THE UNIVERSITY OF LIVERPOOL

Characterization of the influence of the galactoside-binding protein Peanut Agglutinin on endothelial secretion of cytokines in cancer metastasis

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

Weikun Wang

November

2019

ACKNOWLEDGEMENTS

I am grateful and indebted to my supervisor Professor Lugang Yu for his guidance and support throughout my research and for his constructive criticism and excellent advice during the preparation of this thesis. I am also very grateful to my secondary supervisor, Professor Jonathan Rhodes for his kindly advice and help throughout this period. I would like to thank colleagues from our research group, in particular, Dr. Chen Chen, Dr. Qicheng Zhao and Dr. Carrie Duckworth for their technical support and advices.

I am grateful to my father Dr. Yimin Wang, my mother Mrs. Ling Xiong and my husband Mr. Yun Lin. This work would not have been possible without their financial and spiritual support.

AUTHOR'S DECLARATION

All techniques and experiments performed and described in this thesis were undertaken by me as a student working towards the degree of Doctor of Medicine at the University of Liverpool.

Neither this thesis, nor part of it has been submitted in support of an application for another degree or qualification at this or any other university or institute of learning.

Weikun Wang

ABSTRACT

Peanut agglutinin (PNA) is a dietary lectin which accounts for 0.15% of the total weight of peanut *Arachis hypogaea*. PNA is a galactoside-binding protein which binds highly specifically to the tumour-associated Thomsen-Friedenreich blood group antigen. Previous studies demonstrate that PNA rapidly enters the blood circulation after peanut ingestion. PNA interacts with oncofetal TF disaccharide on the transmembrane mucin protein MUC1, causing polarization of MUC1 and thus reveals functional cell surface adhesion molecules. Therefore, circulating PNA has potential effects by inducing cancer cell homotypic and heterotypic adhesion, cell survival and migration, thus potentially promoting cancer metastasis and resistance to anoikis. In addition, earlier studies from our group have shown that a human galactoside-binding galectin-3 also interacts with MUC1-TF potentially inducing cancer metastasis from vascular endothelium. It is also reported that circulating galectin-3 enhances cytokine secretion from endothelial cells, and this interaction is suggested to be responsible for the metastasis-promoting effect of galectin-3. This thesis further explores the PNA effect on endothelial secretion of metastasis-associated cytokines. The study presented here reports that PNA at a concentration similar to that found in the sera of people after eating 200g peanuts causes increase of IL-6 and MCP-1 secretion from both micro-vascular and macro-vascular endothelial cells. The

PNA-induced cytokine secretion was found to enhance endothelial expression of several cell surface adhesion molecules, leading to increased cancer cell-endothelial cell adhesion. The increased cytokine secretion is also shown to promote endothelial cell adhesion, proliferation and angiogenesis. The molecular mechanism of the PNA-induced cytokine secretion is also studied, and two cell surface adhesion molecules MCAM and PECAM were identified as major functional receptors of PNA. Moreover, siRNA-mediated suppression of MCAM and PECAM expression completely abolished the PNA-induced cytokine secretion from endothelial cells. This study also investigated the cell signaling involved in the potential pro-metastasis effect of PNA. The results from this study suggested that the actions of dietary PNA in cancer patients might have impacts on cancer growth and metastasis.

ABBREVIATIONS

Akt	Protein Kinase B (PKB)
ASF	Asialofetuin
ATP	Adenosine triphosphate
BCL2	B-cell lymphoma 2
BRCA1/2	Breast Cancer genes 1 or 2
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CAMs	Cell adhesion molecules
CCL2	Chemokine (C-C motif) ligand 2 (MCP-1)
CCR	C-C motif receptor
CD146	Melanoma cell adhesion molecule (MCAM)
CD23	Fc epsilon RII (FcεRII)
CD31	Platelet endothelial cell adhesion molecule (PECAM)
CD44	Homing cell adhesion molecule (HCAM)
CM	Conditioned Medium
CRC	Colorectal Cancer
CRDs	Carbohydrate recognition-binding domains
CTL	C-type lectins
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix components
EDTA	Ethylenediaminetetraacetic acid

EGF	Epithelial growth factor
EGM	Endothelial growth media
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
Gal	Galactose
Glc	Glucose
G-CSF	Granulocyte colony-stimulating factor
GlcNAc	N-acetylglucosamine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCAM	Homing cell adhesion molecule
hEGF	Human Epidermal Growth Factor
HMVEC-Ls	Human micro vascular lung endothelial cells
HUVEC	Human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
Ig-CAM	Immunoglobulin-like Cell Adhesion Molecules
IgSF	Immunoglobulin superfamily
IKK	IκB kinase
IL-6	Interleukin 6
JAK	Janus kinase
KSHV	Kaposi's sarcoma-associated herpesvirus
MAPK	Mitogen-activated protein kinases
MCAM	Melanoma Cell Adhesion Molecule
MCP-1	Chemokine (C-C motif) ligand 2 (CCL2)
MEK	Mitogen-activated protein kinase
MHC	Major Histocompatibility Complex
MIP	Macrophage Inflammatory Proteins
MMP	Matrix metalloproteinase

MMR	Mismatch repair
MUC1	Membrane-associated sialomucin episialin
NAC	N-acetylcysteine
NF-Kappa B	Nuclear factor Kappa B
NIH	National Institute of Health
NK	Natural killer cell
PAGE	Polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cells
PECAM	Platelet endothelial cell adhesion molecule
PNA	Peanut agglutinin
Poly-HEMA	Poly-2-hydroxyethyl methacrylate
RGD	Arginylglycylaspartic acid (Arg-Gly-Asp)
SDS	Sodium dodecyl sulphate
SiRNA	Small interfering RNA
STAT	Signal Transducers and Activators of Transcription
TEMED	Tetramethylethylenediamine
TF	Thomsen-Friedenreich oncofetal carbohydrate antigen (N-acetyl-D-galectosamine linked to protein)
TNF-α	Tumour necrosis factor-alpha
TNM	The TNM Classification of Malignant tumour (, Lymph Nodes, Metastasis)
cTNM	Clinical TNM
pTNM	Pathological testing
UICC	Union International Cancer Control
UV	Ultraviolet light
VEGF	Vascular endothelial growth factor
VCAM-1	Vascular cell adhesion protein 1
WHO	World Health Organization

TABLE OF CONTENT

CHAPTER 1 Introduction

1.1 Cancer

1.1.1 General

The words cancer and carcinoma come from the Latin (*cancer*) and Greek words *Karkinos (carcinos)* for crab. This was first used by the Greek physician Hippocrates investigating the appearance of the cut surface of a removed malignant tumour with "veins stretched on all sides as the crab has its feet". [1] In addition, he even recorded several types of cancer including breast cancer at around 460-370 B.C. but the earliest evidence of cancer is from mummies which contained evidence of human bone cancer in ancient Egypt. [2] An ancient manuscript, the Papyrus Ebers, described cancer without naming it, and even includes the first description of a surgical treatment of breast cancer with a tumour removal procedure using cauterization at around 1600BC. [3] The theories of cancer have changed over the past two thousand years, along with the developing understanding of cancer. [4]

Cancer is a cluster of diseases and can happen in any part of the body. From the data published by the World Health Organization (WHO), 1.7 million people die from lung cancer together with trachea and bronchus cancers each year, increasing from less than 1 million deaths in 2002, making it the 6th leading cause of deaths in the world in 2016. Cancer overall is the 2nd leading cause of death

worldwide in 2018. The World Health Organization reports 18.1 million people developing cancers and 9.6 million dying from cancer worldwide in 2018. This amounts to about 1 in 5 in men and 1 in 6 in women dying from cancers around the world. The latest cancer statistics from Cancer Research UK states that 1 in 2 UK residents will have cancer diagnosed in their lifetimes. The most common cancers are lung, breast, colorectal, prostate, skin and stomach cancer, and the lung, breast, colorectal, stomach, and liver are the top five causes of cancer death. [5]

There are over 200 different types of cancer and all start with abnormal behaviour in a single cell or group of cells. A normal cell has a controlled cell cycle including division, duplication and cell death which regulates cell growth and differentiation, invasiveness and apoptosis. [6] It was first suggested by Peter Nowell in 1976 that genetic instability is the key to cellular evolution and mutation to cancer. [7] The transformation from normal cells to cancer cells involves a multistage process. [8] This is determined by the interaction between individual genetic factors and external factors which may be physical, chemical or biological. [9] When these changes happen in any cell, the cancer cell becomes uncontrollable and grows and divides unstoppably. When the cancer cells grow and accumulate into a solid tissue, the lump is then called a tumour.

Genetic abnormalities may lead cancer cells to behave differently from the normal cell through many biological functions such as cell proliferation, migration, adhesion and survival. It is also shown by microscopy that cancer cells have

different shapes from normal cells. [10] Cancers are classified pathologically by the tissue origin into five main types, carcinoma, sarcoma, leukaemia, and lymphoma and brain/spinal cord cancers. The Cancer Research UK has reported that epithelial malignancies account for 85% of cancers in UK. Carcinomas are more frequent in adults than in children and this frequency increases with age. [11] Carcinomas are also classified according to their organ of origin: eg colorectal cancer, breast cancer and lung cancer. In addition, modern genomics studies also allow a molecular-based classification system.[12]

With huge amount of research into treatments for different types of cancer, the death rate of cancer has declined over the past two decades. It has been reported that improved cancer treatment and especially screening has contributed greatly to this decreased death rate for cancers such as colorectal, breast and cervical cancer. [13]

Staging is an important and essential step in preparation for further treatment of any types of cancer. It was first proposed by Pierre Denoix between 1943 and 1952 that a Tumour, Node, Metastasis (TNM) staging system should be used for categorization of malignant tumours. [14] This TNM classification is continuously edited by the Union International Cancer Control (UICC) with clinical data collected worldwide. The TNM classification describes a cancer as T1-T4 for increasing size of primary tumour, N1-N3 for increasing spread to regional lymph nodes and M0 or M1 for absence or presence of distant metastasis. [15]

Additionally, TNM is separated into two types of classification, the clinical TNM (cTNM) which refers to cancer staging before treatment, and pathological TNM (pTNM) which refers to cancer staging after therapy or after pathological testing in the laboratory. It is also common to combine the TNM results into four stages such as stages I, II, III and IV. [1] For example, carcinoma in situ is grouped to Stage I cancer, and Stage II describes an invading tumour which might spread to regional lymph nodes. Progression through grades 1-4 is associated with increasing abnormality of tumour cells comparing to normal cells. [16] After staging, cancer treatment is selected depending on the individual specific cancer type. Until recently, the main treatments for cancer have been surgery and radiotherapy. Modern cancer treatment with high technology now involves many methods such as chemotherapy, hormone therapy and, increasingly, gene therapy and immunotherapy. Above all, the choice of cancer treatment depends on good communication between the medical team and the cancer patient. [2, 17]

1.1.2 Genetics and Physiology

Cancer is a genetic disease characterized by the emergence of deranged versions of normal cells, born out of aberrant molecular biology. Normal cells have a nucleus containing chromosomes which in total contain about 25,000 genes. A gene is a segment of deoxyribonucleic acid (DNA) that codes for an individual protein, and these proteins then control and modulate cell activities. Genes contain

unique messages that relate to cell types, functions, division and death. [8, 10, 18] Tumour development involves both promotion and escape from restraining forces. Uncontrolled cell-cycle progression is produced by this interaction. [19] Research into rare autosomal dominant monogenic cancer syndromes has greatly helped the understanding of cancer biology. The familial cancers may result from rare germline mutations in highly penetrant genes such as the BRCA1/2 mutation in breast cancer. [20] Alternatively, cancers result from acquired mutation, so-called sporadic cancer, and express few or no germline mutations. [21] Earlier studies have shown sporadic cancers such as breast cancer are influenced by environmental factors including diet and tobacco, and are associated with various genetic polymorphisms. [22] Chronic inflammation also plays an important role in the development of many carcinomas and this can involve infectious agents such as *Helicobacter pylori* in stomach cancer. [23]

Cancer is a complicated disease arising by a multistep process involving clonal expansion, genetic alteration and clonal selection with clone evolution within tissue ecosystems that follow the proven Darwinian evolution theory. [24] The cancer clone evolution involves selective sub-clone expansion. Darwinian natural selection can be advantageous to cancer cells, empowering clone expansion with characteristics of extensive proliferation, resistance to apoptosis, immortality, angiogenesis and metastasis. [25] It was first suggested by Douglas Hanahan and Robert A. Weinberg that there are six hallmarks of cancer. [26] These

six common biological capabilities involve tumour cell sustaining proliferative signals, irrespective of growth suppressors, resistance to apoptosis, unrestricted replicative potential (immortality), sustained angiogenesis and ability to invade surrounding tissue and metastasize. In addition, recent studies demonstrate the Warburg effect in which cancer cells favour metabolism of glycolysis with increased ATP production. This and tumour-promoting inflammation have been recorded as the seventh and eighth hallmark features. [27, 28]

1.2 Cancer Metastasis

1.2.1 General

The mortality of cancer is usually associated with cancer metastasis. Metastasis is the transport of cancer cells detached from the original tumour site to other parts of the body and formation of secondary tumours. [29] Metastasis is a multistep interrelated process. Through mutation, de-differentiated cancer cells are maintained by specific survival factors that prevent cancer cell death by apoptosis (programmed cell death). [30] Metastases are initiated through the overgrowth of cancer cells into a proliferative state, forming a tumour. Then, blood vessels form and penetrate the tumour. The tumour cells then break into the blood vessel and invade the circulation. [31] Until now, it has been generally considered that if tumour cells do not spread, the cancer can be cured. In fact, 99% of invading tumour cells will be destroyed by specific apoptotic processes, often involving the immune system. However, a small proportion of tumour cells invade the circulation, form tumour emboli via cell-cell attraction, and adhere to vessel walls, followed by extravasation.[32] In the process of metastasis, many glycoproteins play an important role in tumour cell survival in the circulation and cancer cell homo- and heterotypic adhesion. (Figure 1.2.1[29, 33, 34])

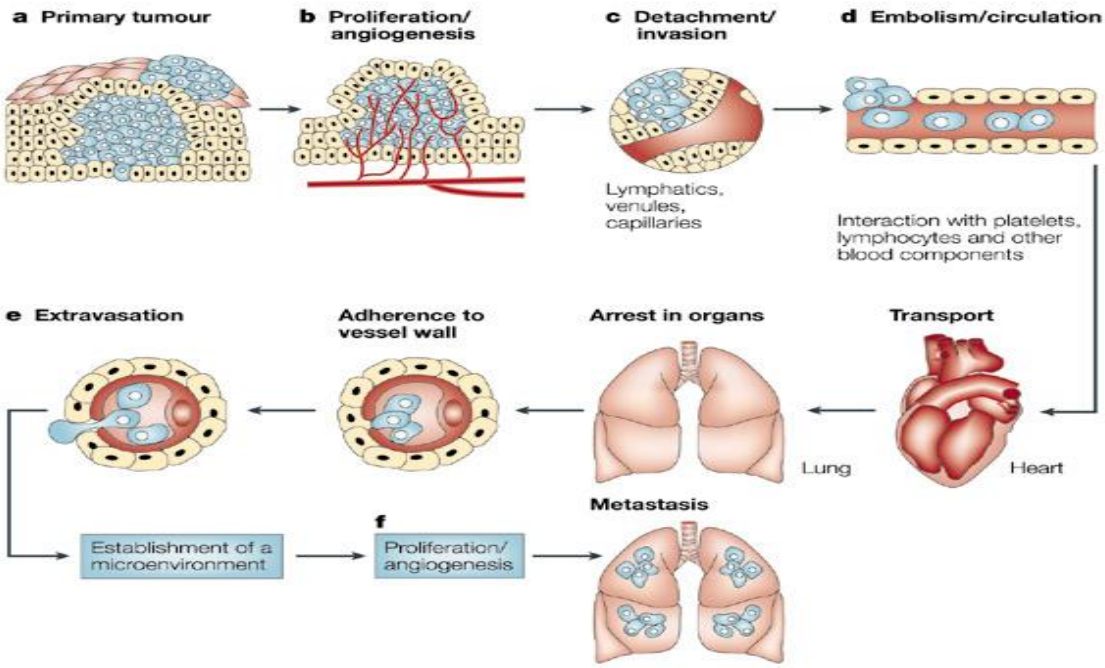


Figure 1.2.1: Major steps of cancer metastasis. (Adapted from *Fidler et al* [34])

Metastasis accounts for around 90% of cancer mortality and has given cancer its reputation as an incurable disease.[35] However, the survival rate of cancer has been rising dramatically in the past decade because of advances in early diagnosis and cancer growth inhibition. [36] Cancer survival rates vary according to the type of cancer, stage of cancer, treatment given and age groups. [5, 37-39]

Metastasis is a complex progress that involves detachment, migration, invasion and adhesion. [40] During angiogenesis, human endothelial progenitor cells are generated from both blood and bone marrow and differentiate into endothelial cells with functions in cell proliferation, tubulogenesis and neovascularization.[41, 42] Cell adhesion including cell-cell adhesion, and

adhesion to extracellular matrix (ECM) are important. [43] Normally, cells are kept within their defined boundary through cell adhesion which helps maintain their stability. In cancer metastasis, dysfunction of cell adhesion allows tumour cells to disseminate from their primary site to different locations through the circulatory system. [44] Cell adhesion is also an essential part of cell proliferation and survival pathways. Cancer adhesion molecules can be mainly divided into two groups: the calcium-dependent group including cadherin, integrin or selectins, and the calcium-independent group including members of the immunoglobulin superfamily and a homing cell adhesion molecule (HCAM) known as CD44. [45] The calcium-dependent cadherins include more than 100 members and are responsible for different locations like the E-cadherins at the surface of epithelial cell and the N-cadherin expressed in neuron differentiation. [46] Another major group of cell surface proteins is the immunoglobulin superfamily (IgSF) cell adhesion molecules known as Ig-CAM, and are characterized by their shared extracellular domains with immunoglobulins. Ig-CAM are calcium-independent transmembrane glycoproteins, and only have one Ig-like domain at the N-terminal which interacts with other Ig-like domains, integrins and carbohydrates. [45] The Ig-CAM can be classified into three classes: members with only a Ig-like domain; members with an Ig-like domain and at the other terminus an extracellular domain; members with an Ig-like domain and motifs. [47] The second group with C-terminal intracellular domains usually activate the extracellular interaction

within cytoskeletal or adaptor proteins, and can lead to cell signaling which might lead to tumour genesis. [48] It is also suggested that IgSFs can be used as biomarkers for cancer metastasis progression. For example, the melanoma cell adhesion molecule (MCAM), also known as CD146, is a novel prognostic biomarker for melanoma and breast cancer that also affects tumour cell adhesion and angiogenesis. [49] MCAM is highly expressed in the endothelial adherent junction, and affects blood vessel growth thus promoting neoplastic progression from benign to malignant states. [50] Another member of the IgSF, platelet endothelial CAM (PECAM), is also expressed in endothelial cell and hematopoietic cells. Recent studies suggested that soluble PECAM can be used in the diagnosis of late-stage metastatic progression related to its effect in regulation of endothelial cell adhesion and migration. [40]

1.2.2 Inflammation and Cancer metastasis

In the tumour microenvironment, inflammation can cause infiltration by innate immune cells such as macrophages, dendritic cells and myeloid-derived suppressor cells. It is also found that cancer-associated fibroblasts which mediate the tumour-induced inflammation, produce cytokines, chemokines and growth factors which as a result assist tumorigenesis by enhancing cancer cell angiogenesis, proliferation and invasion. [51] In the past decades, the study of interaction between inflammation and tumorigenesis has produced much

interesting evidence including genetic, pharmacological and epidemiological aspects. [52] The relationship between inflammation and cancer metastasis is now well established. Inflammation has an effect on tumour development's every aspect and might in a similar way influence cancer therapies' efficacy. [53] For example, inflammation is shown to take part in the pathways that lead to sporadic colorectal cancer (CRC). The mechanisms of cancer development mediated by inflammation remain unknown, and might vary in different forms of colon cancer. [54] Moreover, the function of cytokines, immune cells and other immune receptors has been explored by recent studies in colorectal tumorigenesis, and is involved in colorectal carcinoma initiation and metastasis. One of the most important risk factors in developing colorectal cancer is inflammatory bowel disease. [55] Anti-inflammatory drugs like anti-IL-6 antibody, STAT3 and NF-kB inhibitors, may potentially downsize the CRC risk, and clinical trials are ongoing. [56] However, anti-inflammatory drugs usually target mature myeloid cells and lymphoid cells, that won't develop oncogenic mutations. [57] It is suggested that a combination of anti-inflammatory therapy with other regular therapies like chemo- or radiotherapy, may increase the therapies' efficacy when preventing or treating colorectal cancer and colitis-associated cancer. [58-60]

Inflammation also contributes in the cancer microenvironment directly with a multifunctional effect on cancer cell proliferation and migration by secreting metastasis-provoking cytokines. It is known that cytokines including

chemokines, interleukins and their receptors are associated with risk for invasion, migration and metastasis. [61] Cytokines are small secreted proteins consisting of proteins, peptides and glycoproteins in different parts of human body. The molecular weights of most cytokines are around 5-20kDa, and they have fundamental functions in signaling pathways. Cytokines are highly inducible and play a key role in mediating intercellular signals in the immune system. [62] Cytokines can be categorized into various families such as tumour necrosis factors (TNF), interleukins, interferons, chemokines, lymphocytes and colony-stimulating factors. [63] Recent studies have shown that some of these proteins as well as their receptors act as mediators and influence numerous cell activities under physiological, pharmacological and pathological conditions. [64] Cytokines produced in infection, and inflammation in the tumour microenvironment, play an important role in carcinogenesis and metastasis. For example, in breast cancer, inflammatory cytokines play an important role in the regulation of angiogenesis and consequent prognosis. These pro-angiogenic cytokines have become a potential target for cancer therapy. [65] Recent data of pro-inflammatory cytokines including IL-1, IL-6 and TGF-β, demonstrate crosslinking between inflammation and cancer. [66]

Inflammation also has an impact on the activation of cytokine receptors and intracellular signal pathway by NF-kB which participates in tumour progression. [53] NF-kB transcription factors are critical regulators of innate

immune responses and inflammation. As a key mediating factor in inflammatory signals, NF-kB has received additional consideration as well as its upstream kinase, IKK, in associating between inflammation and cancer development. [67] Many proinflammatory stimuli activate NF-kB, mostly through IKK-dependent phosphorylation and degradation of inhibitor of kB (IkB) proteins. [68] Once activated, NF-kB dimers stimulate transcription of genes encoding cytokines, growth factors, chemokines, and anti-apoptotic factors. [69, 70] In inflammation, NF-kB plays a key role in mediating most effects of activation and organization of both innate immune system and the adaptive immune system. [71] NF-kB can be found in nearly all cell types in animals and is engaged in cellular development. [68, 72] Many structural studies found a structural homology with a Rel homology domain on the N-terminus in most NF-kB family members. This structural homology with retroviral oncoproteins known as v-Rel, has classified NF-kB as NF-kB/Rel proteins. Among the NF-kB, there are five members discovered in mammals: NF-kB1, NF-kB2, RelA, RelB and c-Rel. [73] NF-kB has two signaling pathways, the canonical and the non-canonical pathway. [74] The canonical pathway is initiated by binding and inhibited by the IkB kinase secreted from cytoplasm, and is predominantly applicable to the p50/RelA and c-Rel dimers. [75, 76] NF-kB has also been shown to have an essential effect in the activation of many proinflammatory cytokines secreted from several different cell types such as macrophages, lymphocytes and epithelial cells. Following conditional deletions of

IKKb, NF-kB is activated in the tumour microenvironment, and mediates the cytokine secretion, and therefore activates pro-carcinogenic pathways in tumour cells. [77] When NF-kB dimers are activated, numerous gene transcription of encoding cytokines including IL-6 and GM-CSF, chemokines including MCP-1 and MIP-1, adhesion molecules including ICAM and VAM, and several anti-apoptotic factors occurs. [78-81] Recent studies have shown that NF-kB acts as an evolutionarily conserved regulator of the genes coding for antimicrobial peptides, including beta defensins. [82] Given its function in gene coding molecule expression, NF-kB has a significant effect on the adaptive immune response involving the major histocompatibility complex (MHC) proteins, costimulatory and pro-inflammatory cytokines such as IL-6 and IFN-beta. [83] The activation of NF-kB is responsible for the secretion of some chemokines and cytokines which participate in lymphocyte migration and maturation

In the past decade, numerous studies have indicated that pro-inflammatory cytokines especially interleukins may stimulate tumour cell growth and survival of tumour cells. Recent studies have demonstrated the important contribution of interleukins, especially with their pleiotropic influence on tumour cell proliferation and metastasis. Past study on the expression of soluble interleukin-2 receptor α in nasopharyngeal cancer patients shows significant increase in patients with distant metastasis. It is known that IL-6 mediates estrogen synthesis and regulate aromatase activity, and promotes breast cancer metastasis. Recent

study shows that IL-6 acts as a genetic factor in breast cancer metastasis with a single nucleotide polymorphism (SNP) in IL-6. The SNP in IL-6: rs1800795 is suggested in monitoring breast cancer patients with distant metastasis.[84](IL-6 variant is associated with metastasis in breast cancer patients) [85] IL-6 also contributes to pro-tumorigenic activity in bone marrow microenvironment enhancing bone metastasis, and promotes cancer progression. The effect of IL-6 is associated with its multifunctional role in several signaling pathways. (Interleukin-6 regulation of transforming growth factor (TGF)-beta receptor compartmentalization and turnover enhances TGF-beta1 signaling) It is known that cancer progression involves abnormal cell proliferation, invasion and evasion from the immune system. Interleukins are well-known as mediators in stimulating immune response, and in relation to tumour growth and metastasis, members of the interleukin family such as IL-6 and IL-10 have different roles in the acute phase responses and immune reactions. [86] *In vitro* studies showed that interleukins promote tumour cell growth in many cancer types such as ovarian cancer, kidney cancer and skin cancer, via autocrine- and paracrine- dependent manners. [87, 88] Moreover, interleukins secreted by immune system cells will mediate the activity of immune system and the stage of malignancy. Recent studies suggested that interleukins have an impact on the treatment effectiveness and cancer survival rates. [89] In the past decades, it has been shown that other inflammatory cytokines such as tumour necrosis factor also play an important role

in cancer progression. It is reported that transforming growth factor-β (TGF- β) act as regulator in tumour cell proliferation and migration in many types of cancer such as breast cancer, colon cancer and pancreatic cancer. *In vivo* study has shown that IL-6 trans-signaling interacts with TGF-β signaling and prevents tumour progression and may be a target for cancer therapy. This relationship between TGF-β and interleukin-6 (IL-6) shows the importance of cytokine-cytokine interaction in cancer progression. [90]

1.2.3 The endothelium and cancer metastasis

Cancer cell adhesion to the endothelium is an important stage during cancer metastasis and it is therefore very important to understand the mechanisms that underlie it. [91] The endothelium is a thin layer between circulating blood and the vessel wall. It is therefore seen as the last barrier against tumour cell extravasation. Previous study using Arg-Gly-Asp (RGD) analogues, has shown that they interfere with the interaction between endothelial cells and metastatic cancer cells. The RGD peptide is a core sequence found in fibronectin. RGDs are ligands for αvβ3 integrin which is overexpressed on the surface of angiogenetic endothelial cells and many types of cancer cells. In lung and liver cancer, tumour metastasis is inhibited significantly by this analogue polypeptide without interfering with tumour cell proliferation. [92, 93] The RGD-αvβ3 integrin interaction on endothelial cell surface is therefore relevant to cancer therapy and

diagnosis and also has an important role in angiogenesis. Moreover, studies of chemotherapy combined with anti-adhesin therapy show a significant reduction of tumour metastasis compared to those with chemotherapy alone. [94] It is found that interaction of endothelium-cancer metastasis plays a positive effect on increased secretion of cell adhesion molecules which has an anti-tumour role in immune response, thus also preventing cancer metastasis. [95] Earlier studies demonstrated that tumour metastasis is decreased dramatically in B7-transfected melanoma cells. Thus, targeting the interaction of endothelial cells and tumour cells is a novel strategy in regulation and prevention of cancer metastasis. [96] Interaction between endothelium and tumour cells plays an important role in both hematogenous and lymphatic dissemination during cancer metastasis. This process is activated through complex pathways involving various multifunctional cytokines such as E-selectin, VCAM-1 and IgSF. [97, 98]

Cytokines can be produced by endothelial cells and can also regulate endothelial cell functions, and these highly inducible polypeptide mediators act as expression signals for leukocytes in various tissues and organs. Inflammatory cytokines like interleukin-1 and TNF modulate haemostasis and inflammation and potentially affect endothelial cell functions. The pleiotropic IL-1 acts as an effective inducer of procoagulant activity and is temporarily expressed in the endothelial cell. [99] Recent studies showed that IL-1 and TNF also mediate expression of endothelial adhesive molecules such as ICAM-1, and VACM-1. These inducible

glycoproteins which belong to the immunoglobulin (Ig) superfamily were found greatly increased by IL-1 and TNF and reached peak levels at about 6-8 h. Cytokine-mediated inflammatory activation in endothelial cells, induces the expression of adhesion molecules involving VCAM-1 and ICAM-1 which has an effect on the inflammatory cell adhesion leading to an adhesion cascade. Endothelial cells are activated by inflammatory cytokines and contribute in leukocyte recruitment which strongly raises the affinity of receptors for ICAM-1 and VCAM-1. These were found to be influenced by the conformational changes of these ligands which are members of integrin family and mediated by its cytoplasmic domains of the beta-2 chain. [100]

1.3 Lectins and Cancer

1.3.1 General and structure

Over the last decade, many studies have illustrated the presence of galactoside-binding proteins, especially various galactoside-specific lectins which bind to sugar complexes attached to protein and lipids, on the surface of various normal cells and tumour cell lines. Increasingly studies have shown that normal cells express endogenous galactoside-binding lectins as mediators of cell adhesion, such as hepatocytes and Kupffer liver endothelial cells. [101] Since 1988, lectins have been studied with a view to drug delivery. [102] The principle of lectin-mediated drug targeting is simple. As diseased cells, such as cancerous cells, express different glycans compared to normal cells, lectins are used as carrier molecules that depend on a specific protein-sugar interaction. [103] This concept can be applied not only for the gastrointestinal tract but also for other biological sites. [104] In recent studies our group has been interested in the interaction between galactoside-binding lectins, such as galectin-3 and PNA, and the Thomsen-Friedenreich (TF) carbohydrate antigen which is the core 1 structure of *O*-linked mucin type glycans in cancer progression and metastasis. [105]

Lectins are multivalent proteins that bind specific carbohydrates which may be displayed on the cell surface. Lectins play important roles in cell-cell interaction and communication based on their selective recognition of specific carbohydrates. Such carbohydrates include simple monosaccharides and

structures containing multiple carbohydrates, also called oligosaccharides. [106] Both types of carbohydrates are important ligands for lectins on cell surface glycoproteins. [107] Lectins were first discovered in 1888 in plants and are abundant in many foods. [108] Lectins help regulation of cell adhesion to glycoproteins. Many lectins participate in sugar-specific agglutinins in red blood cells and so are widely used to identify blood-type specificity. [109] Similar to the interaction of antigen and antibody, lectins can be inactivated by specific carbohydrate protruding from soluble glycoproteins. Characterization of the primary structural homology between animal lectins and plant lectins is not well defined, but they all show specific preferential binding to various mono and oligosaccharides. [110] It is possible that lectins from animals and plants may be coevolutionary. In recent years, lectins have been studied for their primary and three-dimensional structure and their biological functions in animal, plants, bacteria and viruses. [108] It was noticed that there is no significant sequence similarity in lectin families, but they shared high structural similarity. Thus, all lectins have specific lectin-carbohydrate binding activity restricted by amino acid residues, in the region known as carbohydrate recognition domain (CRD). The CRD typically identifies the non-reducing oligosaccharides of glycolipids and cell membrane glycoproteins. [111]

Lectins are characterized into a range of structurally distinct families. According to their different functional groups, they are separated into intracellular

lectins including P-type, M-type, L-type and the calnexin family; and the extracellular lectins involving C-type, R-type, siglecs, selectins and galectins. [112] With the help of identification of CRD amino acid sequences, the majority of lectins can be categorized into several structural superfamilies. C-type lectins (CTL) have a calcium-dependent CRD and are well-established animal lectins. The CTL superfamily members use a calcium ion to bind to carbohydrate through interaction with OH groups, the calcium also helps connecting the carbohydrate and glutamates. [113] On the other hand, galectins, or the so-called S-type lectins, are relatively much less abundant. Galectins are non-glycosylated, Ca2+-independent proteins and soluble galactose-binding lectins are found both intra- and extracellularly. The galectin family bind specifically to β-galactosides, such as N-acetyllactosamine (Galβ1-3GlcNAc or Galβ1-4GlcNAc). [111] Currently, there are 15 galectins that have been discovered in animals, and most are also identified in humans. Galectins have various functions in cell activities such as cell-cell adhesion, inflammation, immune response, angiogenesis, cellular proliferation, signalling and survival. [114] Galectin-3 expression changes dramatically with neoplastic progression in tumour cells of the head, neck, stomach, thyroid and nervous system. Nevertheless, the expression of galectin-3 in cells is also down-regulated in many cancers such as breast cancer, cervical cancer and uterine cancer. [115] In conclusion, modified galectin-3 expression in malignancies may affect cancer cell-cell interactions and cancer cell-normal cell interactions, and

therefore might have an effect on cancer cell angiogenesis and metastasis. [111]

Legume lectins, also known as L-type lectins, are found in the seeds of most legume plants. They form one of the largest lectin families and are homologous carbohydrate binding proteins. Legume lectins can be classified into several types occurring to their monosaccharide specificity. (Table 1.3.1) [116] [116] [116] [116] [116] Concanavalin A (Con A) is a mannose specific lectin found from Jack bean seed, and acts as a T-cell mitogen. Con A was showed to inhibit hepatoma growth in mice. *In vitro* study also showed that Con A inhibits cell growth of breast cancer cell and melanoma cells. [117, 118]The galactose/N-acetylgalactosamine specific binding lectin PNA, amaranth lectin (ACA) and jacalin are all TF antigen binding proteins. ACA and PNA showed increased colon cancer cell growth whilst jacalin inhibits colon cancer cell proliferation. In addition, jacalin mediated TNF-α and TGF-β secretion by colon homogenates.[119, 120] The N-acetylglucosamine specific lectin wheat germ agglutinin (WGA) was also found to bind to many different cancer cells such as colon cancer, pancreatic cancer and stomach cancer. With its binding in gastric carcinoma, WGA expression has been suggested as a useful prognostic indicator in stomach cancer. [121, 122]

Lectin	Origin	Carbohydrate specificity	Ligand	Reference
Concanavalin A (ConA)	Canavalia ensiformis	Mannose	α-D-mannosyl or α-D-glycosyl residues	[123]
Jacalin (AIL)	Artocarpus integrifolia	Galactose/N-acetylgalactosamine	Galβ1-3GalNAcα1-Ser/Thr (TF-Antigen)	[124]
Peanut agglutinin (PNA)	Arachis hypogaea	Galactose/N-acetylgalactosamine	Galβ1-3GalNAcα1-Ser/Thr (TF-Antigen)	[125]
Amaranth caudatus agglutinin (ACA)	Amaranthus caudatus	Galactose/N-acetylgalactosamine	Galβ1-3GalNAcα1-Ser/Thr (TF-Antigen)	[126]
Wheat Germ Agglutinin (WGA)	Triticum vulgaris	N-acetylglucosamine	GlcNAc or NeuNAc (sialic acid)	[127]

Table 1.3.1: Plant lectins and their carbohydrate binding specificities and ligands.

