, hTERT-LEC and HMVEC-dLy Neo with IL1-β did not show any significant altered CLEVER-1 expression. Such results, in light of the adhesion assay results, suggest that although CLEVER-1 may be an important lymphatic specific adhesion molecule, it may not be a principal regulator of lymphatic vessel invasion mediated via IL-1β. The expression of ICAM-1 and VCAM-1 were also studied. Results showed that both hMEC-1 and hTERT-LEC expressed ICAM-1 and VCAM-1 on their surface with higher expression observed on hMEC-1 cells. Upon stimulation with IL-1β, both ICAM-1 and VCAM-1 expression increased significantly on hMEC-1 and hTERT-LEC suggesting that these adhesion molecules might be responsible, at least in part, for the regulation of lymphatic vessel invasion. ICAM-1 and VCAM-1 knockdown in lymphatic and blood vascular endothelial cells would be required to further examine the role that these molecules play in regulating tumour cell trafficking via lymphatic vessels. It would also be of interest to examine expression of other adhesion molecules associated with lymphatic endothelium (mentioned in Section 1.5 –Chapter 1) and determine if their expression is altered by exposure to IL-1β or macrophage conditioned media.

In the scratch wound migration assay, TNF-α and IL-1β-stimulation was shown to increase the migratory ability of all tumour cell lines used in this study (MDA-MB-231, MCF7, MeWo and SKMEL-30), which was observed at numerous time points and these observations are in agreement with other studies (Hidalgo et

al., 2005; Katerinaki et al., 2003). In the in-vivo settings, tumour cells that detach from the tumour mass migrate through the extracellular matrix components present in the tumour microenvironment such as collagen, fibronection, laminin and others. Therefore, it would be potentially interesting to examine tumour cell migration by coating the tissue culture wells with extracellular matrix substrates first before plating the control and IL-1β stimulated cells. By doing this, the effect of extracellular matrix on control and cytokine stimulated cells could be studied in detail, using conditions which better mimick the in-vivo migration process.

Tumour transendothelial migration follows migration and adhesion steps and therefore was also off interest to this study. Experiments comparing transendothelial migration of tumour cell lines; MDA-MB-231 and MeWo across blood and lymphatic endothelial cells in a single experiment have not been previously reported. As with adhesion assays, most transendothelial migration assays have used large vein endothelial cells (HUVEC). In the current study, MDA-MB-231 cells and MeWo show similar transmigratory abilities across both blood and lymphatic endothelial cells. However, MDA-MB-231, being the more aggressive cell type, transmigrated in higher number compared to MeWo cells. Upon stimulation with IL-1β, there was a significant increased in the number of MDA-MB-231 cells that transmigrated across both hMEC-1 and hTERT-LEC with similar affinity to both endothelial cell types; blood and lymphatic endothelium. Similarly, upon stimulation with IL-1β, there was an increased in the transmigratory ability of MeWo cells across hMEC-1 and hTERT-LEC which was notably observed across hMEC-1 cells.

The adhesion, migration and transmigration assays in this study were also carried out using tumour conditioned media and macrophage conditioned media in an attempt to determine the potential source of IL-1β that might influence the initial metastatic processes. Results from IL-1β ELISA's showed that no significant IL-1β expression was detectable in the tumour conditioned media. When adhesion assays were conducted using such tumour conditioned media, no significant changes were observed in the adhesive patterns of tumour cells

to the endothelium of either blood or lymphatics compared to the unstimulated controls. Similarly, tumour cells stimulated with their own respective tumour conditioned media showed no significant changes in their migratory ability compared to their unstimulated counterparts. However, the use of macrophage conditioned media, generated from LPS-γ stimulated macrophages, increased the number of adhered MDA-MB-231 cells compared to the unstimulated controls. In the transwell migration assay, there was an increased in the transmigratory ability of MDA-MB-231 cells when the endothelial cells were stimulated with such macrophage supernatant. In both adhesion and transmigration assays, the increased adhesion and transmigration ability were both higher across hTERT-LEC compared to hMEC-1 cells and was statistically significant in one donor in particular i.e. donor 2. When both hMEC-1 and hTERT-LEC cells were exposed to macrophage conditioned media generated from LPS-γ+ICE inhibitors stimulated macrophages, the number of cells adhered and transmigrated to/across both endothelial cell types significantly decreased. IL-1β ELISA results showed high IL-1β expression in LPS-γ stimulated macrophages which was reduced when ICE inhibitor was used. ICE is a cysteine protease responsible for proteolytic activation of the biologically inactive IL-1β precursor to the proinflammatory active cytokine (Livingston, 1997). Therefore, current results suggest that IL-1β might be involved in the regulation of tumour cell adhesion and transmigration across the endothelial cells. The fact that it was detected in macrophage conditioned media and not the tumour conditioned media suggested that macrophage may be the main source of the IL-1β that is capable of regulating the initial metastatic processes.

To further evaluate whether IL-1β a regulator of invasion, specific blocking of IL-1β receptors could be carried out by using the IL-1β receptor antagonist (IL-1Ra). IL-1Ra is a naturally occurring inhibitor of IL-1β that binds to IL-1β receptors with the same affinity as IL-1β but without triggering a biochemical response in cells (Vidal-Vanaclocha et al., 1994). The role of IL-1β could be studied by culturing the endothelial cells in the presence and absence of IL-1β receptor blockade. Furthermore, co-culture experiments of endothelial cells and macrophages could also be carried out to assess the influence of macrophage

in IL-1β production thus influencing adhesion and transmigration of tumour cells in metastasis.

The lack of IL-1β in the tumour derived lysate is interesting as tumour cells can also produce IL-1β. Studies using melanoma, non small cell carcinoma and colon cancer cell lines have shown that these cell lines exhibited significantly increased copy number of IL-1β (Elaraj et al., 2006; Lewis et al., 2006). However, macrophages and monocytes also produce IL-10, an anti-inflammatory cytokine, which has been shown to inhibit LPS-induced IL-1β mRNA and protein production in human monocytes and neutrophils (Jenkins et al., 1994). IL-10 has also been shown to enhance the level of IL1Ra mRNA in LPS-induced monocytes resulting in an increased ratio of IL1Ra to IL-1β (Bogdan et al., 1992). In addition, IL-6 might also inhibit the production of IL-1β through the activation of IL-10 and IL1Ra. Therefore, based on the results of this study, IL-6 and IL-10 ELISA's could be carried out on the macrophage supernatant and tumour conditioned media to confirm the expression of these cytokines, and determine how IL-1β is regulated in these cells.

The conclusion for this part of the project was that macrophage associated cytokines, TNF-α and more importantly IL-1β could regulate melanoma and breast cancer cell adhesion, migration and transmigration in-vitro.

IL-1β expression in breast tissue

Based on the in-vitro results generated, the third part of the current study dealt with immunohistochemical investigation of IL-1β expression in 1511 early stage breast cancer specimens. In term of lymphatic vessel invasion, high IL-1β expression in the cytoplasm of tumour cells was significantly associated with the absence of lymphatic vessel invasion - this observation differs to the in-vitro adhesion, migration and transmigration results observed. IL-1β is initially synthesised as an immature 31kDa proIL-1β precursor which remains in the cytoplasm until it is cleaved into a mature 17kDa IL-1β that is secreted into the cellular compartment through a membrane channel. In the in-vitro assays, it was the mature IL-1β that was used to stimulate endothelial and tumour cells

but yet, the anti-IL-1β antibody used to stain breast tumour specimens detects both immature and mature forms of IL-1β. Such differences might account for the differences observed between the in-vitro observations and the tumour specimens.

The mode of presentation of the active IL-1β within the tumour cells and the microenvironment can also determine its effect in the malignant process (Song et al., 2003). It was shown from the studies of Song and colleagues that the secreted form of mature IL-1β in the extracellular compartment but not the mature cytosolic IL-1β could increase tumour growth, invasion and metastasis. The degree of invasiveness of fibrosarcoma cells (Song et al., 2003) and lung carcinoma cells (Saijo et al., 2002a) may depends on the concentration of secreted IL-1β. However, results from the current breast tumour specimens were based on cytoplasmic IL-1β and this might also account for the differences observed in the in-vitro assays.

As with the suggestions for further in vitro work, mentioned above, it would also be of interest to assess expression of IL-6 and IL-10 in the breast tumour sections. Interleukin 10 which is also known as cytokine synthesis inhibitory factor (CSIF) is an anti-inflammatory cytokines capable of inhibiting the production of other cytokines such as IFN-γ, IL-1α, IL-1β, IL-6, IL-8 and TNF-α. It was shown that IL-10 could reduce the synthesis of IL-1β by up to 90% following the optimal activation of monocytes by LPS (de Waal Malefyt et al., 1991). Therefore, it would be interesting to stain patients tissue samples to study any correlation that IL-1β has with IL-10 and whether IL-10, being anti-inflammatory cytokines and negative regulator of IL-1β could reduce tumour cells invasion especially to the lymphatic vessels.

Furthermore, results from the in vitro component of the project concluded that macrophages are a main producer of IL-1β that may have a positive effect in tumour cell invasion especially to the lymphatic circulation. Therefore, IL-1β staining on macrophages (using a double immunohistochemical staining technique) should be evaluated to study the correlation of this parameter with lymphatic vessel invasion and other clinicopathological criteria. A further area

of future interest, in terms of potential histopathology based studies, might be to look at macrophage subtyping i.e. to determine whether it was M1 or M2 associated macrophage that were associated with lymphatic vessel invasion. Each of these macrophage subtypes produce a different spectrum of cytokines and so such information may narrow the spectrum of factors to be examined.

In summary:

In conclusion, this study showed that (1) lymphatic vessel invasion occurred predominantly in melanoma although lymphatic vessel density was detected in lower number compared to microvessel density in the same tissue samples, (2) the use of immunohistochemical marker, D2-40 increased the accuracy of lymphatic vessel invasion detection compared to the use of conventional staining and (3) the presence of high macrophage count was associated with the presence of lymphatic vessel invasion which might indicate the role of macrophage in the regulation of this process.

In the in-vitro settings, this study has shown that IL-1β present in macrophage conditioned media can regulate the adhesion, migration and transmigration processes as shown in the static adhesion assays, haplotaxis scratch wound migration assay and the Boyden chamber transmigration assays respectively. Further studies are needed to validate the role of IL-1β in the regulation of lymphatic vessel invasion by using IL-1β receptor antagonist or carrying out co-culture experiments with tumour associated macrophages as discussed above. Based on these results, questions arise on how IL-1β regulates the initial metastatic processes? In this study, it was shown that the surface expression of ICAM-1 and VCAM-1 increased significantly in both blood and lymphatic endothelial cell models used indicating that these adhesion molecules might be the regulator of lymphatic vessel invasion. To address this, ICAM-1 and VCAM-1 knockdown would be required to further examine the role that this molecule plays in mediating tumour cell trafficking via lymphatic vessels. Although current results suggest that CLEVER-1 may not be involved it would also be of interest to examine expression of other ‘lymphatic specific’ adhesion molecules such as junctional adhesion molecules, sphingosine 1 phosphate receptor and mannose

receptor (as reviewed in Section 1.6). All the results mentioned in this study were based on static assays. It would be interesting to study the effect of flow on adhesion, migration and transmigration of tumour cells. Using a flow based assay is important as it better represents the in vivo process.

The current study has yielded important results, shedding some light on our understanding of the lymphatic vasculature and the modulation of lymphatic vessel invasion. Understanding these processes is crucial to enable translation of research into research for patient benefit and identification of therapeutic targets.

REFERENCES

Abi-Raad, R., Boutrus, R., Wang, R., Niemierko, A., Macdonald, S., Smith, B., and Taghian, A.G. (2011). Patterns and risk factors of locoregional recurrence in T1-T2 node negative breast cancer patients treated with mastectomy: implications for postmastectomy radiotherapy. Int J of Radiation Oncol Biol Physics 81, e151-e157.

Achen, M.G., and Stacker, S.A. (2008). Molecular control of lymphatic metastasis. Annals of the New York Academy of Sciences 1131, 225-234.

Adachi, H., and Tsujimoto, M. (2002). FEEL-1, a novel scavenger receptor with in vitro bacteria-binding and angiogenesis-modulating activities, pp. 34264-34270.

Algars, A., Irjala, H., Vaittinen S, Huhtinen H, Sundström J, Salmi M, Ristamäki R, and S., J. (2011). Type and location of tumor-infiltrating macrophages and lymphatic vessels predict survival of colorectal cancer patients. Int J Cancer.