1.3.2 Glycosylation and Cancer

Glycosylation is one of the most significant post-translational alterations affecting a large field of biological interactions. These include cell-cell, cell-extracellular matrix interaction, and glycan-protein, glycan-lipid interactions. [128] Deficiencies in glycosylation in humans and their associations with disease have demonstrated that the mammalian glycobiome contains an impressive range of biological information. [129] Glycosylation, as a post-translational alteration, is characterized into five categories of glycans: *N*-linked glycans, *O*-linked glycans, *C*-

linked glycans, phosphoglycans and glypiation. [130] Characterizing each glycan's biological role, as well as those of glycan-binding proteins including sialic acid-binding immunoglobulin-type lectins and galectins, has been shown to make significant contributions to the cancer field. Each type of glycosylation interacts with specific cancer cell activity as well as with the tumour microenvironment. Altered epithelial glycosylation has been discovered in precancerous adenomatous polyps, inflammation and colonic disease. The *O*-linked mucin-type glycans that are initiated by GalNAc alpha-Ser/Thr are particularly affected. Changes include *O*-glycan shortening, increased sialylation, decreased sulphation and enhanced expression of oncofetal glycans including the Thomsen-Friedenreich antigen. [131] Till now, the mechanism for these glycosylation alterations is not well understood. *O*-glycans in carcinoma cells often carry a core 1 structure, while normal cells mostly carry *O*-glycans with the core 3 structure. In colon cancer, the GlcNAc-transferase activity is reduced, as a result, the synthesis of the core 3 structure becomes reduced. [132] The increased expression in colon cancer of high molecular weight splice variants of cell surface glycoprotein CD44 showing higher expression of unsialylated TF antigen, the PNA receptor, is found. [133]

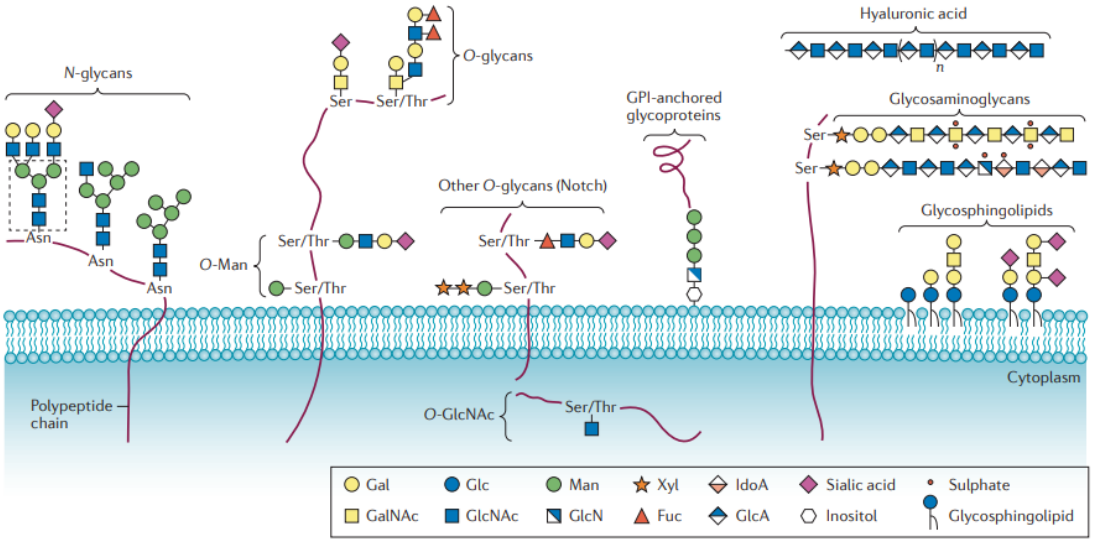


Figure 1.3.2: Glycosylation progression (Adapted from *Pinho et al* [129])

Glycosylation is an enzymatic process which results in linkages between glycans to proteins, peptides, lipids and other saccharides. (Table 1.3.2) The process of glycosylation forms glycoconjugates. According to the nature of their non-glycoside part, glycoconjugates are defined as glycoproteins, glycopeptides, glycolipids and lipopolysaccharides. Glycoproteins contain multiple oligosaccharide chains (glycans) covalently attached to a polypeptide structure. Glycosylation includes *N*-glycosylation, initiated by *N*-acetylglucosamine to asparagine and *O*-glycosylation, initiated by *N*-acetylgalactosamine to serine or threonine via attachment through nitrogen or oxygen respectively. [129] Recent studies have shown that altered glycosylation on the surface of cancer cells may act as a cancer biomarker. Quantification of such glycoconjugates in the serum of cancer patients has also been used effectively in serological surveillance for cancer diagnosis and monitoring. However, the application of these cancer biomarkers is

limited due to their wide expression by different types of cancer cells. Moreover there are problems with specificity as similar changes can also be found in some inflammatory diseases and non-neoplastic conditions. [134]

Malignant transformation can be associated with various kinds of altered cell glycosylation patterns that may affect glycosphingolipids, glycoproteins and glycolipids[135] Mechanisms for the altered glycosylation may include altered glycosyltransferase activity, altered substrate availability and Golgi disarrangement. [136]

Mucins are epithelial glycoproteins of large molecular weight and can be classified into different types with their unique structure and functions. The two main classes of mucins are secreted gel-forming mucins (e.g MUC2, MUC5AC) and transmembrane mucins (e.g. MUC1, MUC16). [137] The expression of mucins in colon cancer are altered. MUC1 overexpression in colon cancer correlates with cancer progression, while MUC2 expression is decreased in non-mucinous colorectal adenocarcinoma. [138] Intestinal mucins participate in the function of mucosal immune system. Mucin *O*-glycosylation starts in the Golgi when glycan (GalNAc) is added on to the hydroxyl domains of amino acid Ser or Thr. This results in the formation of GalNAca1-Ser/Thr (Tn). Tn is elongated into four core mucin-type *O*-glycans including TF antigen (GalNAc-beta1,3GalNAc-Ser/Thr, core-1). These core glycan structures are then varyingly extended by further glycosylation, sialylation and sulphation. [139] In colorectal cancer, MUC1 carries much shorter

and less branched oligosaccharides in comparison to normal epithelium. [140]

1.3.3 Galectin-3

Galectin-3 is a member of the galectin family and is a mammalian beta-galactoside-binding protein. There are 15 galectins expressed in mammals in many cell types. Galectins consist of a carbohydrate-recognition domain, containing 130-140 amino acids. [141] All of the galectin proteins bind to carbohydrates with terminal galactose residues. Galectins are expressed by various cell types, particularly epithelial cells, immune cells and endothelial cells. [142] Among the 15 proteins, galectins are divided into three groups: proto-type, chimera type and tandem-repeat type. Galectin-3 is the only member of the chimera type that contains a non-lectin N-terminal region of 120 amino acids connected to the only carbohydrate-binding side, which is similar to the proto-type. [143] However, it can form various polymers with this unique structure. It consists of three domains: one carbohydrate binding domain which is also called the COOH-terminal domain, a repeated collagen alpha-like sequence and a nonlectin NH2-terminal domain which is the action site of serine phosphorylation. Ligands bind to the carbohydrate binding sides and galectin-3 polymerization then occurs. [144] Galectin-3 is found in various locations both inside and outside the cell. It has various functions depending on its binding ligands. For example, when it binds to Bcl-2 in a variety of carcinomas, it prevents apoptosis and cell growth.

Circulating galectin-3 is a key factor in tumour cell adhesion and metastasis. Previous studies have shown that the expression of galectin-3 is increased under cancer conditions, and it is involved in the regulation of cancer cells in blood vessels through interacting with the Membrane-associated sialomucin episialin (MUC1) protein. Galectin-3 is increased up to 30-fold in the sera of various cancer patients. [145, 146]

1.3.4 Galectin-3-MUC1 interaction in cancer metastasis

Metastasis is a multistep processes. This involves progressive growth of the primary tumour, vascularization, invasion, survival in the circulation, adhesion, extravasation and finally progressive growth into secondary tumour. [147] Galectin-3 plays an important role on the tumour cell survival in the circulation and cancer cell homo- and heterotypic adhesion. Recent studies in our laboratory have demonstrated that the increased circulation of galectin-3 is an important metastasis promoter of cancer cell haematogenous dissemination. This galectin-3 effect is attributed partly by the galectin-3 interaction with the TF antigen on the large transmembrane mucin protein MUC1, which is also overexpressed in cancer cells. MUC1 is normally expressed around the surface of epithelial cells and, through its large size, blocks access to other cell surface adhesion molecules. [148] When circulating galectin-3 binds to the TF antigen of MUC1-mediated cancer cells, the smaller adhesion molecules or ligands on the cancer cell surface become

exposed as a result of MUC-1 polarization. These can then lead to tumour cell-cell aggregation and embolus formation. Secondly, the small adhesion molecules or ligands help epithelial tumour cell-vascular endothelial cell adhesion followed by cancer cell extravasation and growth into metastasis. (Figure 1.3) In addition, cancer cell adhesion and aggregation partly consequent to the galectin-3 interaction with vascular endothelial cells, also results in secretion of several metastasis-promoting cytokines by the endothelial cells. [149]

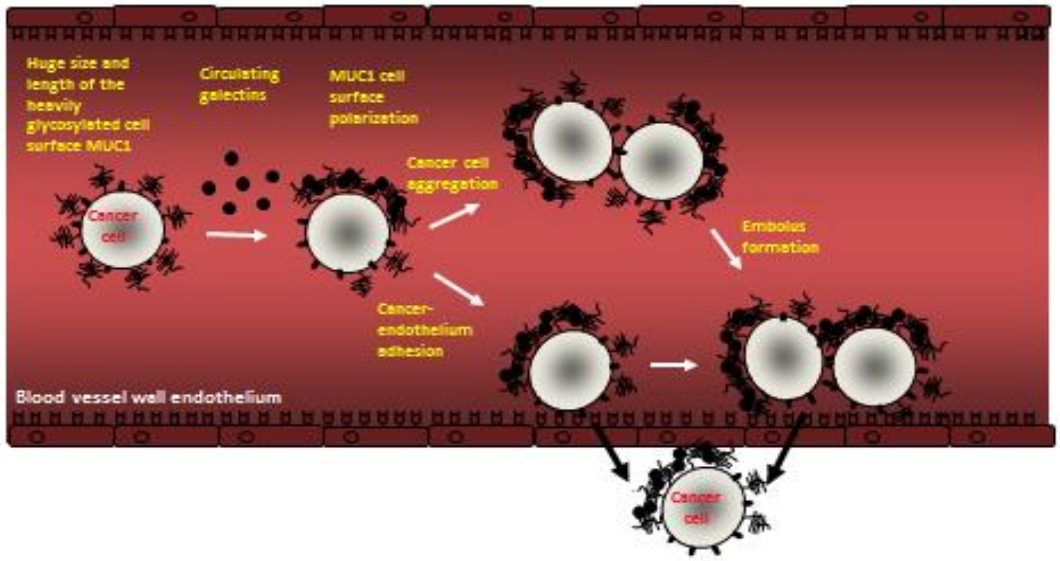


Figure 1.3: Galectin-3-MUC1 interactions in the sera of patients with metastatic cancer. (Adapted from *Chen et al* [150])

Galectin-3 in the blood circulation binds to TF-antigen carried by the large transmembrane glyocoprotein MUC1 that is expressed around the surface of cancer cells, and causes MUC1 polarization and thus exposes adhesion receptors on the surface of tumour cells. [151] These can then lead to tumour cell-cell aggregation and embolus formation. Secondly, interaction between the adhesion molecules and their ligands lead

to epithelial tumour cell-vascular endothelial cell adhesion followed by cancer cell extravasation and growth into a metastasis. [152]

1.3.5 Galectin-3 induces endothelial cytokine secretion

Many cytokines, in particular proinflammatory cytokines, such as IL-6, are well-known for their prometastatic promotion of cancer cell-endothelial cell adhesion. A previous study also showed that increased circulation of galectin-3 induces the secretion of four cytokines: IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF), and intercellular adhesion molecular 1 (ICAM-1) both *in vitro* and *in vivo*. (Figure 1.4, [153]) It is shown from previous study that galectin-3 induced secretion of cytokines by microvascular endothelial cells (HMVEC-Ls) is essential for galectin-3-induced adhesion of MUC-1 negative cancer cells to endothelial cells. Galectin-3 also induces endothelial cell surface adhesion molecule expression and is associated with autocrine or paracrine actions of galectin-3-mediated endothelial cytokine secretion. These actions are responsible for various important steps of the cancer metastasis cascade such as endothelial cell migration and tubule formation. In summary, circulating galectin-3 also has a MUC-1 independent action on cancer metastasis as a consequence of stimulating secretion of metastasis-

promoting cytokines. [153]

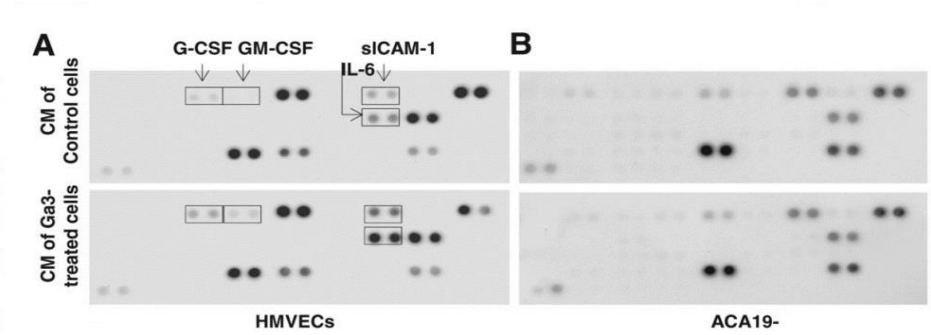


Figure 1.4: Galectin-3 induces secretion of IL-6, G-CSF, GM-CSF, and sICAM-1 from endothelial but not cancer cells. (Adapted from *Chen et al* [153]

Galectin-3 induces secretion of IL-6, G-CSF, GM-CSF, and sICAM-1 from endothelial but not cancer cells. Cytokine profile in the conditioned medium (CM) of 24-hour 1 μg/ml galectin-3 or BSA-treated HMVECs (A) or ACA19- cells (B). Treatment of H galectin-3 (1 mg/mL) for 24 hours resulted in increased concentrations of 4 cytokines: IL-6 (2.1-fold), G-CSF (2.2-fold), GM-CSF (3.2-fold), and sICAM-1 (2.3-fold) in the culture medium, whereas treatment of ACA19 cells with galectin-3 had no significant effect on cytokine abundances in the culture medium when the cytokine profiles were analyzed using a human cytokine assay array. This suggests that galectin-3 enhances cytokine secretion from HMVECs but not ACA19 cells. [153]

1.4 Peanut Agglutinin

1.4.1 General and structure

Peanut agglutinin (PNA) is a common dietary lectin and is derived from the peanut, *Arachis hypogea*. The molecular weight of PNA is about 110 kDa and it is a homo-tetrameric protein with four parallel chains each of 27 kDa. [154] PNA accounts for 0.15% of the total peanut weight and is highly resistant to degradation during cooking or digestion by gastric acid and enzymes in the small intestine. [155] Consequently it can be recovered in active form from faeces. Moreover, and remarkably, PNA rapidly enters the blood circulation in intact form after peanut ingestion. After ingestion of peanuts, intact PNA can be detected at 5μg/ml in the systemic circulation at 1-hour time point. [156] It presumably gains entry by binding to epithelial surface ligands followed by internalisation but the exact site and mechanism are unknown. The overall effect of the uptake of intact dietary lectins into the circulation might lead to important interactions with circulation and cellular glycoproteins and, therefore, affect cell-to-cell adhesion and proliferation. [157-159]

Previous studies have provided considerable epidemiological evidence for the role of diet in colorectal cancer causation. Patients who consumed 100g peanuts per day for five days were found on rectal biopsy to show considerable increase in epithelial proliferation. PNA remains active in roasted peanuts.[160] Moreover there is increased expression of TF antigen in hyperplastic and

neoplastic colonic epithelial cells *in vitro* and *in vivo*. [161]

PNA is shown to bind to many abnormal glycosylation sites on the tumour cell surface leading to homotypic and heterotypic cell adhesion that could potentially promote cancer metastasis. [162] In colon cancer, alterations in epithelial cell surface carbohydrate expression are common and are significant examples of abnormal neo-expression of oncofetal antigens. [163] These changes also happen in hyperplasia and malignancy. [161, 164] All these variations can be detected by different specific patterns of lectin-ligand binding. Previous study by our group has shown that PNA has mitogenic effects on normal colorectal epithelium, if low level TF expression is present, and on HT29 colorectal cancer cells. In addition, PNA has also been studied in lymphoid and some vascular muscle cells showing mitogenic effects. [165] PNA binding to endothelial cells is increased in colonic cancer and adenomas, whereas the binding site is concealed by further sialylation in healthy individuals. [166] Earlier study from our group has shown that PNA enhances both melanoma and colonic cancer cell growth and adhesion to endothelial cells, suggesting that PNA could act as a significant promotor in cancer metastasis. [167, 168]

PNA is not the major allergen responsible for peanut allergy. Allergy to peanut is usually lifelong and occurs in early age.[169] Peanut allergy is one of the most common food allergies and affects 2% of children in the UK. [170] The allergic process is initiated with activation of immunoglobulin E involving a series of

functional and regulator proteins. Mammalian IgE provides the first barrier against pathogens. [171] [172, 173] There are two fundamental receptors for IgE, the high-affinity receptor for immunoglobulin E (FcεRI) and the low-affinity receptor CD23. [174] In the human body, FcεRI is initially expressed on the cell surface of mast cells, basophils, eosinophils, monocytes and Langerhans cells. [175-177] The binding site of FcεRI-IgE is located on the Fcε3 domain of IgE, and is mediated by cross-linking with galectin-3 acting as a secretory protein through their N-glycans. [178] The binding of FcεRI to IgE activates its function and degranulates mast cell and basophils which plays an important effect in type I allergic reactions. [179] CD23 is a soluble calcium-dependent lectin and is expressed by B lymphocytes, monocytes and eosinophils. [180] CD23 acts as the promoter in cell growth of B lymphocytes, and synthesis of IgE. [181] The PNA which in contrast to the peanut allergenic glycoproteins, Ara h1/2, is considered as a minor allergen, is reported to be involved in the Ig-binding complex. [182] Moreover, recent studies have showed that PNA-binding is a new marker for histiocytes which are specific cellular members of the immune system that participate in both immune and non-immune cellular reactions. [183]

1.4.2 PNA binds to the Thomsen-Friedenreich antigen

Previous studies have provided much epidemiological evidence of diet and colorectal cancer causation. [184] Previous studies have shown that lectins like

PNA and galectins that bind to cancer cells such as human melanoma may cause increased metastasis. [185] Peanut (*Arachis hypogaea*) agglutinin, shows specific binding to beta-D-galactose residues, and has high affinity for the Gal (β1-3) GalNAc carbohydrate sequence which is also known as the Thomsen-Friedenreich antigen (TF antigen). As previously mentioned, TF antigen is expressed by the transmembrane mucin protein MUC1 which is overexpressed on cancer cell surface. [186] TF antigen is expressed in up to 90% of human cancers but is rarely expressed on healthy adult tissues. The TF disaccharide is a core carbohydrate structure of *O*-linked oligosaccharides on glycoproteins. [187] It is normally concealed by glycosylation, sialylation or sulphation in the normal epithelium but becomes expressed in pre-malignant and cancerous epithelia in various sites. It behaves as an oncofetal antigen, expressed normally in the foetus then disappearing to be expressed again in hyperplasia or malignancy. The increased expression of TF antigen is one of the commonest glycosylation changes in cancer and probably usually results from Golgi disarray resulting in a change in the site within the Golgi of expression of the β-galactosyl (l-3)-GalNac-α (2, 3) sialyl transferase. This could be a crucial early step in the development of colon cancer. [188] In human melanoma the interaction between PNA and TF antigen occurs with high affinity and contributes to cell proliferation and differentiation. [165]

1.4.3 PNA induces cancer cell proliferation

Previous studies have showed that PNA has mitogenic effects on colon cancer

cells, lymphocytes and on vascular smooth muscle cells. PNA at relevant concentrations achievable *in vivo* causes up to 50% increase in colon cell proliferation *in vitro* and, following peanut consumption, there was a similar (41%) increase in rectal mucosal proliferation *in vivo* in humans. Moreover, after ingestion of peanuts, there was a strong relationship between faecal hemagglutinating activity against TF-expressing desialylated red blood cells and rectal mitotic index. [160, 165] In addition, when combined with epithelial growth factor (EGF), PNA and EGF caused maximal stimulation of proliferation much greater than either agent alone. This indicated that there might be possibilities for interactions between dietary lectins and endogenous growth-related peptide molecules in the colonic epithelium. [155] The mechanism of PNA-induced proliferation is unknown, but the apoptosis-inducing effects are dependent on internalization of the lectins. [166]

1.4.4 PNA as biomarker in cancer

Studies have shown that the effect of PNA on the cell surface is blocked by overexpressed sialic acid thus shielding its ligand. PNA agglutinates human erythrocytes only when the Neu5Ac on the cell surface of erythrocytes has been removed by prior neuraminidase (sialidase) treatment. PNA induced agglutination can be used as a marker for red blood cell polyagglutinability. PNA is also a T cell mitogen. [189]

Binding between PNA and breast cancer cells is greater than PNA binding to normal cells, again due to increased expression of cancer specific TF antigen. [190] PNA-binding has been widely studied as a tumour marker for several malignant cell types including the bladder tumour cells and hematopoietic cells. [191] It has been shown that PNA predominately recognizes the galactosyl (β-1,3) *N*-acetyl-galactosamine (GalNAc) carbohydrate group which is commonly expressed as an O-linked glycan by tumour cells. [192] However it can also bind *N*-acetyllactosamine (LacNAc, Galβ1–4GlcNAc) with lower affinity than the TF antigen. [193] PNA also interacts with the fibronectin isoform HFL-1 and has therefore been suggested as a potential marker for diagnosis and detection of rheumatic disease. [194] Previous studies have use fluorescein labelled PNA as a marker for both B cell and T cell differentiation. [195, 196] Tagged PNA has also been used as a chemiluminescence biomarker in breast cancer histochemistry. [197]

1.5 Diet and Cancer

1.5.1 Epidemiology

Demographic studies have shown that the incidence of cancer is modulated by various changes in different populations. It is suggested that increased prevalence of unhealthy dietary and lifestyle habits are responsible for recent increases especially in colorectal cancer. [198] Diet is of great interest for its key role in both cause and prevention of cancer and in relation to different cultural models between countries. Earlier studies have estimated that diet may account for one quarter of risk factors for cancers in western countries, and is second only to tobacco for importance in the cause and prevention of cancer development. [199] There has been a significant increase in food consumption per person worldwide for the last fifty years although some developing countries such as Somalia and Rwanda remain at a low calorie consumption level. [200] Besides the remarkable improvements of food consumption level globally, the diet-pattern is also changed largely between developing and developed countries. For example, the consumption of calories from vegetable oil, meat and sugar has increased significantly in China during the past fifty years. [201] These diet pattern changes together with reduction in physical activities, will affect the nutrition-related non-communicable diseases especially cancer. The most popular cause of nutrition-mediated disease is overweight and obesity from having unhealthy dietary habits, particularly in the USA and Western European countries. [202]

In addition, several rapidly developing counties such as China, Mexico and Brazil are changing their traditional diets from grains and vegetables, into high fat and sugar meals with easier access to more meat and animal products as a benefit of their high rates of economic development. Globally, both developing and developed countries are experiencing a rapid change in their nutrition, with their various traditional diet cultures affected by the so-called 'westernised' diet pattern (high fat, sugar and salt). [203] As a consequence, the incidence of many diet-related diseases such as diabetes and several types of cancer is increasing dramatically worldwide. [204]

1.5.2 Diet and cancer development

It was first suggested in the 1970s that people having diets high in animal products might have high rates of cancers. [205] Studies of people migrating from one country to another showed that they soon acquired similar cancer rates as their new host country. This showed that in addition to genetic factors, environmental factors, particularly diet, must play a crucial role in determining country to country variations in cancer rates. [199] The dietary pattern of many countries changed substantially between the 1950s and 1990s with increased consumption of meat, vegetable oil and sugar. In parallel with these changes of food consumption, the cancer rates increased significantly in the past four decades. For example, in some countries the consumption from animal products has

increased ten-fold over forty years, while the incidence rate of colorectal cancer increased five-fold. When compared to Japan, people in the UK who consume a western diet pattern show a 35% greater incidence rate of colorectal cancer in men. [206]

Cancer risk may also be related to the ingestion of carcinogens as components of food. For example, a three-fold increase in cancer amongst people who consume bracken fern in Japan. [207] Epidemiological studies have also demonstrated the relationship between consumption of broiled food and reduced gastric cancer incidence rates in Americans. [208] Recent studies on the regulatory effect of consumption of soy isoflavone genistein and blueberry polyphenol on mammosphere formation in breast cancer, indicate that some dietary factors might also be beneficial and should be considered in further novel targeted cancer therapies. [208]

Many natural components have been reported to have potential in cancer chemoprevention and treatment. Natural phytochemicals such as cyanidins and genistein from grapes and soy inhibit angiogenesis, have antioxidant effects, and also affect cancer cell proliferation by interaction with growth factors. [209] Cyanidin, delphinidin, malvidin and pelargonidin are common anthocyanidins. Anthocyanidins are the de-glycosylated forms of anthocyanins. Anthocyanins are plant-extracted flavonoids and can be found in a range of fruits, seeds and vegetables in the forms of intact glycosides. Both *in vitro* and *in vivo* studies have

shown anti-cancer activities of anthocyanins. [210] Recent studies using eleven anthocyanin-rich plant extracts showed anti-proliferative effects in colon cancer. These include decreased cell proliferation by black lentil, sorghum and red grape extracts, and the inhibition of tyrosine kinase by two anthocyanins. [211, 212]

Nut consumption has also been associated with health and disease. Nut consumption has increased significantly along with the concept of balanced diet over the past century. Most of the beneficial effects have been claimed for tree nuts although some studies have also grouped ground nuts such as peanuts, which are legumes, together with tree nuts. Nuts are known to be rich in vitamin, fiber, minerals and high concentration of unsaturated fatty acid. Those bioactive compounds make nuts a unique healthy dietary plant sources in addition to fruit and vegetables. [213] Most previous studies have focused on their beneficial effects on diseases such as cardiovascular disease and type 2 diabetes. Nut consumption was reported to be associated with a reduction of all-cause mortality risk, the relationship between nut consumption and cancer is also studied worldwide. [214]

Most previous studies have focused on their beneficial effects on diseases such as cardiovascular disease and type 2 diabetes. Nut consumption was reported to be associated with a reduction of all-cause mortality risk, the relationship between nut consumption and cancer is also studied worldwide. [215] This result is however different from an epidemiologic study that involved ten European

counties and showed no association between nut consumption and colorectal, colon and rectal cancer in mixed gender. Further analysis of the subgroups in that study showed inverse relationship between colorectal cancer risk and increased nut consumption in women. [216] These results indicates that the relationship between nut consumption and risk of colon cancer and colorectal cancer. Is complicated and further investigation in this area is clearly needed.

Peanuts are rich in proteins and involved in daily diet across the world. Peanut consumption includes the nuts themselves and derivatives such as peanut oil, peanut butter. Peanut shells are also rich in functional polyphenol compounds. Previous study showed that polyphenolic extract of peanut shell reduced epididymal fat and reduced intracellular lipid accumulation and decreased cholesterol concentration in rats. Peanuts contain the polyphenol compound resveratrol which can inhibit colon cancer cell proliferation and vascularization.

The peanut lectin (peanut agglutinin – PNA) binds highly specifically to TF antigen. Other plant-based lectins with similar binding specificity include Jacalin (JAC) from jackfruit, Agaricus bisporus (ABL) from edible mushroom, Amaranthus caudatus (ACA) from amaranth grain, all of which showed to affect cancer proliferation in previous studies. They differ however in their receptor specifcities - ABL, JAC and ACA but not PNA also bind to sialylated-TF antigen. Whilst AMA has a similar proliferative effect to PNA, ABL and JAC inhibit colon cancer cell growth in a dose-dependent manner. Immunoblotting analysis suggested a strong binding

glycoprotein at 90 kDa by ABL, ACA and PNA but weakly for JAC. [119]

The work in this thesis will address the possibility that the peanut lectin, PNA, already known to enter the circulation in intact form after peanut ingestion, could, by mimicking the effects of the naturally occurring lectin galectin-3 have various effects in cancer metastasis. The mechanisms of these effects are also explored.

CHAPTER 2 Hypothesis and Aims

2.1 Hypothesis

PNA in the circulation may mimic the actions of endogenous galectin-3 by interaction with cancer cell-associated MUC1 and thus promote cancer cell adhesion and aggregation in tumour cell spreading. PNA appearance in the circulation may also act similarly to endogenous galectin-3 by interaction with the vascular endothelium and enhance endothelial secretion of metastasis-promoting cytokines, thus promoting metastasis.

2.2 The aims of this study

The aims of this study were to investigate the effect of PNA on endothelial cell secretion of cytokines, the underlying mechanism and the biological consequence in cancer metastasis.

CHAPTER 3 Materials and Methods

3.1 Materials

All T25/T75 cell culture flasks, 96-/24- ELISA plates, chemicals of analytical grade, Asialofetuin, PNA, FITC conjugated PNA and non-enzymatic cell dissociation solution (NECDS) were from Sigma (Poole, UK).

Calcein (AM) cell labelling solution (C3099) was purchased from Invitrogen (Paisly, UK).

Human G-CSF, GM-CSF, IL-6, ICAM-1 and MCP-1 ELISA kits were purchased from Peprotech (London, UK).

Biotin-conjugated PNA was purchased from Vector Laboratories Ltd (Peterborough,UK).

Human cytokine array kit (ARY005), human phospho-kinase array kit (ARY003b), Recombinant galectin-3 (MAB1154), antibodies against CD146/MCAM (MAB932) and CD144/PECAM-1 (BBA7) were purchased from R&D Systems (Abingdon, UK).

Antibodies against AKT (9272), phosph-AKT (Thr308), PRAS (2610), phospho-PRAS40 (Thr246), STAT3 (79D7), phosph-PRAS40 (Tyr705) were purchased from NEB (UK).

3.2 Cell Lines

Human microvascular lung endothelial cells (HMVEC-Ls) and human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Basel,

Switzerland) and cultured in endothelial growth media (EGM)-2 and EGM (Lonza) respectively.

Human colon cancer cells SW620 with MUC1 expression and MUC-1-negative human colon cancer cells HCT116 were obtained from the European Cell Culture Collections via the Public Health Laboratory Services (Porton Down, Wiltshire, UK) and cultured in Dulbecco's modified Eagle's medium (DMEM) and McCoy's 5a medium respectively.

The MUC1 negative human colon cancer cells HCT29-5F7 was kindly provided by Dr. Thecla Lesuffleur (INSERM U560, France) and cultured in DMEM medium.

The MUC1 negative human melanoma A375 cells ACA19- was kindly provided by Dr. John Hilkens (Netherlands Cancer Institute) and cultured in DMEM medium[217].

3.3 Cell Culture Medium

(1) EGM-2 and EGM bulletkits were purchased from Lonza. Complete EGM-2 (500ml/bottle) contains 10ml FBS, 0.2ml hydrocortisone, 2ml hFGF-B, 0.5ml VEGF, 0.5mll R^3-Insulin-like growth factor-1 (R3-IGF-1), 0.5ml ascorbic acid, 0.5mlhEGF and 0.5 gentamicin Sulfate and Amphotericin-B (GA-1000) and 0.5ml heparin. Complete EGM (500ml/bottle) contains 10ml FBS, 0.5ml hydrocortisone, 25ml L-Glutamine, 1ml EnGs, 0.5ml ascorbic acid, 0.5ml rhEGF, 0.5ml GA-100 and 0.5ml heparin. The serum-free EGM-2 and EGM use 25ml fetal bovine serum albumin (BSA) instead of FBS, and the other supplements remain the same.

(2) McCoy's 5a medium and DMEM were purchased from Sigma (Poole, UK). Complete McCoy's 5a medium and DMEM contains 10% FCS, 100U/ml penicillin, 100μg/ml streptomycin and 2mM glutamine. The serum-free medium use 0.5% BSA instead of FCS, and the other supplements remain the same.

3.4 Cell Counting

Cell samples were prepared to be evenly suspended in the culture medium. Cells were loaded 10μl on the hemacytometer slide, and counted under the microscope. The number of cells was obtained with the following equation: cell density (/ml)= number of cells per grid square x 10^4.

3.5 Cell Passage

Cells were incubated at 37℃ in the incubator with 5%CO_2 95% O_2. All cells were grown in T25 flasks with culture medium till reaching 60%-80% confluence. The cells were then released with 1ml trypsin (0.5mg/ml) diluted in PBS, and re-suspended in culture medium. Endothelial cells were then incubated for 2-4 hrs and cancer cells for 24 hrs. When most cells were attached on the bottom of cell culture flasks, cell medium was changed with fresh medium to avoid cell damage from trypsin. Endothelial cells were routinely passage at a 1:3 subculture ratio and cancer at 1:6. Endothelial cells were used within six passages and cancer cells were used within eighteen passages in all experiments.

3.6 Cell Thawing and Plating

The cell culture medium was warmed for 20 min before use in 37℃ water bath. The frozen vials of cells from the liquid nitrogen cell bank were also warmed up in the water bath. For cancer cells, cell supernatant was then added to 10ml of pre-warmed culture medium, and centrifuged for 4 min at 1000 rpm. The supernatant was discarded and cell pellet was re-suspended in 10ml pre-warmed culture medium and seeded into T25 flask. For endothelial cells, cell supernatant was added directly into T25 cell culture flask to avoid cell damage from centrifugation. Cells were then cultured in an incubator with 5%CO_2, 95% O_2 at 37℃. The culture medium was changed every four days.

3.7 Detachment of the Cells with Non-enzymatic Cell Dissociation Solution

The cell culture medium, NECDs were pre-warmed in water bath. Cells with 80% confluence in T25 flask were rinsed three times with 10ml sterilised PBS, and 1ml NECDs was added for 5-10 min at 37℃. When most cells had detached from the flasks, 10ml pre-warmed culture medium was added and cells were re-suspended with repeated aspiration using the pipette. The cell suspension was then used in cell counting or experiments.

3.8 Cell Adhesion Assay

EGM-2 or EGM and trypsin were warmed up 20 min ahead in water bath and conditioned medium were prepared together with endothelial cell monolayer

on Day 1. Endothelial cells with 80% confluence were released from T25 flask by trypsinization and re-suspended at 2x 10^6 /ml in pre-warmed EGM-2 or EGM medium. The cell suspension was then added 200µl/well to two 95-well plates for three hours (one for conditioned medium and the other for endothelial cell monolayer). When cells were attached on plates, culture medium was replaced with fresh EGM-2 or EGM medium to remove residual trypsin. The cells were then cultured in incubator overnight. On Day 2, endothelial cell monolayers were formed. For the conditioned medium plate, treatment of 4µg/ml of PNA or BSA in the presence of 20µg/ml ASF or antibodies against IL-6/MCP-1 were applied for 24 hrs. The cell supernatants were collected as conditioned medium on day 3. For the cell monolayer plate, endothelial cell monolayer was washed with sterilised PBS and conditioned medium were added into each well before assessment of cancer cell adhesion on day 3.

The cancer cells with 80% confluence were washed with PBS and released by NECDS on day 3, as a result, the cancer cell surface proteins would remain intact. Cells were then re-suspended at 5x10^6 /ml in serum-free medium in a 15ml tube before labeling with 10µl/ml Calcein AM for 30 min at 37℃. Cells were then washed with PBS and re-suspended at 1x10^5/ml with serum-free culture medium. Then 100µl/well Calcein AM-labled cancer cells were added into the endothelial monolayer plate for 1hr at 37℃.

The cells were gently washed twice with PBS before measuring the endothelial cell-associated fluorescence using the TECAN infinite F200 fluorescent microplate reader at 485nm excitation /535nm emission.

3.9 Electrophoresis and Lectin/Immunoblotting

Cells with 100% confluence were washed twice with 10ml PBS in T25 flask before adding 1ml sodium dodecyl sulfate(SDS)-sample buffer(prepared as below). The cells were incubated for 20 min at room temperature and cell lysates were collected in a 1.5ml tube and kept in freezer at -20℃.

Table 3.1 SDS-sample buffer:

	2 fold	**4 fold**
Stacking buffer	2.5ml	0
Glycerol	1.0ml (20%)	2ml
Mercaptoethanol	0.5ml (10%)	1ml
20% SDS	1.0ml (4%)	2ml
1% Bromophenol blue	50µl	0.1ml

Before running, the casting frames with glass plates were set up, and running gel (prepared as below) was added up to 1cm below the bottom green edge of the casting frame. Then 80µl saturated butanol was added to cover the gel creating a leveled top. After leaving the gel to set for around 40 min, butanol was then discarded. The gel was rinsed three times with 2ml ELGA water and the stacking gel was added (prepared as below) and comb inserted before leaving to set for around 20 min.

Table 3.2 Resolving buffer (1.5M Tris-HCl buffer):

	4 fold
Tris-base	36.3 g
Deionized water	120ml
2N HCl to pH 8.8	
Make up to	200ml

Table 3.3 Stacking buffer (0.5M Tris-HCl buffer):

	4 fold
Tris-base	12 g
Deionized water	120ml
2N HCl to pH 6.8	
Make up to	200ml

Table 3.4 Running gel preparation:

Separating gel	4%	7.5%	10%	15%
Deionized water	6ml	4.85ml	4.2ml	2.35ml
1.5M Tris-HCl resolving buffer	2.5ml	2.5ml	2.5ml	2.5ml
10% SDS	100µl	100µl	100µl	100µl
30% acrylamide	1.35ml	2.5ml	3.33ml	5ml
TEMED	5µl	5µl	5µl	5µl
10% ammonium persulfate (APS)	65µl	50µl	50µl	50µl

Table 3.5 Stacking gel preparation:

Stacking gel	3.75%	4.00%
Deionized water	3.09ml	3.05ml

0.5M Tris-HCl stacking buffer	1.25ml	1.25ml
10% SDS	50µl	50µl
30% acrylamide	0.625ml	0.665ml
TEMED	10µl	10µl
10% ammonium persulfate	65µl	50µl

When the stacking gel had set, the comb was carefully removed and the glass plates were removed from the casting frame and placed in the running electrode (short plates inwards). The electrode was then placed into running tank with 1X running buffer (prepared as below). Samples were heated for 10 min at 100°C before loading samples into wells of the running gel. The gel was first run for 30 minutes at 70V letting samples run through the stacking gel, and then fun for 90 minutes at 110V till the sample runs to the bottom of the gel.

Table 3.6 Running buffer preparation:

Tris-Base	**30.67g**
Glycine	64.04g
SDS	2.2g
Make up to	4L

After running, the plates were removed and rinsed with 1X transfer buffer to remove SDS. Running gel was removed carefully from the glass plates and transfer sandwich prepared as follows: Negative pole (black) - sponge pad - filter paper - gel - nitrocellulose membrane - filter paper - sponge pad - positive pole (white). Transfer sandwich was placed into transfer electrode in the transfer tank

with 1X transfer buffer (prepared as below). Gel was transferred for 60 min at 110V.

Table 3.7 Transfer buffer preparation:

Tris-Base	**12.12g**
Glycine	57.65g
Methanol	800ml
Make up to	4L

After transferring, the nitrocellulose membrane was carefully removed from transfer sandwich and placed into separate universals with blocking buffer (1% BSA in 1% Tween-20 in PBS). After blocking the membrane for 30 min at room temperature or 4°C overnight, first antibody diluted in blocking buffer was applied to the membrane and incubated on roller for 2hrs at room temperature. The blot was washed for 10 min with 10ml washing buffer (1% Tween-20 in PBSA) on the roller at room temperature. Blot was washed three times and the secondary antibody diluted in blocking buffer was applied for 1hr at room temperature. Blot was washed 5 times with 10ml washing buffer on roller for 10 min each. The washing buffer was replaced with Supersignal west solution (0.5ml of each solution from the kit). The binding on the blot was visualized using the Molecular Imager® Gel Doc™ XR system (Bio-Rad).

3.10 Cell Proliferation Assay

EGM-2 or EGM medium and trypsin were warmed up for 20 min in water bath before use. Conditioned medium was prepared on day 1 as follows:

Endothelial cells with 80% confluence were washed and released by trypsinization and re-suspended at $2x10^5$/ml with pre-warmed culture medium. Cells were then seeded with 2ml/well into the 6-well plates and incubated for 2hrs at 37℃ before replacing with fresh culture medium. Cells were incubated overnight at 37℃. Endothelial cell monolayers formed on day 2, and were treated with 4μg/ml of PNA or BSA in the presence of 20μg/ml asialofetuin (ASF) or antibodies against IL-6 (5ng/ml) or MCP-1 (40ng/ml) in the incubator for 24hr at 37℃. The conditioned medium from each well was collected for future use on day 3.

Endothelial cells used for assessment of cell proliferation assay with 80% confluence were washed and detached with NECDs on day 3. Cells were re-suspended at 5 x 10^4/ml with culture medium before application of 200μl/well to each well to be used in 96-well plates. The medium in each well was replaced with 200μl/well of conditioned medium after 1hr incubation. The cells were incubated at 37℃ for 24hr for the endothelial cell growth. The endothelial cells were labeled with 10μl/ml Calcein AM Cell Labeling Solution at 37℃ for 30 minutes.

Endothelial cells were washed twice with PBS and the endothelial cell-associated fluorescence was measured with the TECAN infinite F200 fluorescent microplate reader at 485nm excitation /535nm emission.

3.11 Sandwich ELISA Assay for Cytokine quantification in Cell Culture Supernatants Samples

EGM-2 or EGM medium and trypsin were warmed 20 min before using in water bath. Endothelial cells with 80% confluence were washed and released by trpsinization and re-suspended at $1x10^5$/ml with culture medium on day 1. Cell suspensions were seeded into 96-well plate and cultured in incubator for 24hrs at 37°C. Endothelial cell monolayers were formed and treated with a certain concentration of PNA or BSA for 24 hr on day 2. The cell supernatant was collected and the concentrations of cytokines in the supernatant were analyzed using Cytokine ELISA kits on day 3. (*Peprotech*)

ELISA half-well 96-plates were coated with 50µl of Cytokine Capture Antibody overnight at room temperature on day 2. The plates were washed three times with 100µl/well washing buffer (0.05% Tween20 in PBS) and 150µl of Reagent Diluent (1% Bovine Serum Albumin (BSA) in PBS, pH 7.2-7.4) was applied to each well for a minimum of 1hr as blocking buffer on day3. The plates were washed three times with 100µl/well washing buffer and 50µl of cell supernatant collected above was added to the wells. The 8 points' serial cytokine standard dilution were prepared and added 50µl to the wells. The plates were incubated for 2-4 hrs at room temperature. Then wells were washed three times with 100µl washing buffer and 50µl/well of detection antibody were added to the wells before incubating for 2-4hrs at room temperature. The plates were washed three times with 100µl washing buffer and 50µl Streptavidin-HRP were added to the wells and incubated for 20 min at room temperature. The plates were washed

three times with 100µl washing buffer before being introduced to 50µl developing solution (Sigma FAST OPD tablets were dissolved in 20ml distilled water) for 20 min at room temperature. When the colour of the wells turned to a yellow/orange colour, 25µl 4M sulphuric acid were added to stop the reaction. The cytokine concentration-associated optical density (OD) was measured with the TECAN infinite F200 microplate reader at 492nm with a reference at 595nm.

3.12 Assessment of Endothelial Cell Surface Adhesion Molecule expression by Flow Cytometry

Endothelial cells with less than 80% confluence were treated with 4µg/ml PNA or BSA for 1hr and released by NECDs. Cells were washed with 10ml PBS and centrifuged for 5 min at 1000 rpm. Supernatants were discarded and, 5ml 2% paraformaldehyde was applied to the cells for 25 min at room temperature. Endothelial cells were washed twice with 10ml PBS and centrifuged at 1000 rpm to remove the supernatants. Cells were counted and re-suspended at $1x10^6$/ml with PBS and blocked with 5% goat serum on the roller for 30 min at room temperature. Supernatants were removed by centrifugation at 1000 rpm for 5 minutes. Cells were washed and re-suspended at $1x10^6$ in blocking buffer (1% goat serum in PBS) before separating into 1.5ml tubes (1ml/tube). Antibodies against CD44 (1mg/ml), α5β1 (1mg/ml), α5β3 (1mg/ml), E-selectin (1mg/ml), VCAM (1mg/ml) and ICAM (1mg/ml) all in 1:400 dilution with PBS were applied to the cell solutions and incubated on the roller for 1 hr at room temperature or overnight at 4°C. Cells were then washed twice with PBS and FITC-conjugated

secondary antibodies were applied for 1hr at room temperature. Cells were washed three times and re-suspended in PBS in 0.5ml/tube. The expression of cell surface adhesion molecules was measured through BD FACSAria III and analysed by ImageJ.

Flow cytometry is a useful tool to measure a range of cell characteristics including cell size, number, and cell surface receptor binding. The procedure of flow cytometry involves several steps. After the cell suspension (10,000 cells per sample was used in this study) were injected into the machine, the cells pass through the laser excitation beam one by one and the fluorescence from the labelled cell surface adhesion molecules were recorded. Flow cytometry results were showed in a graph with cell numbers as against (X axis) fluorescent intensity (Y-axis).

3.13 Silver staining

The silver staining was conducted according to the instructions provided with the kit (R&D system). Tris-glycine gels (10%) were prepared as described in section 3.9. Protein samples from PNA purification assay were separated by SDS-PAGE in 10% gel. After protein separation by electrophoresis, the gel was fixed in 40% methanol/10% acetic acid for 30 mins. Then the gel was treated with oxidizer reagent for 5 mins and washed three time with deionized water for 5 mins each time till all the tallow colour was removed. The gel was then treated with silver reagent for 20 mins followed with washing with deionized water for 1 min. The gel was immersed in developer solution until protein bands appeared. The

reaction was stopped with 5% acetic acid. Gels was photographed using the Molecular Imager® Gel Doc™ XR system (Bio-Rad).

3.14 Mass spectrometry analysis

The Liquid chromatography-mass spectrometry (LC-MS) assay were performed by Dr, Deborah Simpson in the Institute of Integrative Biology with a QExactive quadrupole-Orbitrap mass spectrometer coupled to a Dionex Ultimate 300 RSLC nano-liquid chromatograph. The LC-MS procedure was the same as our previous work. [218] 1 µl of the sample was loaded onto a trapping column with loading buffer (0.1% Trifluoroacetic acid, 2% acetonitrile in water) for 7 mins at 9 µl/min flow rate. Then the trapping column was set in line using an analytical column. Peptides from the sample were eluted by a linear gradient of eluent A (0.1% formic acid in water) and B (0.1 formic acid in acetonitrile) (96.2% A: 3.8% B to 50% A : 50% B over 30 mins) at a low rate of 300 nl/min. The effluent was introduced into the ultrasensitive nano-electrospray ionization source in positive ion model. The data-dependent mass spectrometry was operated and data were analyzed as described in our previous work. The mass spectrometry analysis was conducted by Dr. Deborah Simpson in IIB in the University of Liverpool.

3.15 Statistics

Data were analyzed using independent t test, analysis of variance and one-way ANOVA followed by Bonferroni as appropriate.

CHAPTER 4 Investigation of the effect of PNA on the endothelial secretion of cytokines

4.1 Hypothesis and Aim

4.1.1 Hypothesis

The hypothesis of this part of the study is that the presence of PNA in the circulation after peanut ingestion may like, endogenous TF-binding galectin-3, also interact with vascular endothelial cells to promote endothelial secretion of cytokines that could promote cancer metastasis.

4.1.2 Aim

To assess the effect of PNA on endothelial secretion of metastasis-promoting cytokines.

4.2 Introduction

PNA is one of the commonest dietary galactoside-binding lectins. PNA binds highly specifically to the Thomsen-Friedenreich oncofetal carbohydrate (galactose β1, 3N-acetylgalactosamine, TF) antigen, which is expressed by the transmembrane mucin protein MUC1. [219] The TF disaccharide is a natural ligand of several different lectins and it is the core 1 carbohydrate structure of O-linked oligosaccharides on glycoproteins. TF antigen is expressed in up to 90% of human cancers but is rarely expressed on healthy adult tissues. [220] PNA is a tightly globular protein and is highly resistant to degradation during cooking or digestion by gastric acid and enzymes in the small intestine and can be recovered intact in active form from faeces. Remarkably, PNA can rapidly enter the blood circulation as an intact protein after peanut ingestion. After ingestion of peanut, intact PNA can be detected at 5μg/ml in systemic circulation at 1-hour time point. The mechanism of uptake is uncertain but from its speed of action it seems likely that it occurs in the stomach, presumably by internalisation after binding to cell surface TF-expressing glycoproteins. [155, 156]

Previous study in our group has shown the increased circulation of galectin-3 in various cancers and that this binds to the TF disaccharide on MUC1 on cancer cells and induces MUC1 cell surface polarization. [221] This leads to increased cancer cell adhesion to the vascular endothelium and cancer cell-cell aggregation for increased tumour cell survival in the circulation. It was also

revealed that galectin-3 binding to the TF-disaccharide on MUC1 induces cancer cell-endothelial adhesion. [151] More recently, circulating galectin-3 was found to interact with CD146 (MUC18) enhancing endothelial secretion of cytokines IL-6, G-CSF, GM-CSF and sICAM-1. [149] These cytokines are known to be metastasis-promoting cytokines that increase tumour cell spreading. This part of the study aimed to determine whether PNA can mimic galectin-3 and similarly affect endothelial secretion of cytokines.

4.3 Methods

4.3.1 Assessment of the effect of galectin-3 and PNA on cytokine secretion by microvascular and macrovascular endothelial cells

HUVECs and HMVEC-Ls monolayers prepared as above (Chapter 3.5) were similarly incubated with 4 μg/ml BSA and 1μg/ml, 2μg/ml of PNA, or 1μg/ml, 2μg/ml, 4μg/ml and 8μg/ml PNA for 24 hr at 37℃. For time-dependent assay, 4μg/ml PNA or control 4μg/ml BSA were introduced at 6hr, 12hr, 18hr, 24hr, 30hr, 36hr and 48hr at 37℃. Culture media were collected and levels of cytokines Il-6 and MCP-1 were determined by cytokine ELISA as described in section 3.11.

High-binding 96-well plates were coated with anti-cytokine (IL-6, MCP-1) antibody in coating buffer overnight at room temperature. The plates were washed with washing buffer and incubated with blocking buffer for 1 hr at room temperature. Prepared culture media were introduced to the plates for 2 hrs. Meanwhile, for the cytokine standard curve, pre-coated 8 wells were introduced with diluted standard cytokine with concentration of 0.03125μg/ml, 0.0625μg/ml, 0.125μg/ml, 0.25μg/ml, 0.5μg/ml, 1μg/ml and 2μg/ml for 2 hrs. Then specific cytokine detecting antibody was treated for 1hr at room temperature. After introduction of ExtraAvidin peroxidase (1:10,000 dilutions in blocking buffer) for 30 minutes, the plates were developed with SigmaFAST OPD and the absorbance was immediately read at 492 nm using a microplate reader. Cytokine levels in the culture media were calculated from the standard curve for each cytokine run in each assessment.

4.3.2 Human cytokine array

HMVECs ($1x10^5$ cells/ml) were cultured in 6-well plates pre-coated with 10 mg/ml poly-HEMA, for 24 hr in order to form a firm monolayer. Then the endothelial cells were treated with 4μg/ml PNA or control 4μg/ml BSA for 24 hr at 37℃. The culture media were collected and incubated with the pre-coated Proteome Profiler array membrane overnight at 2-8℃. These membranes can detect the levels of 36 different human cytokines, each in duplicate. The concentration of cytokines in the culture medium was analyzed according to the manufacturer's instructions. The levels of cytokines in the culture medium detected in the arrays were quantified with BioRad Image LabTM software.

4.4 Results

The vasculature in the human body is a very complicated structure. There is a heterogeneous population of endothelium from macrovascular and microvascular vessels in the human body. Galectin-3 and PNA circulate in human body and will adhere to either micro- or macrovascular endothelium. In order to mimic the performance of galectin-3 and PNA in the human blood circulation, both micro- and macro- vascular endothelial cells were used in this study.

4.4.1 Investigation of the effect of galectin-3 presence on cytokine secretion from HMVEC-Ls

Galectin-3 enhanced G-CSF secretion into the culture medium in a dose-responsive way. Galecin-3 at 2μg/ml was 12.52-fold higher, and galectin-3 at 1μg/ml was 4.35-fold higher than the BSA-treated group (P for both <0.001). Similarly, GM-CSF in response to 2μg/ml galctin-3 was 1.96-fold higher than the control group with a P value of 0.0003.

Galectin-3 showed similar dose-responsive stimulation of secretion of IL-6 at 1μg/ml and 2μg/ml galectin-3 treated groups 1.9- and 3.81- fold higher than the control group (P for both <0.001). Similarly, with galectin-3 at 1μg/ml and 2μg/ml, ICAM-1 levels in the culture medium were 2.04- and 2.44- fold higher than the BSA treated group (P<0.001 for both). (Fig 4.4.1)

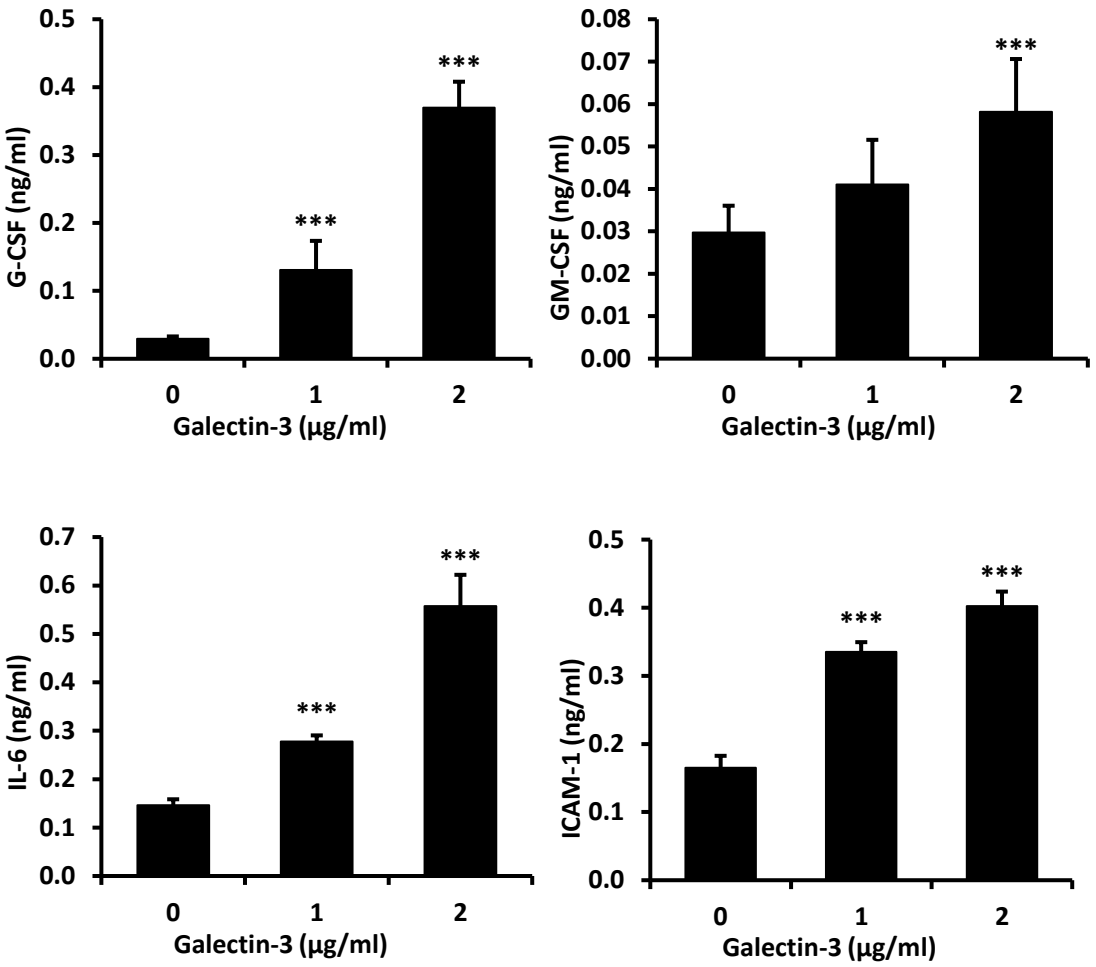


Figure 4.4.1: Galectin-3 induces secretion of G-CSF, GM-CSF, IL-6 and ICAM-1 from HMVECs

After incubation of HMVECs with 1µg/ml BSA, 1µg/ml galectin-3 or 2µg/ml galectin-3 for 24 hr, the levels of G-CSF, GM-CSF, IL-6 and ICAM-1 in the culture media (CM) were analyzed by ELISA. The data are from at least 3 independent experiments, each in triplicate. Data are expressed as mean value ± SD of triplicate. ***$p<0.001$. (One-way ANOVA followed by Bonferroni)

4.4.2 Investigation of the effect of galectin-3 presence on cytokine secretion from HUVECs

Similar dose-responsive cytokine release was demonstrated in response to galectin-3 by HUVECs: G-CSF from 2μg/ml galectin-3 treated cells was 28.92-fold higher, and 5.86-fold higher in the 1μg/ml group than the BSA-control group ($P<0.001$ and $P=0.0013$ respectively). A similar dose-response was shown for GM-CSF: galectin-3 at 1μg/ml and 2μg/ml causing 1.46- and 4.22- fold increase compared with control ($P=0.0067$ and <0.001 respectively), and also for IL-6: 1.8- and 4.58- fold higher than the control group ($P=0.003$ and <0.001 respectively), and for ICAM-1: galectin-3 at 1μg/ml and 2μg/ml inducing 2.51- and 4.75- fold secretion compared with the BSA treated group ($P=0.009$ and <0.001). In conclusion, the cytokine secretion from galectin-3 treated HUVECs was also dose-dependent. (Fig 4.4.2)

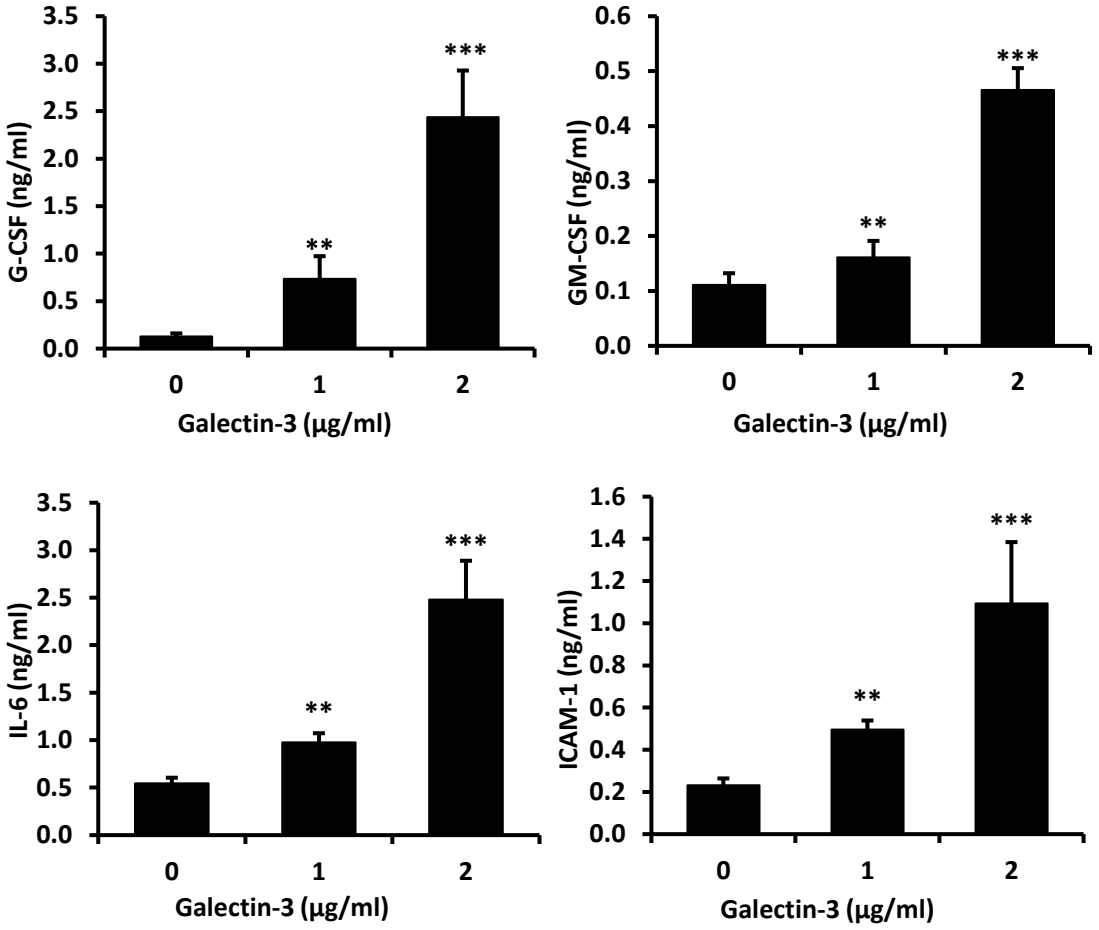


Figure 4.4.2: Galectin-3 induces secretion of G-CSF, GM-CSF, IL-6 and ICAM-1 from HUVECs

After incubation of HUVECs with 1µg/ml BSA, 1µg/ml galectin-3 or 2µg/ml galectin-3 for 24 hr, the levels of G-CSF, GM-CSF, IL-6 and ICAM-1 in the culture media (CM) were analyzed by ELISA. The data are from at least 3 independent experiments, each in triplicate. Data are expressed as mean value ± SD of triplicate. *P<0.05, **P<0.01, ***p<0.001. (One-way ANOVA followed by Bonferroni)

4.4.3 Investigation of the effect of PNA presence on cytokine(s) secretion in HMVEC-Ls

When HMVEC-Ls were treated with 4 μg/ml of PNA for 24 hr,, a concentration that has been found in the circulation after peanut ingestion, cytokine array assay for 36 of the most common human cytokines showed increase of two cytokines: Monocyte Chemoattractant Protein-1 (MCP-1)11.66-fold) and IL-6 (3.9-fold) in the culture media from PNA treated cells in comparison to that from the BSA-treated cells (control group). (Fig 4.4.3)

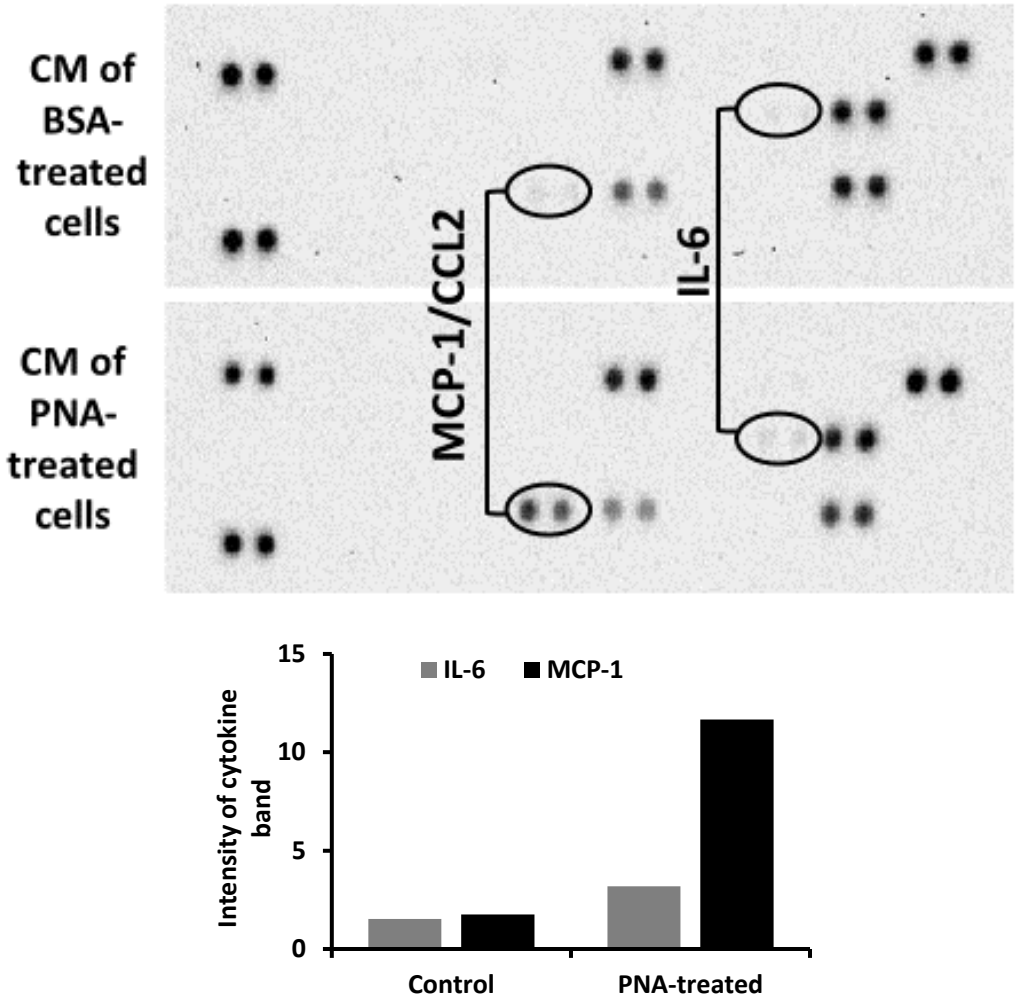


Figure 4.4.3: The presence of PNA induces secretion of two cytokines secreted from HMVEC-Ls

The levels of 36 common cytokines in the culture medium from HMVEC-Ls cells treated with 4 μg/ml PNA or BSA for 24 hr under suspension were assessed by cytokine protein array

4.4.4 Investigation of PNA effects on IL-6 and MCP-1 secretion from HMVEC-Ls

To investigate whether the IL-6 and MCP-1 secretions induced by PNA were dose-dependent, confluent HMVEC-Ls cell monolayers were treated with 4μg/ml BSA as control group, and different concentrations of PNA (1, 2, 4 and 8 μg/ml) for 24hr. Then the level of IL-6 and MCP-1 in the conditional medium were determined by individual cytokine ELISA.