Ammar, A., Mohammed, R.A.A., Marko Salmi, M.P., Emma C. Paish,, and Ian O. Ellis and Stewart G. Martin (2011). Lymphatic expression of CLEVER-1 in breast cancer and its relationship with lymph node metastasis. Analytical Cellular Pathology 34.

Attout, T., Hoerauf, A., Debrah, A.Y., Marfo-Debrekyei, Y., Boussinesq, M., Wanji, S., Martinez, V., Mand, S., Adjei, O., Bain, O., et al. (2009). Lymphatic vascularisation and Involvement of lyve-1 macrophages in the human onchocerca nodule. PLoS ONE 4, e8234.

Azzola, M.F., Shaw, H.M., Thompson, J.F., Soong, S.-j., Scolyer, R.A., Watson, G.F., Colman, M.H., and Zhang, Y. (2003). Tumor mitotic rate is a more powerful prognostic indicator than ulceration in patients with primary cutaneous melanoma. Cancer 97, 1488-1498.

Baay, M., Brouwer, A., Pauwels, P., Peeters, M., and Lardon, F. (2011). Tumor cells and tumor-associated macrophages: secreted proteins as potential targets for therapy.

Balch, C., Soong, S., Smith, T., Ross, M., Urist, M., and Karakousis, C., et al. (2001a). Long-term results of a prospective surgical trial comparing 2 cm vs. 4 cm excision margins for 740 patients with 1–4 mm melanomas. . Ann Surg Oncol 8, 101-108.

Balch, C.M., Gershenwald, J.E., Soong, S.-j., Thompson, J.F., Atkins, M.B., Byrd, D.R., Buzaid, A.C., Cochran, A.J., Coit, D.G., Ding, S., et al. (2009). Final version of 2009 AJCC melanoma staging and classification, pp. 6199-6206.

Balch, C.M., Soong, S.-J., Gershenwald, J.E., Thompson, J.F., Reintgen, D.S., Cascinelli, N., Urist, M., McMasters, K.M., Ross, M.I., Kirkwood, J.M., et al. (2001b). Prognostic factors analysis of 17,600 melanoma patients: validation of the American Joint Committee on Cancer melanoma staging system, pp. 3622-3634.

Baldwin, M.E., Stacker, S.A., and & Achen, M.G. (2002). Molecular control of lymphangiogenesis. . Bioessays 24, 1030-1040.

Baluk, P., Fuxe, J., Hashizume, H., Romano, T., Lashnits, E., Butz, S., Vestweber, D., Corada, M., Molendini, C., Dejana, E., et al. (2007). Functionally specialized junctions between endothelial cells of lymphatic vessels, pp. 2349-2362.

Banerji, S., Ni J, Wang S-X, Clasper S, Su J, Tammi R, Jones M, and DG, J. (1999a). LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. . J Cell Biology 144, 789-801.

Banerji, S., Ni, J., Wang, S.X., Clasper, S., Su, J., Tammi, R., Jones, M., and Jackson, D.G. (1999b). LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. The Journal of cell biology 144, 789-801.

Bao, M., Chen, Z., Xu Y, Zhao Y, Zha R, Huang S, Liu L, and Chen T, L.J., Tu H, He X. (2012). Sphingosine kinase 1 promotes tumour cell migration and invasion via the S1P/EDG1 axis in hepatocellular carcinoma. Liver Int 32, 331-338.

Barbieri, M., Ferrucci, L., Ragno, E., Corsi, A., Bandinelli, S., BonafÃ¨, M., Olivieri, F., Giovagnetti, S., Franceschi, C., Guralnik, J.M., et al. (2003). Chronic inflammation and the effect of IGF-I on muscle strength and power in older persons, pp. E481-E487.

Bardeesy, N., Bastian, B.C., Hezel, A., Pinkel, D., DePinho, R.A., and Chin, L. (2001). Dual Inactivation of RB and p53 Pathways in RAS-Induced Melanomas, pp. 2144-2153.

Bhakdi, S., Muhly, M., Korom, S., and Schmidt, G. (1990). Effects of Escherichia coli hemolysin on human monocytes: cytocidal action and stimulation of interleukin 1 release. The Journal of Clinical Investigation 85, 1746-1753.

Bogdan, C., Paik, J., Vodovotz, Y., and Nathan, C. (1992). Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10, pp. 23301-23308.

Bono, P., Wasenius, V.-M., Heikkil, P., Lundin, J., Jackson, D.G., and Joensuu, H. (2004). High LYVE-1 positive lymphatic vessel numbers are associated with poor outcome in breast cancer, pp. 7144-7149.

Breiteneder-Geleff, S., Soleiman, A., Kowalski H, Horvat R, Amann G, Kreihuber E, Diem K, Weninger W, Tschachler E, Alitalo K, et al. (1999). Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries: podoplanin as a specific marker for lymphatic endothelium. AmJ Path 154 154, 385–394.

Brigati, C., Noonan, D.M., Albini, A., and Benelli, R. (2002). Tumors and inflammatory infiltrates: friends or foes? , pp. 247 - 258.

Burger, R., Grosen, E., Ioli, G., van Eden, M., Brightbill, H., Gatanaga, M., DiSaia, P., Granger, G., and Gatanaga, T. (1994). Host-tumor interaction in ovarian cancer. Spontaneous release of tumor necrosis factor and interleukin-l inhibitors by purified cell populations from human ovarian carcinoma in vitro. . Gynecol Oncol 55.

Camp, R.L., Dolled-Filhart, M., and Rimm, D.L. (2004). X-tile: a new bio-informatics tool for biomarker assessment and outcome-based cut-point optimization. . Clin Cancer Res 10.

Carreira, C.M., Nasser, S.M., di Tomaso, E., Padera, T.P., Boucher, Y., Tomarev, S.I., and Jain, R.K. (2001). LYVE-1 Is not restricted to the lymph vessels, pp. 8079-8084.

Carrire, V., Colisson, R., Jiguet-Jiglaire, C., Bellard, E., Bouche, G., Al Saati, T., Amalric, F., Girard, J.-P., and M'Rini, C. (2005). Cancer cells regulate lymphocyte recruitment and leukocyte-endothelium interactions in the tumor-draining lymph node, pp. 11639-11648.

Cera, M.R., Del Prete, A., Vecchi, A., Corada, M., Martin-Padura, I., Motoike, T., Tonetti, P., Bazzoni, G., Vermi, W., Gentili, F., et al. (2004). Increased DC trafficking to lymph nodes and contact hypersensitivity in junctional adhesion molecule-deficient mice. The Journal of Clinical Investigation 114, 729-738.

Chao, C., Martin, R.C., and Ross, M.I. (2004). Correlation between prognostic factors and increasing age in melanoma. . Ann Surg Oncol 11, 259-264.

Chen, G., and Goeddel, D.V. (2002). TNF-R1 signaling: A beautiful pathway. 296, 1634-1635.

Chen, L.L., Blumm, N., Christakis, N.A., Barabasi, A.L., and Deisboeck, T.S. (2009). Cancer metastasis networks and the prediction of progression patterns. Br J Cancer 101, 749-758.

Chen, Q., Zhang, X., and Massaque, J. (2011). Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. Cancer Cell 20, 538-549.

Chirivi, R.G.S., Garofalo, A., Padura, S., Mantovani, A., and Giavazzi, R. (1993). Interleukin 1 receptor antagonist inhibits the augmentation of

metastasis induced by interleukin 1 or lipopolysaccharide in a human melanoma/nude mouse system. Can Res 53, 5051-5054.

Choo, M., Sakurai, H., Koizumi K, and I., S. (2006). TAK1-mediated stress signaling pathways are essential for TNF-alpha-promoted pulmonary metastasis of murine colon cancer cells. . Int J Cancer 118, 2758-2764.

Claudia, T.M., Daniel, P.Z., Philip Kollmannsberger, Carina Raupach, Ursula Schlötzer-Schrehardt, Tamme Weyert Goecke, Jürgen Behrens, and Fabry, a.B. (2008). Breakdown of the endothelial barrier function in tumor cell transmigration. Biophys J 97, 2832-2846.

Coffelt, S.B., Hughes, R., and Lewis, C.E. (2009). Tumor-associated macrophages: Effectors of angiogenesis and tumor progression. Biochimica et Biophysica Acta 1796, 11-18.

Cuellar, F.A., Vilalta, A., Rull, R., Vidal-Sicart, S., Palou, J., and Ventura, P.J. (2004). Small cell melanoma and ulceration as predictors of positive sentinel lymph node in malignant melanoma patients. . Melanoma Res 14, 277-282.

Cueni, L.N., and Detmar, M. (2009). Galectin-8 interacts with podoplanin and modulates lymphatic endothelial cell functions. Experimental Cell Research 315, 1715-1723.

Curtin, J.A., Busam, K., Pinkel, D., and Bastian, B.C. (2006). Somatic activation of KIT in distinct subtypes of melanoma, pp. 4340-4346.

Dadras, S.S., Paul, T., Bertoncini, J., Brown, L.F., Muzikansky, A., Jackson, D.G., Ellwanger, U., Garbe, C., Mihm, M.C., and Detmar, M. (2003a). Tumor lymphangiogenesis: a novel prognostic indicator for cutaneous melanoma metastasis and survival. Am J Pathol 162, 1951-1960.

Dadras, S.S., Paul, T., Bertoncini, J., Brown, L.F., Muzikansky, A., Jackson, D.G., Ellwanger, U., Garbe, C., Mihm, M.C., and Detmar, M. (2003b). Tumor lymphangiogenesis: A novel prognostic indicator for cutaneous melanoma metastasis and survival, pp. 1951-1960.

Danussi, C., Coslovi, A., Campa, C., Mucignat, M.T., Spessotto, P., Uggeri, F., Paoletti, S., and Colombatti, A. (2009). A newly generated functional antibody identifies Tn antigen as a novel determinant in the cancer cell lymphatic endothelium interaction, pp. 1056-1067.

de Oca, P.M., Macotela, Y., Nava, G., Lopez-Barrera, F., de la Escalera, G.M., and Clapp, C. (2005). Prolactin stimulates integrin-mediated adhesion of circulating mononuclear cells to endothelial cells. Lab Invest 85, 633-642.

de Waal Malefyt, R., Abrams, J., Bennet, B., Figdor, C., and de Vries, J. (1991). Interleukin 10 inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med 174.

Dejana, E., Bertocchi, F., Bortolami, M.C., Regonesi, A., Tonta, A., Breviario, F., and Giavazzi, R. (1998). Interleukin 1 promotes tumor cell adhesion to cultured human endothelial cells. The Journal of Clinical Investigation 82, 1466-1470.

Dinarello, C.A. (1996). Biologic basis for interleukin-1 in disease, pp. 2095-2147.

Dobi, A., Kelemen, G., Kaizer, L., Weiczner, R., Thurza, L., and Kahain, Z. (2011). Breast cancer under 40 years of age: increasing number and worse prognosis. Pathology & Oncology Research 17, 425-428.

Doeden, K., Ma, Z., Narasimhan, B., Swetter, S.M., Detmar, M., and Dadras, S.S. (2009a). Lymphatic invasion in cutaneous melanoma is associated with sentinel lymph node metastasis. J Cutan Pathol 36, 772-780.

Doeden, K., Ma, Z., Narasimhan, B., Swetter, S.M., Detmar, M., and Dadras, S.S. (2009b). Lymphatic invasion in cutaneous melanoma is associated with sentinel lymph node metastasis. Journal of Cutaneous Pathology 36, 772-780.

Domingo-Domenech, J., Castel, T., Auge JM, Garcia-Albeniz XA, Conill C, Puig S, Vilella R, Matas J, Malvehy J, Gascón P, et al. (2007). Prognostic implications of protein S 100 beta serum levels in the clinical outcome of high-risk melanoma patients. Tumour Biol 28, 264-272.

Duncan, L.M. (2009). The classification of cutaneous melanoma. Hematology/Oncology Clinics of North America 23, 501-513.

Eccles, S.A., and Welch, D.R. (2007). Metastasis: recent discoveries and novel treatment strategies. . Lancet 369, 1742-1757.

Egberts, F., Hitschler, W., Weichenthal, M., and A., H. (2009). Prospective monitoring of adjuvant treatment in high-risk melanoma patients: lactate dehydrogenase and protein S-100B as indicators of relapse. Melanoma Res 19, 31-35.

Elaraj, D.M., Weinreich, D.M., Varghese, S., Puhlmann, M., Hewitt, S.M., Carroll, N.M., Feldman, E.D., Turner, E.M., and Alexander, H.R. (2006). The role of interleukin 1 in growth and metastasis of human cancer xenografts, pp. 1088-1096.