Highly significant dose-response to PNA were seen for both cytokines. With PNA 4 μg/ml and 8 μg/ml IL-6 was 4.67- and 5.49- fold higher than the BSA treated control (P for both < 0.001) with lower but still significant (P<0.05) increase at the lower concentrations (1μg/ml and 2μg/ml PNA) tested. Similarly, MCP-1 showed 3.1 to 13- fold increase with 1μg/ml to 8 μg/ml PNA compared to control (P < 0.001). (Fig. 4.4.4)

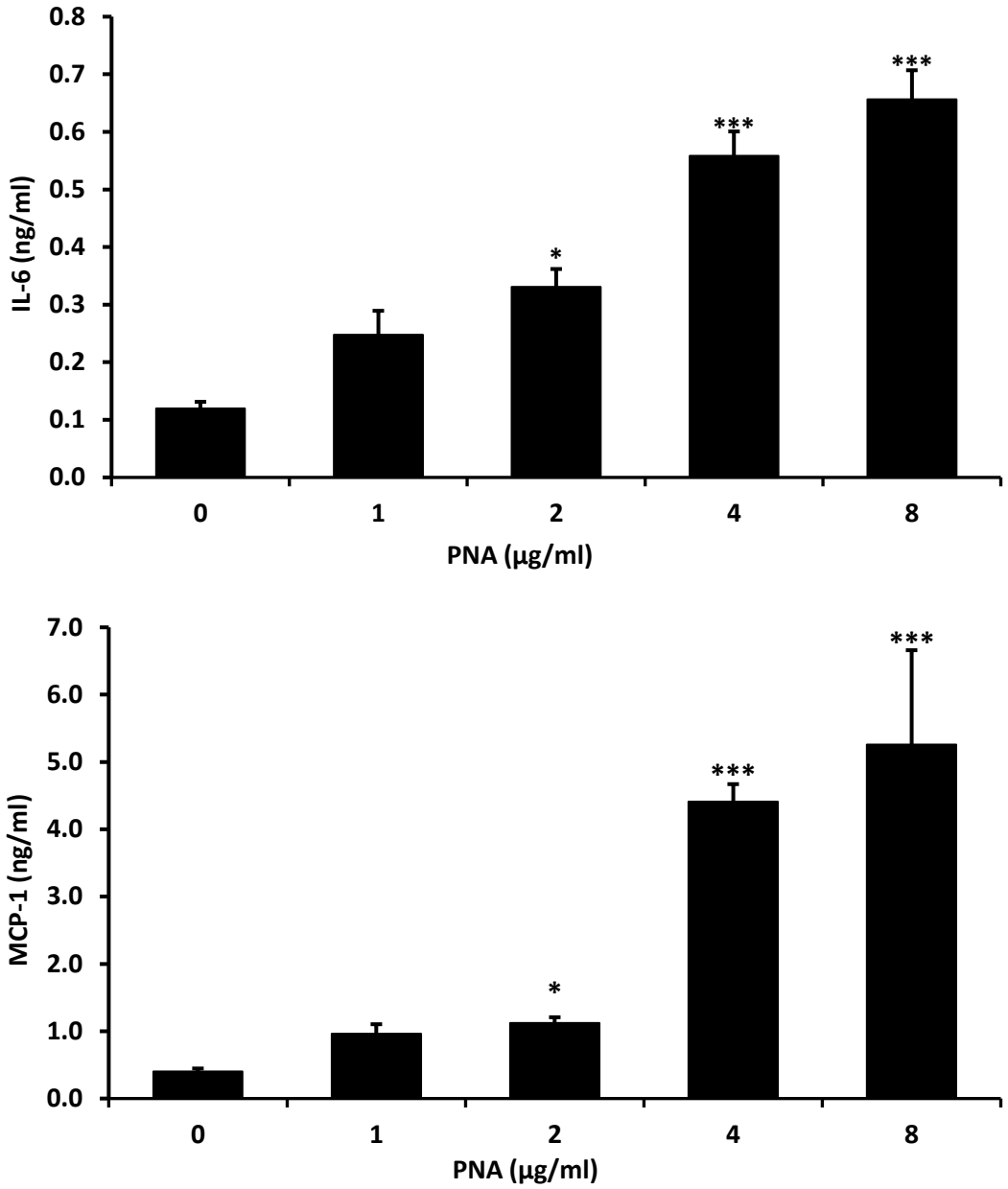


Figure 4.4.4: PNA induces secretion of IL-6 and MCP-1 from human micro-vascular lung endothelial cells (HMVECs)

After incubation of HMVECs with 4µg/ml BSA, 1µg/ml PNA, 2µg/ml PNA, 4µg/ml PNA,8µg/ml PNA for 24 hr, the levels of IL-6 and MCP-1 in the culture media (CM) were analyzed by ELISA. The data are from at least 3 independent experiments, each in triplicate. Data are expressed as mean value ± SD of triplicate. *P<0.05, **P<0.01, ***p<0.001. (One-way ANOVA followed by Bonferroni)

4.4.5 Investigation of PNA effects on IL-6 and MCP-1 secretion from HUVECs

To investigate whether the IL-6 and MCP-1 secretions induced by PNA were dose-dependent, confluent HUVEC cell monolayers were treated with 4μg/ml BSA as control group, and different concentrations of PNA (1, 2, 4 and 8 μg/ml) for 24hr. Then the level of IL-6 and MCP-1 in the culture medium was determined by individual cytokine ELISA. Again, similar dose-response to PNA was seen for both cytokines. (Fig 4.4.5)

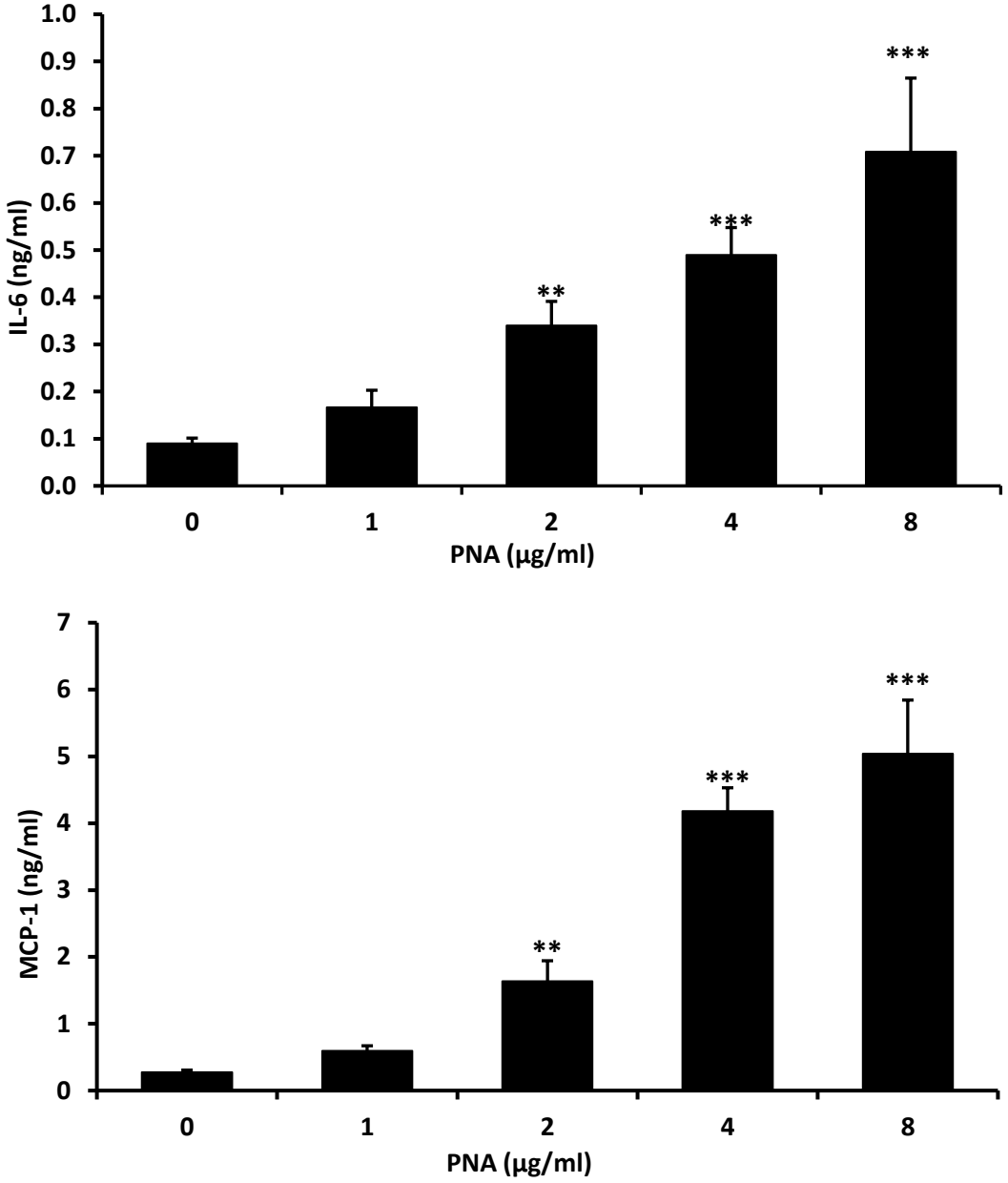


Figure 4.4.5 PNA induces secretion of IL-6 and MCP-1 from HUVEC

After incubation of HUVECs with 4µg/ml BSA, 1µg/ml PNA, 2µg/ml PNA, 4µg/ml PNA, 8µg/ml PNA for 24 hr, the levels of IL-6 and MCP-1 in the culture media (CM) were analyzed by ELISA. The data are from at least 3 independent experiments, each in triplicate. Data are expressed as mean value ± SD of triplicate. *P<0.05, **P<0.01, ***p<0.001. (One-way ANOVA followed by Bonferroni)

4.4.6 Investigation of the time-response of PNA effects on IL-6 and MCP-1 secretion from HMVEC-Ls

To investigate whether the IL-6 and MCP-1 secretions induced by PNA were time-dependent, confluent HMVEC-Ls cell monolayers were treated with 4μg/ml BSA as control group, and 4μg/ml PNA for 9 time points (0hr, 6hr, 12hr, 18hr, 24hr, 30hr, 36hr, 48hr and 72hr). Then the level of IL-6 and MCP-1 in the conditional medium were determined by individual cytokine ELISA. PNA at 4μg/ml induced endothelial secretion of IL-6 and MCP-1 progressively over the 72-hour time course of the incubation. (Fig 4.4.6)

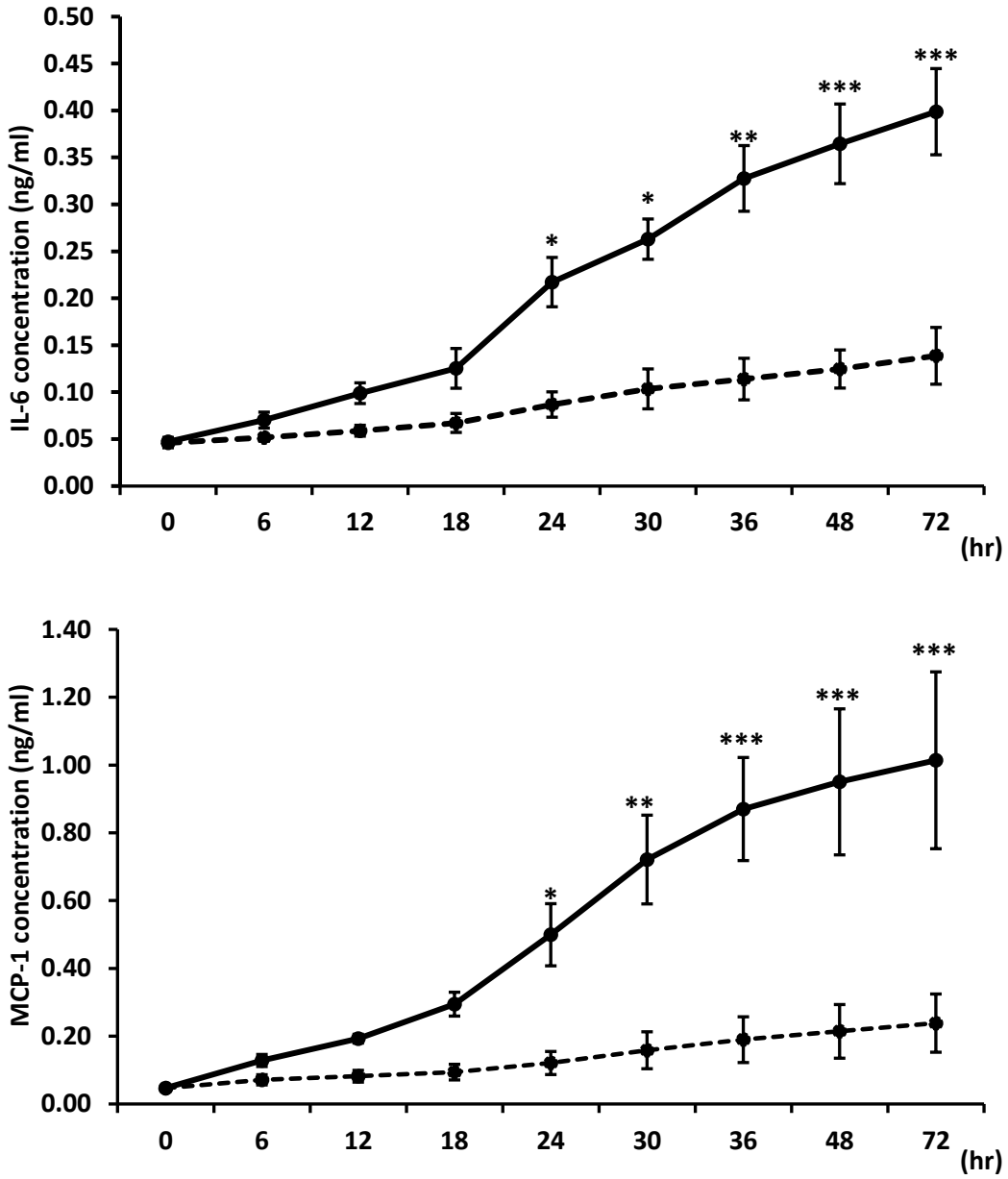


Figure 4.4.6 PNA induces secretion of MCP-1 and IL-6 from HMVEC-L*s*

HMVEC-L*s* were treated with 4μg/ml PNA or BSA for various time points, the cultured medium was collected and levels of IL-6 and MCP-1 in the culture medium were determined by IL-6 and MCP-1 ELISA. The data are from at least 3 independent experiments, each in triplicate. Data are expressed as mean value ± SD of triplicates. The PNA-treated cytokine secretion was analyzed by One-way ANOVA followed by Bonferroni, with comparison to the 0 hr time point *P<0.05, **P<0.01, ***p<0.001.

4.4.7 Investigation of the time-response of PNA effects on IL-6 and MCP-1 secretion from HUVECs

To investigate whether the IL-6 and MCP-1 secretions induced by PNA from HUVECs were also time-dependent, confluent HUVEC cell monolayers were treated with 4μg/ml BSA as control group, and 4μg/ml PNA for 9 time points (0hr, 6hr, 12hr, 18hr, 24hr, 30hr, 36hr, 48hr and 72hr). A similar time course was again seen for the cytokine response to PNA as in the microvascular cells. (Fig. 4.4.7)

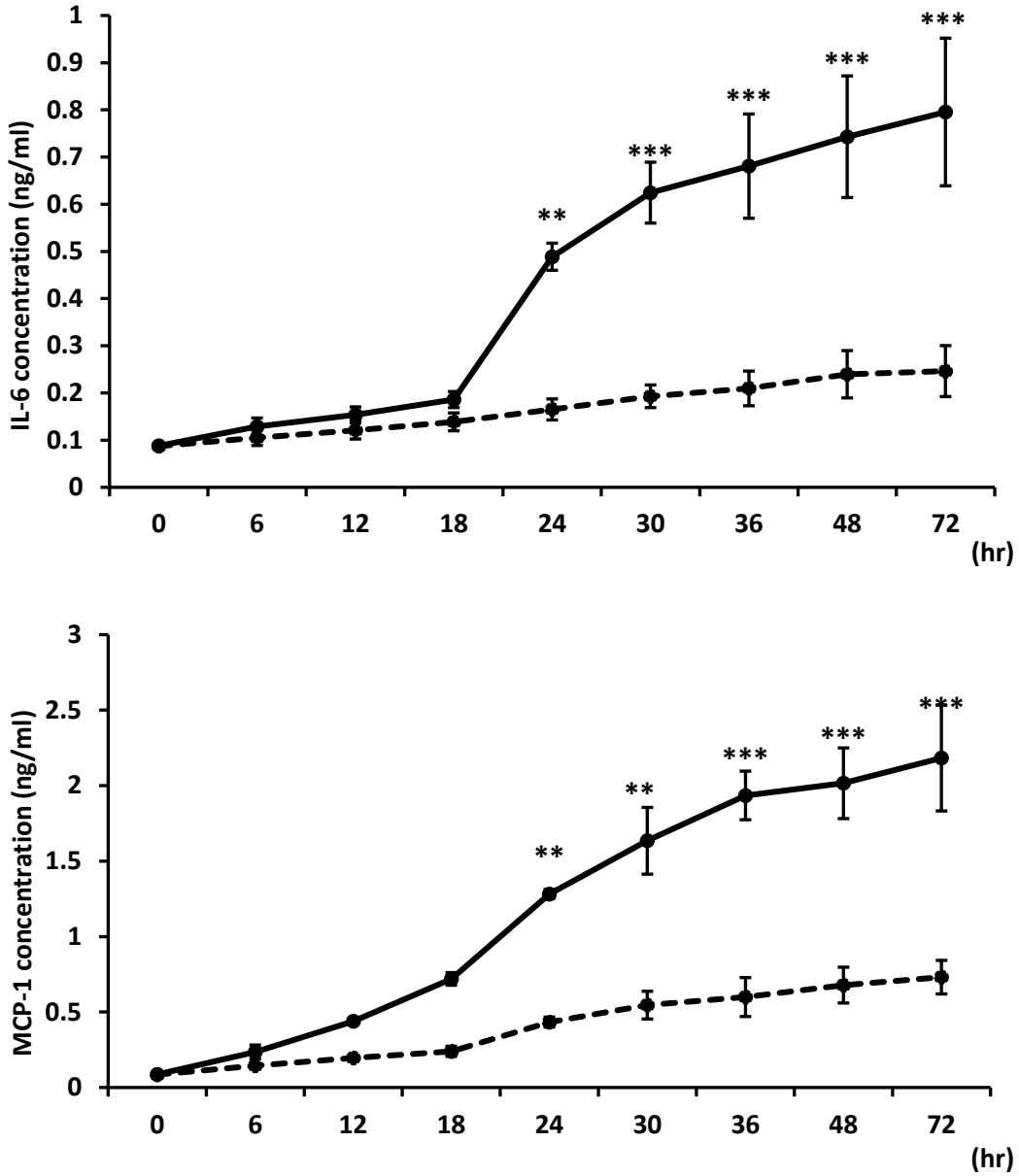


Figure 4.4.7 PNA induces secretion of MCP-1 and IL-6 from HUVECs

HUVEC were treated with 4μg/ml PNA or BSA for various time points, the cultured medium were collected and levels of IL-6 and MCP-1 in the culture medium were determined by IL-6 and MCP-1 ELISA. The data are from at least 3 independent experiments, each in triplicate. Data are expressed as mean value ± SD of triplicate.*P<0.05, **P<0.01, ***p<0.001. (One-way ANOVA followed by Bonferroni)

4.5 Discussion

This study confirms that the galactoside-binding protein galectin-3 induced secretion of G-CSF, GM-CSF, IL-6 and ICAM-1 in both micro-vascular and macro-vascular endothelial cells. It was found that the TF-binding protein PNA at concentrations found in the circulation after peanut ingestion induces dose- and time-dependent secretion of IL-6 and MCP-1 from both micro-vascular and macro-vascular blood endothelium *in vitro.* It is believed that circulating PNA binds to its receptor(s) on the surface of endothelial cells, and induces signaling leading to increased production of IL-6 and MCP-1. The mechanism of this interaction is unclear. It is known that cytokine secretion can be up-regulated following interactions with the cell surface protease-activated receptor (PAR1) which is a G protein-coupled receptor.

It is known that inflammatory and immune responses play important roles in the cause of malignancy. Changes in the inflammatory microenvironment of tumours are also believed to be important in cancer metastasis. [222] Since 1863 when cancer initiation was first reported to occur at sites of chronic inflammation, the relationship between inflammation, immune system and cancer is a major part in the understanding of cancer development. [223] It is postulated that tumour cells interact with various cytokines of the immune system such as chemokines and interleukins, in the promotion of metastasis. [224] Previous studies have proposed for example that IL-6 is a critical regulator in tumorigenesis and metastasis. [225] In this study, both of the two Thomsen-Friedenreich antigen-specific proteins, galectin-3 and PNA, induced the pro-inflammatory cytokine IL-

6. In addition, PNA also induced endothelial secretion of MCP-1 which is a chemoattractant cytokine.

Human IL-6 is a glycosylated protein which is around 21-26 kDa with 212 amino acids. [226] It is a multifunctional cytokine that is secreted, particularly by macrophages, in inflammatory, immune responses and infectious disease. Pathogen-associated molecular patterns (PAMPs) activate Toll-like receptors (TLRs) causing intracellular signaling which induces the production of different inflammatory cytokines on the cell surface and intracellular compartments. [227] IL-6 was first discovered as a B cell differentiation factor which induces the maturation of B cells into antibody-producing cells. [228] Besides its role in immune regulation, it plays an important role in the maintenance of hepatocytes, haematopoietic progenitor cells, the skeleton, the placenta, the cardiovascular system and the endocrine as well as nervous system. In addition, IL-6 is found to promote the expansion of progenitor cells by stimulating cells from the resting stage to enter the GI phase. [229] IL-6 also supports various physiological functions by acting as a hepatocyte stimulatory factor and by inducing acute-phase protein synthesis. [230] It is also known to stimulate osteoclast formation, induce bone resorption and is responsible for neural differentiation. [231] Thus, IL-6 is an important inflammatory cytokine with numerous functions on almost all the immune cells. [232] It plays an important role in regulating chronic inflammation, and thus mediates the tumour microenvironment and cancer cell growth. [233]

Recent studies have shown that IL-6 production is correlated with cancer development and metastasis so IL-6 blockade has been evaluated for cancer

treatment. [226] IL-6 blockade has also been used in recent novel treatment for various diseases such as rheumatoid arthritis, autoimmune diseases, chronic inflammatory diseases and especially many types of cancer including myeloma, pancreatic, cervical and colon cancers. [234] It is found that there is a particularly high level of IL-6 in the serum of multiple myeloma patients. Additionally, IL-6 acts as a major inhibitor of plasma cell apoptosis and is a significant cytokine in paracrine tumour-associated cell growth in multiple myeloma. [235] IL-6 is also seen as a potential immunomodulatory target in immunotherapy of pancreatic cancer. [236] IL-6 is overexpressed in the microenvironment of cervical cancer where it has a positive influence on the tumour angiogenesis and thus is beneficial to cervical cancer development. [237] In the most common and lethal gynaecological malignancy, ovarian cancer cells produce IL-6/IL-6R signalling which has an important impact on the ovarian tumour microenvironment. [238] In addition, IL-6 is also overexpressed in gastric cancer where it plays a similar effect by inducing angiogenesis and metastasis. It is suggested that increased plasma IL-6 can be used as a predictor in metastasis of gastric cancer. [239] Earlier data demonstrated that IL-6 stimulates cancer cell proliferation of colorectal cancer. [240] In conclusion, the multifunctional cytokine IL-6 secreted by the malignant tumours and inflammatory tissues, has a significant stimulatory effect on cancer cell growth and angiogenesis of many cancer types. Furthermore, previous studies in many cancer types, show that IL-6 secreted by human endothelial cells stimulates tumour cell growth and induces cell apoptosis. [241]

MCP-1 is a monomeric polypeptide protein which is about 13kDa with 76 amino acids. MCP-1 acts as a regulatory chemokine in infiltration and migration of

macrophages and monocytes, and is thought to be involved in many diseases such as cardiovascular disease, atherosclerosis, inflammatory bowel disease and also cancer. [242] Mice genetically lacking the receptor for MCP-1, show a significant decrease in arterial lipid deposition in an atheroma model. [243] It seems that MCP-1 has an important role in atherosclerosis through helping fat-laden monocytes transfer to the sub-endothelium. [244] Augmented plasma level of MCP-1 that follows coronary's balloon angioplasty arteries anticipates early restenosis. [245] A polymorphism in MCP-1 inducer has been shown to be related to a raised risk of coronary artery disease[246] and MCP-1 is over expressed from macrophage-rich atherosclerotic plaques in primates and humans. [247] Moreover, oxidized low-density lipoproteins, promote MCP-1 secretion by smooth muscle vascular cells. [248] MCP-1 can be viewed as a potential molecular connection between recruitment of foam cells and oxidized lipoproteins to the blood vessel wall.

MCP-1's overexpression in tissues results in a localized infiltration of macrophages and monocytes. [249] Thus experimental and clinical inflammatory bowel diseases show upregulation of MCP-1 in mucosal tissues. [250] In addition, previous studies show a comparable distribution of MCP-1 secretion from macrophages, vascular muscle and endothelium. [251] Drug therapy with the MCP-1 inhibitor bindarit has been shown to ameliorate trinitrobenzene sulfonic acid-mediated colitis in mice. [252]

MCP-1 and its receptors are found in most tumours and it participates in many host activities which impact on cancer development. [253] MCP-1 has

significant effects on tumour infiltration by macrophages and on angiogenesis and progression. MCP-1 directly mediates endothelial cell angiogenesis both in vitro and in vivo. [254] Immunohistochemistry results also showed high expression of MCP-1 in breast cancer cells, and proposed to have significant effect in macrophage recruitment and matrix metalloproteinases (MMPs) activation. [255] The serum levels of MCP-1 from primary ovarian cancer and recurrent ovarian cancer, is significantly higher than healthy women. It is suggested that MCP-1 acts both as a mediator of cancer cell growth and also of tumour-associated macrophage infiltration. [256] MCP-1 is also overexpressed in ovarian cancer xenografts from nude mice. [257] It is also suggested that MCP-1 can be used as prognostic marker in pancreatic cancer and has functional effect in tumour macrophage infiltration and tumour proliferation. [258] Recent studies using human gastric cancer cell line TMK-1, MCP-1 transfectants show that is has an important role in tumorigenicity leading to increased lymph node metastasis and also macrophage recruitment leading to cancer cell angiogenesis. [259] MCP-1 is also over-expressed in prostate cancer where it has functional effects on proliferation and invasion. [260] Serum MCP-1 is affected by diet - significantly higher in individuals taking a high-fat diet than either high-carbohydrate diet or control diet and this is thought to be relevant to prostate cancer development via activation of MCP-1/CCR2/Akt signalling. [261]

Given the various actions of IL-6 and MCP-1 in promoting metastasis, the increased secretion of IL-6 and MCP-1 from vascular endothelium in response to PNA could have an important impact on metastasis in cancer patients. It is also with great interest to study other plant lectins like jacalin which is also specific to

the TF-antigen, to see whether they will have influence on cytokine secretion from endothelial cells. Recent studies have looked into the interaction between jacalin and the macrophage surface, and found induced expression of five cytokines including IL-6 secretion induced in a dose-dependent manner. Also the mechanism of the increased cytokine secretion by PNA-stimulated endothelial cells remains unknown. It is suggested to study the intracellular cytokine signalling pathway and possible cell surface receptor(s) which might be responsible for the up-regulated cytokine expression.

In this study, we used both micro- and macro vascular endothelial cells to study the role of galectin-3 and PNA in blood circulation. The interior surface of blood vessels is a monolayer of endothelial cells - the endothelium. The endothelium is an important barrier and participates in inflammatory signalling pathways in the process of cancer metastasis especially extravasation. Microvascular endothelial cells (such as HMVEC-Ls) are found in small vessels such as the capillaries and macrovascular endothelial cells (such as HUVEC) are typically derived experimentally from the umbilical vein. HUVECs are commonly used in cell culture because of their stability, but HMVECs are more sensitive and show higher cell-cell variability. It is suggested that with their quick respond to pathology-related tumour microenvironmental alterations, HMVECs should also be selected to study cancer cell-related cell activities.

There has so far been little study of the impact of diet on metastasis although there are many studies linking dietary habits to cancer causation. There is incomplete evidence on how single nutrients, combinations of nutrients, over

nutrition, and energy imbalance, or the amount and distribution of body fat at particular stages of life, can influence risk for specific caners. Until more is known current recommendations for general health are to consume a mostly plant-based diet that includes at least five servings of vegetables, and fruits each day, to choose whole-grain carbohydrate sources over refined sources, and to limit saturated fat, alcohol, and excess calories. [262, 263] The possible impact of diet on metastasis risk in patients with pre-existing cancer clearly deserves much more attention

CHAPTER 5 Investigation of the effects of PNA-induced cytokine secretion on cancer and endothelial cell behaviour

5.1 Hypothesis and Aim

5.1.1 Hypothesis

PNA-induced secretion of IL-6 and MCP-1 from the endothelium may affect cancer and endothelial cell behaviour relevant to cancer metastasis.

5.1.2 Aim

To investigate the effect of PNA-mediated IL-6 and MCP-1 endothelial secretion on cancer cell-endothelial adhesion, endothelial cell growth, endothelial cell tubule formation and expression of cell surface adhesion molecules.

5.2 Introduction

As shown in the previous chapter, the tumour microenvironment produces a mixture of cytokines which may have direct or indirect effects in cancer metastasis. In particular, PNA was shown to induce increased secretion of IL-6 and MCP-1 from endothelial cells. These two cytokines participate in many cell activities such as cell adhesion, growth and migration. Previous studies in our group have shown that increased expression of the TF-binding lectin galectin-3 leads to increased IL-6 secretion that increases cancer cell-endothelial cell adhesion by increasing expression of endothelial cell surface adhesion molecules. Moreover, patients with metastatic cancer show increased expression of G-CSF, sICMA-1 and I-6, all of which are secreted from the endothelium in response to galectin-3. [149]

As a multifunctional pro-inflammatory cytokine, IL-6 shows a key role in many metastatic cancer types including breast cancer, prostate cancer, renal cancer and colorectal cancer. Clinical studies in untreated metastatic breast cancer showed the serum levels of IL-6 were higher in patients with more than one metastatic site than those with only one metastasis site. The level of IL-6 was higher in patients with liver metastasis, pleural effusion or predominant visceral disease. Patients with high serum level of IL-6 had a worse survival rate. [264] In renal and cervical cancer, IL-6 stimulates tumour cell growth in vitro. [265, 266] Activated Kupffer cells release cytokines including IL-6 in association with significant increase of colorectal cancer cell adhesion to both cancer cell and

endothelial cells, but the IL-6 alone does not have any effect on cancer cell adhesion to endothelial cells. [267]

As a member of the C-C chemokine family, endothelial MCP-1 promotes monocyte adhesion to the endothelium and accelerates arteriolar remodelling. [268] MCP-1 has a chemotactic effect on both HUVECs and HMECs that is dose-dependent. In the same study, MCP-1 was also shown to induce secretion of angiogenic factors including epidermal growth factor. Antibody-mediated inhibition of MCP-1 in mice implanted with human breast carcinomas led to increased survival time. [254] MCP-1 also has angiogenic effects by stimulating infiltrating macrophages to produce angiogenic cytokines. This is important since inflammatory recruitment of macrophages occurs in many tumours. [269] MCP-1 also promotes the inflammatory response by inducing cytokines including IL-6 and cell surface adhesion molecules. [270]

Studies in the previous chapter showed that the presence of PNA induces IL-6 and MCP-1 secretion from vascular endothelial cells. The study presented here aimed to investigate the potential impact of IL-6 and MCP-1 secretion induced by PNA on cancer and endothelial cell activity and behaviours. In addition, in order to clarify the PNA effect, we add a specific PNA inhibitor asialofetuin (ASF) in this study. ASF is a multivalent glycoprotein and contains both TF antigen and N-acetyllactosamine making it an inhibitor for PNA. In the experiment, ASF bind to PNA in the cell supernatant and inhibit PNA binding to endothelial cell.

5.3 Methods

5.3.1 Preparation of conditioned medium

HUVECs or HMVEC-Ls ($1.5x10^5$ cells/ml) were cultured in 96-well plates at 37°C overnight. When a firm monolayer was formed, 4μg/ml PNA was added to two groups (three wells/each), 4μg/ml BSA was added to one group (three wells, and 4μg.ml PNA + 20μg/ml ASF to another group (three wells) for 24 hrs at 37°C overnight. To one of PNA treated group and one of the BSA treated group (three wells), 5ng/ml anti-IL-6 antibody and 40ng/ml anti-MCP-1 antibody were added and incubated for 30 min. The conditioned media were then collected.

5.3.2 Assessment of cancer cell-endothelial cell adhesion

It has previously been shown that PNA-MUC1 interaction has a great impact on cancer cell-endothelial cell adhesion by PNA binding to TF on cancer-associated MUC-1. Both MUC-1 negative cancer cells ACA19- and HCT116 and MUC-1 overexpressed cancer cells SW620 HT29-5F7 were used here. ACA19-, HCT116, SW620 and HT29-5F7 cancer cells were detached using NECDS (to keep cell surface adhesion proteins intact.) After washing with PBS and resuspension at 5x106 cells/ml in DMEM, cells were labelled with 10μg/ml Calcein AM for 30 min at 37°C. Then cells were re-suspended at $1x10^5$ cells/ml with serum-free DMEM before application to new plates containing HUVEC monolayer with prepared conditioned medium. To one group of PNA-treated conditioned media, antibodies against IL-6 (10ng/ml) and MCP-1 (80 ng/ml) were introduced. The

cells were maintained at 37°C for 1 hr. After twice washing with PBS to remove unbound cancer cells, the monolayer was lysed with 0.25% SDS. The endothelial cell-associated fluorescence was read by the fluorescence microplate reader at 485nm excitation and 535nm emission.

5.3.3 Assessment of endothelial cell growth

HUVEC or HMVEC-Ls ($5x10^4$ cells/ml) were cultured in 96-well plates at 37°C for 1hr. The medium was then replaced with the conditioned medium and cultured for 24 hr at 37°C. Endothelial cells were then labelled with 2µl of Calcein AM for 30 min at 37°C. After three PBS washes, the endothelial cell-associated fluorescence was read using a fluorescence microplate reader at 485nm excitation and 535nm emission.

5.3.4 Assessment of endothelial cell tubule formation

HUVECs were seeded in a T25 tissue culture flask until 80% confluence. Phenol red free BD Matrigel matrix was thawed at 2-4℃ overnight in a refrigerator. The tips and 96-well plates to be used were also kept in the refrigerator overnight. Fifty µl Matrigel were aliquoted into each well of a 96-well plate on ice. The plate was incubated at 37℃ for 1 hr to allow gel formation and make sure no air bubbles were trapped in the Matrigel.

A T25 flask of HUVECs was released with 1ml trypsin and washed with PBS. After centrifugation at 1000 rpm for 5 minutes, the cells were re-suspended at a concentration of $1x10^5$ cells/ml with conditioned medium and seeded at 100μl per well to a 96-well plate for 16-24 hr at 37°C.Tubule formation was recorded using a Leica dissecting microscope. Micro-photographs of the whole wells of each group were taken and the number of tubules and nodules as well as the length of each tubule were measured and recorded. Tubule length and branch points were calculated using Image J software.

5.3.5 Assessment of expression of cell surface adhesion molecules by flow cytometry

HMVEC-Ls were treated with conditioned medium for 24hr before they were released by NECDs. Cells were then washed and fixed with 1ml 2% paraformaldehyde. After fixing, cells were washed again and re-suspended at $1x10^6$/ml with PBS. Cells were then incubated with 5% goat serum and washed again before incubating with antibodies against CD44 (1mg/ml), α5β1 (1mg/ml), α5β3 (1mg/ml), E-selectin (1mg/ml), VCAM (1mg/ml) and ICAM (1mg/ml) all in 1:400 dilution with PBS. Cells were then washed and incubated with FITC-conjugated secondary antibodies. Cells were washed and re-suspended in PBS in small 0.5ml tubes. The cell surface expression of CD44, α5β1, α5β3, E-selectin, VCAM and ICAM was analyzed by BD FACSAria III and analysed by ImageJ.

5.4 Results

Previous studies from our group have shown that interaction between PNA and TF antigen carried by MUC1 cause several cell activities involving enhanced cell adhesion and proliferation. Therefore, in order to assess the impact of the PNA-MUC1 interaction, the MUC1 negative cancer cell line ACA19- and HCT116 were used together with MUC1 positive cancer cell lines SW620 and HT29-5F7. In this study, conditioned media is used on same condition (passage, cell number) of endothelial cells. It is known that after treating with PNA, endothelial cell proliferation will increase. In order to test the effect of PNA-mediated cytokine secretion on endothelium, cell supernatant is prepared ahead with PNA treatment, and applied on fresh endothelial cell monolayer with same passage and cell numbers. This avoids the higher number of endothelial cells in PNA-treated group, and other possible element activated by PNA binding to TF antigen on endothelial cell surface.

5.4.1 PNA-induced cytokine secretion increases cancer cell heterotypic adhesion with microvascular endothelial cells

The conditioned medium obtained from PNA-treated HMVEC-Ls increased adhesion of ACA19- and HCT116 cells adhesion to HMVEC-Ls monolayers by 100±13% and 72±10% in comparison to the BSA-treated control group. This PNA-induced increase of cancer cell adhesion was mostly inhibited by ASF, and partially inhibited with the present of combination of antibodies to IL-6 (5ng/ml) and MCP-1 (40ng/ml). The PNA-pretreated group also induced adhesion of SW620 and HT29-5F7 cells to HMVEC-Ls by 129±11% and 141±20% when compared with the

control. The PNA-induced cancer cell adhesion was fully inhibited by ASF and partially inhibited by IL-6/MCP-1 antibodies. (Fig 5.4.1)

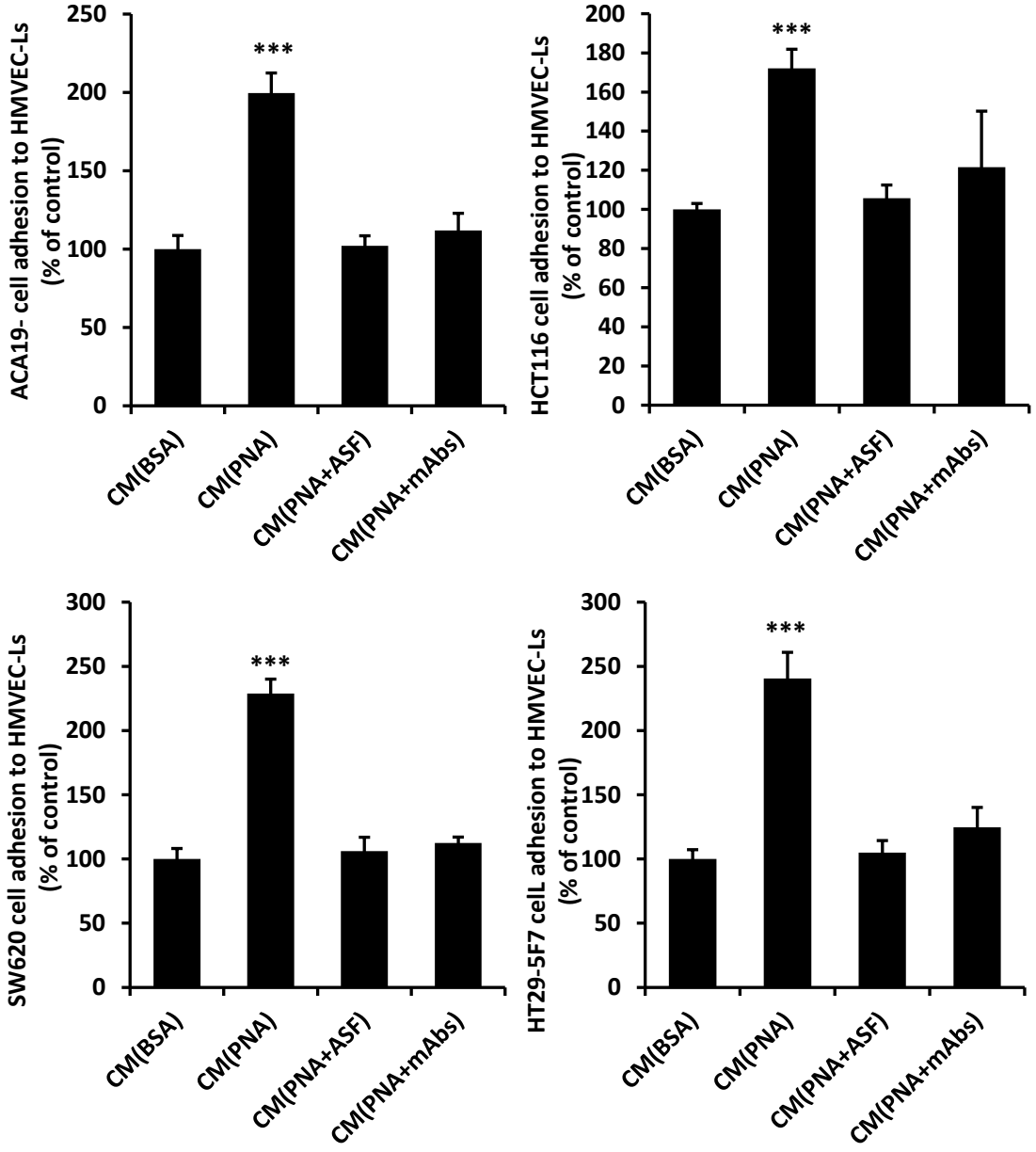


Figure 5.4.1: PNA-mediated endothelial cytokine secretion increases heterotypic cancer cell adhesion with microvascular endothelial cells (HMVEC-Ls)

HMVEC-Ls monolayers were prepared with conditioned medium. Cancer cells ACA19-, HCT116, SW620 and HT29-5 adhesion to fresh HUVECs with or without a combination of neutralizing antibodies against IL-6 (5ng/ml) and MCP-1 (40ng/ml). The data are expressed as percentage compared with BSA-treated controls from at least 3 independent experiments, each in triplicate. Data are expressed as mean value ± SD of triplicate. *P<0.05, **P<0.01, ***P<0.001. (One-way ANOVA followed by Bonferroni).

5.4.2 PNA-induced cytokine secretion increases cancer cell heterotypic adhesion with macrovascular endothelial cells

The conditioned medium obtained from PNA-treated HUVECs increased adhesion of ACA19- and HCT116 cells to HUVEC monolayers by 105±12% and 94±9% in comparison to the BSA-treated control group. This PNA-induced increase of cancer cell adhesion was mostly inhibited by ASF, and partially inhibited in the presence of a combination of antibodies to IL-6 and MCP-1. The ACA19- and HCT116 cell adhesion assays were repeated six times and each experiment in triplicate. The PNA-pretreated group induced SW620 and HT29-5F7 cell adhesion to HUVECs by 147±26% and 164±26% when compared with control. The PNA-induced cancer cell adhesion was fully inhibited by ASF and partially inhibited by the combination of antibodies to IL-6 and MCP-1. The SW620 and HT29-5F7 cell adhesion experiments were repeated five times with each experiment in triplicate. (Fig. 5.4.2)

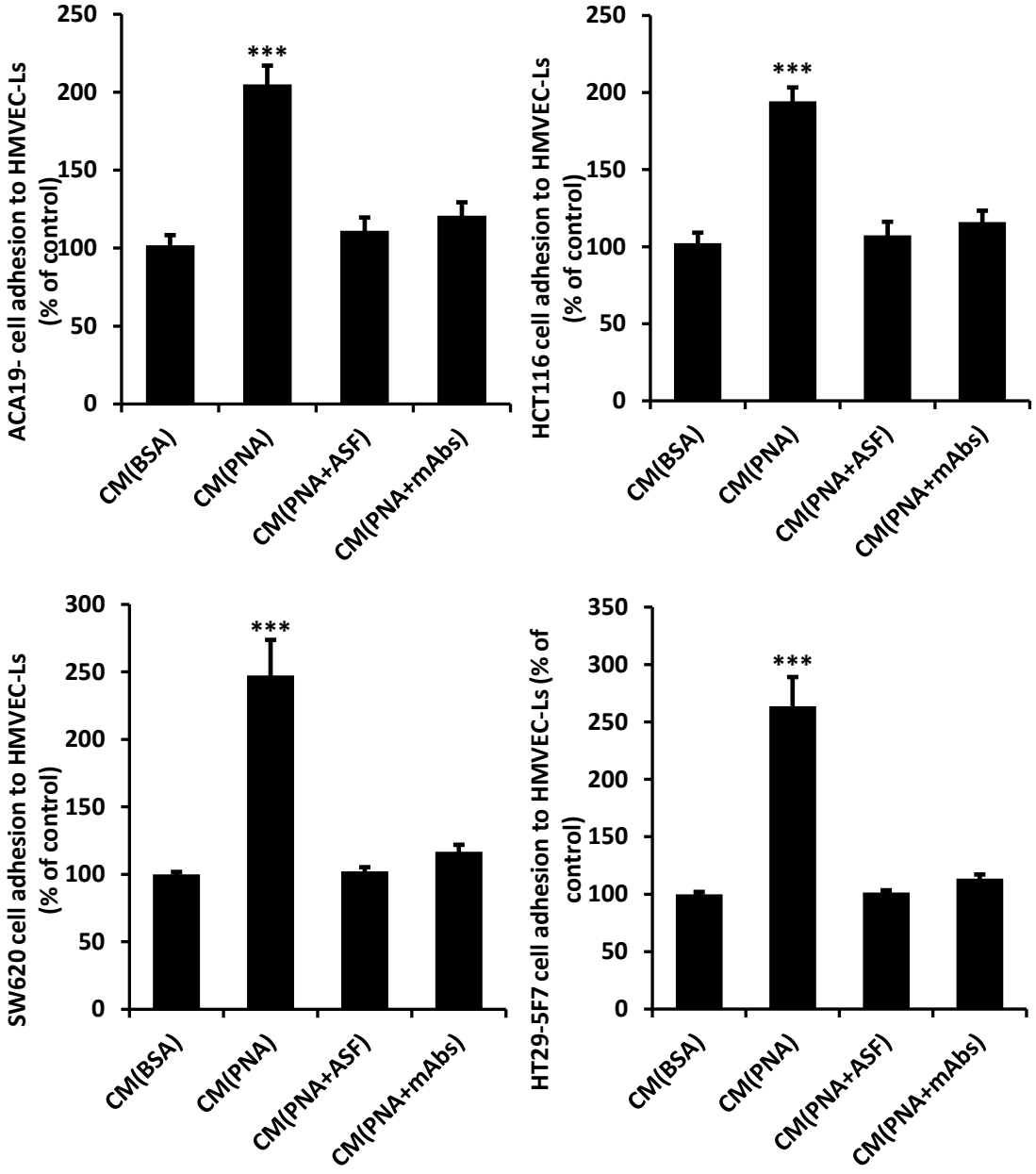


Figure 5.4.2: PNA-induced cytokine secretion promotes endothelial cell migration.

Conditioned medium was collected after overnight incubation of HUVECs monolayers with 4µg/ml PNA, 4µg/ml BSA, 20µg/ml ASF and a combination of antibodies against IL-6 (5ng/ml) and MCP-1 (40ng/ml). Cancer cells ACA19-, HCT116, SW620 and HT29-5 adhesion to fresh HUVECs was assessed with or without a combination of neutralizing antibodies againstIL-6 (5ng/ml) and MCP-1 (40ng/ml). The data are

expressed as percentage compared with BSA-treated controls from five independent experiments, each in triplicate. Data are expressed as mean value ± SD of triplicate. *P<0.05, **P<0.01, ***p<0.001. (One-way ANOVA followed by Bonferroni).

5.4.3 PNA-induced cytokine secretion induces endothelial cell growth

Both microvascular endothelial cells and macrovascular endothelial cells were prepared and incubated with conditioned medium mentioned above for 24hr. Endothelial cell growth was then analysed by calcein AM fluorescence and showed an increase in both HMVEC-Ls and HUVECs cell growth by 143±16% and 166±7% respectively in comparison with the BSA-treated control group. (Fig. 5.4.3)

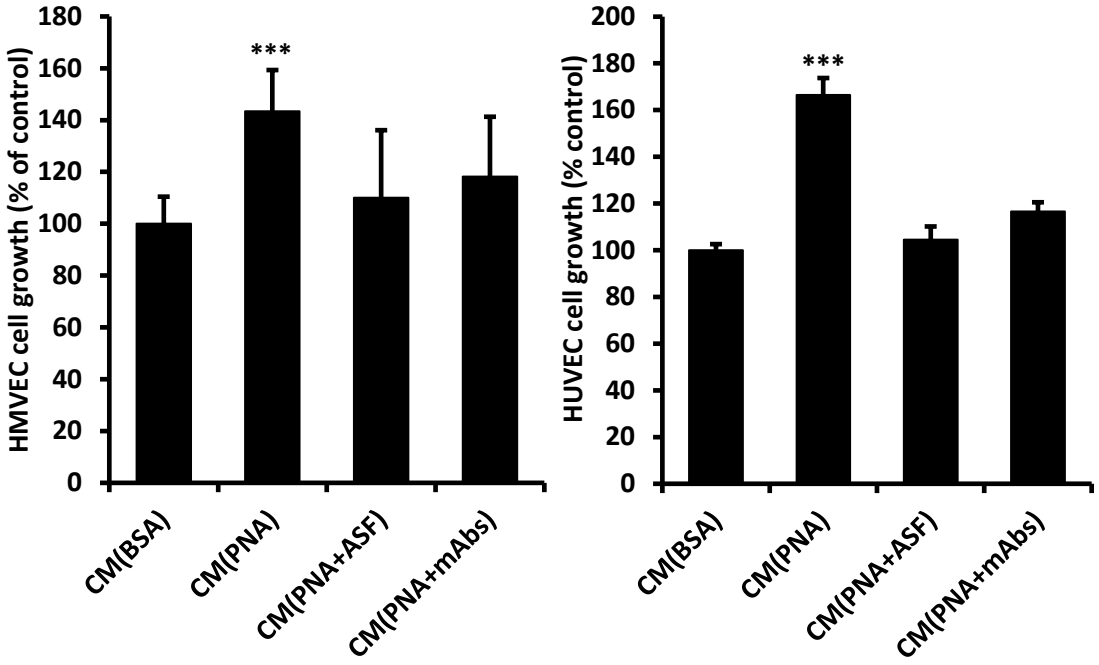


Figure 5.4.3: PNA-mediated endothelial cytokine secretion induces endothelial cell growth

Both HMVEC-Ls and HUVECs were prepared and incubated with conditioned medium for 24 hr. The cell growth was analysed by fluorescent labelling the endothelial cells. The data are expressed as percentage compared with BSA-treated controls from three independent experiments, each in sextuplicate. Data are expressed as mean value ± SD of triplicate. *P<0.05, **P<0.01, ***p<0.001. (One-way ANOVA followed by Bonferroni).

5.4.4 PNA-induced endothelial secretion of cytokines promotes endothelial cell tube formation (angiogenesis)

The effect of PNA-induced IL-6 and MCP-1 secretion on angiogenesis was assessed by measuring HHUVEC tubule formation. Conditioned medium from the PNA (4μg/ml) -treated group increased the total length of endothelial cell tubule formation by 125±48% compared to the control group. ASF is a glycoprotein that carries several O- and N-linked glycans including TF and sialyl-TF. It is a very known binding ligand of PNA and is used as a competitive binding inhibitor for PNA binding to cell surface. The effect of PNA on cytokine secretion was inhibited by the presence of ASF and also by the presence of antibodies against IL-6 and MCP-1. These results illustrated that PNA-induced endothelial secretion of IL-6 and MCP-1 promotes endothelial cell tube formation in angiogenesis. (Fig 5.4.4) In this experiment, conditioned medium is also used in order to analyse the effect of PNA-mediated cytokine secretion on endothelial cells.

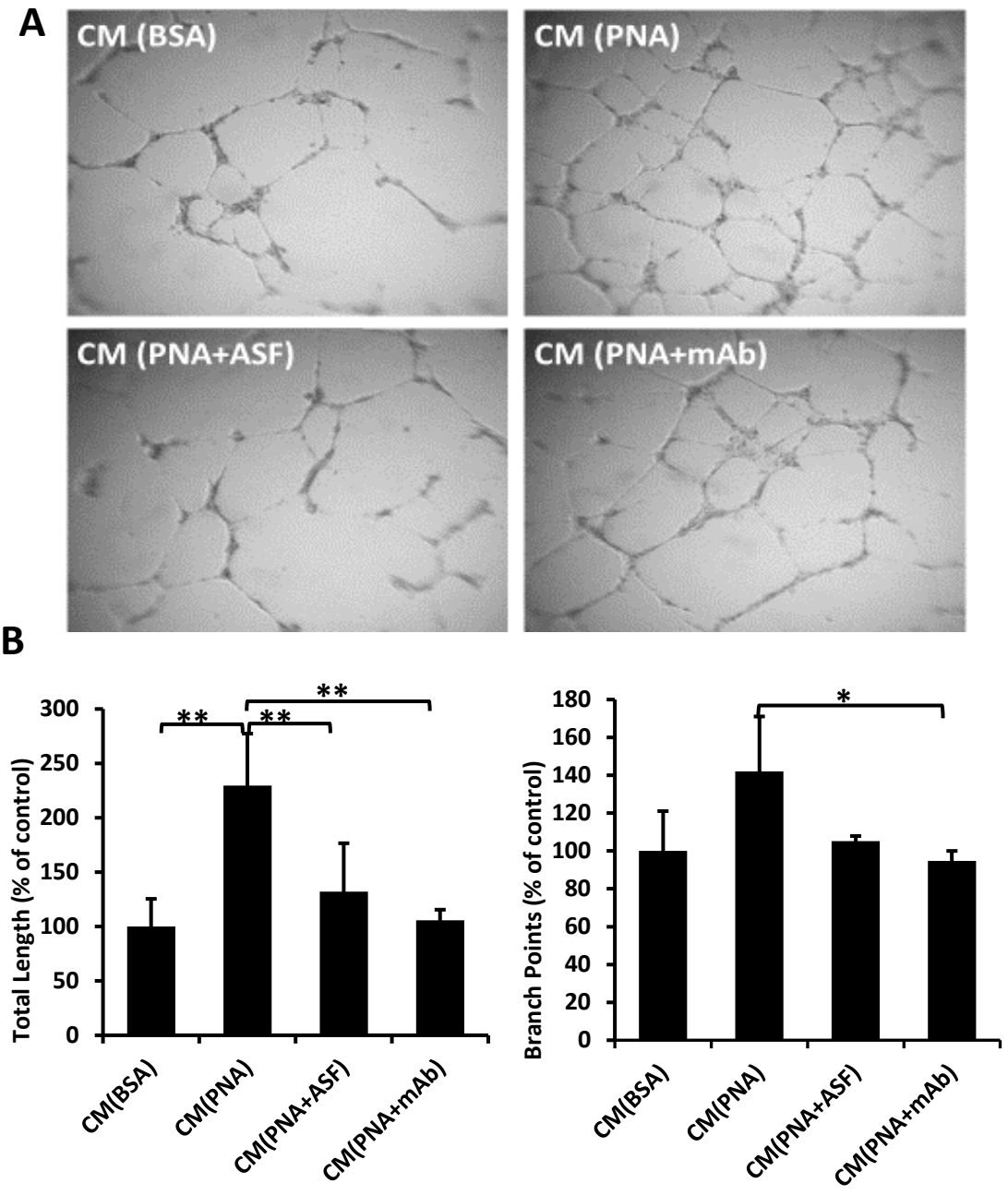


Figure 5.4.4: PNA-induced cytokine secretion promotes endothelial tubule formation

HUVECs were seeded on matrix gel with 4μg/ml PNA or BSA in the presence or absence of 20μg/ml ASF or 5ng/ml anti-IL-6 antibody and 40ng/ml anti-MCP-1 antibody for 24 hr. The cell images were taken (A) and the tubular length was quantified (B). Data

are expressed as mean value ± SD of triplicate. *P<0.05, **P<0.01. (One-way ANOVA followed by Bonferroni).

5.4.5 Investigation of the effect of PNA treatment on expression of cell surface adhesion molecules by HUVECs

To gain an insight into the mechanism of the PNA-induced, cytokine-mediated cell adhesion, we analysed the expression of several common cell surface adhesion molecules on HUVECs after treatment of the cells with conditional medium. HUVEC cells were treated with PNA (4μg/ml) for 24 hr before the expression of endothelial cell surface adhesion molecules (Integrinα5β1, Integrinα5β3, CD44, E-selectin, ICAM and VCAM) were analysed by flow cytometry.

It was found that HUVECs treated with PNA (4 μg/ml) for 24 hr increased expression of cell surface Integrinα5β1 (29.6%), Integrinα5β3 (33.4%) E-selectin (20%), VCAM-1 (45.3%), CD44 (18.6%) and ICAM-1 (13.3%) whereas the expression of cell surface CD44 and integrinα5β3 was not affected (n=3). The presence of antibodies against IL-6 and MCP-1 caused 79±14% reduction of PNA-mediated increase of VCAM-1 expression.

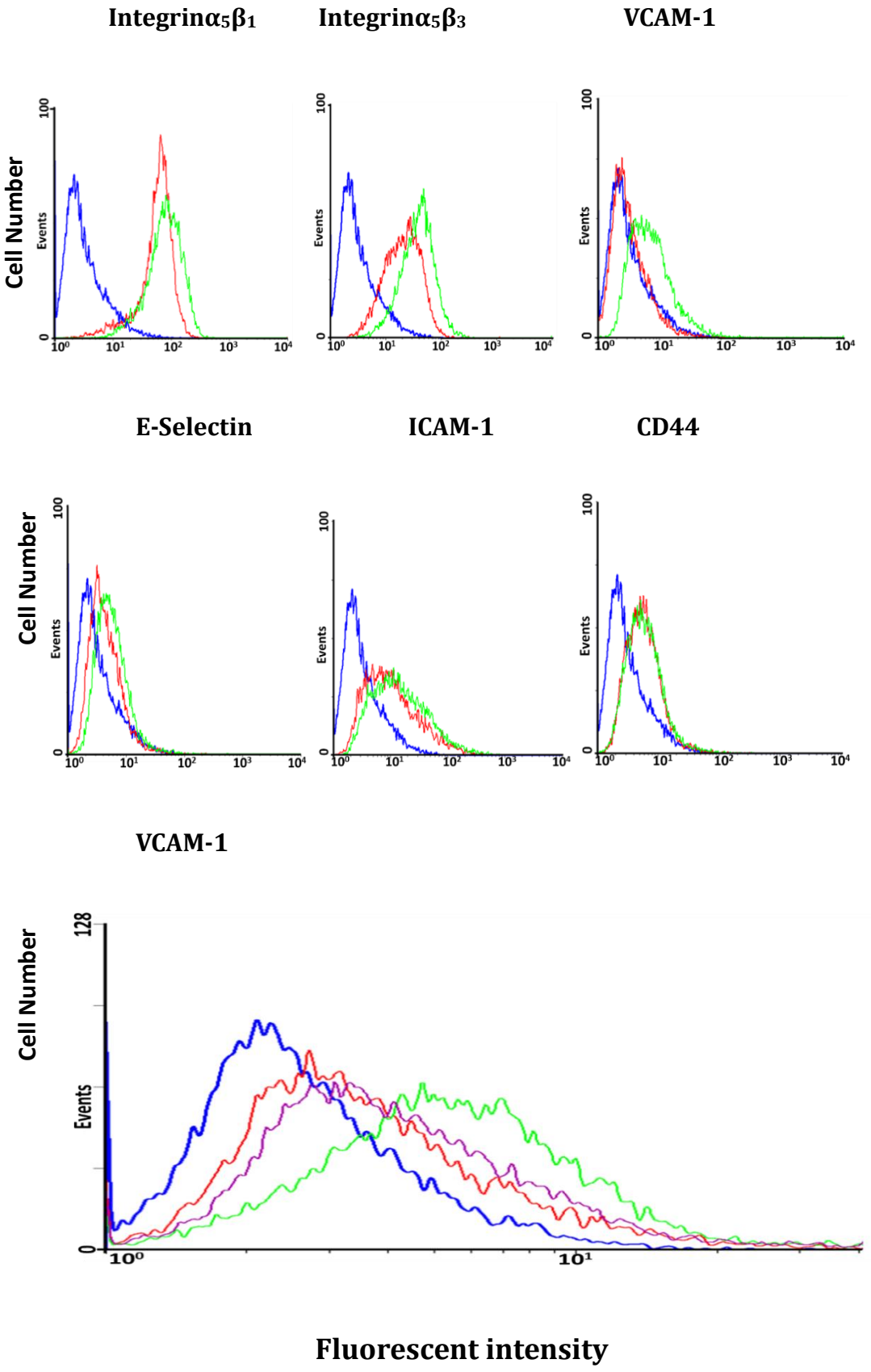

Integrinα5β1
Integrinα5β3
VCAM-1
E-Selectin
ICAM-1
CD44
VCAM-1
Cell Number
Events
Fluorescent intensity

Figure 5.4.5: PNA induces expression of cell surface Integrinα5β1, Integrinα5β3, E-selectin and VCAM but not CD44 and ICAM1

Fresh HMVEC-Ls were incubated with conditioned medium from endothelial cells treated with 4μg/ml PNA (green) or without (red), or anti-IL-6/MCP-1 antibodies (purple) for 24 hr. The cells were released and probed with antibodies against Integrinα5β3, integrinα5β1, VCAM-1, ICAM-1, E-selectin or CD44. After application of FITC-conjugated secondary antibody, expression of these cell adhesion molecules was analysed by flow cytometry. *The Ig G control is shown in blue (n=3)*

5.5 Discussion

This study shows that PNA-induced secretion of IL-6 and MCP-1 from endothelial cells increases cancer cell-endothelial cell adhesion of both MUC1-negative ACA19-, HCT116 and MUC1-positive SW620, HT29-5F7 cancer cells. Growth of both microvascular and macrovascular endothelial cells is also promoted by this PNA-mediated cytokine secretion. Additional evidence of stimulation of angiogenesis is shown by increased tubule length and branch points. PNA-induced cytokine secretion also increases expression of several cell surface adhesion molecules integrinαvβ1, integrinαvβ3, E-selectin and VECAM-1, but not CD44 and ICAM-1. The PNA-mediated increase of VCAM-1 expression is inhibited by the presence of antibodies against MCP-1 and IL-6. Thus, the increase tumour cell adhesion to endothelium by PNA is likely attributed by the increased expression of the cell surface adhesion molecules through IL-6 and MCP-1.

Tumour cell adhesion to vascular endothelium and endothelial tubule formation are two important steps in cancer metastasis. Metastatic cancer cells from renal and lung cancers and melanomas have been shown to have much stronger ability to bind homotypically to each other and to form potential tumour emboli- or heterotypically to the endothelium in comparison with cancer cells with lower metastatic ability. [271-273] We have previously shown that cancer cell-endothelial cell adhesion can be promoted by the TF-binding glycoprotein galectin-3. [274] Such interactions between cancer cells and the endothelium are a crucial step in cancer growth and metastasis. [91] Expression of pro-inflammatory cytokine IL-8 and Gro-β is induced by the interaction between

endothelial cells and invasive cancer cells. [275] In breast cancer it has been shown that this cytokine induction can increase endothelial barrier permeability and thus helps cancer cell adhesion in metastasis. [276]

The tube or tubule formation assay is a well-known method for measuring angiogenesis which was first used in 1988. [277] Previous studies on microvascular endothelial cells have shown that recombinant human IL-8 increases HUVECs cell growth and tubule formation. The in vivo control of angiogenesis is incompletely understood. It is proposed that expression of IL-8 receptors (CXCR1 and CXCR2) are important. [278] There is also evidence for an important role of VEGF in gliomas [279] It is thought that inhibitors of angiogenesis may have great potential in cancer treatment although earlier enthusiasm has yet to be confirmed by convincing translation into clinical practice. [280] Previous studies of angiogenesis inhibitors Anginex, endostatin and TNP-470 had shown that angiogenesis is reduced by all three inhibitors with shorter tubule and fewer branch points. [281]

IL-6 is a pleiotropic cytokine with tumour-promoting effects. Recent studies have proposed that IL-6 and E-cadherin are possible factors important in breast cancer metastasis formation. It has been reported that up-regulation of IL-6 may be involved in the high invasiveness and metastatic capability of oestrogen receptor-negative tumours. Further, in patients for whom Microscopic polyangiitis (MPA) treatment was effective, the IL-6 level prior to treatment was clearly low. [282] Moreover, IL-6 induced kinase pathways have roles in regulating pathogenic bone remodeling, inflammation, cell survival, proliferation

and pro-tumorigenic effect. Accumulating data indicates that overexpressed IL-6 in cancer correlates with metastasis to bone. [283] IL-6 is also associated with auto and paracrine stimulation of tumour cell proliferation, with the upregulation of anti-apoptotic proteins, and with the induction of pro-angiogenic cytokines including VEGF. These IL-6-mediated effects are all relevant to cancer metastasis. [264] Expression of IL-6 mRNA is increased during ovarian folliculogenesis and maternal decidual vascularization, two important independent angiogenic processes. This is further evidence that IL-6 has an important role in angiogenesis. [284] In vitro studies using vascular tumours in syngenic mice showed that inhibition of IL-6 causes decreased endothelial cell proliferation and which could be regained, dose-dependently, by addition of recombinant IL-6. [285] Blockage of IL-6 results in reductions in tumour burden and metastasis in experiment models so humanized blocking antibodies such as tocilizumab against IL-6R, are already Food and Drug Administration-approved following clinical trials. Delineation of the regulatory mechanisms that promote IL-6 secretion might promote the further development of targeted therapies. [286]

Chemokines and their receptors mediate acute inflammation chemokines, induced at sites of inflammation, act as chemotactants providing directional cues during the migration of leukocytes to damaged or infected tissues. [287] However, elevated expression of chemokines, leading to alterations in chemokine-receptor signalling, can contribute to chronic inflammation and malignancy. Cancer cells and host stromal cells in the tumour microenvironment including endothelial cells, fibroblasts, mesenchymal stem cells and infiltrating leukocytes produce a wide range of chemokines that exert numerous biological functions during tumour

progression and metastasis. [224] Of these, the C-C chemokine subfamily member, MCP-1 together with its cognate receptor CCR2 have been shown to play key roles in cancer metastasis by sustaining cancer cell proliferation and survival, stimulating cancer cell migration and invasion, and inducing deleterious inflammation and angiogenesis. [253] MCP-1 has also been shown to be important for the recruitment of monocytes to tumours and to be involved in breast cancer development. [288] In vivo studies using metastatic breast cancer cells have shown increased secretion of IL-8, VEGF and MCP-1. These high levels of cytokines have been shown to contribute to the increased adhesion of breast cancer cells to HUVECs. E-selectin plays a large part in this adhesion interaction between tumour cell and endothelial cells. [276] Previous studies demonstrate MCP-1 with very low concentration, has chemotactic effect on both microvascular and umbilical vein endothelial cells. In addition, Matrigel studies have shown that inhibition of MCP-1 leads to reduction in endothelial cell angiogenesis. [254] Many breast cancers secrete MCP-1, and applying neutralizing anti-MCP-1 antibodies suppresses experimental lung metastasis by the human MDA-MB-231 breast cancer cells. [255] Furthermore, oestrogen receptor-negative breast cancer cells show upregulated surface expression of cadherin, which induces lung metastasis through regulation of MCP-1 expression. Thus MCP-1 is a key contributor to lung metastasis by breast cancer cells. By contrast, the role played by MCP-1 in breast cancer metastasis to bone remains largely unknown. [289]

It is well-known that cell adhesion molecules have an important role in cell activities including proliferation, adhesion, migration and invasion, as well as angiogenesis. Our studies show that expression of integrinαvβ1, integrinαvβ3, E-

selectin, slightly ICAM-1 and VECAM-1 are induced by PNA-induced cytokines acting on endothelial cells. These cell surface adhesion molecules from the most common four groups were selected in this study. Integrins are transmembrane receptors that participate in cell-extracellular matrix adhesion. Previous studies showed that integrin αvβ1 is overexpressed in melanoma metastases, and a high level of integrin αvβ1 expression in melanoma metastasis patients correlates with a low survival rate. [290] Similar to integrin αvβ1, αvβ3 is also upregulated in melanoma metastasis and thought to have important role in melanoma tumorigenesis. Increased expression of αvβ3 vitronectin receptor during melanoma progression modulates collagenase and promotes tumour cell invasion. [91, 291] Moreover, integrin αvβ3 also mediates tumour-associated angiogenesis. [292] E-selectin also has a key role in the cytokine-induced cancer cell-endothelial cell adhesion in several cancer types such as breast cancer and colonic cancer. [293, 294] Other soluble adhesion molecules, ICAM-1 and VCAM-1 are also expressed highly in both colorectal cancer and breast cancer. These three cell surface adhesion molecules all correlate with tumour progression and have been shown to enhance cancer cell heterotypic adhesion to endothelial cells and also to play a role in host immune anti-tumour defense. [295] High serum level of these soluble adhesion molecules (E-selectin, ICAM-1 and VCAM-1) are reported in both colorectal cancer and breast cancer where they correlate with decreased survival. [296, 297]

In conclusion, as tumour cell adhesion to endothelium and endothelial tube formation are important steps in metastasis and both are enhanced by PNA-induced cytokine secretion, the presence of intact PNA in the circulation after

peanut consumption may plausibly contribute to metastasis by inducing secretion of IL-6 and MCP-1. The mechanism of action is not known, the expression of several cell surface adhesion molecules increased by PNA in endothelial cells contributes to the PNA-induced cancer cell-endothelial cell adhesion. In this study, ASF was not tested in the expression of cell surface adhesion molecules due to lack of cells number. It is known that glycoprotein ASF bind to tumour cell surface and induced tumour cell homotypic aggregation, the interaction was also mentioned as the initial indications of lectin activities on cell surface. While, these is no clear evidence of ASF interaction with cell surface adhesion molecules such as Integrinα5β1, Integrinα5β3 and E-selectin. It is with great interest to test the effect of ASF on endothelial cell surface separately and with PNA in future work. [298] In addition, as this study has shown a combination of IL-6 and MCP-1 secretion by PNA from endothelial cells, individual cytokine effects will need to be analysed in future research. A recent study has shown that IL-6 works together with MCP-1 causing increased vascular inflammation. During the recruitment of monocytes into the adventitia, IL-6 secretion is induced and mediates monocyte differentiation. Meanwhile activated macrophages up-regulate MCP-1 secretion which invade into the media and help further monocyte recruitment. In that way, IL-6 and MCP-1 form a loop that greatly increases vascular inflammation. [299]

CHAPTER 6 Identification of the PNA binding receptor and the underlying mechanism of PNA-induced cytokine secretion

6.1 Hypothesis and Aim

6.1.1 Hypothesis

The mechanism of the interaction between PNA and endothelial secretion of cytokine involves the binding receptor for PNA on cell surface of endothelial cells.