Ellis, I., Coleman, D., and Wells, C.e.a. (2006). Impact of a national external quality assessment scheme for breast pathology in the UK. . J Clin Pathol 59, 138-145.

Ellis, I., Galea, M., Broughton, N., Locker A, Blamey RW, and CW., E. (1992). Pathological prognostic factors in breast cancer. II. histological type. relationship with survival in a large study with long-term follow-up. Histopathology 20, 479-489.

Emmett, M.S., Symonds, K.E., Rigby, H., Cook, M.G., Price, R., Metcalfe, C., Orlando, A., and Bates, D.O. (2010). Prediction of melanoma metastasis by the Shields index based on lymphatic vessel density. BMC Cancer 10, 208.

Essner, R. (2006). Sentinel lymph node biopsy and melanoma biology, pp. 2320s-2325s.

Etienne-Manneville, S. (2008). Polarity proteins in migration and invasion. Oncogene 27, 6970-6980.

Etienne-Manneville, S., and Hall, A. (2001). Integrin-mediated activation of CDC42 controls cell polarity in migrating astrocytes through PKC. Cell 106, 489-498.

Fallowfield, M., and Cook, M. (1989). Vascular invasion in malignant melanoma: An independent prognostic variable? . American J of Surg Pathol 13.

Fargnoli, M.C., Gandini, S., Peris, K., Maisonneuve, P., and Raimondi, S. (2010). MC1R variants increase melanoma risk in families with CDKN2A mutations: A meta-analysis. European journal of cancer 46, 1413-1420.

Fecher, L.A., Cummings, S.D., Keefe, M.J., and Alani, R.M. (2007a). Toward a Molecular Classification of Melanoma, pp. 1606-1620.

Fecher, L.A., Cummings, S.D., Keefe, M.J., and Alani, R.M. (2007b). Towards a molecular classification of melanoma, pp. 1606-1620.

Feldman, M., Stanford, R., Catcheside, A., and Stotter, A. (2002). The use of prognostic table to aid decision making on adjuvant therapy for women with early breast cancer. EJSO 28, 615-619.

Fidler, I.J. (1997). Molecular biology of cancer: Invasion and metastasis. . In Cancer: Principles and Practice of Oncology (Philadelphia, PA. , Lippincott-Raven), pp. 135-152.

Fisher, B., Bauer, M., Wickerham DL, Redmond CK, Fisher ER, Cruz AB, Foster R, Gardner B, Lerner H, and Margolese R (1983). Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. An NSABP update. Cancer 52, 1551-1557.

Flanagan, J.M., Cocciardi, S., Waddell, N., Johnstone, C.N., Marsh, A., Henderson, S., Simpson, P., da Silva, L., Khanna, K., Lakhani, S., et al. (2010). DNA methylome of familial breast cancer identifies distinct profiles defined by mutation status. American journal of human genetics 86, 420-433.

Fohn, L.E., Rodriguez, A., Kelley, M.C., Ye, F., Shyr, Y., Stricklin, G., and Robbins, J.B. (2011). D2-40 lymphatic marker for detecting lymphatic invasion in thin to intermediate thickness melanomas: association with sentinel lymph node status and prognostic value-a retrospective case study. J Am Acad Dermatol 64, 336-345.

Forman, S.B., Ferringer, T.C., Peckham, S.J., Dalton, S.R., Sasaki, G.T., Libow, L.F., and Elston, D.M. (2008). Is superficial spreading melanoma still the most common form of malignant melanoma? Journal of the American Academy of Dermatology *58*, 1013-1020.

Fox, S.B., Leek, R.D., Smith, K., Hollyer, J., Greenall, M., and Harris, A.L. (1994). Tumor angiogenesis in node-negative breast carcinomas--relationship with epidermal growth factor receptor, estrogen receptor, and survival. Breast Cancer Res Treat *29*, 109-116.

Fox, S.B., Leek, R.D., Weekes, M.P., Whitehouse, R.M., Gatter, K.C., and Harris, A.L. (1995). Quantitation and prognostic value of breast cancer angiogenesis: comparison of microvessel density, Chalkley count, and computer image analysis. The Journal of pathology *177*, 275-283.

Franco-Barraza, J., Valdivia-Silva, J.E., Zamudio-Meza, H., Castillo, A., Garcaa-Zepeda, E.A., Benatez-Bribiesca, L., and Meza, I. (2010). Actin cytoskeleton participation in the onset of IL-1 induction of an invasive mesenchymal-like phenotype in epithelial MCF-7 Cells. Archives of medical research *41*, 170-181.

Friedl, P., and Wolf, K. (2003). Tumour-cell invasion and migration: diversity and escape mechanisms. Nat Rev Cancer *3*, 362-374.

Gahl, J., Hohenberger, W., and Merkel, S. (2009). Malignes Melanom. Der Chirurg *80*, 559-568.

Galea, M., Blamey, R., Elston, C., and Ellis, I. (1992). The Nottingham prognostic index in primary breast cancer. Breast Cancer Research and Treatment *22*, 207-219.

Gao, P., Zhou, G.Y., Zhang, Q.H., Xiang, L., Zhang, S.L., Li, C., and Sun, Y.L. (2008). Clinicopathological significance of peritumoral lymphatic vessel density in gastric carcinoma, pp. 223 - 230.

Garicochea, B., Morelle, A., Andrighetti, A.E., Cancella, A., Bós, Â., and Werutsky, G. (2009). Age as a prognostic factor in early breast cancer. Rev Saúde Pública *43*, 1-6.

Genestie, C., Zafrani, B., Asselain, B., Fourquet, A., Rozan, S., Validire, P., Vincent-Salomon, A., and Sastre-Garau, X. (1998). Comparison of the prognostic value of Scarff-Bloom-Richardson and Nottingham histological grades in a series of 825 cases of breast cancer: major importance of the mitotic count as a component of both grading systems. Anticancer Res *18*, 571-576.

Gershenwald, J.E., and Fidler, I.J. (2002). Targeting lymphatic metastasis, pp. 1811-1812.

Ghislin, S., Obino, D., Middendorp, S., Boggetto, N., Alcaide-Loridan, C., and Deshayes, F. (2011). Junctional adhesion molecules are required for

melanoma cell lines transendothelial migration in vitro. Pigment Cell & Melanoma Research *24*, 504-511.

Gimotty, P.A., Van Belle, P., Elder, D.E., Murry, T., Montone, K.T., Xu, X., Hotz, S., Raines, S., Ming, M.E., Wahl, P.*, et al.* (2005). Biologic and prognostic significance of dermal Ki67 expression, mitoses, and tumorigenicity in thin invasive cutaneous melanoma, pp. 8048-8056.

Giorgadze, T.A., Zhang, P.J., Pasha, T., Coogan, P.S., Acs, G., Elder, D.E., and Xu, X. (2004). Lymphatic vessel density is significantly increased in melanoma. Journal of Cutaneous Pathology *31*, 672-677.

Goerdt, S., Walsh, L.J., Murphy, G.F., and Pober, J.S. (1991). Identification of a novel high molecular weight protein preferentially expressed by sinusoidal endothelial cells in normal human tissues, pp. 1425-1437.

Gonzalez-Vela, M., Garijo MF, Fernandez F, and JF., V.-B. (2001). MIB1 proliferation index in breast infiltrating carcinoma: comparison with other proliferative markers and association with new biological prognostic factors. Histol Histopathol *16*, 399-406.

Gout, S., Morin, C., Houle, F., and Huot, J. (2006). Death receptor-3, a new E-selectin counter-receptor that confers migration and survival advantages to colon carcinoma cells by triggering p38 and ERK MAPK activation, pp. 9117-9124.

Granada, J.F., Ensenat, D., Keswani, A.N., Kaluza, G.L., Raizner, A.E., Liu, X.-m., Peyton, K.J., Azam, M.A., Wang, H., and Durante, W. (2005). Single perivascular delivery of mitomycin C stimulates p21 expression and inhibits neointima formation in rat arteries, pp. 2343-2348.

Gunn, M.D., Tangemann, K., Tam, C., Cyster, J.G., Rosen, S.D., and & Williams, L.T. (1998). A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naive T lymphocytes. Proc Natl Acad Sci USA *95*, 258-263.

Hacham, M., White, R., Argov, S., Segal, S., and Apte, R. (2004). Interleukin-6 and interleukin-10 are expressed in organs of normal young and old mice. European Cytokine Network *15*, 37-46.

Hagemann, T., Wilson, J., Kulbe, H., Li, N.F., Leinster, D.A., Charles, K., Klemm, F., Pukrop, T., Binder, C., and Balkwill, F.R. (2005). Macrophages induce invasiveness of epithelial cancer cells via NF-kappa B and JNK, pp. 1197 - 1205.

Hagi-Pavli, E., Farthing, P.M., and Kapas, S. (2004). Stimulation of adhesion molecule expression in human endothelial cells (HUVEC) by adrenomedullin and corticotrophin, pp. C239-C246.

Hahne, M., Jaeger, U., Isenmann, S., Hallmann, R., and Vestweber, D. (1993). Five tumor necrosis factor-inducible cell adhesion mechanisms on the

surface of mouse endothelioma cells mediate the binding of leukocytes, pp. 655-664.

Hanahan, D., and Weinberg, R.A. (2000). The Hallmarks of Cancer. Cell *100*, 57-70.

Hanauske, A., Degen D, Marshall MH, H.S., and Banks P, S.J., Leahy M, Von Hoff DD: (1992). Effects of recombinant interleukin-la on clonogenic growth of primary human tumors in vitro. . J Immunother *11*.

Harpio, R., and Einarsson, R. (2004). S100 proteins as cancer biomarkers with focus on S100B in malignant melanoma. Clinical Biochemistry *37*, 512-518.

Haybittle, J., Blamey, R., and Elston, C.e.a. (1982). A prognostic index in primary breast cancer. . Br J Cancer *45*, 361-366.

Hayes, H.F., and Seigel, G.M. (2009). Immunoreactivity of ICAM-1 in human tumors, metastases and normal tissues. Int J Clin Exp Pathol *2*, 553-560.

Hewett, P.W., and Murray, C. (1996). Modulation of human endothelial cell procoagulant activity in tumour models in vitro. Int J Cancer *66*, 784-789.

Hidalgo, K., Rojas, I., AB, P., and MI., R. (2005). TNF-α increases in vitro migration of human HPV18-positive SW756 cervical carcinoma cells. Biocell *29*, 303-311.

Holness, C., and Simmons, D. (1993). Molecular cloning of CD68, a human macrophage marker related to lysosomal glycoproteins. Blood *81*, 1607-1613.

Hong, Y., Shin, J., and Detmar, M. (2004a). Development of the lymphatic vascular system: a mystery unravels. . Dev Dyn *231*, 462-473.

Hong, Y.K., Lange-Asschenfeldt, B., Velasco, P., Hirakawa, S., Kunstfeld, R., Brown, L.F., Bohlen, P., Senger, D.R., and Detmar, M. (2004b). VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. Faseb J *18*, 1111-1113.

Hussein, M.R. (2006). Tumour-associated macrophages and melanoma tumourigenesis: integrating the complexity. International Journal of Experimental Pathology *87*, 163-176.

Incardona, F., Lewalle, J., Morandi, V., Lambert, S., Legrand, Y., Foidart, J., and C., L. (1995). Thrombospondin modulates human breast adenocarcinoma cell adhesion to human vascular endothelial cells. . Cancer Res *55*, 166-173.

Irigoyen, M., Anso, E., Martinez, E., Garayoa, M., Martinez-Irujo, J.J., and Rouzaut, A. (2007). Hypoxia alters the adhesive properties of lymphatic

endothelial cells. A transcriptional and functional study. Biochim Biophys Acta *1773*, 880-890.

Irjala, H., Alanen, K., Grenman, R., Heikkila, P., Joensuu, H., and Jalkanen, S. (2003). Mannose receptor (MR) and common lymphatic endothelial and vascular endothelial receptor (CLEVER)-1 direct the binding of cancer cells to the lymph vessel endothelium. Cancer Res *63*, 4671-4676.

Iwaki, K., Ohashi, K., Ikeda, M., Tsujioka, K., Kajiya, F., and Kurimoto, M. (1997). Decrease in the amount of focal adhesion kinase (p125 FAK) in interleukin-1-stimulated human umbilical vein endothelial cells by binding of human monocytic cell lines, pp. 20665-20670.

Jackson, D.G. (2004). Biology of the lymphatic marker LYVE-1 and applications in research into lymphatic trafficking and lymphangiogenesis. APMIS *112*, 526-538.

Jaffe, E., Nachman, R., CG, B., and CR, M. (1973). Culture of human endothelial cells derived from umbilical veins: identification by morphologic and immunologic criteria. . J of Clinic Invest *52*, 2745-2756.

Jenkins, J., Malyak, M., and Arend, W. (1994). The effects of interleukin-10 on interleukin-1 receptor antagonist and interleukin-1 beta production in human monocytes and neutrophils. Lymphokine Cytokine Res *13*, 47-54.

Jensen, T.O., Schmidt, H., Mueller, H.J., Maniecki, M.B., Sjoegren, P., Christensen, I.J., and Steiniche, T. (2009). Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer Stage I/II Melanoma, pp. 3330-3337.

Jeon, B.-H., Jang, C., Han, J., Kataru, R.P., Piao, L., Jung, K., Cha, H.J., Schwendener, R.A., Jang, K.Y., Kim, K.-S.*, et al.* (2008). Profound but dysfunctional lymphangiogenesis via vascular endothelial growth factor ligands from CD11b+ macrophages in advanced ovarian cancer, pp. 1100-1109.

Ji, R.C. (2006). Lymphatic endothelial cells, tumor lymphangiogenesis and metastasis: New insights into intratumoral and peritumoral lymphatics. Cancer Metastasis Rev (2006) 25:677–694 *25*, 17.

Ji, R.C., Eshita, Y., and Kato, S. (2007). Investigation of intratumoural and peritumoural lymphatics expressed by podoplanin and LYVE-1 in the hybridoma-induced tumours. International Journal of Experimental Pathology *88*, 257-270.

Johnson, L.A., Clasper, S., Holt, A.P., Lalor, P.F., Baban, D., and Jackson, D.G. (2006). An inflammation-induced mechanism for leukocyte transmigration across lymphatic vessel endothelium, pp. 2763-2777.

Johnson, L.A., and Jackson, D.G. (2008). Cell traffic and the lymphatic endothelium. Annals of the New York Academy of Sciences *1131*, 119-133.

Joimel, U., Gest, C., Soria, J., Pritchard, L.-L., Alexandre, J., Laurent, M., Blot, E., Cazin, L., Vannier, J.-P., Varin, R.*, et al.* (2010). Stimulation of angiogenesis resulting from cooperation between macrophages and MDA-MB-231 breast cancer cells: proposed molecular mechanism and effect of tetrathiomolybdate, pp. 375.

Joukov, V., Pajusola, K., Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, and K, A. (1996). A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt-4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. . EMBO J *15*, 290-298.

Jurisic, G., Lolyeva, M., Proulx, S.T., Halin, C., and Detmar, M. (2010). Thymus cell antigen 1 (Thy1, CD90) is expressed by lymphatic vessels and mediates cell adhesion to lymphatic endothelium. Experimental Cell Research *316*, 2982-2992.

Kahn, H.J., and Alexander, M. (2002). A new monoclonal antibody, D2-40, for detection of lymphatic invasion in primary tumors. Laboratory Investigation *82*.

Kaiserling, E. (2004). Immunohistochemical identification of lymph vessels with D2-40 in diagnostic pathology. . Pathologe *25*, 362-374.

Karpanen, T., Wirzenius, M., Makinen, T., Veikkola, T., Haisma, H.J., Achen, M.G., Stacker, S.A., Pytowski, B., YlÃ¤-Herttuala, S., and Alitalo, K. (2006). Lymphangiogenic growth factor responsiveness is modulated by postnatal lymphatic vessel maturation. The American journal of pathology *169*, 708-718.

Kashani-Sabet, M., Sagebiel, R.W., Ferreira, C.M.M., Nosrati, M., and Miller Iii, J.R. (2001). Vascular involvement in the prognosis of primary cutaneous melanoma, pp. 1169-1173.

Katerinaki, E., Evans, G., PC, L., and S., a.M. (2003). TNF-α increases human melanoma cell invasion and migration in vitro:the role of proteolytic enzymes. . British J of Cancer *89*, 1123-1129.

Kawai, Y., Kaidoh, M., Yokoyama Y, K, S., and T., a.O. (2009). Chemokine CCL2 facilitates ICAM-1-mediated interactions of cancer cells and lymphatic endothelial cells in sentinel lymph nodes. . Cancer Sci *100*, 419-428.

Kerjaschki, D. (2005). The crucial role of macrophages in lymphangiogenesis. . J Clin Invest *115*, 2316-2319.

Kesmodel, S.B., Karakousis, G.C., Botbyl, J.D., Canter, R.J., Lewis, R.T., Wahl, P.M., Terhune, K.P., and Spitz, F.R. (2004). Mitotic rate as a predictor of

sentinel lymph node positivity in patients with thin melanomas. . Annals of Surgical Oncol *12*, 1-10.

Kim, H., Kim, J., Han EH, Hwang YP, Choi JH, BH, P., and HG., a.J. (2010). Metallothionein-2A overexpression increases the expression of matrix metalloproteinase-9 and invasion of breast cancer cells. . FEBS Letters *585*, 421-428.

Kim, R.H., Takabe, K., Milstien, S., and Spiegel, S. (2009). Export and functions of spingosine-1 phosphate. Biochimica et Biophysica Acta *1791*, 15.

Kim, S.J., Kim, N.S., and Lee, J.L. (1993). Effect of cytokines on the expression of cell adhesion molecule and on the adhesion of melanoma cells to endothelial cells. J Korean Med Sci *8*, 41-52.

Kirkwood, J., Strawderman, M., Ernstoff, M., Smith, T., Borden, E., and Blum, R. (1996). Interferon alfa-2b adjuvant therapy of high-risk resected cutaneous melanoma: the Eastern Cooperative Oncology Group Trial EST 1684. . J Clin Oncol *14*, 7-17.

Koscielny, S., Arriagada, R., Adolfsson, J., Fornander, T., and Bergh, J. (2009). Impact of tumour size on axillary involvement and distant dissemination in breast cancer. Br J Cancer *101*, 902-907.

Koskivuo, I., Talve, L., Vihinen, P., MÃ¤ki, M., Vahlberg, T., and Suominen, E. (2007). Sentinel Lymph Node Biopsy in Cutaneous Melanoma: A Case-Control Study. Annals of Surgical Oncology *14*, 3566-3574.

Kuchelmeister, C., Schaumburg-Lever, G., and Garbe, C. (2000). Acral cutaneous melanoma in caucasians: clinical features, histopathology and prognosis in 112 patients. British Journal of Dermatology *143*, 275-280.

Kuroda, H., Nakai, M., Ohnisi K, Ishida T, S, K., and S, a.I. (2010). Vascular invasion in triple-negative carcinoma of the breast identified by endothelial lymphatic and blood vessel marker. . Int J Surg Pathol *18*, 324-329.

Kuwashima, N. (1997). Organ-specific adhesion of neuroblastoma cells in vitro: correlation with their hepatic metastasis potential. . J of Pediatric Surgery *32*, 546-551.

Kzhyshkowska, J., Gratchev, A., and Goerdt, S. (2006). Stabilin-1, A novel scavenger receptor with multiple functions. J Cell Mol Med *10*, 635-649.

Kzhyshkowska, J., Gratchev, A., Schmuttermaier, C., Brundiers, H., Krusell, L., Mamidi, S., Zhang, J., Workman, G., Sage, E.H., Anderle, C*., et al.* (2008). Alternatively activated macrophages regulate extracellular levels of the hormone placental lactogen via receptor-mediated uptake and transcytosis, pp. 3028-3037.

Laakkonen, P., Waltari, M., Holopainen, T., Takahashi, T., Pytowski, B., Steiner, P., Hicklin, D., Persaud, K., Tonra, J.R., Witte, L*., et al.* (2007). Vascular

endothelial growth factor receptor 3 is involved in tumor angiogenesis and growth. . cancer Res *67*, 593-599.

Laakkonen, P., Zhang, L., and Ruoslahti, E. (2008). Peptide targeting of tumor lymph vessels. Annals of the New York Academy of Sciences *1131*, 37-43.

Lama, P.J., and Fechtner, R.D. (2003). Antifibrotics and wound healing in glaucoma surgery. Survey of ophthalmology *48*, 314-346.

Langer, H., Orlova, V., Xie, C., Kaul, S., Schneider D, Lonsdorf AS, Fahrleitner M, Choi EY, Dutoit V, Pellegrini M*, et al.* (2011). A novel function of junctional adhesion molecule-C in mediating melanoma cell metastasis. Cancer Res *71*, 4096-4105.

Langerod, A., Zhao H, and O, B. (2007). TP53 mutation status and gene expression profiles are powerful prognostic markers of breast cancer. . Breast Cancer Res *9*, R30.

Lazovich, D., Vogel, R.I., Berwick, M., Weinstock, M.A., Anderson, K.E., and Warshaw, E.M. (2010). Indoor tanning and risk of melanoma: A case-control study in a highly exposed population, pp. 1557-1568.

Leber, M., and Efferth, T. (2009). Molecular principles of cancer invasion and metastasis (Review). . International J of Oncol *34*, 881-895.

Lens , M., Dawes, M., Goodacre, T., and Bishop, J. (2002). Excision margins in the treatment of primary cutaneous melanoma: a systematic review of randomized controlled trials comparing narrow vs wide excision. . Arch Surg *137*, 1101-1105.

Lewis, A., Varghese, S., Xu, H., and Alexander, H.R. (2006). Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment, pp. 48.

Lewis, A.M., Varghese, S., Xu, H., Alexander, H.R. (2006). Interleukin-1 and cancer progression: the emerging role of interleukin-1 receptor antagonist as a novel therapeutic agent in cancer treatment. J of Trans Med *4*.

Li, C.I., Uribe, D.J., and Daling, J.R. (2005). Clinical characteristics of different histologic types of breast cancer. Br J Cancer *93*, 1046-1052.

Li, G., Satyamoorthy, K., and Herlyn, M. (2002). Dynamics of cell interactions and communications during melanoma development, pp. 62-70.

Li, Y.-H., and Zhu, C. (1999). A modified Boyden chamber assay for tumor cell transendothelial migration in vitro. Clinical and Experimental Metastasis *17*, 423-429.

Liang, M., Zhang, P., and J., F. (2007). Up-regulation of LOX-1 expression by TNF-alpha promotes trans-endothelial migration of MDA-MB-231 breast cancer cells. . Cancer Letters *258*, 31-37.

Liang, X.S., Pfeiffer, R.M., Wheeler, W., Maeder, D., Burdette, L., Yeager, M., Chanock, S., Tucker, M.A., Goldstein, A.M., and Yang, X.R. (2011). Genetic variants in DNA repair genes and the risk of cutaneous malignant melanoma in melanoma-prone families with/without CDKN2A mutations. International Journal of Cancer, n/a-n/a.

Lin, W.-W., and Karin, M. (2007). A cytokine-mediated link between innate immunity, inflammation, and cancer. J of Clinic Invest *117*, 1175-1183.

Lin, Y.-C., Shun, C.-T., Wu M-S, and C-C., C. (2006). A novel anticancer effect of Thalidomide: inhibition of intercellular adhesion molecule-1 mediated cell invasion and metastasis through suppression of nuclear factor-kB. . Clin Cancer Res *12*, 7165–7173.

Linehan, S. (2005). The mannose receptor is expressed by subsets of APC in non-lymphoid organs, pp. 4.

Livingston, D.J. (1997). In vitro and in vivo studies of ICE inhibitors. Journal of Cellular Biochemistry *64*, 19-26.

Longatto-Filho, A., Pinheiro, C., Pereira, S.M., Etlinger, D., Moreira, M.A., Jube, L.F., Queiroz, G.S., Baltazar, F., and Schmitt, F.C. (2007). Lymphatic vessel density and epithelial D2-40 immunoreactivity in pre-invasive and invasive lesions of the uterine cervix, pp. 45 - 51.

Lose, F., Lovelock, P., Chenevix-Trench, G., Mann, G., Pupo, G., and Spurdle, A. (2006). Variation in the RAD51 gene and familial breast cancer, pp. R26.

Louise, A., Clasper, J.S., Holt, A.P., F., P., B, L., and J, a.D.G. (2006). An inflammation-induced mechanism for leukocyte transmigration across lymphatic vessel endothelium. JEM *203* 2763-2777.

Mackie, R.M., Hauschild, A., and and M, E.A.M. (2009). Epidemiology of invasive cutaneous melanoma. Annals of Oncology *20*, vi1-vi7.

Maddaluno, L., Verbrugge, S.E., Martinoli, C., Matteoli, G., Chiavelli, A., Zeng, Y., Williams, E.D., Rescigno, M., and Cavallaro, U. (2009). The adhesion molecule L1 regulates transendothelial migration and trafficking of dendritic cells, pp. 623-635.