6.1.2 Aim

To identify the PNA binding receptor on the endothelial cell surface responsible for PNA-induced cytokine secretion and to study the molecular mechanism of PNA-mediated cytokine secretion

6.2 Introduction

It is likely that PNA-induced cytokine secretion is mediated by an interaction with glycans expressed on the endothelial membrane. PNA is a lectin which has been shown to be generally non-toxic to cells.[300] It is known that the interaction between lectins and cell surface ligands contributes to lectin internalization into various cell types, and induces different cell activities such as proliferation [301, 302] PNA is a galactose-binding lectin with high specificity for the cell surface TF antigen (β-galactosyl(l-3)-α-N-acetylgalactosamine).[303, 304] PNA has important mitogenic effects on various human colon cancer cells.[160, 219, 305] The mechanism of these effects is unknown, but PNA binds to TF antigen expressed on high molecular weight splice variants of the transmembrane glycoprotein CD44v6 on colon cancer cells, and this interaction initiates phosphorylation of c-Met receptor and therefore activates the p44/p42 MAPK cell signaling pathway.[306] PNA also interacts with thymocyte surface glycoproteins such as CD8 and CD45, and thus mediates intracellular cell signaling affecting development of both mature and immature thymocytes.[307-309]

Circulating galectin-3 is overexpressed in most types of cancer, and plays an important role in cancer development with its effects on promoting cancer cell adhesion, migration and metastasis.[221] Studies from our group have shown that galectins including galectin-3 and galectin-1 strongly bind to the cell surface oncofetal TF antigen which is overexpressed on the tumour-associated transmembrane mucin protein MUC1, and increases cancer cell adhesion, migration and metastasis.[151, 310] Galectin-3 also interacts with various cell

surface N-glycans.[311, 312] Previous study from our group has established that circulating galectin-3 induces endothelial secretion of metastasis-promoting cytokines. The mechanism of this interaction is related to four cell membrane glycoproteins, of which CD146 was found to be the major cell surface receptor for galectin-3. [149, 218]

Studies reported in the previous chapter showed that PNA-induced IL-6 and MCP-1 secretion plays an important role in cell adhesion, proliferation, angiogenesis and expression of cell surface adhesion molecules. The study presented here aimed to investigate the molecular mechanism of the PNA-induced cytokine secretion. It is known that both galectin-3 and PNA bind to TF antigen on endothelial cell surface. It is interestig to study whether they have the same binding site or not. In this study, a flow cytometry study and cell adhesion assay were performed to observe if galectin-3 and PNA have the same receptors on cell surface. Then cytokine secretion ELISA assay was done to analyse their relationship in cytokine secretion. Previous studies showed that MCAM is the cell surface receptor of galectin-3. The PNA receptor would be extracted by PNA purification assay and analysed further. In this study, lactose was used as a competitive inhibitor for PNA binding.

6.3 Methods

6.3.1 Assessment of PNA binding to HUVECs by histochemistry

A T25 flask of 75% confluent HUVECs was released with 1ml trypsin and washed with PBS. After incubation for 5 minutes, the cells were suspended at $1x10^5$ cells/ml with EGM medium before seeding 250µl per well to the 24-well plate inserted with glass coverslips. The cells were cultured overnight at 37 °C before treatment with 4µg/ml FITC-PNA for 1 or 24 hr. The cells were fixed with 2% paraformaldehyde and imaged under fluorescence microscope. For each glass coverslip, 10 images were randomly taken

6.3.2 Assessment of PNA-FITC binding to HUVEC cell surface in the presence of galectin-3

Endothelial cells (HUVEC) were cultured in five T-25 tissue culture flasks (A to E) until 80% confluence. The cells were washed with PBS, and one flask (flask A) was introduced with 4µg/ml PNA and the other four (flasks B-E) with 4 µg/ml BSA (control) for 24 hr at 37℃. All flasks were washed once with lactose (10µM) (to remove any endogenous cell surface galectin-3 binding in B-E), and twice with PBS before the cells were released with trypsin, washed once with PBS before fixation with 2% paraformaldehyde for 20min. The cells were then washed once with PBS before suspension in 1% BSA/PBS for 30 min at room temperature before putting into five individual tubes. The cells in Flask A were also washed with lactose (to remove PNA binding), released by trypsin and put into a tube. The

tubes were the added with PBS, TITC-conjugated anti-mouse antibody (1:500), or 4μg/ml FITC-PNA without or with 20μg/ml ASF or 4 μg/ml galectin-3 for 1 hr at room temperature. The cells were washed once with PBS before analysis by flow cytometry to assess the PNA-FITC cell surface binding. The flow cytometry assay was operated as described in section 3.12.

6.3.3 Assessment of the effect of PNA-induced cytokine secretion on cell adhesion in the presence of galectin-3

ACA19- and HCT 116 cancer cells were detached using NECDs NECDS (to keep cell surface adhesion proteins intact.) After washing with PBS and resuspension at $5x10^6$ cells/ml in DMEM, cells were labelled with 10μg/ml calcein AM for 30 min at 37°C. Then cells were re-suspended at $1x10^5$ cells/ml with serum-free DMEM before application to a new plate of HUVEC monolayer with prepared conditioned medium. The prepared conditioned medium was divided into five groups with different treatments of: 4μg/ml BSA; 4μg/ml PNA; 4μg/ml PNA + 20μg/ml ASF; 4μg/ml PNA + 4μg/ml Galectin-3 and 4μg/ml BSA + 4μg/ml Galectin-3. The cells were then maintained at 37°C for 1 hr. After twice washing with PBS to remove unbound cancer cells, the monolayer was lysed with 0.25% SDS. The endothelial cell-associated fluorescence was read by the fluorescence microplate reader at 485nm excitation and 535nm emission.

6.3.4 Assessment of PNA-mediated cytokine secretion in the presence of galectin-3

HUVECs with $1.5x10^5$ cells/ml were cultured in 96-well plates overnight for monolayer formation. 4μg/ml BSA, 4μg/ml PNA, 4μg/ml recombinant galectin-3 (Gal3F) or C-terminally truncated galectin-3 (Gal3C) and a combination of 4μg/ml PNA and 4μg/ml Gal3F or Gal3C were introduced to the culture medium for 24 hr at 37°C. Culture medium was collected and levels of cytokines IL-6, MCP-1, G-CSF, GM-CSF and ICAM-1 were determined by cytokine ELISA.

6.3.5 Extraction of PNA-binding proteins by PNA affinity purification

PNA-agarose (3ml) was washed three times with 20ml PBS and centrifuged at 800 rpm for 5 min before use. HUVECs were cultured in T25 flasks overnight till 100% confluent. Confluent HUVECs were washed three times with PBS before being scraped by a cell scraper and then stored at -80 °C till use. After collection of ten T25 flask of HUVECs, the HUVECs were defrosted on ice and the supernatant was then removed by centrifugation at 2000 rpm before application of 10ml of cell protein extraction buffer. Then the cells were sonicated for 20 seconds for 3 times on salt ice. The lysate was then centrifuged at 15,000 rpm for 10 min at 4 °C, and supernatant was collected and applied onto the PNA-agarose beads. The supernatant-beads mixture was left on a roller overnight at 4 °C to allow PNA binding to its ligands. PNA-agarose beads were washed with 20ml PBS to remove nonspecific binding. The PNA binding proteins were eluted with 5 ml elution solution. The collected elute was then dialyzed against H_2O in snake skin fibre

overnight to remove galactose, and freeze-dried before analysis by electrophoresis. The eluate is also analysed by mass spectrometry.

6.3.6 Assessment of PNA and MCAM co-localization on HUVEC cell surface.

Sub-confluent HUVECs were seeded into 12-well plates inserted with glass coverslips. After 24 hr incubation at 37 °C, the cells were washed twice with PBS and fixed with 4% paraformaldehyde without detergent. Then cells were incubated with 1% BSA for 1hr before application of 4μg/ml FITC-PNA with anti-MCAM (1:1000) in 2% BSA/PBS (w/v) for 1 hr at room temperature. After three washes with PBS, the cells were incubated with Texas Red-Avidin (1:2000) for 1 hr at room temperature. The cells were washed five times in PBS before being mounted with DAPI-containing fluorescent mounting media (Vector Laboratories, Burlingame, CA). The slides were analysed using a 3i confocal microscope (Marianas SDC, 3i Imaging) and Slidebook 6 Reader version 6.0.4 (intelligent-imaging).

6.3.7 SiRNA protein knockdown

80% HUVECs were cultured in 12-well plated for 24 hr. MCAM , PECAM siRNA (150pmol) (Human MCAM/4162, Human PECAM/5175) and control non-target siRNA (non-targeting siRNA #1) were designed by and purchased from the Thermo Fisher company. HUVECs were introduced with 20nM siRNA to MCAM,

PECAM and non-target siRNA in 100µl serum free medium for 5min. 2.5µl of DharmaFect Transfection Reagent-4 (Thermo Fisher) were dissolved in 100µl serum free medium before introducing to HUVECs with 800µl EGM medium for 20 min at room temperature. HUVECs were incubated for 16 hr at 37 °C before replacing the transfection medium with fresh EGM medium. Cells were then incubated for 24 hr at 37 °C and were used in two experiments. For immunoblotting, cells were lysed by SDS-sample buffer before expression of MCAM and PECAM were analysed. For ELISA, cells were introduced with PNA for 24 hr at 37 °C before concentration of IL-6 and MCP-1 were tested in the culture media.

6.4 Results

6.4.1 PNA shows binding to the surface of HUVECs

75% confluent HUVECs were treated with 4µg/ml FITC-PNA for 1hour and 24 hours. PNA binding to the endothelial cells can be seen after 1hr and increased binding can be seen after 24 hours.(Fig. 6.4.1)

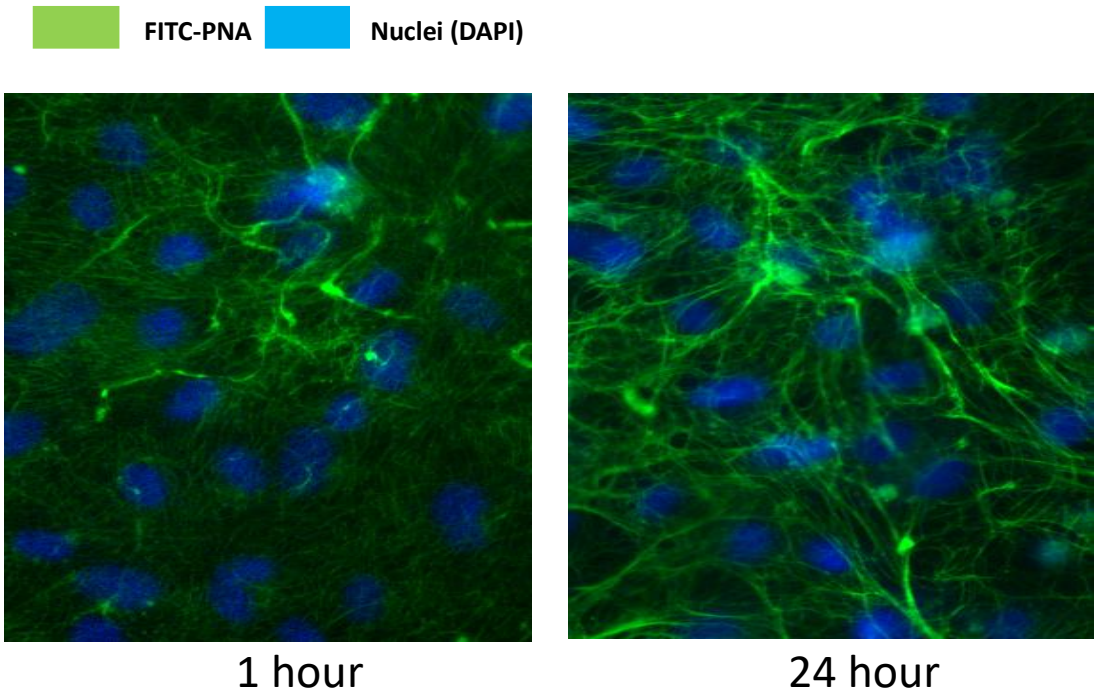


Figure 6.4.1: PNA binding to HUVECs after 24 hours

HUVECs cells were seeded into 24-well plates with glass coverslips, treated with 4µg/ml FITC-PNA for 1 hour and 24 hours before fixed and images taken under fluorescence microscope. The experiments were repeated three times. Representative images are shown in Fig 6.4.1. PNA (green) showed strong binding to HUVEC and the binding was still present after 24 hr. *Cell nuclei were stained by DAPI (blue).*

6.4.2 PNA-FITC binding to endothelial cell surfaces is partly inhibited by galectin-3

In order to gain an insight of relationship between PNA and galectin-3 binding to endothelial cell surface, the PNA binding to HUVECs was analysed with or without introduction of galectin-3 and the PNA binding ligand ASF that expresses TF-antigen. 80% confluent HUVECS were used here and 4μg/ml PNA (for the PNA pre-treated group, to test if PNA treatment affects expression of its cell surface binding receptors) or BSA were introduced to HUVECs for 24 hr. After washing with lactose to remove any endogenous galectin-3 binding (or unconjugated PNA binding), the cells were released and fixed. The fixed cells were then treated with 4μg/ml FITC-PNA in the presence of 20μg/ml ASF or 4μg/ml galectin-3. The flow cytometry analysis showed that PNA binding to HUVECs was 57.7%. The presence of ASF or galectin-3 caused 59% and 25% reduction respectively of PNA binding to the cells. (Fig 6.4.2). Pre-treatment of the cells with PNA also results in an increase of PNA cell surface binding (56.2%) (Fig 6.4.2)

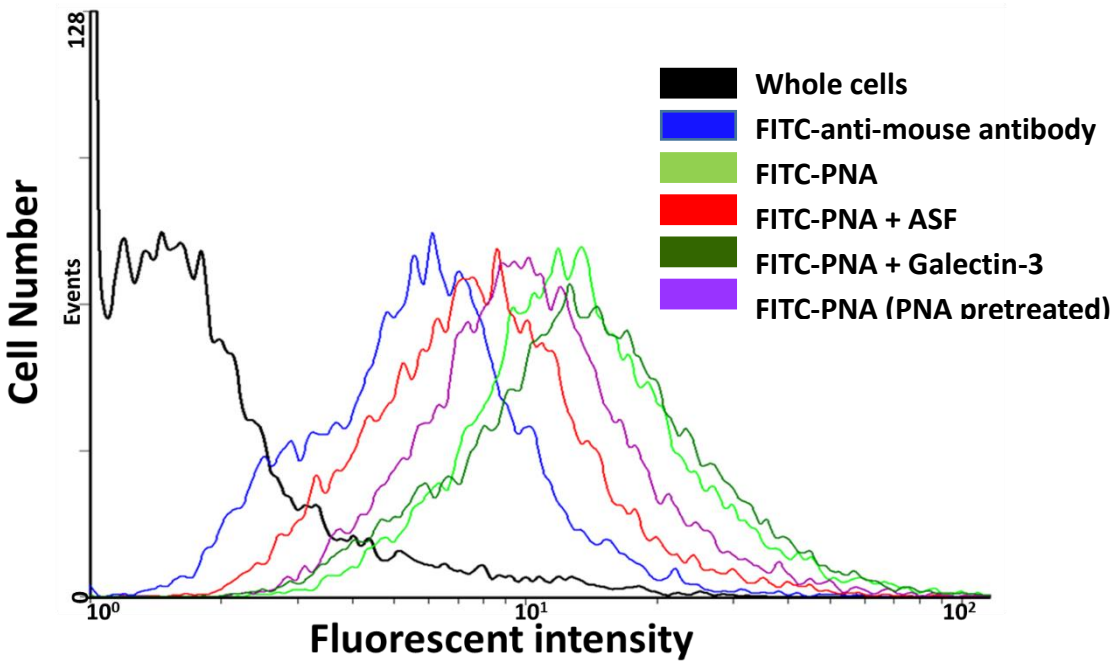


Figure 6.4.2: PNA binding to the surface of HUVECs is partly inhibited by the presence of asialofetuin and galectin-3.

The HUVECs were treated with 4µg/ml PNA (PNA pretreated group) or 4µg/ml BSA for 24 hours. Then the BSA treated cells were collected and fix followed by different treatment: 4µg/ml FITC-PNA, 4 µg/ml FITC-PNA+ 20µg/ml ASF, 4µg/ml FITC-PNA+ 4µg/ml Galectin-3. The PNA binding to the HUVECs was 59% inhibited by ASF and 25% by galectin-3. *The IgG negative control is shown in blue.*

6.4.3 Investigating the effect of PNA-mediated cytokine secretion on cancer cell-endothelial cell adhesion in the presence of galectin-3

In order to avoid higher number of endothelial cells in PNA-treated group, conditioned medium was used in this cell adhesion assay. The conditioned medium obtained from PNA-treated HUVECs increased adhesion of ACA19- and HCT 116 cells to endothelial cell monolayers by 85±5% and 56±18% in comparison to the BSA-treated control group. This PNA-mediated increase of cancer cell adhesion was mostly inhibited by ASF, and partially inhibited with the present of galectin-3. In addition, the galectin-3 treated group also caused an increase in both ACA19- (56±15%) and HCT116 (30±18%) cell adhesion to HUVECs when compared to the control group. (Fig 6.4.3)

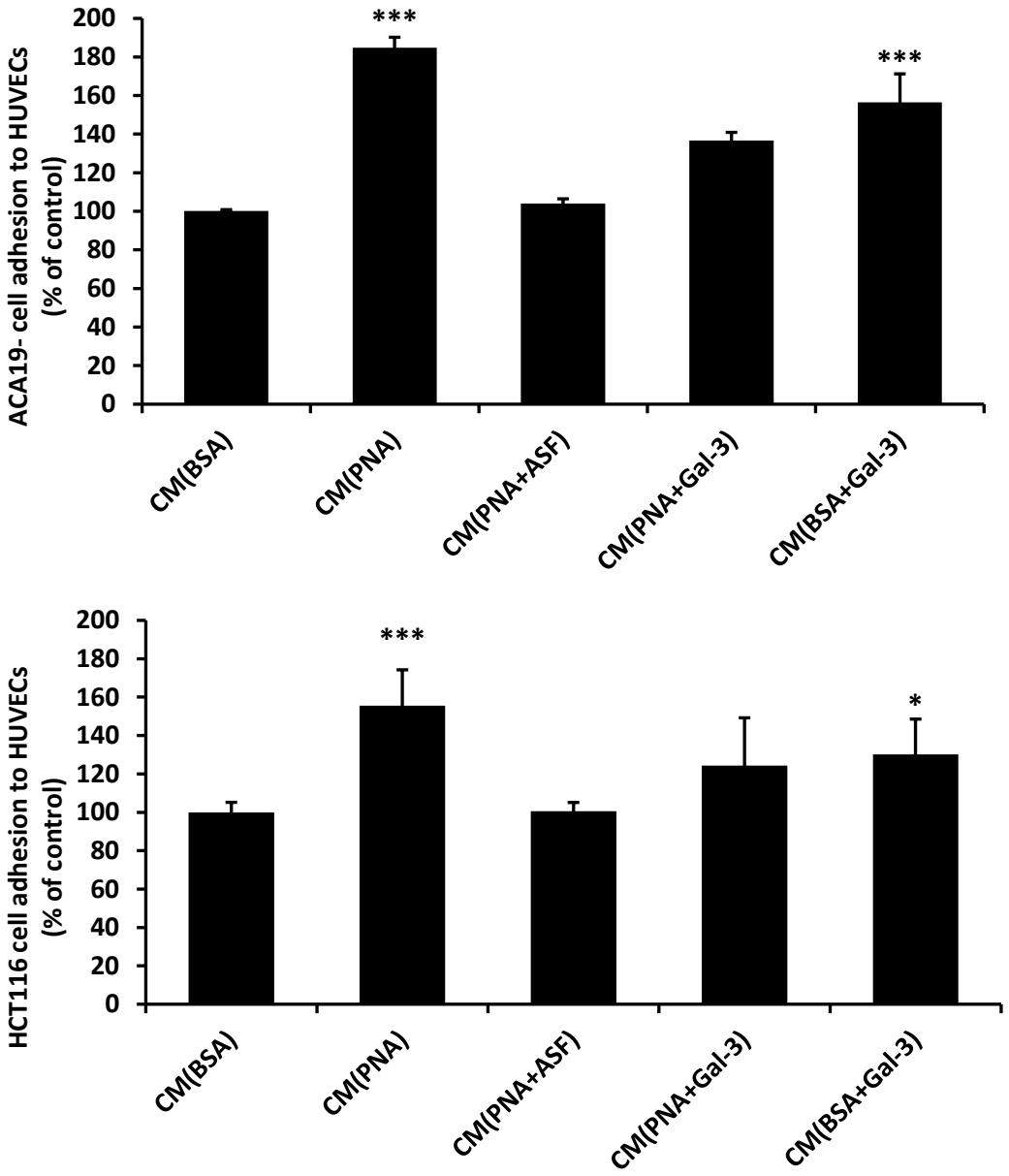


Figure 6.4.3: The effect of PNA-induced cytokine secretion on heterotypic cancer cell adhesion with HUVECs can be inhibited by the presence of galectin-3

HUVECs monolayers were prepared with conditioned medium. Cancer cells ACA19- and HCT116 adhesion to fresh HUVECs with or without galectin-3 (4μg/ml). The data are expressed as percentage compared with BSA-treated controls from at least 3 independent experiments, each in triplicate. Data are expressed as mean value ± SD of triplicate. **P<0.05, ***p<0.001. (One-way ANOVA followed by Bonferroni)*

6.4.4 The effect of galectin-3 on PNA-induced cytokine secretion from HUVECs.

Confluent HUVECs cell monolayer were treated with 4μg/ml BSA, 4μg/ml PNA, 4μg/ml PNA+ 20μg/ml ASF, 4μg/ml recombinant galectin-3 (Gal-3F) or C-terminally truncated galectin-3 (Gal-3C) and a combination of 4μg/ml PNA and 4μg/ml Gal-3(F) or Gal-3(C). The level of IL-6 in the PNA treated cells were 226% ±22% higher than BSA-treated group in HUVECs. This increase was fully inhibited by ASF and mostly inhibited by Gal-3(C).

The level of G-CSF in the Gal-3(F) treated cell were 407% ± 13% higher than BSA-treated group in HUVECs. The galectin-3-induced G-CSF increase was partially inhibited by PNA. Similar changes happened with GM-CSF, the Gal-3(F) treated cells were 182% ± 3% higher than BSA-treated group. Galectin-3 induced G-CSF increase was also inhibited by PNA. Moreover, ICAM-1 secretion was increased 238% ± 3% by Gal-3(F) comparing to BSA-treated group, and this increase was again partially inhibited by PNA.

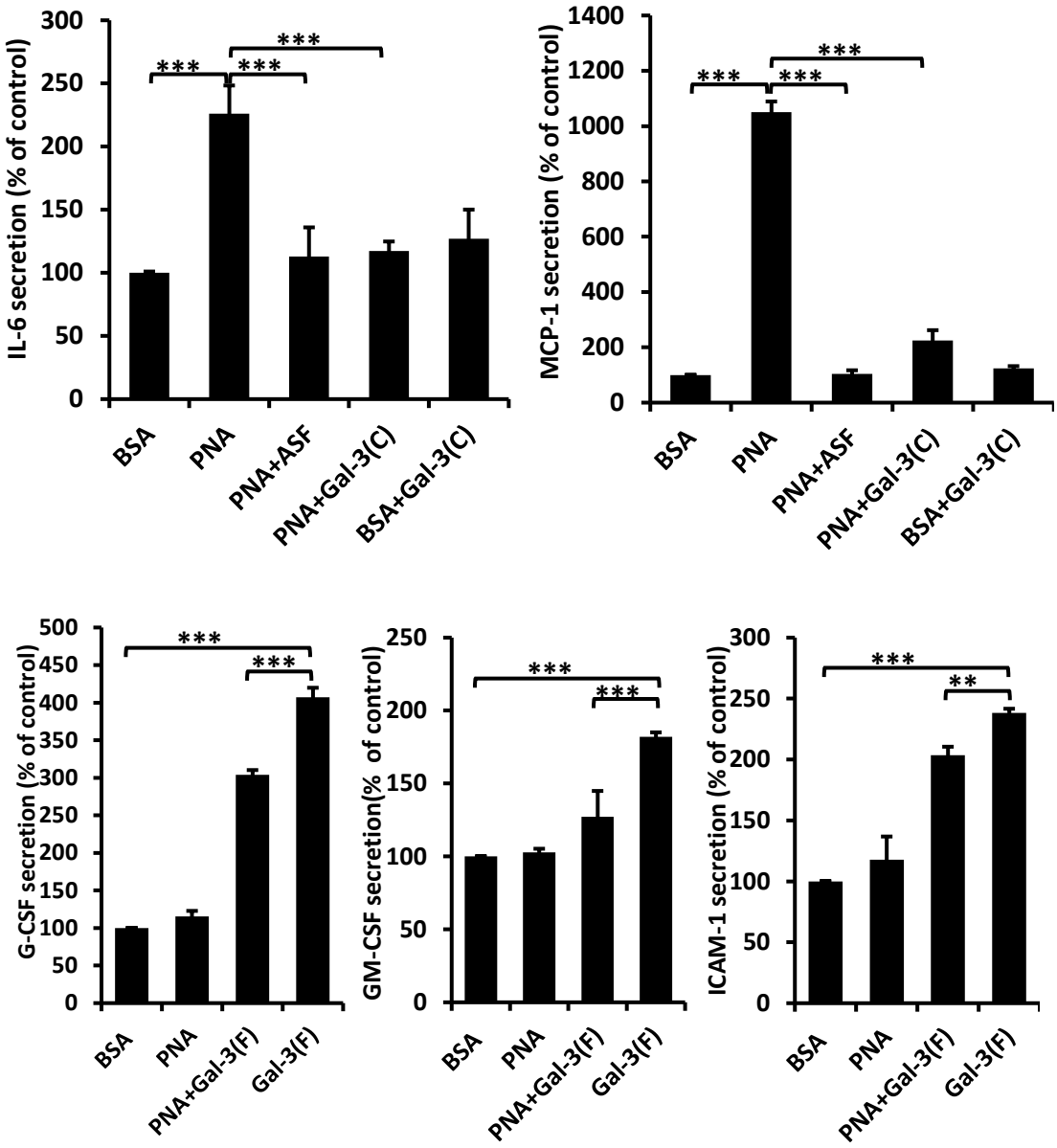


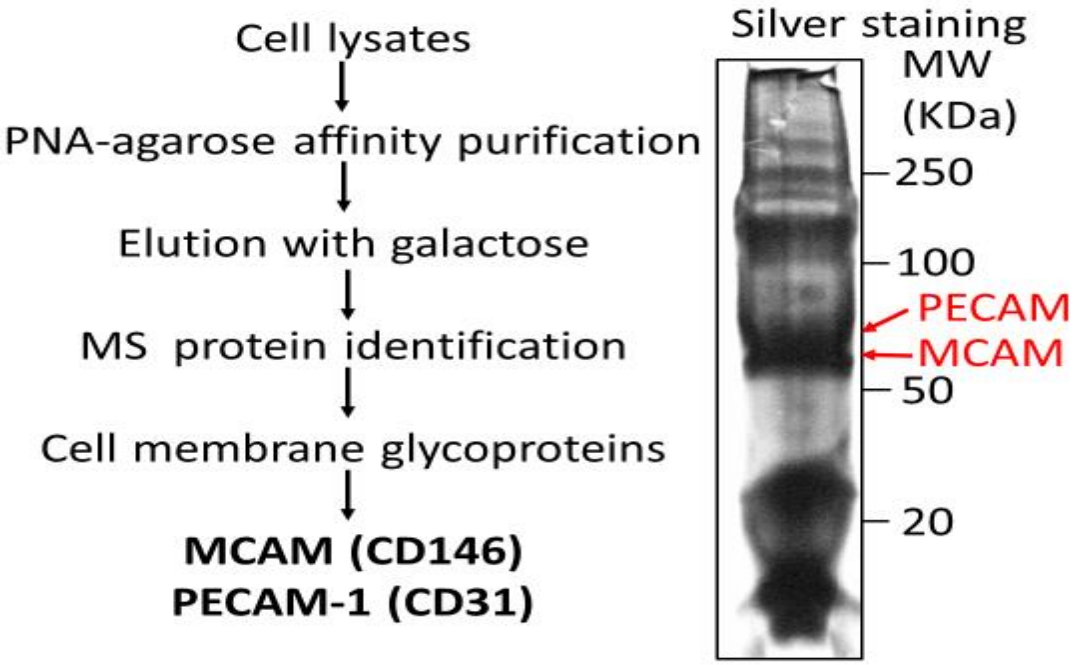
Figure 6.4.5: Partial competition between the effects of PNA and galectin-3 on cytokine secretion by endothelial cells

HUVECs were treated with 4μg/ml PNA or BSA in the presence or absence of 20μg/ml ASF, 4μg/ml recombinant galectin-3(Gal-3F) or C-terminally truncated galectin-3 (Gal-3C) for 24 hr before the concentration of cytokines in the culture medium were analyzed by ELISA. Data are presented as mean ± SD in

comparison to control, from 3 independent experiments. ****p<0.001. (One-way ANOVA followed by Bonferroni)*

6.4.5 Identification of PNA binding ligands on endothelial cell surface by PNA affinity purification and mass spectrometry

HUVECs were collected by cell scraper and treated with cell protein extraction buffer before sonication. After ultra-centrifugation, the supernatants were applied to the PNA-agarose beads. Bound proteins were then eluted from the beads with galactose (2M). The eluted proteins were dialyzed against H_2O to remove galactose. The proteins were then freeze-dried and analysis by electrophoresis and silver staining, and mass spectrometry. The silver staining showed several strong bands between 20 and 250kDa. PNA affinity purification followed by Mass spectrometry analysis identified a number of proteins that were extracted by PNA affinity purification. The highest scored cell membrane glycoproteins in the hit list were MCAM and PECAM. The silver staining also showed proteins in that range. (Fig 6.4.6)



Description	Score	Coverage	MW [kDa]	calc. pI
Cell surface glycoprotein MUC18 OS=Homo sapiens GN=MCAM PE=1 SV=2 - [MUC18_HUMAN]	225.25	15.17%	71.6	5.76
Platelet endothelial cell adhesion molecule OS=Homo sapiens GN=PECAM1 PE=1 SV=1 - [PECA1_HUMAN]	172.94	8.67%	82.5	6.99
Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	158.23	9.49%	62.3	7.74
Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5 - [HS90A_HUMAN]	148.78	10.52%	84.6	5.02
78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2 - [GRP78_HUMAN]	128.15	9.48%	72.3	5.16
Src substrate cortactin OS=Homo sapiens GN=CTTN PE=1 SV=2 - [SRC8_HUMAN]	125.11	11.09%	61.5	5.4
Heterogeneous nuclear ribonucleoprotein L OS=Homo sapiens GN=HNRNPL PE=1 SV=2 - [HNRPL_HUMAN]	110.17	14.09%	64.1	8.22

Figure 6.4.6: Identification of endothelial cell surface PNA-binding proteins

PNA affinity purification followed by mass spectrometry identify MCAM and PECAM as PNA binding cell surface proteins from HUVECs. PNA blotting with silver staining shows similar binding of PNA to a number of proteins in HUVECs. Mass spectrometry results showed MCAM and PECAM were possible PNA binding proteins. *MW, molecular weight.*

6.4.6 MCAM is the one of the major PNA-binding proteins on the endothelial cell surface

80% confluent HUVECs were treated with FITC-PNA and anti-MCAM antibody for 1hr. The cell surface adhesion molecule MCAM was labelled by using the Texas Red-Avidin before endothelial cells being mounted by DAPI-containing fluorescent mounting medium. Substantial colocalization of PNA binding to endothelial cells with MCAM is shown (due to time constrains, co-localization of PNA with PECAM was not studied in this study).

(Fig 6.4.7)

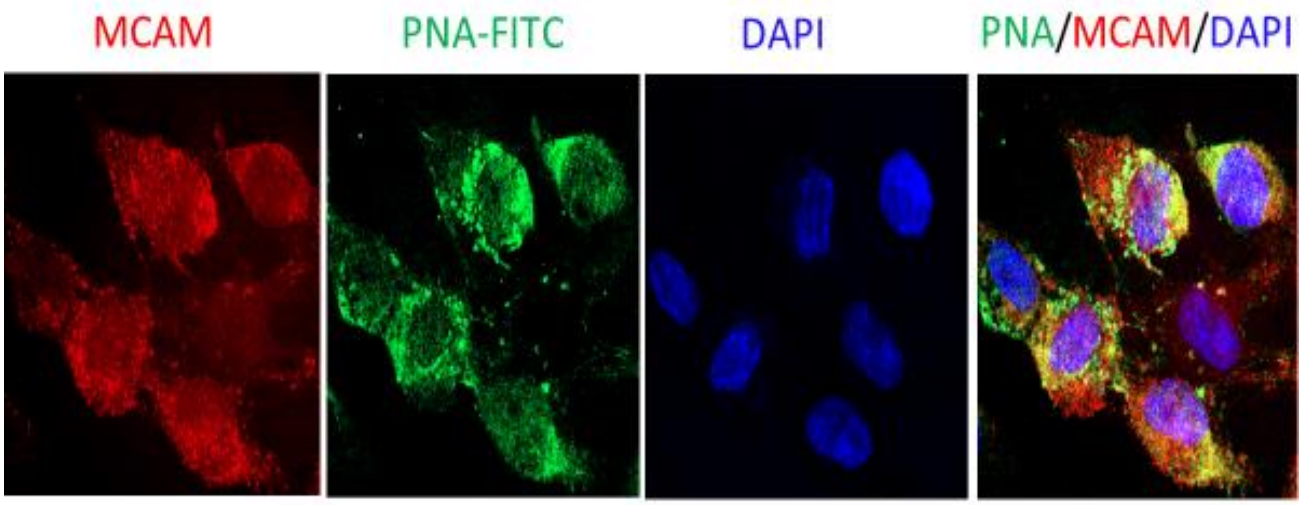


Figure 6.4.7: Co-localization of PNA with MCAM in HUVECs

After treatment of HUVECs with 4μg/ml PNA for 60 min, the cells were labelled, and MCAM (red) and PNA (green) immunohistochemistry was conducted by confocal microscopy. *Cell nuclei were stained by DAPI (blue).*

6.4.7 Investigation of the effect of PNA binding proteins MCAM and PECAM on the endothelial secretion of PNA-mediated cytokine

To determine whether interaction between PNA and cell membrane proteins MCAM or PECAM are responsible for PNA-mediated cytokine secretion, we treated the HUVECs with 4μg/ml BSA, 4μg/ml PNA, 20μg/ml ASF, and 2μg/ml of anti-MCAM or anti-PECAM antibody for 24 hr. Then the cultured medium was collected and analysed by ELISA. PNA binds to TF antigen carried on ASF, the PNA effects should therefore be inhibited by ASF. The level of IL-6 released by the PNA treated cells was 236±18% higher than the BSA-treated group and the increase was fully inhibited by ASF (99±12%). PNA-mediated increase of IL-6 secretion was shown to be partially inhibited by the presence of anti-MCAM (57±14%) and anti-PECAM antibody (48±17%). PNA treatment resulted in increase of MCP-1 secretion by 405±15% comparing to the BSA-treated group and the increase was inhibited by ASF (82±1%) and partially by anti-MCAM antibody (60±19%) and anti-PECAM antibody (57±44%). (Fig 6.4.8) It is known that MCAM is also the receptor for galectin-3.

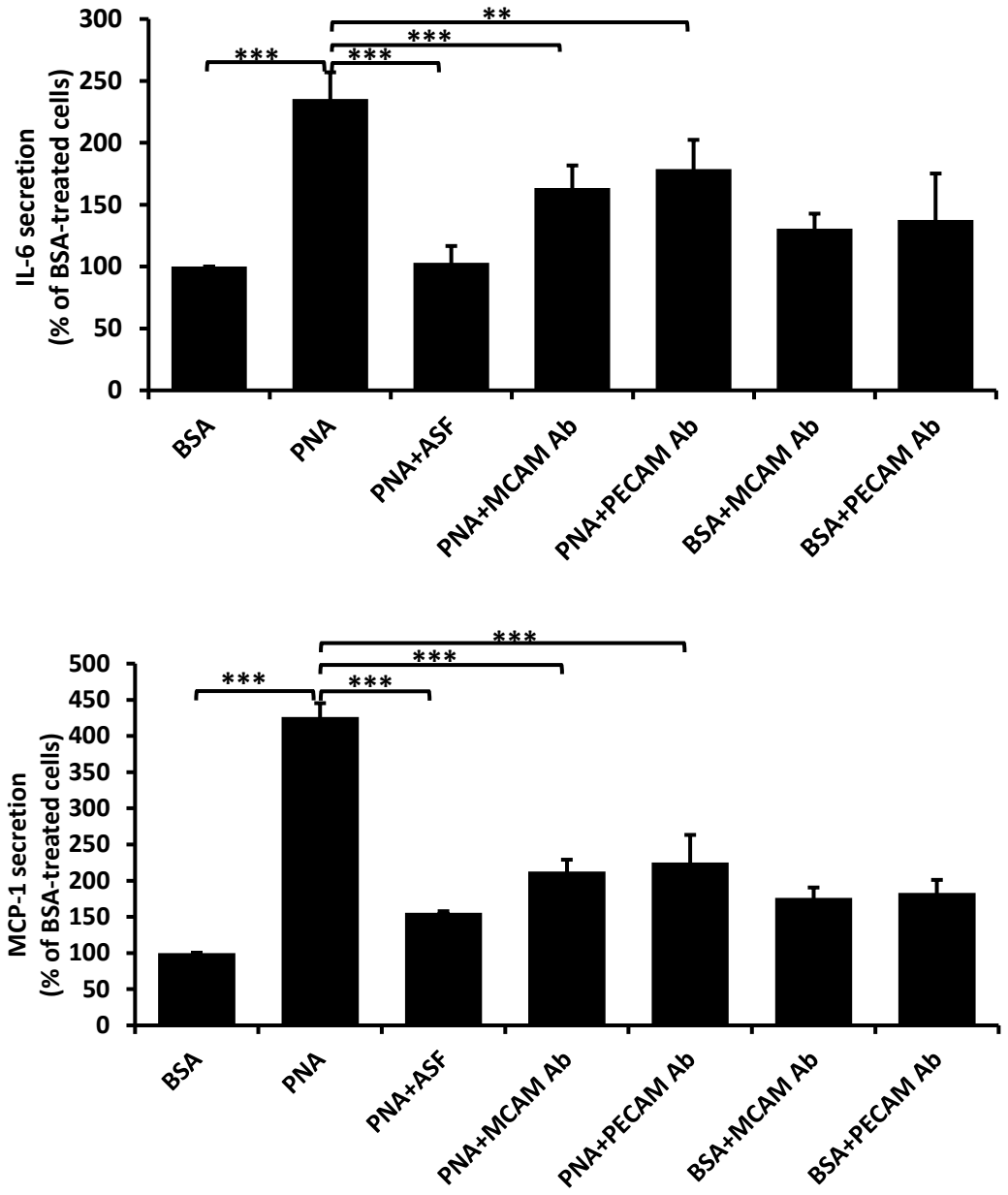


Figure 6.4.8: PNA binding to cell surface MCAM and PECAM are responsible for PNA-mediated cytokine secretion

HUVECs were treated with 4ug/ml PNA or BSA in the presence or absence of 20μg/ml ASF, antibodies against MCAM (2μg/ml) or PECAM (2μg/ml) for 24hr followed by ELISA analysis of the concentration of cytokines in the culture medium. Data are presented as Mean ± SD in comparison to control, from 3 independent experiments. ****p<0.001. (One-way ANOVA followed by Bonferroni)*

6.4.8 Suppression of MCAM and PECAM expression on endothelial cells

To determine whether MCAM and PECAM are the major receptors for PNA, the expression of MCAM and PECAM from HUVECs was suppressed by siGENOME siRNA. SiRNA treatment showed a 53% blockage of cellular MCAM expression, and 79% blockage of cellular PECAM expression when comparing to cells treated with control siRNA (Fig 6.9.9).

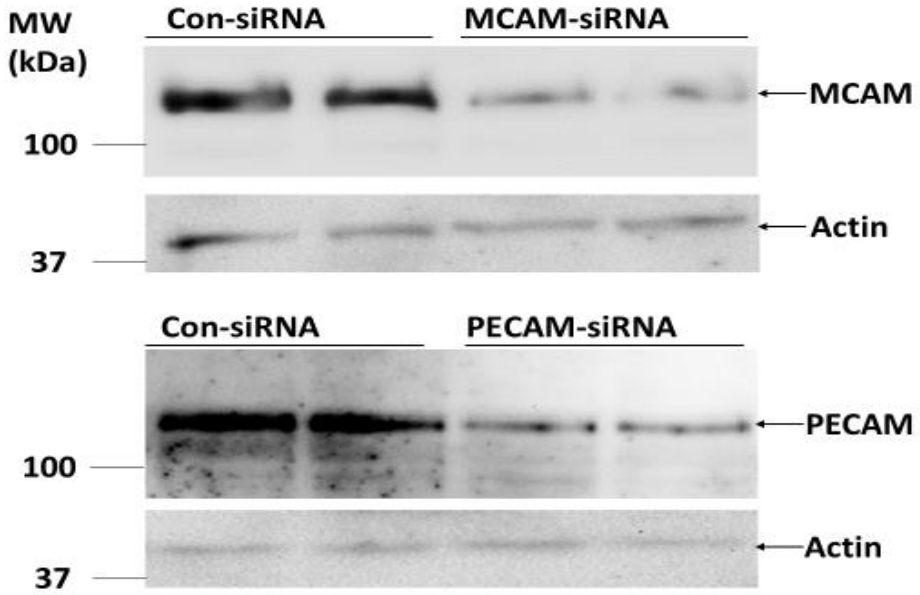


Figure 6.4.9: Suppression of MCAM and PECAM expression in endothelial cell

HUVECs were treated with 150pmol of control siRNA, MCAM siRNA or PECAM siRNA for 36 hr. The cells were lysed and analyzed for MCAM and PECAM expression by immunoblotting. The cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were incubated with primary antibodies against MCAM (1:500), PECAM (1:500) or actin (1:1000) as control for 24 hr. After secondary antibody, the blots were washed and developed Super Signal kit. The protein bands on the blots were visualized with the Molecular Imager Gel Doc™ XR system (Bio-Rad).

6.4.9 Investigation of the influence of suppression of MCAM or PECAM expression on PNA-induced endothelial cytokine secretion

To investigate whether MCAM and PECAM are the functional ligands responsible for PNA-induced cytokine secretion by the endothelium, siRNA treatment was applied to the endothelial cells and suppression of MCAM and PECAM expression was confirmed by immunoblotting. The suppressed cells were then introduced with 4μg/ml PNA and IL-6 and MCP-1 concentrations in the culture media then determined by ELISA. Treatment with 4μg/ml PNA increased IL-6 concentration to 5.61ng/ml (± 0.7ng/ml), a 3.96-fold increase in the control-siRNA treated HUVECs cells compared with BSA. The MCAM siRNA+PNA group showed marked inhibition of IL-6 secretion (67±5%) in comparison to the control cells treated with PNA. The PECAM siRNA+PNA group also showed full blockage of IL-6 endothelial secretion (71±5%). Within the control siRNA cells, PNA induced MCP-1 secretion to 16.93±2.41ng/ml which is 3.3-fold higher than the control group. MCAM and PECAM siRNA suppressed cells treated with PNA showed major reduction of MCP-1 secretion (83±5%; 81±8%). In conclusion, suppression of either MCAM or PECAM resulted in loss of the PNA effect on IL-6 and MCP-1 secretion. (Figure 6.4.10)

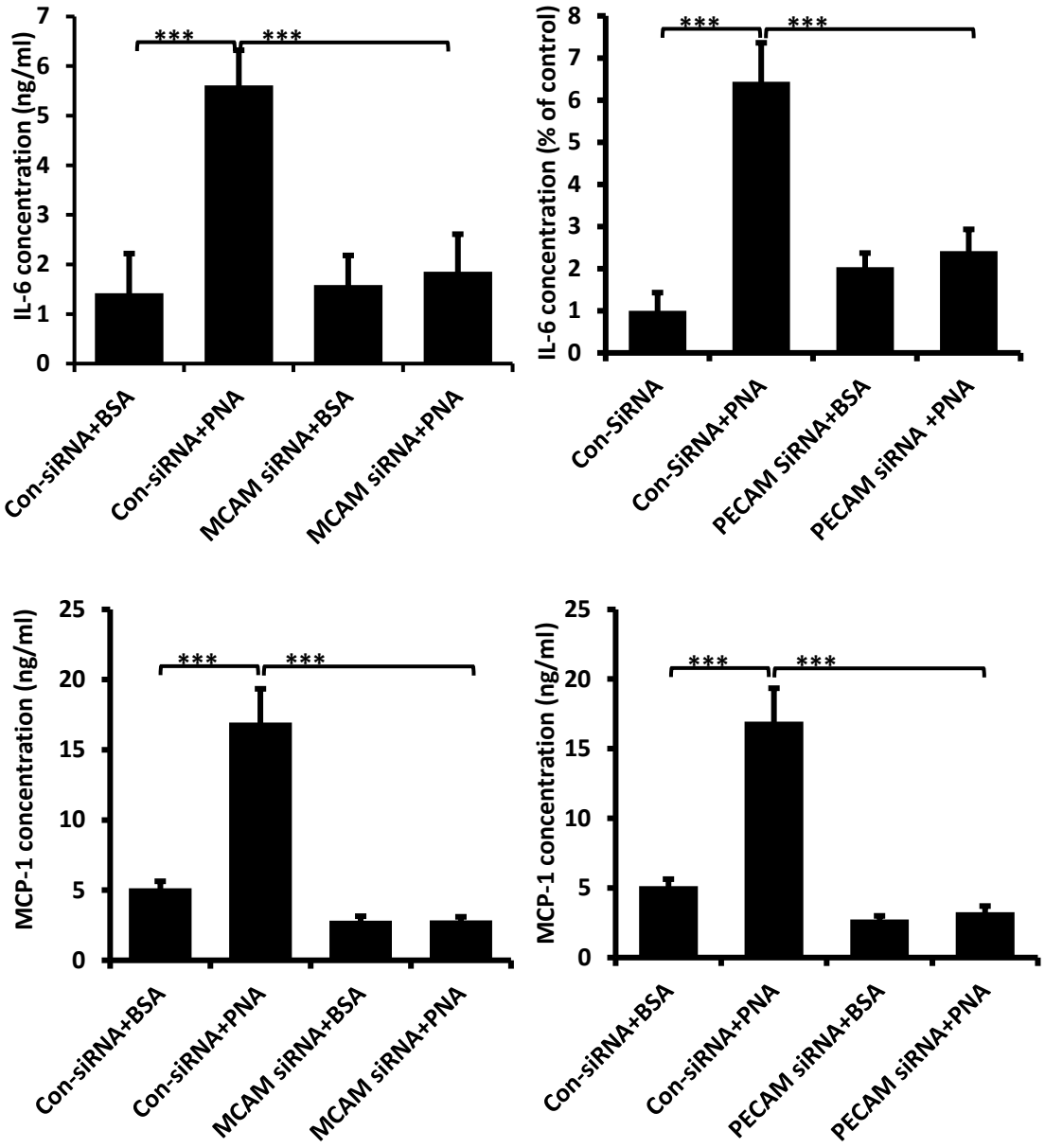


Figure 6.4.10: Suppression of MCAM and PECAM expression in endothelial cells blocks PNA-induced cytokine secretion

HUVECs were treated with 150pmol of control siRNA, MCAM siRNA or PECAM siRNA for 36 hr. The cells were treated with 4μg/ml BSA or 4μg/ml PNA and the concentration of IL-6 and MCP-1 in the culture medium was analyzed by ELISA. Data are presented as mean ± SD in comparison to control, from 3 independent experiments. ****p<0.001. (One-way ANOVA followed by Bonferroni)*

6.5 Discussion

In this part of the study, the PNA histochemistry analysis showed strong PNA binding to the endothelial cell surface. Flow cytometry analysis showed that PNA binding to the surface of endothelial cells was partly inhibited by ASF (59%) and galectin-3 (25%). PNA-agarose affinity purification followed by mass spectrometry analysis identified MCAM and PECAM as two PNA binding cell surface glycoproteins. PNA-induced cytokine secretion was shown to be partly inhibited by the presence of anti-MCAM or anti-PECAM antibody. PNA-mediated cytokine secretion was largely lost when MCAM or PECAM expression was suppressed by siRNA treatment. To test whether PNA and MCAM were co-localized on the cell surface, confocal microscopy was performed without addition of detergents such as Triton-100. ASF was used as a competitive inhibitor of binding by PNA. Galectin-3 which also binds to TF antigen was also used here as a potential competitive PNA-binding inhibitor. The presence of galectin-3 caused 25% reduction of PNA binding to the cell surface, indicating that PNA and galectin-3 share some but not all cell surface binding receptors. PNA pre-treatment was shown to increase subsequent PNA binding on the endothelial cell surface when compared with the non-pretreated group. This indicates that PNA-induced cytokine secretion likely increases the expression of PNA endothelial cell surface receptors.

Melanoma cell adhesion molecule (MCAM) also known as CD146 or cell surface glycoprotein MUC18, is a cell surface adhesion molecule with a molecular weight of 113kDA and is identified as a marker for cancer diagnosis, prognosis,

and response to treatment. [313] MCAM is a membrane glycoprotein consisting of an extracellular domain containing 8 putative N-glycosylation sites, a single transmembrane domain and a cytoplasmic tail. [314, 315] The glycosylation of MCAM comprises 35% of the total molecular weight. MCAM was viewed as an endothelial cell marker previously [316] but is expressed by melanoma cells and plays an important role in melanoma metastasis. [317, 318] The MCAM protein is composed of two isoforms, CD146 long and CD146 short isoforms. These two isoforms can be identified by their different lengths of intracellular domain. [319] The CD146 long form consists of a 63-aa intracellular fragment including 5 protein kinase binding sites and 2 putative endocytic domains, while the CD146-short form consists of a truncated cytoplasmic tail that lacks endocytosis motifs and a protein kinase site. [320] Both the short and long forms are expressed by endothelial cells while melanoma cells express mainly the long isoform. On the vascular endothelial cell surface, MCAM is identified as an immunoglobulin superfamily member of endothelial cell surface adhesion molecules with a key effect on cell adhesion. [321] MCAM is also involved in cell-matrix interactions and various kinds of cell-cell interaction in vascular endothelial cell activities such as angiogenesis, migration and engagement in outside-in signals. Although the natural MCAM ligand continues to be elusive, its engagement plays an important role in activation of several signalling pathways including p38, PKC and PI3K/AKT. Recent studies have shown that the interaction between MCAM and VEGFR-2 is the key to activation of p38 and AKT signalling on the endothelial cell surface, and therefore promotes cell migration. [322] In addition, MCAM interaction with Laminin-411 allows Th17 cell entry to the central nervous system and promotes

inflammation. [323] There is also evidence that MCAM binding to Netri-1 activates downstream signalling, thus again enhancing cell proliferation, adhesion, migration and angiogenesis. [320] Recent studies have shown interaction between MCAM and exogenous galectin-1 with inhibition of galectin-1-induced endothelial cell apoptosis. [324]

Platelet endothelial cell adhesion molecule (PECAM) is also known as CD31 with a molecular weight of approximately 130kDa. [325] PECAM is a transmembrane glycoprotein and is a member of the immunoglobulin superfamily of cell adhesion molecules. [326] Due to its differing glycosylation, the molecular weight of PECAM varies between different cell types. [327] PECAM was first characterized as a cell-surface protein before being identified as an endothelial cell junction molecule. Recent studies have found that PECAM is expressed on the cell surface of many cell types including platelets, monocytes, and neutrophils. [328] PECAM is commonly expressed at the intercellular junctions on endothelial cells and on T cells. Previous studies have shown that PECAM plays an important role in leukocyte migration through vascular endothelial cells. [329, 330]PECAM is composed of a large extracellular fragment of 574 amino acids containing nine N-glycosylation sites, a single trans-membrane region of 18 hydrophobic compounds and a cytoplasmic tail of 118 amino acids. [327] Its glycosylation accounts for 40% of the molecular weight. The cDNA of PECAM has an approximately around 70% homology between murine and human sequence. [331] A structure containing six Ig-like homology domains is presented on its extracellular region and this is believed to play a critical role in cell adhesion. The structure of this domain 2 carries an identification sequence for

glycosaminoglycan binding which is found to play a key role in PECAM-1 heterotypic adhesion. In endothelial cell culture, earlier data showed an inhibited cell-cell interaction in the presence of anti-PECAM antibody. [332] In inflammation, the leukocyte-endothelial interaction activates a cascade of cell activities involving cytokines, chemoattractant and cell adhesion molecules. In vivo and in vitro studies have suggested that PECAM has an inhibitory effect at inflammatory sites for neutrophils. [333] Like MCAM, PECAM has also been identified as a biomarker for different stages of cancer metastasis. Both MCAM and PECAM are associated with metastasis and progression of melanoma, breast and prostate cancer. As a member of the Ig superfamily, PECAM is involved in angiogenesis, and has a potential role in cell migration, polarity and extravasation. For example, the expression of PECAM is stimulated by the VEGF secreting from tumour cells, and PECAM acts as a regulator in cell migration by its function on modulating cell-matrix and cell-cell interactions. [334, 335] Previous study also showed that PECAM expressed from melanoma cells participated in cancer cell-vascular endothelial cell adhesion, and promotes metastasis.

As mentioned above, the cell surface adhesion molecules MCAM and PECAM are both reported to interact with VEGFR in endothelial cells, and their interaction affects AKT signalling which could regulate various cytokine secretion such as IL-6. [336] This study shown that the PNA-induced cytokine secretion of IL-6 and MCP-1 were partially inhibited with the present of antibodies against MCAM and PECAM. In addition, the endothelial secretion of IL-6 and MCP-1 were markedly reduced in MCAM/PECAM siRNA suppressed endothelial cells. It seems that the interaction between PNA and its cell surface receptors MCAM and PECAM

involves several cell signalling mechanisms such as PI3K-AKT signalling in human endothelial cells. It remains to be investigated how MCAM and PECAM mediate PNA-induced endothelial cytokine secretion, and activation of various signal transduction pathways may positively regulate these MCAM/PECAM-PNA interactions.

The discovery in this study that galectin-3 can substantially inhibit PNA–induced cytokine secretion suggests that PNA and galectin-3 might share binding to some of the same endothelial cell surface glycoproteins. Both MCAM and PECAM were found in the extracts from galectin-3 affinity purification but only MCAM was shown to interact directly with galectin-3. [218] Inhibition of either MCAM or PECAM on their own only partially inhibits PNA-mediated IL-6 and MCP-1 secretion indicating that, in contrast to the action of galectin-3, both MCAM and PECAM may be involved in PNA-mediated cytokine secretion. In addition, this suggested that we should also look into the interaction between PNA and MCAM/PECAM, this protein-protein interaction can be analysed by co-immunoprecipitation.

CHAPTER 7 Investigating the mechanism of PNA interaction with MCAM and PECAM in endothelial cell signaling

7.1 Hypothesis and Aim

7.1.1 Hypothesis

Induction of endothelial cytokine secretion by PNA following interaction with MCAM and PECAM will involve one or more cell signaling pathways.

7.1.2 Aim

To study the cell signalling pathways involved in the interaction of PNA with MCAM and PECAM in endothelial cells

7.2 Introduction

Studies reported previously in this thesis have shown that PNA induces endothelial secretion of metastasis-associated cytokines IL-6 and MCP-1 and this effect is related to PNA binding to cell surface MCAM and PECAM. It is shown that the PNA-induced cytokine secretion can be inhibited with the present of antibodies against MCAM and PECAM. [337] IL-6 also acts predominantly in the activation of Janus Kinase (JAK) signaling which contributes to initiation of the Signal Transducer and Activator of Transcription protein (STATS) family. [338, 339] In addition, IL-6 is also affected by the mitogen-activated protein kinase (MAPK) signaling cascade which mediates IL-6 mRNA stability. [340] Furthermore, the chemotactic cytokine MCP-1 is also shown to mediate various cell signal transduction pathways by inducing kinase activity and having multifunctional effects on endothelial cell chemotactic activity. [341]

The PNA receptors MCAM and PECAM are also reported to participate in some important cell signalling pathways. [342] MCAM acts as a downstream target of the CD44 signalling pathway which leads to inhibition of breast cancer cell invasion. [343] In melanoma cells, MCAM expression increases phosphorylation of AKT. The expression of MCAM is also strongly linked to the activation of AKT. In contrast, phosphorylation of MAPK was reduced after activation of MCAM in melanoma cells. [344] In hepatocellular carcinoma, PECAM expression mediated by integrin β1, acts as a promoter of cell invasion and metastasis. The expression of PECAM is responsible for the downstream activation of FAK/AKT signalling pathway. [345] Recent studies also showed that

PECAM expression has a regulatory effect on phosphorylated STAT signal transduction. [346]

This part of the study aims to investigate the signalling pathways induced by PNA interaction with MCAM and PECAM in the PNA-mediated secretion of IL-6 and MCP-1.

7.3 Methods

7.3.1 Assessment of PNA related signaling pathway by protein phosphor-kinase array

Sub-confluent HUVECs were treated with 4μg/ml PNA or BSA for 24 hr. Cells were washed, lysed and analysed using the Proteome Profiler human phosphor-kinase array kit according to the instructions of the manufacturer. The phosphor-array kit provided membranes with antibodies against 43 common kinase phosphorylation sites. Following the instruction, the membranes were prepared, washed and incubated with cell lysed samples before being visualized by the Molecular Imager® Gel Doc™ XR system and analysed using Image Lab software version 5.2.1 (Bio-Rad).

7.3.2 Assessment of the effect of PNA treatment on cell protein tyrosine phosphorylation

Sub-confluent HUVECs were cultured in 12-well plates overnight to form a monolayer. Then HUVECs were treated with different concentrations of PNA (0, 1, 2, 4, 8 μg/ml) for 1 hour at 37°C. Cells were washed in ice cold PBS and lysed in SDS-sample buffer with beta-mercaptoethanol. The samples were then analysed by SDS-PAGE and western blotting with antibodies (1:1000) against AKT, phosphor-AKT, PRAS40 and phosphor-PRAS40. After three washes with 0.05% Tween 20/PBS, the blots were incubated with peroxidase-conjugated anti-rabbit antibodies (1:2000) as secondary detecting antibody. After development and

imaging, the immunoblotting membranes were then stripped using stripping buffer for 30 minutes in a warm water bath, and then re-probed with actin antibody (1:1000) and anti-mouse antibody (1:4000) for validation of protein loading. The blots were visualized with the Molecular Imager® Gel Doc™ XR system and analysed using Image Lab software version 5.2.1 (Bio-Rad).

7.3.2 Assessment of the effect of protein kinase inhibitors on PNA-mediated cytokine secretion from endothelial cells

HUVECs with $1.5x10^5$ cells/ml were cultured in 96-well plates overnight for monolayer formation. The cells were divided into two groups and treated with 4μg/ml PNA or 4μg/ml BSA. Each group was then treated with 5μM PI3K inhibitor Wortmanin, 20μM LY294002, 20μM MEK1/2 inhibitor PD98059, and 50 μM STAT3 inhibitor DPP individually for 24 hr at 37 °C. The cell culture medium was collected and levels of cytokines IL-6 and MCP-1 were determined by cytokine ELISA.

7.3.3 Assessment of the effect of protein kinase inhibitors on the PNA-mediated endothelial cell growth

HUVECs $5x10^5$ cells/ml were cultured in 96-well plates at 37°C for 1hr. The cells were then treated with 4μg/ml BSA, 4μg/ml PNA, 4μg/ml BSA with four individual kinase inhibitors (5μM Wortmanin, 20μM LY294003, 20μM PD98059 and 50μM DPP) and 4μg/ml PNA with the same four individual kinase inhibitors. Endothelial cells were then cultured for 24 hr at 37°C before labelling with 2μl of Calcein AM for 30 min at 37°C. After three PBS washes, the endothelial cell-associated fluorescence was read using a fluorescence microplate reader at 485nm excitation and 535nm emission (infinite 200, TECAN).

7.4 Results

7.4.1 PNA increases phosphorylation of AKT, STAT3, PRAS40 and RSK in endothelial cells

Treatment of HUVECs with PNA was shown to enhance the phosphorylation of several key cell signalling proteins STAT3, PRAS40, AKT1/2/3 and RSK1/2/3. STAT3 phosphorylation at Y705 and S727 was increased by 2.86- and 3.32-fold respectively. AKT1/2/3 phosphorylation at T308 site was increased by 2.81-fold. RSK1/2/3 phosphorylation at S380, S586, S377 was increased by 1.91-fold, and the PRAS40 phosphorylation at T246 site was elevated 1.86-fold. (Fig 7.4.1)

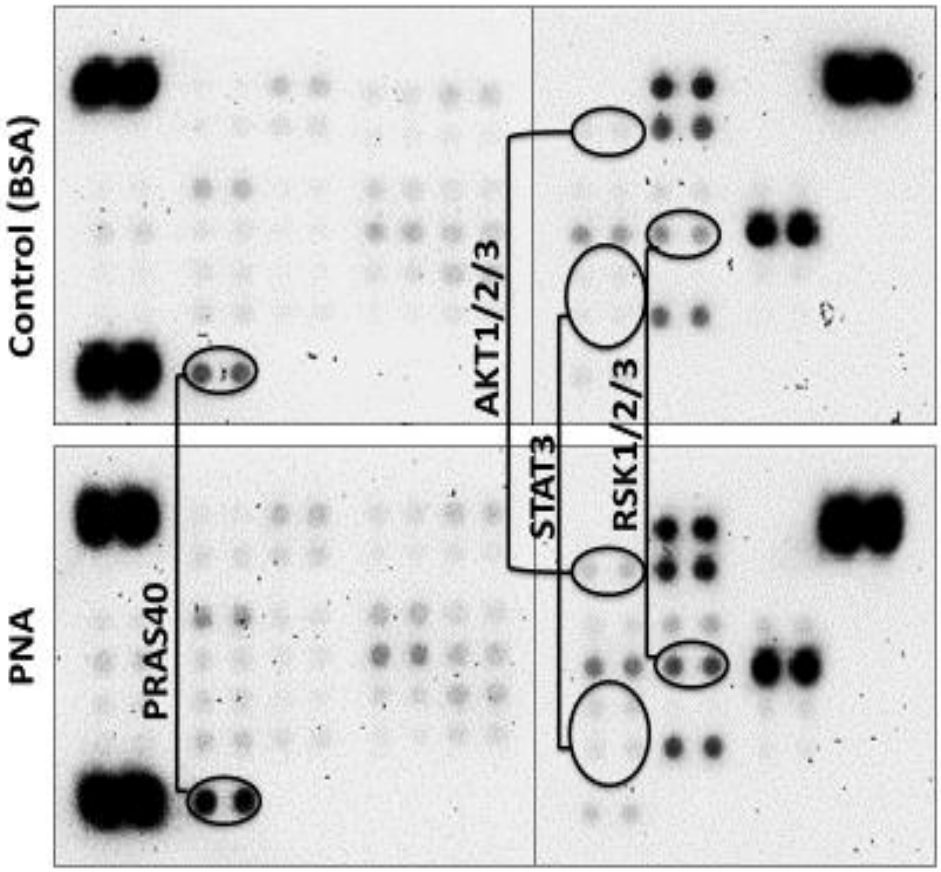


Figure 7.4.1: The presence of PNA increases tyrosine phosphorylation of several key signalling proteins in HUVECs

HUVECs were treated with 4 μg/ml PNA or 4 μg/ml BSA (control) for 30 minutes before analysed by the Human Phosphor-Kinase Array. PNA presence increased the phosphorylation of AKT, TAT3, RSK and PRAS40 when compared to the BSA-treated control group.

7.4.2 Assessments of PNA effects on AKT and PRAS40 phosphorylation

The phosphor-kinase array analysis showed PNA effects on phosphorylation of four kinases at respective phosphorylation sites. In order to further study the mechanism of PNA-induced kinase phosphorylation, the effect of different concentrations of PNA on these kinases was studied by immunoblotting. HUVECs were treated with different concentrations of PNA (0, 1, 2, 4, 8μg/ml) and the cells were collected and analysed by immunoblotting with antibodies (1:1000) against AKT, phosphor-AKT, PRAS40, phosphor-PRAS40, STAT3 and phosphor-STAT3.

The immunoblotting results show that PNA increased the level of phosphorylated AKT compared to the expression of AKT. The PNA treatment also induces a similar dose-dependent increase in PRAS40 phosphorylation. STAT3 phosphorylation showed no significant changes after PNA treatment. (Fig 7.4.2)

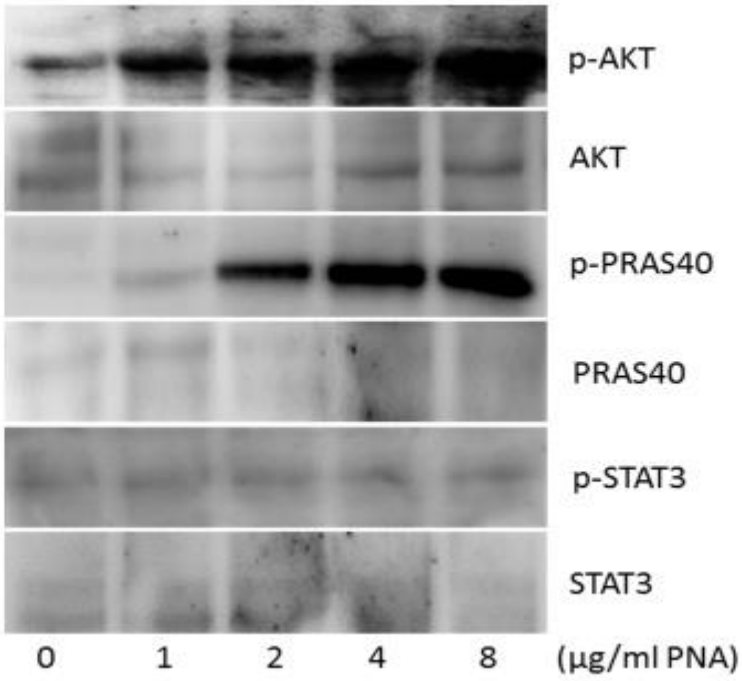


Figure 7.4.2: PNA activates AKT, PRAS40 and STAT3 signaling

HUVECs were treated with 0, 1, 2, 4 and 8µg/ml PNA and lysed with 2% sample buffer. The samples were then analysed by immunoblotting with antibody (1:1000) against AKT, phosphor-AKT, PRAS40, phosphor-PRAS40, STAT3 and phosphor-STAT3. PNA is shown to increase the levels of phosphorylated AKT (60 kDa), PRAS40 (40 kDa) and STAT3 (80 kDa).

7.4.3 PNA-induced cytokine secretion is inhibited by the presence of protein kinase inhibitors against PI3K, MEK1/2 and STAT3

To confirm the involvement of these kinases in PNA-induced cytokine secretion from HUVECs, the HUVECs were treated with PNA in the presence or absence of 4 different kinase inhibitors, PI3K inhibitor Wortmanin (5μM), LY294002 (20 μM), MEK1/2 inhibitor PD98059 (20μM), and STAT3 inhibitor DPP (50μM). The level of IL-6 in the PNA treated cells was 440±27% higher comparing to the BSA-treated control group. This increase was significantly inhibited by the present of each of these kinase inhibitors Wortmanin (70%), LY294002 (97%), PD98059 (67%) and DPP (54%). PNA-treatment increased MCP-1 level by 838±390% and this increase was inhibited by the present of Wortmanin (86%), LY294002 (96%), PD98059 (87%) and also DPP (74%). (Fig 7.4.3)

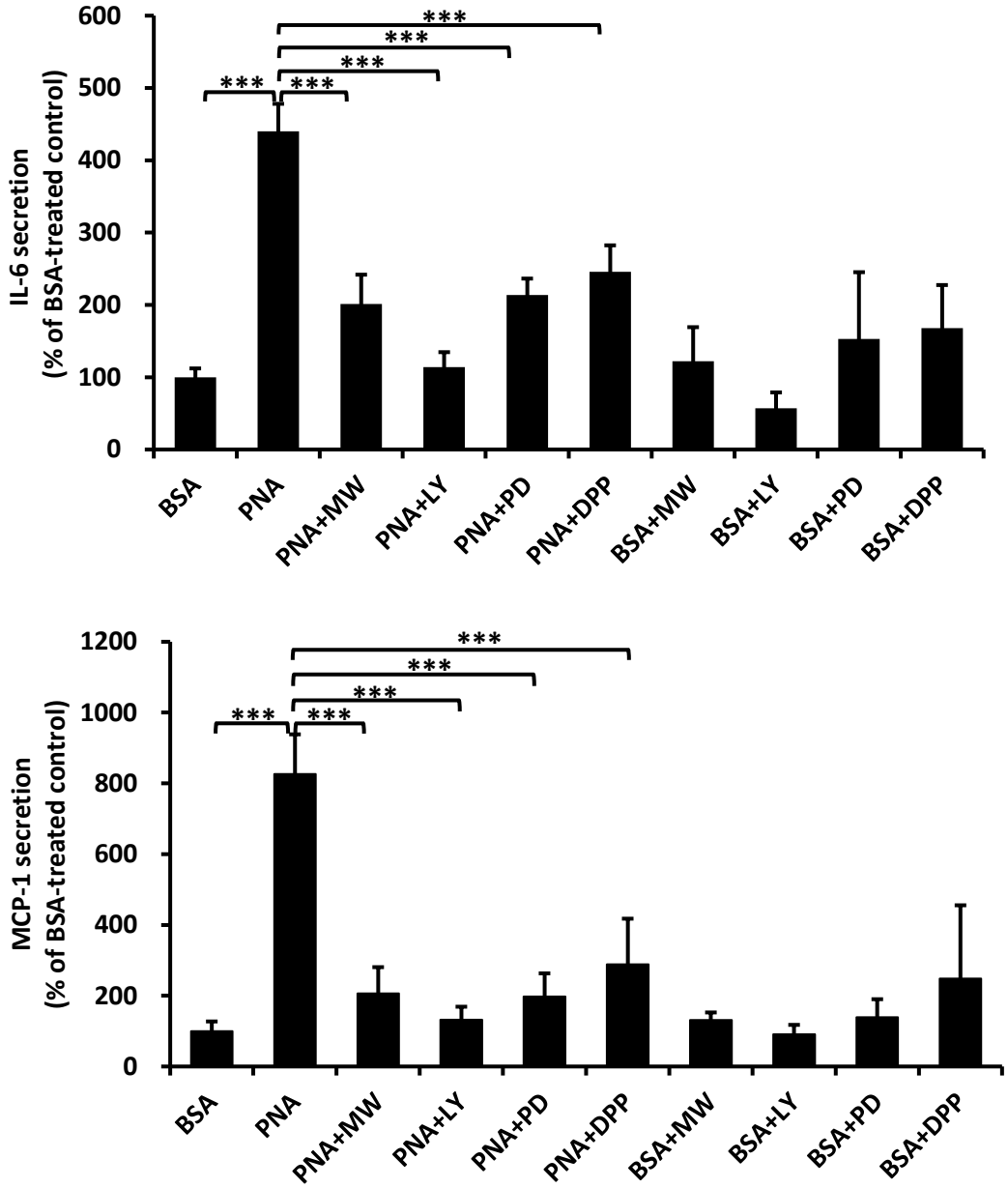


Figure 7.4.3: PNA induced secretion of IL-6 and MCP-1 from HUVECs was inhibited by inhibitors of P13K, MEK1/2 and STAT3 kinases.