Maisonpierre, P.C., Suri, C., Jones, P.F., Bartunkova, S., Wiegand, S.J., Radziejewski, C., Compton, D., McClain, J., Aldrich, T.H., Papadopoulos, N.*, et al.* (1997). Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. . Science *277*, 55-60.

Mandriota, S.J., Jussila, L., Jeltsch, M., Compagni, A., Baetens, D., Prevo, R., Banerji, S., Huarte, J., Montesano, R., Jackson, D.G.*, et al.* (2001).

Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. EMBO J *20*, 672-682.

Mantovani, A., Allavena, P., Sica, A., and Balkwill, F. (2008). Cancer-related inflammation. Nature *454*, 436-444.

Marghoob, A.A., Koenig, K., Bittencourt, F.V., Kopf, A.W., and Bart, R.S. (2000). Breslow thickness and Clark level in melanoma. Cancer *88*, 589-595.

Marinho, V., Metze K, Fernanda SF, and Gobbi, H. (2008). Lymph vascular invasion in invasive mammary carcinomas identified by the endothelial lymphatic marker D2-40 is associated with other indicators of poor prognosis. BMC Cancer *8*.

Marks, A., Sutherland, D., and Bailey, D.e.a. (1999). Characterization and distribution of an oncofetal antigen (M2A antigen) expressed on testicular germ cell tumours. . Br J Cancer *80*, 569-578.

Marttila-Ichihara, F., Turja, R., Miiluniemi, M., Karikoski, M., Maksimow, M., NiemelÃ¤, J., Martinez-Pomares, L., Salmi, M., and Jalkanen, S. (2008). Macrophage mannose receptor on lymphatics controls cell trafficking, pp. 64-72.

Massi, D., Puig, S., Franchi, A., Malvehy, J., Vidal-Sicart, S., Gonzalez-Cao, M., Baroni, G., Ketabchi, S., Palou, J., and Santucci, M. (2006). Tumour lymphangiogenesis is a possible predictor of sentinel lymph node status in cutaneous melanoma: a case-control study. J Clin Pathol *59*, 166-173.

Matloubian, M., Lo, C.G., Cinamon, G., Lesneski, M.J., Xu, Y., Brinkmann, V., Allende, M.L., Proia, R.L., and Cyster, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature *427*, 355-360.

Matsuo, M., Koizumi, K., Yamada S, Tomi M, Takahashi R, Ueda M, Terasaki T, Obinata M, Hosoya K, O, O.*, et al.* (2006). Establishment and characterization of conditionally immortalized endothelial cell lines from the thoracic duct and inferior vena cava of tsA58/EGFP double-transgenic rats. . Cell Tissue Res *326*, 749–758.

McCready, D., Chapman, J., Hanna, W., Kahn, H., Yap, K., Fish, E., and Lickley, H. (2000). Factors associated with local breast cancer recurrence after lumpectomy alone: postmenopausal patients. Annals of Surgical Oncology *7*, 562-567.

McKenna, J.K., Florell, S.R., Goldman, G.D., and Bowen, G.M. (2006). Lentigo Maligna/Lentigo Maligna Melanoma: Current State of Diagnosis and Treatment. Dermatologic Surgery *32*, 493-504.

McSherry, E., Brennan, K., Hudson, L., Hill, A., and AM., H. (2011). Breast cancer cell migration is regulated through junctional adhesion molecule-A-mediated activation of Rap1 GTPase. Breast Cancer Res *13*, R31.

Miller, L., Kurtzman, S., Anderson, K., and Wang, Y. (2000). Interleukin 1 family expression in human breast cancer: Interleukin 1 receptor antagonists. Cancer Investigation *18*, 293-302.

Mladkova, J., Sanda, M., Matouskova, E., and Selicharova, I. (2010). Phenotyping breast cancer cell lines EM-G3, HCC1937, MCF7 and MDA-MB-231 using 2-D electrophoresis and affinity chromatography for glutathione-binding proteins, pp. 449.

Mochizuki, H., Matsubara, A., Teishima, J., Mutaguchi, K., Yasumoto, H., Dahiya, R., Usui, T., and and Kamiya, K. (2004). Interaction of ligand-receptor system between stromal-cell-derived factor-1 and CXC chemokine receptor 4 in human prostate cancer: a possible predictor of metastasis. . Biochemical and Biophysical Res Comm *320*, 656-663.

Mohammed, R., Ellis, I., Somaia Elsheikh, Emma C. Paish, and Martin, S.G. (2008). Lymphatic and angiogenic characteristics in breast cancer :morphometric analysis and prognostic implications. Breast Cancer Res Treat.

Mohammed, R., Martin, S., Muhammad S Gill, Andrew R Green, Paish, E.C., and Ellis, a.I.O. (2007). Improved methods of detection of lymphovascular invasion demonstrate that it is the predominant method of vascular invasion in breast cancer and has important clinical consequences. Am J Surg Pathol *31*, 9.

Mohammed, R.A.A., Martin, S.G., Mahmmod, A.M., Macmillan, R.D., Green, A.R., Paish, E.C., and Ellis, I.O. (2011). Objective assessment of lymphatic and blood vascular invasion in lymph node-negative breast carcinoma: findings from a large case series with long-term follow-up. The Journal of Pathology *223*, 358-365.

Moore, R.J., Owens, D.M., Stamp, G., Arnott, C., Burke, F., East, N., Holdsworth, H., Turner, L., Rollins, B., Pasparakis, M.*, et al.* (1999). Mice deficient in tumor necrosis factor-[alpha] are resistant to skin carcinogenesis. Nat Med *5*, 828-831.

Morales, S.-V., MM, , Llopis, G.A., Lacasana Navarro M, Agudo Ferrándiz J, and L., S.C. (1992). An epidemiological study of the distinct histological types of cutaneous melanoma in relation to other variables of the disease. Rev Clin Esp *190*, 169-176.

Morandini, R., Boeynaems, J., Hedley SJ, MacNeil S, and G, G. (1998). Modulation of ICAM-1 expression by alpha-MSH in human melanoma cells and melanocytes. . J Cell Physiol *175: 276–282*, 276-282.

Morton, D., Thompson, J., and Essner, R., et al. (1999). Validation of the accuracy of intraoperative lymphatic mapping and sentinel lymphadenectomy for early stage melanoma: a multicenter trial. Multicenter Selective Lymphadenectomy Trial Group. . Ann Surg 1999 *230*, 453-463.

Muller, A.M., Hermanns, M.I., Skrzynski, C., Nesslinger, M., Muller, K.-M., and Kirkpatrick, C.J. (2002). Expression of the Endothelial Markers PECAM-1, vWf, and CD34 in Vivo and in Vitro. Experimental and Molecular Pathology *72*, 221-229.

Muller, W., and Luscinskas, F. (2008). Assays of transendothelial migration in vitro. Methods Enzymol *443*, 155-176.

Murakami, S., Morioka, T., Nakagawa Y, Suzuki Y, M, A., and T., a.O. (2001). Expression of adhesion molecule by cultures human glomerular endothelial cells in response to cytokines: comparison to human umbilical vein and dermal microvascular endothelial cells. . Microvascular Res *62*.

Murali, R., Moncrieff, M., Hong, J., Cooper, C., Shingde, M., Samuel, D., Thompson, J., and Scolyer, R. (2010). The prognostic value of tumor mitotic rate and other clinicopathologic factors in patients with locoregional recurrences of melanoma. Annals of Surgical Oncology *17*, 2992-2999.

Nathanson, S.D. (2003). Insights into the mechanisms of lymph nodes metastasis. . Cancer *98*, 413-423.

Niakosari, F., Kahn, H.J., Marks, A., and From, L. (2005a). Detection of lymphatic invasion in primary melanoma with monoclonal antibody D2-40: a new selective immunohistochemical marker of lymphatic endothelium. Arch Dermatol *141*, 440-444.

Niakosari, F., Kahn, H.J., Marks, A., and From, L. (2005b). Detection of lymphatic invasion in primary melanoma with monoclonal antibody D2-40: A new selective immunohistochemical marker of lymphatic endothelium, pp. 440-444.

Niakosari, F., Kahn, H.J., McCready, D., Ghazarian, D., Rotstein, L.E., Marks, A., Kiss, A., and From, L. (2008). Lymphatic invasion identified by monoclonal antibody D2-40, younger age, and ulceration: predictors of sentinel lymph node involvement in primary cutaneous melanoma. Arch Dermatol *144*, 462-467.

Nickoloff, B. (1991). The human progenitor cell antigen (CD34) is localized on endothelial cells, dermal dendritic cells, and perifollicular cells in formalin-fixed normal skin, and on proliferating endothelial cells and stromal spindle-shaped cells in Kaposi's sarcoma. . Arch Dermatol *127*, 523-529.

Nisato, R.E., Harrison, J.A., Buser, R., Orci, L., Rinsch, C., Montesano, R., Dupraz, P., and Pepper, M.S. (2004). Generation and characterization of telomerase-transfected human lymphatic endothelial cells with an extended life span. . Am J Pathol *165*, 11-24.

Nizamutdinova, I., Lee, G., Lee JS, Cho MK, Son KH, Jeon SJ, Kang SS, Kim YS, Lee JS, Seo HG*, et al.* (2008). Tanshinone I suppresses growth and invasion of human breast cancer cells, MDA-MB-231, through regulation of adhesion molecules. . Carcinogenesis *29*, 1885-1892.

Nobes, C.D., and Hall, A. (1999). Rho GTPases control polarity, protrusion, and adhesion during cell movement, pp. 1235-1244.

Norval, M., Lucas, R.M., Cullen, A.P., de Gruijl, F.R., Longstreth, J., Takizawa, Y., and van der Leun, J.C. (2011). The human health effects of ozone depletion and interactions with climate change. Photochemical & Photobiological Sciences *10*, 199-225.

Nowecki, Z., Rutkowski, P., and Michej, W. (2008). The survival benefit to patients with positive Sentinel Node Melanoma after completion lymph node dissection may be limited to the subgroup with a primary lesion Breslow thickness greater than 1.0 and less than or equal to 4 mm Annals of Surgical Oncology *15*, 2223-2234.

Ohsie, S.J., Sarantopoulos, G.P., Cochran, A.J., and Binder, S.W. (2008). Immunohistochemical characteristics of melanoma. Journal of Cutaneous Pathology *35*, 433-444.

Okada, T., Okuno, H., and Mitsui, Y. (1994). A novel in vitro assay system for transendothelial tumor cell invasion: significance of E-selectin and alpha 3 integrin in the transendothelial invasion by HT1080 fibrosarcoma cells. Clin exp mets *12*, 305-314.

Oliver, G. (2004). Lymphatic vasculature development. Nat Rev Immunol *4*, 35-45.

Oliver, G., and Detmar, M. (2002). The rediscovery of the lymphatic system: old and new insights into the development and biological function of the lymphatic vasculature, pp. 773-783.

Olle, B., Mikael, D., and Sjogren, a.H.-O. (2000). Vaccination with B16 melanoma cells expressing a secreted form of interleukin-1 induces tumor growth inhibition and an enhanced immunity against the wild-type B16 tumor Cancer gene therapy *7*, 1365-1374.

Omachi, T., Kawai, Y., Mizuno, R., Nomiyama, T., Miyagawa, S., Ohhashi, T., and Nakayama, J. (2007). Immunohistochemical demonstration of proliferating lymphatic vessels in colorectal carcinoma and its clinicopathological significance. Cancer letters *246*, 167-172.

Padera, T.P., Kadambi, A., di Tomaso, E., Carreira, C.M., Brown, E.B., Boucher, Y., Choi, N.C., Mathisen, D., Wain, J., Mark, E.J.*, et al.* (2002). Lymphatic Metastasis in the Absence of Functional Intratumor Lymphatics, pp. 1883-1886.

Paget, S. (1889). The distribution of secondary growths in cancer of the breasts. . The Lancet *133*, 571-573.

Parveen, S., and Shahid, M. (1997). Prognostic factors in stage-I breast cancer: a retrospective study. J Pak Med Assoc *47*, 117-118.

Paschos, K.A., Canovas, D., and Bird, N.C. (2009). The role of cell adhesion molecule in the progression of colorectal cancer and the development of liver metastasis. . Cellular signalling *21*, 665-674.

Pepper, M.S. (2001). Lymphangiogenesis and Tumor Metastasis: Myth or reality, pp. 462-468.

Pepper, M.S., and Skobe, M. (2003). Lymphatic endothelium, pp. 209-213.

Petersson, F., Diwan, A.H., Ivan, D., Gershenwald, J.E., Johnson, M.M., Harrell, R., and Prieto, V.G. (2009). Immunohistochemical detection of lymphovascular invasion with D2-40 in melanoma correlates with sentinel lymph node status, metastasis and survival. J Cutan Pathol *36*, 1157-1163.

Petitt, M., Allison, A., Shimoni, T., Uchida, T., Raimer, S., and Kelly, B. (2009). Lymphatic invasion detected by D2-40/S-100 dual immunohistochemistry does not predict sentinel lymph node status in melanoma. J Am Acad Dermatol *61*, 819-828.

Pfeifer, G.P., and Besaratinia, A. (2011). UV wavelength-dependent DNA damage and human non-melanoma and melanoma skin cancer. Photochemical & Photobiological Sciences.

Phan, A., Touzet, S., Dalle, S., Ronger-Savlé, S., Balme, B., and Thomas, L. (2007). Acral lentiginous melanoma: histopathological prognostic features of 121 cases. British Journal of Dermatology *157*, 311-318.

Phan, G.Q., Messina, J.L., Sondak, V.K., and Zager, J.S. (2009). Sentinel lymph node biopsy for melanoma: indications and rationale. . Can Control *16*, 234-240.

Plate, K.H. (2001). From angiogenesis to lymphangiogenesis. Nat Med *7*, 151-152.

Podgrabinska, S., Braun, P., Velasco, P., Kloos, B., Pepper, M.S., Jackson, D.G., and Skobe, M. (2002). Molecular characterization of lymphatic endothelial cells, pp. 16069-16074.

Pollard, J.W. (2008). Macrophages define the invasive microenvironment in breast cancer, pp. 623-630.

Privratsky, J.R., Paddock, C.M., Florey, O., Newman, D.K., Muller, W.A., and Newman, P.J. (2010). Relative contribution of PECAM-1 adhesion and signaling to the maintenance of vascular integrity, pp. 1477-1485.

Qian, B.-Z., and Pollard, J. (2010). Macrophage Diversity Enhances Tumor Progression and Metastasis. Cell *141*, 39-51.

Rajer, E.L., Bridgeford, E.P., and and W, O.D. (2005). Cutaneous Melanoma: Update on Prevention, Screening, Diagnosis, and Treatment. American Family Physician *72*, 269-276.

Rakha, E.A., Martin, S., Lee, A.H.S., Morgan, D., Pharoah, P.D.P., Hodi, Z., MacMillan, D., and Ellis, I.O. (2011). The prognostic significance of lymphovascular invasion in invasive breast carcinoma. Cancer, n/a-n/a.

Ramirez, M.I., Millien, G., Hinds, A., Cao, Y., Seldin, D.C., and Williams, M.C. (2003). T1alpha, a lung type I cell differentiation gene, is required for normal lung cell proliferation and alveolus formation at birth. Dev Biol *256*, 61-72.

Rangnekar, V., Waheed, S., and Rangnekar, V. (1992). Interleukin-linducible tumor growth arrest is characterized by activation of cell type- specific “early” gene expression programs. J Biol Chem *267*.

Rass, K., and Reichrath, J. (2008). UV damage and DNA repair in malignant melanoma and nonmelanoma skin cancer sunlight, vitamin D and skin cancer. In (Springer New York), pp. 162-178.

Renyi-Vamos, F., Tovari, J., Fillinger, J., Timar, J., Paku, S., Kenessey, I., Ostoros, G., Agocs, L., Soltesz, I., and Dome, B. (2005). Lymphangiogenesis correlates with lymph node metastasis, prognosis, and angiogenic phenotype in human non-small cell lung cancer, pp. 7344-7353.

Ribatti, D., Mangialardi, G., and Vacca, A. (2006). Stephen Paget and the seed and soil theory of metastatic dissemination. Clin Exp Med *6*, 145-149.

Rice, C., and Merchant, R. (1992). Systemic treatment with murine recombinant interleukin-lp inhibits the growth and progression of malignant glioma in the rat. . J Neur Oncol *13*.

Rosso, S., Gondos, A., Zanetti, R., Bray, F., Zakelj, M., Zagar, T., Smailyte, G., Ponti, A., Brewster, D.H., Voogd, A.C*., et al.* (2010). Up-to-date estimates of breast cancer survival for the years 2000-2004 in 11 European countries: The role of screening and a comparison with data from the United States. European journal of cancer (Oxford, England : 1990) *46*, 3351-3357.

Rutkowski, P., Nowecki, Z.I., Zdzienicki, M., Michej, W., Symonides, M., Rosinska, M., Dziewirski, W., Bylina, E., and Ruka, W. (2010).

Cutaneous melanoma with nodal metastases in elderly people. International Journal of Dermatology *49*, 907-913.

Saalbach, A., Wetzel, A., Haustein, U.-F., Sticherling, M., Simon, J.C., and Anderegg, U. (2005). Interaction of human Thy-1 (CD 90) with the integrin [alpha]v[beta]3 (CD51//CD61): an important mechanism mediating melanoma cell adhesion to activated endothelium. Oncogene *24*, 4710-4720.

Sahai, E. (2007). Illuminating the metastatic process. Nat Rev Cancer *7*, 737-749.

Sahni, D., Robson, A., Orchard, G., Szydlo, R., Evans, A.V., and Russell-Jones, R. (2005). The use of LYVE-1 antibody for detecting lymphatic involvement in patients with malignant melanoma of known sentinel node status. J Clin Pathol *58*, 715-721.

Saijo, Y., Tanaka, M., Miki, M., Usui, K., Suzuki, T., Maemondo, M., Hong, X., Tazawa, R., Kikuchi, T., Matsushima, K.*, et al.* (2002a). Proinflammatory cytokine IL-1 promotes tumor growth of Lewis lung carcinoma by induction of angiogenic factors: in vivo analysis of tumor-stromal interaction, pp. 469-475.

Saijo, Y., Tanaka, M., Miki, M., Usui, K., Suzuki, T., Maemondo, M., Hong, X., Tazawa, R., Kikuchi, T., Matsushima, K.*, et al.* (2002b). Proinflammatory Cytokine IL-1Î² Promotes Tumor Growth of Lewis Lung Carcinoma by Induction of Angiogenic Factors: In Vivo Analysis of Tumor-Stromal Interaction, pp. 469-475.

Salmi, M., Koskinen, K., Henttinen, T., Elima, K., and and Jalkanen, S. (2004). CLEVER-1 mediates lymphocyte transmigration through vascular and lymphatic endothelium. . Blood *104*, 3849-3857.

Santoso, S., Orlova VV, Song K, Sachs UJ, CL, A.-S., and T, a.C. (2005). The homophilic binding of junctional adhesion molecule C mediates tumour cell-endothelial cell interactions. . J of Biol Chem *280*, 36326-36333.

Schacht, V., Dadras, S.S., Johnson, L.A., Jackson, D.G., Hong, Y.K., and & Detmar, M. (2005a). Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. . Am J Pathol *166*, 913-921.

Schacht, V., Dadras, S.S., Johnson, L.A., Jackson, D.G., Hong, Y.K., and Detmar, M. (2005b). Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. The American journal of pathology *166*, 913-921.

Schacht, V., Ramirez, M.I., Hong, Y.K., Hirakawa, S., Feng, D., Harvey, N., Williams, M., Dvorak, A.M., Dvorak, H.F., Oliver, G.*, et al.* (2003).

T1alpha/podoplanin deficiency disrupts normal lymphatic vasculature formation and causes lymphedema. The EMBO journal *22*, 3546-3556.

Schledzewski, K., Falkowski, M., Moldenhauer, G., Metharom, P., Kzhyshkowska, J., Ganss, R., Demory, A., Falkowska-Hansen, B., Kurzen, H., Ugurel, S.*, et al.* (2006). Lymphatic endothelium-specific hyaluronan receptor LYVE-1 is expressed by stabilin-1+, F4/80+, CD11b+ macrophages in malignant tumours and wound healing tissue in vivo and in bone marrow cultures in vitro: implications for the assessment of lymphangiogenesis. The Journal of pathology *209*, 67-77.

Scholzen, T., and Gerdes, J. (2000). The Ki-67 protein: from the known and the unknown. Journal of Cellular Physiology *182*, 311-322.

Schoppmann, S.F., Fenzl, A., Nagy, K., Unger, S., Bayer, G., Geleff, S., Gnant, M., Horvat, R., Jakesz, R., and Birner, P. (2006). VEGF-C expressing tumor-associated macrophages in lymph node positive breast cancer: impact on lymphangiogenesis and survival. Surgery *139*, 839-846.

Senger, D.R., Perruzzi, C.A., Streit, M., Koteliansky, V.E., de Fougerolles, A.R., and Detmar, M. (2002). The [alpha]1[beta]1 and [alpha]2[beta]1 integrins provide critical support for vascular endothelial growth factor signaling, endothelial cell migration, and tumor angiogenesis. The American Journal of Pathology *160*, 195-204.

Sheikh, S., Gale, Z., Rainger, G.E., and Nash, G.B. (2004). Methods for exposing multiple cultures of endothelial cells to different fluid shear stresses and to cytokines, for subsequent analysis of inflammatory function. . J Immunol Methods *288*, 35-46.

Shetty, S., Weston, C.J., Oo, Y.H., Westerlund, N., Stamataki, Z., Youster, J., Hubscher, S.G., Salmi, M., Jalkanen, S., Lalor, P.F.*, et al.* (2011). Common lymphatic endothelial and vascular endothelial receptor-1 mediates the transmigration of regulatory T cells across human hepatic sinusoidal endothelium, pp. 4147-4155.

Shields, J.D., Borsetti, M., Rigby, H., Harper, S.J., Mortimer, P.S., Levick, J.R., Orlando, A., and Bates, D.O. (2004). Lymphatic density and metastatic spread in human malignant melanoma. Br J Cancer *90*, 693-700.

Singer, C., Kronsteiner, N., Hudelist, G., Marton, E., Walter, I., Marion Kubista, K.C., Martin Schreiber, M.S., and, and Kubista, E. (2003). Interleukin 1 system and sex steroid receptor expression in human breast Ccancer: interleukin 1 protein secretion is correlated with malignant phenotype. Clin Cancer Res *9*, 4877-4883.

Singletary, S.E., Allred, C., Ashley, P., Bassett, L.W., Berry, D., Bland, K.I., Borgen, P.I., Clark, G., Edge, S.B., Hayes, D.F.*, et al.* (2002). Revision of the American Joint Committee on Cancer Staging System for breast cancer, pp. 3628-3636.

Skobe, M., Hawighorst, T., Jackson, D.G., Prevo, R., Janes, L., Velasco, P., Riccardi, L., Alitalo, K., Claffey, K., and Detmar, M. (2001). Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. Nat Med *7*, 192-198.

Sondak, V.K., Taylor, J.M., and Sabel, M.S. (2004). Mitotic rate and younger age are predictors of sentinel lymph node positivity: lessons learned from the generation of a probabilistic model. . Ann Surg Oncol *11*, 247-258.

Song, X., Voronov, E., Dvorkin, T., Fima, E., Cagnano, E., Benharroch, D., Shendler, Y., Bjorkdahl, O., Segal, S., Dinarello, C.A.*, et al.* (2003). Differential effects of IL-1alpha and IL-1beta on tumorigenicity patterns and invasiveness, pp. 6448-6456.

Sporn, M. (1997). The war on cancer: A review. Annals of the New York Academy of Sci *833*, 137-146.

Stacker, S.A., Baldwin, M.E., and Achen, M.G. (2002). The role of tumor lymphangiogenesis in metastatic spread, pp. 922-934.

Stacker, S.A., Caesar, C., Baldwin, M.E., Thornton, G.E., Williams, R.A., Prevo, R., Jackson, D.G., Nishikawa, S.-i., Kubo, H., and Achen, M.G. (2001). VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. Nat Med *7*, 186-191.

Storr, S.J., Safuan, S., Mitra, A., Elliott, F., Walker, C., Vasko, M.J., Ho, B., Cook, M., Mohammed, R.A.A., Patel, P.M.*, et al.* (2011). Objective assessment of blood and lymphatic vessel invasion and association with macrophage infiltration in cutaneous melanoma. Mod Pathol.

Straume, O., and Akslen, L.A. (2004). Lymphatic vessel density and prognosis in cutaneous melanoma. Br J Cancer *91*, 1224-1225.

Straume, O., Jackson, D.G., and Akslen, L.A. (2003). Independent prognostic impact of lymphatic vessel density and presence of low-grade lymphangiogenesis in cutaneous melanoma. Clin Cancer Res *9*, 250-256.

Sun, W., Pitson, S., and CS., a.B. (2010). Tumour necrosis factor-induced neutrophil adhesion occurs via sphingosine kinase-1 dependent activation of endothelial α5β1 integrin. . American J of Pathol *177*, 436-446.

Swamydas, M., Nguyen, D., Allen, L.D., Eddy, J., and Dracau, D. (2011). Progranulin stimulated by LPA promotes the migration of aggressive breast cancer cells, pp. 119-130.

Swerlick, R., Lee, K., Li LJ, Sepp NT, SW, C., and TJ., a.L. (1992). Regulation of vascular cell adhesion molecule 1 on human dermal microvascular endothelial cells. . J of Immunol *149*, 698-705.

Talmadge, J.E. (2007). Clonal selection of metastasis within the life history of a tumor. Cancer Res *67*, 11471-11475.

ten Kate, M., Hofland, L., Koetsveld PM, J, J., and CHJ, a.E. (2006). Pro-inflammatory cytokines affect pancreatic carcinoma cell-endothelial cell interactions. J pancreas *7*, 454-464.

Thelen, A., Jonas, S., and al., B.C.e. (2009). Tumor-associated lymphangiogenesis correlates with prognosis after resection of human hepatocellular carcinoma. . Ann Surg Oncol *16*, 1222-1230.

Thody, A.J., Higgins, E.M., Wakamatsu, K., Ito, S., Burchill, S.A., and Marks, J.M. (1991). Pheomelanin as well as Eumelanin Is Present in Human Epidermis. J Investig Dermatol *97*, 340-344.

Topar, G., Eisendle, K., Zelger, B., and Fritsch, P.O. (2006). Sentinel lymph node status in melanoma: a valuable prognostic factor? . British J of Dermathol *154*, 1080-1087.

Valencak, J., Heere-Ress, E., Kopp, T., Schoppmann, S.F., Kittler, H., and Pehamberger, H. (2004). Selective immunohistochemical staining shows significant prognostic influence of lymphatic and blood vessels in patients with malignant melanoma. Eur J Cancer *40*, 358-364.

van Brocklyn, J.R. (2011). Regulation of cancer cell migration and invasion by sphingosine-1-phosphate. World J Biol Chem *1*, 307-312.

van der Sangen, M., Voogd, A., and van de Poll-Franse, L.e.a. (2008). Breast cancer in young women: epidemiology and treatment dilemmas. . Ned Tijdschr Geneeskd *152*, 2495-2500.

van Grevenstein, W., Hofland, L., van Rossen MEE, van Koetsveld PM, J, J., and CHJ, a.v.E. (2007). Inflammatory cytokines stimulate the adhesion of colon carcinoma cells to mesothelial monolayers. . Dig Dis Sci *52*, 2775-2783. .

van Veer, L., Dai, H., van de Vijver., He., Y.D., Hart, A.A.M., Mao, M., Peterse, H.L., van deer Kooy, K., Marton, M.J., and Witteveen, A.T. (2002). Gene expression profiling predicts clinical outcome of breast cancer. Nature *415*, 530-535.

van Zijl, F., Krupitza, G., and Mikulits, W. (2011). Initial steps of metastasis: Cell invasion and endothelial transmigration. Mutation Research/Reviews in Mutation Research *728*, 23-34.

Veronesi, U., Paganelli, G., Viale, G., Luini, A., Zurrida, S., Galimberti, V., Intra, M., Veronesi, P., Maisonneuve, P., Gatti, G*., et al.* (2006). Sentinel-lymph-node biopsy as a staging procedure in breast cancer: update of a randomised controlled study. The Lancet Oncology *7*, 983-990.

Viale, G., Giobbie-Hurder, A., Gusterson, B.A., Maiorano, E., Mastropasqua, M.G., Sonzogni A., Mallon, E., and Coates, A.S. (2010). Adverse

prognostic value of peritumoral vascular invasion: is it abrogated by adequate endocrine adjuvant therapy? Results from two International Breast Cancer Study Group randomized trials of chemoendocrine adjuvant therapy for early breast cancer. Annals of Oncol *21*, 245-254.

Vidal-Vanaclocha, F., Amezaga, C., Aintzane, A., Gilles, K., and and Charles, A.D. (1994). Interleukin-1 receptor blockade reduces the number and size of murine B16 melanoma hepatic metastases. Cancer Res *54*, 2667-2672.

Voronov, E., Shouval, D.S., Krelin, Y., Cagnano, E., Benharroch, D., Iwakura, Y., Dinarello, C.A., and Apte, R.N. (2003). IL-1 is required for tumor invasiveness and angiogenesis, pp. 2645-2650.

Voura, E.B., Sandig, M., Kalnins, V.I., and and Siu, C. (1998). Cell shape changes and cytoskeleton reorganization during transendothelial migration of human melanoma cells. . *Cell Tissue Res*, 375-387.

Wajant, H., Pfizenmaier, K., and Scheurich, P. (2003). Tumor necrosis factor signaling. Cell Death Differ *10*, 45-65.

Walter-Yohrling, J., Cao, X., Callahan, M., Weber, W., Morgenbesser, S., Madden, S., Wang, C., and and Teicher, B. (2003). Identification of genes expressed in malignant cells that promote invasion. . Cancer Res *63*, 8939-8947.

Walters, R., Groben, P., Busam, K., Millikan, R., Rabinovitz H, Cognetta A, Mihm MC Jr, Prieto VG, Googe PB, King R*, et al.* (2007). Consumption of the epidermis: a criterion in the differential diagnosis of melanoma and dysplastic nevi that is associated with increasing breslow depth and ulceration. Am J Dermatopathol *29*, 527-533.

Wang, X.-L., Fang, J.-P., Tang, R.-Y., and Chen, X.-M. (2010). Different significance between intratumoral and peritumoral lymphatic vessel density in gastric cancer: a retrospective study of 123 cases, pp. 299.

Wang, X., and Lin, Y. (2008). Tumor necrosis factor and cancer, buddies or foes. Acta Pharmacol Sin *29*, 1275-1288.

Watabe, D., Kanno, H., Yoshida, A., Kurose, A., Akasaka, T., and Sawai, T. (2007). Adhesion of peripheral blood mononuclear cells and CD4+ T cells from patients with psoriasis to cultured endothelial cells via the interaction between lymphocyte function-associated antigen type 1 and intercellular adhesion molecule 1. British Journal of Dermatology *157*, 259-265.

Watanabe, T., Hashimoto, T., Sugino, T., Soeda, S., Nishiyama, H., Morimura, Y., Yamada, H., Goodison, S., and Fujimori, K. (2012). Production of IL1-beta by ovarian cancer cells induces mesothelial cell beta1-integrin expression facilitating peritoneal dissemination. Journal of Ovarian Research *5*.

Weidner, N., Semple, J.P., Welch, W.R., and Folkman, J. (1991). Tumor angiogenesis and metastasis - correlation in invasive breast carcinoma, pp. 1 - 8.

Weigelt, B., Horlings, H., B Kreike, MM Hayes, M Hauptmann, LFA Wessels, D de Jong, MJ Van de Vijver, LJ Van't Veer, and Peterse, a.J. (2008). Refinement of breast cancer classification by molecular characterization of histological special types. J Pathol *216*, 141-150.

Weigelt, B., Peterse, J.L., and van't Veer, L.J. (2005). Breast cancer metastasis: markers and models. Nat Rev Cancer *5*, 591-602.

Weinberg, R.A. (2007). The biology of cancer (Garland Science, Taylor & Francis Group, LLC).

Wetterwald, A., Hoffstetter, W., and Cecchini, M.e.a. (1996). Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. . Bone *18*, 125-132.

Wheatley, K., Ives, N., Hancock, B., Gore, M., Eggermont, A., and Suciu, S. (2003). Does adjuvant interferon-alpha for high-risk melanoma provide a worthwhile benefit? A meta-analysis of the randomised trials. . Cancer Treat Rev *29*, 241-252.

Wigle, J.T., Harvey, N., Detmar, M., Lagutina, I., Grosveld, G., Gunn, M.D., Jackson, D.G., and and Oliver, G. (2002). An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. . EMBO J *21*, 1505-1513.

Williams, C.S., Leek, R.D., and al., R.A.M.e. (2003). Absence of lymphangiogenesis and intratumoural lymph vessels in human metastatic breast cancer. . J Pathol *200*, 195-206.

Wilting, J., Papouts, i.M., and al., C.B.e. (2002). The transcription factor Prox1 is a marker for lymphatic endothelial cells in normal and diseased human tissues. . Faseb J *16*, 1271-1273.

Wu, Q.D., Wang, J.H., Condron, C., Bouchier-Hayes, D.a., and Redmond, H.P. (2001). Human neutrophils facilitate tumor cell transendothelial migration. . Am J Physiol Cell Physiol *280*, C814-822.

Wu, Z.-s., Wu, Q., Wang, C.-q., Wang, X.-n., Wang, Y., Zhao, J.-j., Mao, S.-s., Zhang, G.-h., Zhang, N., and Xu, X.-c. (2010). MiR-339-5p inhibits breast cancer cell migration and invasion in vitro and may be a potential biomarker for breast cancer prognosis, pp. 542.

Wyckoff, J.B., Jones, J.G., Condeelis, J.S., and Segall, J.E. (2000). A critical step in metastasis: in vivo analysis of intravasation at the primary tumor, pp. 2504-2511.

Xie, S., Luca, M., Huang, S., Gutman, M., Reich, R., Johnson, J.P., and Bar-Eli, M. (1997). Expression of MCAM/MUC18 by human melanoma cells leads to increased tumor growth and metastasis, pp. 2295-2303.

Xu, X., Gimotty, P.A., Guerry, D., Karakousis, G., Van Belle, P., Liang, H., Montone, K., Pasha, T., Ming, M.E., Acs, G.*, et al.* (2008). Lymphatic invasion revealed by multispectral imaging is common in primary melanomas and associates with prognosis. Hum Pathol *39*, 901-909.

Yano, A., Fujii, Y., and al., I.A.e. (2006). Glucocorticoids suppress tumor lymphangiogenesis of prostate cancer cells. . Clin Cancer Res *12* 6012-6017.

Yokota, J. (2000). Tumour progression and metastasis. . Carcinogenesis *21*, 497-503.

Young, N., and van Brocklyn, J.R. (2007). Roles of sphingosine-1-phosphate (S1P) receptors in malignant behavior of glioma cells. Differential effects of S1P2 on cell migration and invasiveness. Exp Cell Res *313*, 1615-1627.

Yuan, K., Kucik, D., Singh , R., Listinsky, C., Listinsky, J., and GP, a.S. (2008). Alterations in human breast cancer adhesion-motility in response to changes in cell surface glycoproteins displaying alpha-L-fucose moieties. Int J Oncology *32*, 797-807.

Yunping Luo, He Zhou, Jörg Krueger, Charles Kaplan, Sung-Hyung Lee, Carrie Dolman, Dorothy Markowitz, Wenyuan Wu, Cheng Liu, A., R.*, et al.* (2006). Targeting tumor-associated macrophages as a novel strategy against breast cancer. J Clin Invest *116*, 2132-2141.

Zhang, R., Chen, H.J., Wei, B., Zhang, H.Y., Pang, Z.G., Zhu, H., Zhang, Z., Fu, J., and Bu, H. (2010). Reproducibility of the Nottingham modification of the Scarff-Bloom-Richardson histological grading system and the complementary value of Ki-67 to this system. Chin Med J (Engl) *123*, 1976-1982.

Zhang, S.-q., Yu, H., and Zhang, L.-l. (2009). Clinical implications of increased lymph vessel density in the lymphatic metastasis of early-stage invasive cervical carcinoma: a clinical immunohistochemical method study, pp. 64.

Zhu, N., Eves, P., Katerinaki E, Szabo M, Morandini R, Ghanem G, Lorigan P, MacNeil S, and J, H. (2002). Melanoma cell attachment, invasion and integrin expression is upregulated by tumour necrosis factor-a and suppressed by a-melanocyte stimulating hormone. . J Invest Dermatol *119*, 1165-1171.

APPENDIX A: SUPPLEMENTARY INFORMATION FOR CHAPTER 1

Table A1: TNM staging categories for cutaneous melanoma. Reproduced and adapted from (Balch et al., 2009). Copyright© 2009 by American Society of Clinical Oncology with permission conveyed through Copyright Clearance Center Inc.

Tumour (T)	*Breslow thickness (mm)*	*Ulceration status/mitoses*
T0	NA	NA
T1	$\leq$ 1.00	a. without ulceration and mitosis <1/mm^2 b. with ulceration or mitoses $\geq$1/mm^2
T2	1.01-2.00	a. without ulceration b. with ulceration
T3	2.01-4.00	a. without ulceration b. with ulceration
T4	>4.00	a. without ulceration b. with ulceration
Nodes (N)	*No of metastatic nodes*	*Nodal metastatic burden*
N0	0	NA
N1	1	a. micrometastasis* b. macrometastasis+
N2	2-3	a. micrometastasis* b. macrometastasis+ c. in transit metastases/satellites without metastatic nodes
N3	4+	metastatic or matted nodes, or in transit metastases/ satellites with metastatic nodes
Metastases (M)	*Site*	*Serum lactate dehydrogenase*
M0	no distant metastases	NA
M1a	distant/subcutaneous/nodal	normal
M1b	lung metastases	normal
M1c	all other visceral metastases	Normal
	any distant metastasis	Elevated

Abbreviations: NA-not applicable
*micrometastases are diagnosed after sentinel lymph node biopsy
+macrometastases are defined as clinically detectable nodal metastases confirmed pathologically

Table A2: Anatomic stage groupings (clinical and pathologic stage) in cutaneous melanoma. Reproduced and adapted from (Balch et al., 2009). Copyright© 2009 by American Society of Clinical Oncology with permission conveyed through Copyright Clearance Center Inc.

Clinical staging*				***Pathological staging****				***5 years survival (%)***
Stage	**T**	**N**	**M**	Stage	**T**	**N**	**M**	
0	Tis	N0	M0	0	Tis	N0	M0	99.9
IA	T1a	N0	M0	IA	T1a	N0	M0	≥90
IB	T1b	N0	M0	IB	T1b	N0	M0	
	T2a	N0	M0		T2a	N0	M0	
IIA	T2b	N0	M0	IIA	T2b	N0	M0	40-85
	T3a	N0	M0		T3a	N0	M0	
IIB	T3b	N0	M0	IIB	T3b	N0	M0	
	T4a	N0	M0		T4a	N0	M0	
IIC	T4b	N0	M0	IIC	T4b	N0	M0	
III	Any T	N>N0	M0	IIIA	T1-4a	N1a	M0	25-60
					T1-4a	N2a	M0	
				IIIB	T1-4b	N1a	M0	
					T1-4b	N2a	M0	
					T1-4a	N1b	M0	
					T1-4a	N2b	M0	
					T1-4a	N2c	M0	
				IIIC	T1-4b	N1b	M0	
					T1-4b	N2b	M0	
					T1-4b	N2c	M0	
					Any T	N3	M0	
IV	Any T	Any N	M1	IV	Any T	Any N	M1	9-18

*Clinical staging includes microstaging of primary melanoma and clinical evaluation of metastases. It is used after complete excision of the primary melanoma.

+Pathological staging includes microstaging of primary melanoma and pathologic information about the regional lymph nodes after lymphadenectomy.

APPENDIX B: SUPPLEMENTARY INFORMATION FOR CHAPTER 2

Table B1: TNM staging categories for breast cancer. Reproduced and adapted from (Singletary et al., 2002). Copyright© 2002 byAmerican Society of Clinical Oncology with permission conveyed through Copyright Clearance Center Inc,

Tumour (T)	
TX	primary tumour cannot be assessed
T0	no evidence of primary tumour
Tis	Carcinoma in situ: ductal carcinoma in situ, lobular carcinoma in situ Paget disease of the nipple with no tumour
T1	Tumour ≤2cm in greatest dimension
T1mic	≤0.1cm
T1a	>0.1cm but ≤0.5cm
T1b	>0.5cm but ≤1.0cm
T1c	>1.0cm but ≤2.0cm
T2	>2.0cm but ≤5.0cm
T3	>5cm
T4	Tumour of any size extend to the (a) chest wall or (b) skin
T4a	Extend to chest wall not including the pectoralis muscle
T4b	Edema/ulceration of the breast
T4c	T4a + T4b
T4d	Inflammatory carcinoma
Regional lymph nodes (N)	
NX	Cannot be assessed
N0	No regional lymph node metastasis
N1	Metastasis in movable ipsilateral axillary lymph node
N2	
N2a	Metastasis in ipsilateral axillary lymph nodes fixed to one another (matted) or to other structures
N2b	Metastasis only in clinically apparent* ipsilateral internal mammary nodes and in the absence of clinically evident axillary lymph node metastasis
N3	
N3a	Metastasis in ipsilateral infraclavicular lymph node and axillary lymph node
N3b	Metastasis in ipsilateral internal mammary lymph node and axillary lymph node
N3c	Metastasis in ipsilateral supraclavicular lymph node
Distant Metastasis (M)	
MX	cannot be assessed
M0	no distant metastases
M1	distant metastasis
M1c	all other visceral metastases any distant metastasis

Table B2: Stage grouping for breast cancer. Reproduced and adapted from (Singletary et al., 2002). Copyright© 2002 by American Society of Clinical Oncology with permission conveyed through Copyright Clearance Center Inc,

Stage	***T***	***N***	***M***
0	Tis	N0	M0
I	T1	N0	M0
IIA	T0	N1	M0
	TI	N1	M0
	T2	N0	M0
IIB	T2	N1	M0
	T3	N0	M0
IIIA	T0	N2	M0
	TI	N2	M0
	T2	N2	M0
	T3	N1	M0
	T3	N2	M0
IIIB	T4	N0	M0
	T4	N1	M0
	T4	N2	M0
IIIC	Any T	N3	M0
IV	Any T	Any N	M1

APPENDIX C: SUPPLEMENTARY INFORMATION FOR CHAPTER 3

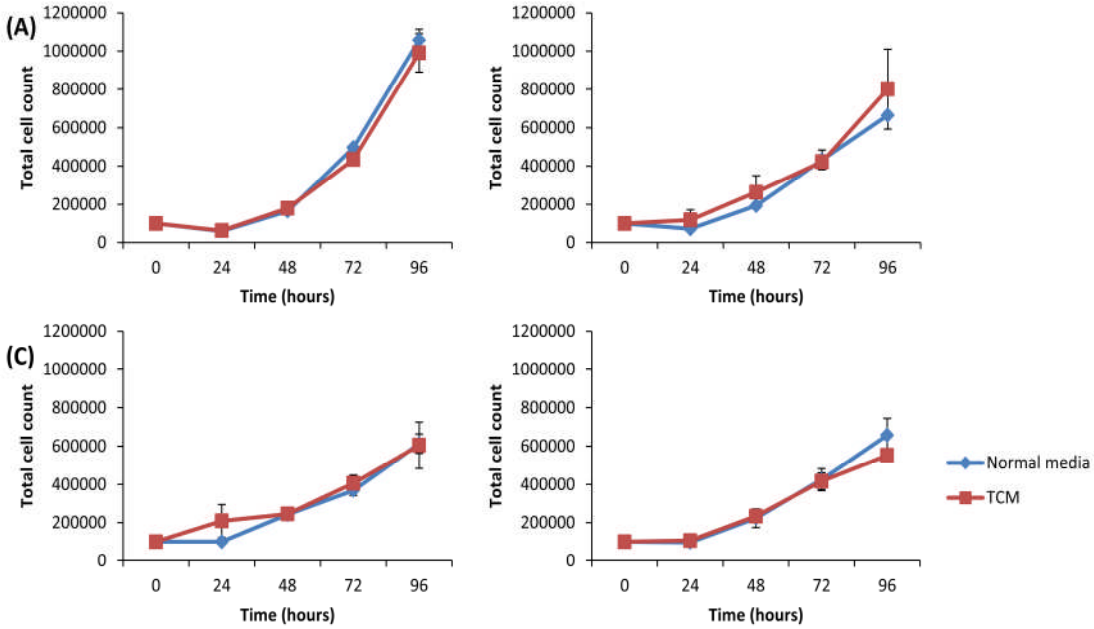


Figure C1: Proliferation assay comparing cells growth cultured with normal cell culture media and tumour conditioned media generated as in Chapter 3 -Section 3.3.5 used in adhesion and migration assay. there was no difference in cell count between both media across MDA-MB-231 (A), MCF7 (B), MeWo (C) and SKMEL-30 (D), showing that the tumour conditioned media used is still bioreactive. Experiments were carried out twice, each in duplicate (n=4).

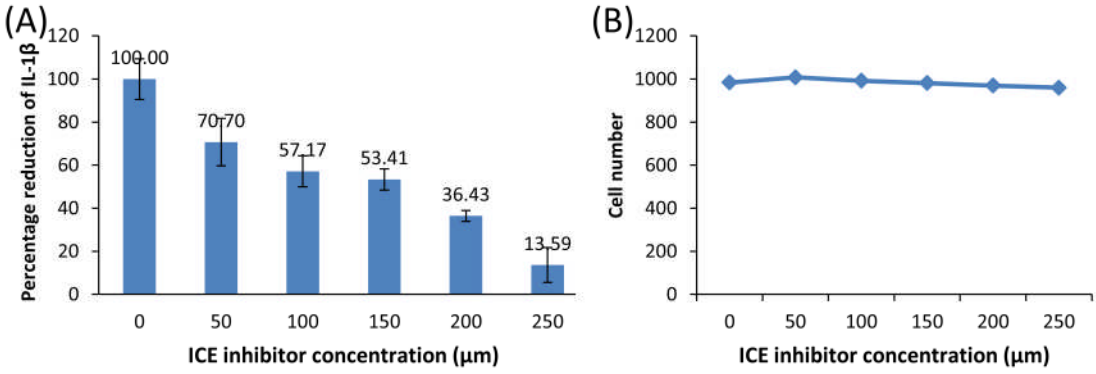


Figure C2: Optimisation of ICE inhibitor concentration used to stimulate macrophages to generate macrophage conditioned media (A). 250µm of ICE inhibitor was used in subsequent assay as at this concentration, 86% inhibition was observed which would not effect macrophages viability (B).

APPENDIX D: SUPPLEMENTARY INFORMATION FOR CHAPTER 5

		IL1Beta
N	Valid	1511
	Missing	0
Mean		111.48
Std. Error of Mean		1.500
Median		125.00
Std. Deviation		58.290
Range		230
Minimum		0
Maximum		230

Figure D1: Descriptive statistic of cytoplasmic IL-1β H-scored of breast tissue microarray.

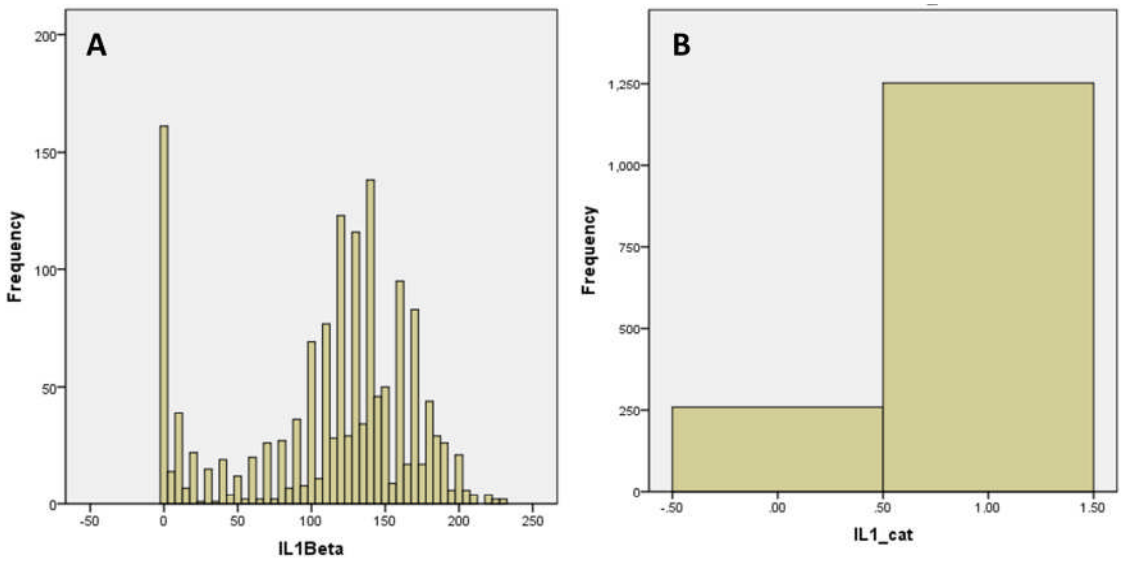


Figure D2: Histogram showing the distribution of cytoplasmic IL-1β H-scored of breast tissue microarray from 1511 patients (A). IL-1β had a H-score cut off point of 35 with 259 (17.1%) cases with low score and 1252 (82.9%) cases having high score (B).