The HUVECs were treated with 4 μg/ml PNA or BSA for 24 hr in the presence and absence of four kinase inhibitors, PI3K inhibitor 5μM Wortmanin (MW), 20μM LY294002 (LY), 20μM MEK1/2 inhibitor PD98059 (PD), and 50μM STAT3 inhibitor DPP (DPP) before the concentrations of IL-6 and MCP-1 in the culture medium were analysed by ELISA. Data are presented as Mean ± SD in comparison to control, from 3 independent experiments. ****p<0.001. (One-way ANOVA followed by Bonferroni)*

7.4.4 PNA-induced endothelial cell growth is inhibited in the presence of kinase inhibitors

The results shown the endothelial cell growth was 160±5% higher in the PNA-treated group than the BSA-treated control. This induced endothelial cell growth is mostly inhibited with the present of four kinase inhibitors, Wortmain (74%), LY294002 (92%), PD98059 (70%) and DPP (63%). (Fig 7.4.4)

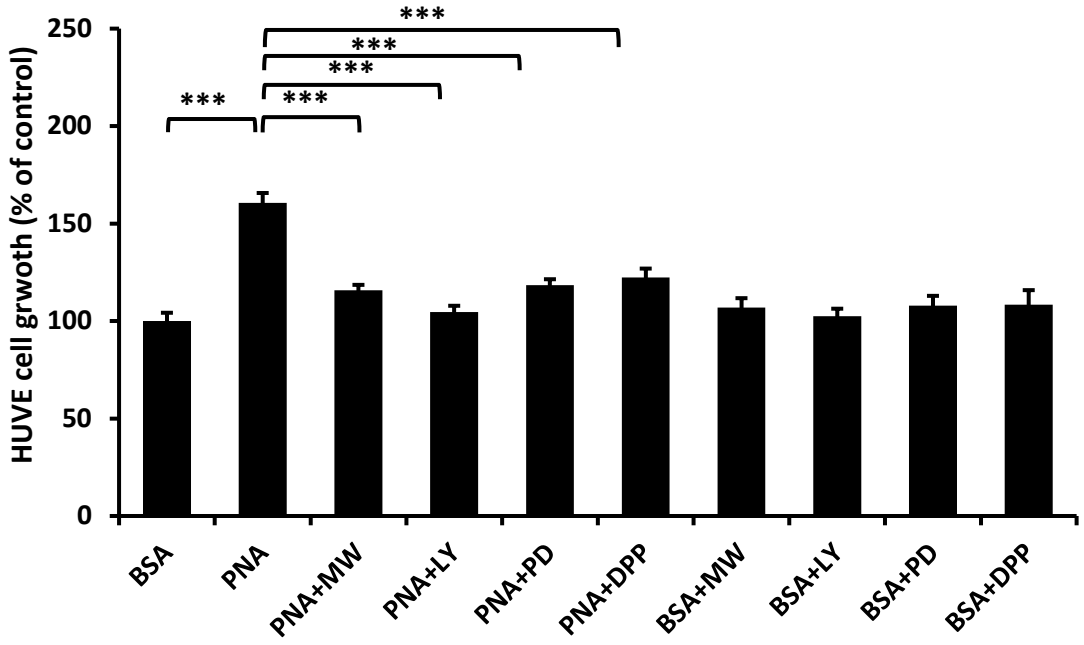


Figure 7.4.4: PNA-mediated endothelial cell growth is inhibited in the presence of kinase inhibitors

Endothelial cells were prepared and incubated with 4μg/ml BSA and 4μg/ml PNA with or without four kinase inhibitors, 5μM Wortmanin, 20μM LY294002, 20μM PD98059 and 50μM DPP for 24hr. Endothelial cells were labelled with Calcein AM and the HUVECs cell survival was measured using a fluorescence microplate reader. The data are expressed as percentage compared with BSA-treated controls from three independent experiments, each in sextuplicate. Data are expressed as mean value ± SD of triplicate. ****p<0.001. (One-way ANOVA followed by Bonferroni)*

7.5 Discussion

This part of the study shows that PNA treatment of HUVECs increases cell phosphorylation of AKT, STAT3, PRAS40 and RSK in a PNA-dose-dependent way and the increase was inhibited by the presence of four kinase inhibitors PI3K inhibitor Wortmanin, LY294002, MEK1/2 inhibitor PD98059, and STAT3 inhibitor DPP.

AKT, also known as protein kinase B, is a serine/threonine protein kinase that has three isoforms AKT1, AKT2 and AKT3. AKT is well known for its regulation of a number of cell functions such as metabolism, proliferation, apoptosis and angiogenesis. [347] It is reported that AKT plays a key role in multiple cell signalling pathways and in determining cell death and survival. [348] The AKT's effect varies in different ways. For examples, AKT stimulates cell growth factors to promote cell survival, and inactivates pro-apoptotic proteins to prevent apoptosis. [349] AKT is composed of an N-terminal domain, a C-terminal domain and a central domain. A structure of 100 amino acids similar to the 3-phosphinositide binding molecules on its N-terminal domain helps its interaction with membrane lipid ligands involving the phosphatidylinositol-3,4,5-triphosphate as called as PIP3 which is associated with the PI3K downstream signaling pathway. [350-352]

PI3Ks are a family of enzymes identified with protein and lipid kinase activity which is activated by different membrane receptors. Even though the PI3Ks have different structure, expression or even substrate specificity, they all share a common function which catalysis the phosphorylation of different

membrane proteins. PI3Ks can be classified into three families by their protein structure and substrate specificity. [353, 354] The most studied family is the four Class I PI3K isoforms which is PI3Kα, β, γ, δ. There are three isoforms in Class II PI3K (PI3KC2α, C3β, C3γ) and also the single class III PI3K. [355] Class I PI3Ks participated in immune regulation and play an important role in related signaling pathways. [356] In dendritic cells, PI3K signaling induced by CD80/CD86, stimulates pro-inflammatory cytokine secretion through downstream AKT phosphorylation and mediates IL-6 expression. [357] It is shown by many studies that the PI3K signal pathway is one of the most common signaling pathways pathogenically affected in cancer, suggesting that PI3K can be used as a target for cancer treatment. Earlier studies in head and neck squamous cell carcinoma (HNSCC) showed that MEK- and PI3K-associated signal pathways play a critical role in hepatocyte growth factor (HGF) inducing expression of angiogenesis factors like IL-8 and vascular endothelial growth factors, leading to increased angiogenesis thus promoting cancer development. [358, 359]

PRAS40 is a substrate of AKT and has a molecular weight of 40kDa. PRAS40 acts as a mammalian target of the heterotrimeric rapamycin (mTOR) protein kinase. [360] After activation of mTORC1 kinase, the phosphorylation of PRAS40 is increased [361] Phosphorylation of various substrates results from activation of mTOR in reaction to growth factors and nutrients, including S6 kinase phosphorylation mediated by mTORC1, and AKT phosphorylation by mTORC2. [362] Modifications in mTOR action and AKT have been associated with progression of various diseases including type 2 diabetes and cancer. [363] Investigation of mTOR-binding partners characterize PRAS40 as both substrate

and also a component of mTORC1. PRAS40's phosphorylation is activated by AKT and results in PRAS40's dissociation from mTORC1 and an inhibitory control on mTORC1 action. [364] Gene silencing studies suggest that PRAS40 is in a similar way required for the mTORC1 complex's activity. Consequently, the role of PRAS40 in the AKT-mTOR signalling pathway is likely to play an important role in disease and health.

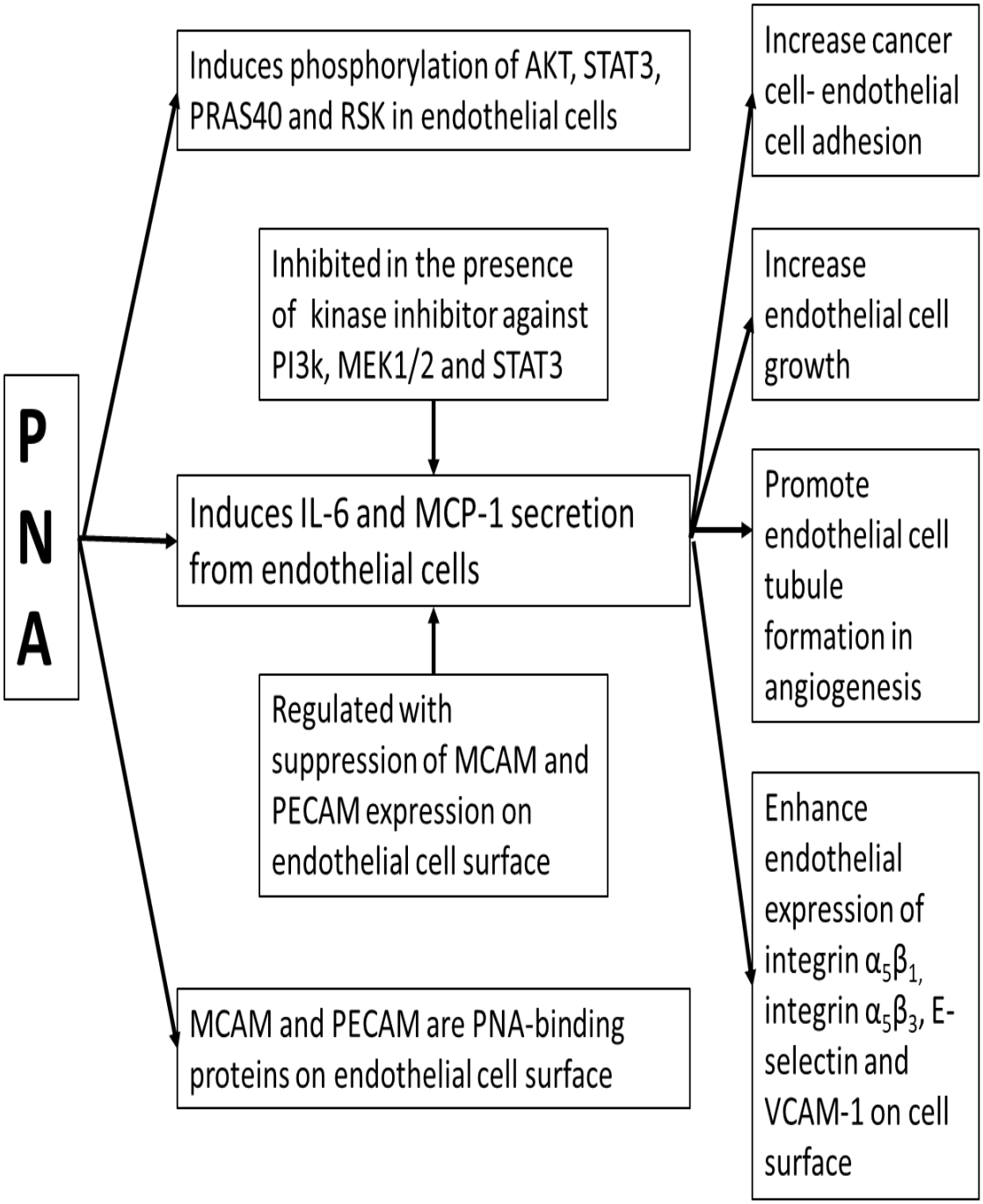
This study showed that PNA induces phosphorylation of all these four kinases AKT, STAT3, PRAS40 and RSK. The presence of kinase inhibitors against PI3K, MEK1/2, and STAT3 inhibits PNA induced phosphorylation of these kinases and prevents the increase of IL-6 and MCP-1 production by PNA. In addition, the PNA-mediated endothelial cell growth is reduced in the presence of these kinase inhibitors. These data indicate that AKT, STAT3, PRAS40 and RSK signalling are involved in the increased secretion of IL-6 and MCP-1 from endothelial cells mediated by interaction of PNA with the endothelial cell surface proteins MCAM and PECAM. It is not yet clear whether the signalling pathways mentioned above are induced indirectly by increased cytokine secretion of IL-6 and MCP-1 from PNA-treated endothelial cells, or are induced more directly by interaction between PNA and its receptors MCAM and PECAM.

CHAPTER 8 Summary of the main findings

- Galectin-3 at pathological concentrations induces dose-dependent secretion of four cytokines (G-CSF, GM-CSF, IL-6 and sICAM-1) from both micro-vascular and macro-vascular endothelia cells.
- PNA at pathological concentrations induced cytokine secretion of IL-6 and MCP-1 from both microvascular and macrovascular endothelial cells.
- The PNA-induced secretion of IL-6 and MCP-1 from endothelial cells in both dose-dependent and time-dependent manner.
- The PNA-induced secretion of IL-6 and MCP-1 increases cancer cell heterotypic adhesion with endothelial cells
- The PNA-induced secretion of cytokines increases endothelial cell growth.
- The PNA-induced secretion of cytokines promotes endothelial cell tubule formation in angiogenesis.
- The PNA-induced secretion of cytokines enhances the endothelial expression of cell surface adhesion molecules including integrin $\alpha_5\beta_1$, integrin $\alpha_5\beta_3$, E-selectin and VCAM-1 on cell surface of HUVECs.
- PNA shows strong binding on the endothelial cell surface after 24 hr.
- The PNA-induced cytokine secretion from endothelial cells is partially inhibited by galectin-3.

- The PNA-induced cancer cell-endothelial cell adhesion is partially inhibited by galectin-3.
- The PNA induced endothelial secretion of IL-6 and MCP-1 is partially inhibited by galectin-3.
- Galectin-3 also shows binding to cancer cells and induces tyrosine phosphorylation.
- PNA-agarose affinity purification extracts cell surface glycoprotein MCAM and PECAM
- MCAM and PECAM are the major PNA-binding proteins on endothelial cell surface.
- The PNA-induced cytokine secretion is inhibited by a combination of anti-MCAM/anti-PECAM antibodies.
- PNA-induced secretion of IL-6 and MCP-1 is regulated with suppression of MCAM and PECAM expression on endothelial cell surface.
- PNA induces phosphorylation of AKT, STAT3, PRAS40 and RSK in endothelial cells.
- PNA-induced cytokine secretion of IL-6 and MCP-1 is inhibited in the presence of kinase inhibitors against PI3k, MEK1/2 and STAT3.

Figure 8: The schematic diagram of main findings


PNA
Induces phosphorylation of AKT, STAT3, PRAS40 and RSK in endothelial cells
Inhibited in the presence of kinase inhibitor against PI3k, MEK1/2 and STAT3
Induces IL-6 and MCP-1 secretion from endothelial cells
Regulated with suppression of MCAM and PECAM expression on endothelial cell surface
MCAM and PECAM are PNA-binding proteins on endothelial cell surface
Increase cancer cell- endothelial cell adhesion
Increase endothelial cell growth
Promote endothelial cell tubule formation in angiogenesis
Enhance endothelial expression of integrin $\alpha_5\beta_1$, integrin $\alpha_5\beta_3$, E-selectin and VCAM-1 on cell surface

CHAPTER 9 General discussion and implications for future studies

9.1 Discussion

The results in this thesis show that PNA at levels similar to that found in the circulation of people after eating 250g peanuts induces secretion of IL-6 and MCP-1 from both human microvascular and macrovascular endothelial cells. The possible relevance of this to human health is further supported by the known marked resistance of peanut lectin to degradation by proteases or cooking. [160] The PNA-induced secretion of these cytokines promotes the expression of several endothelial cell surface adhesion molecules such as E-selectin and VCAM-1, that support adhesion of cancer cells to blood vascular endothelium. PNA-induced secretion of these cytokines also increases endothelial cell migration and tubule formation in angiogenesis. Two cell surface glycoproteins MCAM and PECAM were identified to be the functional PNA ligands responsible for PNA induced secretion of these cytokines. PNA binding to MCAM and PECAM was shown to be partially inhibited by galectin-3, suggesting they share some but not all binding ligands. These discoveries indicate that PNA, like galectin-3, can interact with endothelium and enhances endothelial secretion of cytokines which in turn promotes tumour cell adhesion, migration and angiogenesis in metastasis. Thus, in addition to its direct effect on tumour cell adhesion and aggregation by interaction with cancer associated MUC1 shown in our earlier study, PNA presence in the circulation also

promotes metastasis indirectly by inducing endothelial secretion of metastasis cytokines.

PNA and galectin-3 are both shown to induce IL-6 secretion. Galectin-3 also induces secretion of G-CSF, GM-CSF and ICAM-1 while PNA induces MCP-1 secretion. The difference in the effects of galectin-3 and PNA on secretion of these cytokines is in keeping with the partial competition of galectin-3 with PNA binding on endothelial cells and on PNA-induced cytokine secretion. This is also in keeping with the difference in effect of galectin-3 and PNA on the expression of endothelial cell adhesion molecules. The secretion of cytokines induced by PNA and galectin-3 both increase the expression of α5β1 integrin, E-selectin, VCAM-1 and ICAM-1. While the cytokines induced by PNA also increases α5β3 integrin expression, those induced by galectin-3 have no effect on α5β3 integrin expression. The integrin α5β1 is known as a fibronectin receptor, was first identified as a cell adhesion molecule and acts as receptor for ECM proteins and transmembrane proteins. (220) With its regulation function in intracellular signaling and cell activities including proliferation, differentiation and migration, α5β1 is also a therapeutic target in ovarian cancer and glioma. (221, 222, 223) E-selectin activated by cytokines in endothelial cell is reported previously as a mediator of cell adhesion and inflammation. (224, 225). In the cancer microenvironment, E-selectin is a potential biomarker for detection and monitoring of lung, breast and colon cancer (226, 227, 228) Circulating E-selectin was reported to induce cancer

cell migration and tumour growth through interaction with CD44. Alteration of ICAM-1 expression is correlated with metastasis in breast cancer. (230) (229) ICAM-1 was shown by a previous study to bind to the cell surface MUC1, which is a natural high affinity galectin-3 ligand and carries TF antigen that is the specific binding structure of PNA. These cell surface receptors were activated by PNA-mediated cytokines and contribute to many important cellular and pathological functions. The increased expression of these cell surface adhesion molecules by PNA-induced cytokines thus could have fundamental impact on cancer metastasis

Lectins in plants such as legumes have been known for over a century. Earlier studies on lectins were more focused on their roles in immune and inflammation responses. More recently, interaction between lectins and cancer cells started to attract attention. Lectins with their specific binding to carbohydrates on cell membrane are involved in cell aggregation and adhesion. (214) Animal studies have shown that Viscum album agglutinin-1 (VAA-1) from mistletoe extracts, increase IL-12 secretion and stimulate cytotoxic activity of natural killer (NK) cells. (213), supporting an anticancer function of VAA-1. (214)

Previous studies have shown that PNA stimulates proliferation of colon, melanoma and breast cancer cells (208, 209, 211, 212). IL-6 and MCP-1 are known to affect a range of cancer cell activities such as stimulating cancer-related inflammation from cancer cells or endothelial cells and enhance cell growth, invasion and metastasis. (215, 216, 217, 218, 219) The increased secretion of IL-6

and MCP-1 by PNA would be expected to have an impact of metastasis. Thus, in addition to the direct influence of PNA in the circulation on metastasis by interaction with cancer-associated MUC1, PNA also has an indirect influence on cancer metastasis by interaction with the blood vascular endothelium to induce secretion of IL-6 and MCP-1 cytokines. This provides further support to the hypothesis that regular consumption of peanuts by cancer patients might increase the risk of metastasis and mortality. Although further studies are needed, these data indicate that the presence of PNA in the blood stream of cancer patients who regularly eat peanuts could have a detrimental effect.

9.2 Future studies

It is still unclear how PNA interaction with the cell surface glycoproteins MCAM and PECAM induces endothelial cytokine secretion. Further investigation of their interaction, for example the carbohydrate structure that PNA interacts with on MCAM and PECAM, is needed. *In vivo* studies of the effect of PNA-induced cytokine secretion on metastasis will also be needed to further validate the PNA actions for future potential diet recommendation to patients. Plant lectins similar to PNA with the same carbohydrate recognition domains, will also be studied for their roles in cancer metastasis. Observation studies such as cohort studies on the relationship between nut consumption and the prognosis of colorectal cancer or lung cancer are also suggested.

More broadly, this study suggests an impact of dietary lectin PNA on cancer metastasis which is a largely unexplored area in cancer research. It is possible that other dietary lectins may also have direct or indirect influence on tumour spreading hence affect the overall survival of patients. This is an area that deserves more attention and research in future.

CHAPTER 10 References

1. *Cancer registration: principles and methods.* IARC Sci Publ, 1991(95): p. 1-288.

2. Sudhakar, A., *History of Cancer, Ancient and Modern Treatment Methods.* J Cancer Sci Ther, 2009. **1**(2): p. 1-4.

3. David, A.R. and M.R. Zimmerman, *Cancer: an old disease, a new disease or something in between?* Nat Rev Cancer, 2010. **10**(10): p. 728-33.

4. Hajdu, S.I., *A note from history: landmarks in history of cancer, part 1.* Cancer, 2011. **117**(5): p. 1097-102.

5. Bray, F., et al., *Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries.* CA Cancer J Clin, 2018. **68**(6): p. 394-424.

6. Hartwell, L.H. and M.B. Kastan, *Cell cycle control and cancer.* Science, 1994. **266**(5192): p. 1821-8.

7. Nowell, P.C., *The clonal evolution of tumor cell populations.* Science, 1976. **194**(4260): p. 23-8.

8. Gorgoulis, V.G., et al., *Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions.* Nature, 2005. **434**(7035): p. 907-13.

9. Harris, C.C., *Chemical and physical carcinogenesis: advances and perspectives for the 1990s.* Cancer Res, 1991. **51**(18 Suppl): p. 5023s-5044s.

10. Spill, F., C. Bakal, and M. Mak, *Mechanical and Systems Biology of Cancer.* Comput Struct Biotechnol J, 2018. **16**: p. 237-245.

11. McGuire, S., *World Cancer Report 2014. Geneva, Switzerland: World Health Organization, International Agency for Research on Cancer, WHO Press, 2015.* Adv Nutr, 2016. **7**(2): p. 418-9.

12. Hoadley, K.A., et al., *Multiplatform analysis of 12 cancer types reveals molecular classification within and across tissues of origin.* Cell, 2014. **158**(4): p. 929-944.

13. Siegel, R.L., K.D. Miller, and A. Jemal, *Cancer statistics, 2018.* CA Cancer J Clin, 2018. **68**(1): p. 7-30.

14. Sobin, L.H., *TNM: evolution and relation to other prognostic factors.* Semin Surg Oncol, 2003. **21**(1): p. 3-7.

15. Ferretti, S., et al., *[TNM classification of malignant tumours, VII edition 2009. Changes and practical effects on cancer epidemiology].* Epidemiol Prev, 2010. **34**(3): p. 125-8.

16. Ross, M.I., *New American Joint Commission on Cancer staging system for melanoma: prognostic impact and future directions.* Surg Oncol Clin N Am, 2006. **15**(2): p. 341-52.

17. in *Delivering High-Quality Cancer Care: Charting a New Course for a System in Crisis*, L. Levit, et al., Editors. 2013: Washington (DC).

18. Cairns, J., *Mutation selection and the natural history of cancer.* Nature, 1975. **255**(5505): p. 197-200.

19. Bertram, J.S., *The molecular biology of cancer.* Mol Aspects Med, 2000. **21**(6): p. 167-223.

20. Brankovic-Magic, M., J. Dobricic, and A. Krivokuca, *Genetics of breast cancer: contribution of BRCA1/2 genes alterations to hereditary predisposition.* Vojnosanit Pregl, 2012. **69**(8): p. 700-6.

21. Piccolo, S.R. and L.J. Frey, *Somatic mutation signatures of cancer.* AMIA Annu Symp Proc, 2008: p. 202-6.

22. Hirshfield, K.M., T.R. Rebbeck, and A.J. Levine, *Germline mutations and polymorphisms in the origins of cancers in women.* J Oncol, 2010. **2010**: p. 297671.

23. Sepulveda, A.R., *Helicobacter, Inflammation, and Gastric Cancer.* Curr Pathobiol Rep, 2013. **1**(1): p. 9-18.

24. Pepper, J.W., et al., *Cancer research meets evolutionary biology.* Evol Appl, 2009. **2**(1): p. 62-70.

25. Houston, M., *Cancer: the evolutionary legacy.* BMJ, 2000. **321**(7256): p. 305A.

26. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.

27. Colotta, F., et al., *Cancer-related inflammation, the seventh hallmark of cancer: links to genetic instability.* Carcinogenesis, 2009. **30**(7): p. 1073-81.

28. Liberti, M.V. and J.W. Locasale, *The Warburg Effect: How Does it Benefit Cancer Cells?* Trends Biochem Sci, 2016. **41**(3): p. 211-218.

29. Poste, G. and I.J. Fidler, *The pathogenesis of cancer metastasis.* Nature, 1980. **283**(5743): p. 139-46.

30. Liotta, L.A., *Tumor invasion and metastases--role of the extracellular matrix: Rhoads Memorial Award lecture.* Cancer Res, 1986. **46**(1): p. 1-7.

31. Folkman, J., *How is blood vessel growth regulated in normal and neoplastic tissue? G.H.A. Clowes memorial Award lecture.* Cancer Res, 1986. **46**(2): p. 467-73.

32. Su, Z., et al., *Apoptosis, autophagy, necroptosis, and cancer metastasis.* Mol Cancer, 2015. **14**: p. 48.

33. Nicolson, G.L., *Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites.* Biochim Biophys Acta, 1988. **948**(2): p. 175-224.

34. Fidler, I.J., *The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited.* Nat Rev Cancer, 2003. **3**(6): p. 453-8.

35. Seyfried, T.N. and L.C. Huysentruyt, *On the origin of cancer metastasis.* Crit Rev Oncog, 2013. **18**(1-2): p. 43-73.

36. Printz, C., *Cancer death rate declines 25% after 1991 peak.* Cancer, 2017. **123**(14): p. 2593.

37. Fineberg, H.V., *Cancer care for the whole patient.* Medscape J Med, 2008. **10**(9): p. 213.

38. Siegel, R., et al., *Cancer treatment and survivorship statistics, 2012.* CA Cancer J Clin, 2012. **62**(4): p. 220-41.

39. Miller, K.D., et al., *Cancer treatment and survivorship statistics, 2016.* CA Cancer J Clin, 2016. **66**(4): p. 271-89.

40. Guan, X., *Cancer metastases: challenges and opportunities.* Acta Pharm Sin B, 2015. **5**(5): p. 402-18.

41. Fidler, I.J., *The organ microenvironment and cancer metastasis.* Differentiation, 2002. **70**(9-10): p. 498-505.

42. Flamini, V., et al., *Significance and therapeutic implications of endothelial progenitor cells in angiogenic-mediated tumour metastasis.* Crit Rev Oncol Hematol, 2016. **100**: p. 177-89.

43. Moh, M.C. and S. Shen, *The roles of cell adhesion molecules in tumor suppression and cell migration: a new paradox.* Cell Adh Migr, 2009. **3**(4): p. 334-6.

44. MacDonald, N.J. and P.S. Steeg, *Molecular basis of tumour metastasis.* Cancer Surv, 1993. **16**: p. 175-99.

45. Zetter, B.R., *Adhesion molecules in tumor metastasis.* Semin Cancer Biol, 1993. **4**(4): p. 219-29.

46. Harmon, R.M., B.V. Desai, and K.J. Green, *Regulatory roles of the cadherin superfamily.* F1000 Biol Rep, 2009. **1**: p. 13.

47. Wai Wong, C., D.E. Dye, and D.R. Coombe, *The role of immunoglobulin superfamily cell adhesion molecules in cancer metastasis.* Int J Cell Biol, 2012. **2012**: p. 340296.

48. Afratis, N.A., et al., *Syndecans - key regulators of cell signaling and biological functions.* FEBS J, 2017. **284**(1): p. 27-41.

49. Li, Y., et al., *Immunoglobulin superfamily genes are novel prognostic biomarkers for breast cancer.* Oncotarget, 2017. **8**(2): p. 2444-2456.

50. Rapanotti, M.C., et al., *Minimal residual disease in melanoma: circulating melanoma cells and predictive role of MCAM/MUC18/MelCAM/CD146.* Cell Death Discov, 2017. **3**: p. 17005.

51. Joyce, J.A. and J.W. Pollard, *Microenvironmental regulation of metastasis.* Nat Rev Cancer, 2009.

9(4): p. 239-52.

52. Reuter, S., et al., *Oxidative stress, inflammation, and cancer: how are they linked?* Free Radic Biol Med, 2010. **49**(11): p. 1603-16.

53. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-99.

54. Terzic, J., et al., *Inflammation and colon cancer.* Gastroenterology, 2010. **138**(6): p. 2101-2114 e5.

55. Kim, E.R. and D.K. Chang, *Colorectal cancer in inflammatory bowel disease: the risk, pathogenesis, prevention and diagnosis.* World J Gastroenterol, 2014. **20**(29): p. 9872-81.

56. Sangha, S., M. Yao, and M.M. Wolfe, *Non-steroidal anti-inflammatory drugs and colorectal cancer prevention.* Postgrad Med J, 2005. **81**(954): p. 223-7.

57. Iqbal, A.J., E.A. Fisher, and D.R. Greaves, *Inflammation-a Critical Appreciation of the Role of Myeloid Cells.* Microbiol Spectr, 2016. **4**(5).

58. Brooks, P., *Use and benefits of nonsteroidal anti-inflammatory drugs.* Am J Med, 1998. **104**(3A): p. 9S-13S; discussion 21S-22S.

59. Janakiram, N.B. and C.V. Rao, *The role of inflammation in colon cancer.* Adv Exp Med Biol, 2014. **816**: p. 25-52.

60. Sarlos, P., et al., *Genetic update on inflammatory factors in ulcerative colitis: Review of the current literature.* World J Gastrointest Pathophysiol, 2014. **5**(3): p. 304-21.

61. Oppenheim, J. and H. Fujiwara, *The role of cytokines in cancer.* Cytokine Growth Factor Rev, 1996. **7**(3): p. 279-88.

62. Arango Duque, G. and A. Descoteaux, *Macrophage cytokines: involvement in immunity and infectious diseases.* Front Immunol, 2014. **5**: p. 491.

63. Curfs, J.H.A.J., J.F.G.M. Meis, and J.A.A. HoogkampKorstanje, *A primer on cytokines: Sources, receptors, effects, and inducers.* Clinical Microbiology Reviews, 1997. **10**(4): p. 742-+.

64. Zhang, J.M. and J. An, *Cytokines, inflammation, and pain.* Int Anesthesiol Clin, 2007. **45**(2): p. 27-37.

65. Esquivel-Velazquez, M., et al., *The role of cytokines in breast cancer development and progression.* J Interferon Cytokine Res, 2015. **35**(1): p. 1-16.

66. Akdis, M., et al., *Interleukins (from IL-1 to IL-38), interferons, transforming growth factor beta, and TNF-alpha: Receptors, functions, and roles in diseases.* J Allergy Clin Immunol, 2016. **138**(4): p. 984-1010.

67. Karin, M., *NF-kappaB as a critical link between inflammation and cancer.* Cold Spring Harb Perspect Biol, 2009. **1**(5): p. a000141.

68. Liu, T., et al., *NF-kappaB signaling in inflammation.* Signal Transduct Target Ther, 2017. **2**.

69. Greten, F.R., et al., *IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer.* Cell, 2004. **118**(3): p. 285-96.

70. Maeda, S., et al., *IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis.* Cell, 2005. **121**(7): p. 977-90.

71. Ghosh, S. and M. Karin, *Missing pieces in the NF-kappaB puzzle.* Cell, 2002. **109 Suppl**: p. S81-96.

72. Baud, V. and E. Derudder, *Control of NF-kappaB activity by proteolysis.* Curr Top Microbiol Immunol, 2011. **349**: p. 97-114.

73. Oeckinghaus, A. and S. Ghosh, *The NF-kappaB family of transcription factors and its regulation.* Cold Spring Harb Perspect Biol, 2009. **1**(4): p. a000034.

74. Zheng, C., Q. Yin, and H. Wu, *Structural studies of NF-kappaB signaling.* Cell Res, 2011. **21**(1): p. 183-95.

75. Li, Z.W., et al., *The IKKbeta subunit of IkappaB kinase (IKK) is essential for nuclear factor kappaB activation and prevention of apoptosis.* J Exp Med, 1999. **189**(11): p. 1839-45.

76. Park, M.H. and J.T. Hong, *Roles of NF-kappaB in Cancer and Inflammatory Diseases and Their Therapeutic Approaches.* Cells, 2016. **5**(2).

77. Zhang, W. and S.I. Grivennikov, *Top Notch cancer stem cells by paracrine NF-kappaB signaling in breast cancer.* Breast Cancer Res, 2013. **15**(5): p. 316.

78. Shin, W.G., et al., *Infection of human intestinal epithelial cells by invasive bacteria activates NF-kappaB and increases ICAM-1 expression through NOD1.* Korean J Intern Med, 2018. **33**(1): p. 81-90.

79. Huang, H., et al., *Infection of endothelial cells with Trypanosoma cruzi activates NF-kappaB and induces vascular adhesion molecule expression.* Infect Immun, 1999. **67**(10): p. 5434-40.

80. Wang, V.Y., et al., *The transcriptional specificity of NF-kappaB dimers is coded within the kappaB DNA response elements.* Cell Rep, 2012. **2**(4): p. 824-39.

81. Chen, F., V. Castranova, and X. Shi, *New insights into the role of nuclear factor-kappaB in cell growth regulation.* Am J Pathol, 2001. **159**(2): p. 387-97.

82. Diamond, G., et al., *The roles of antimicrobial peptides in innate host defense.* Curr Pharm Des, 2009. **15**(21): p. 2377-92.

83. Wang, L., et al., *IL-6 induces NF-kappa B activation in the intestinal epithelia.* J Immunol, 2003. **171**(6): p. 3194-201.

84. Setrerrahmane, S. and H. Xu, *Tumor-related interleukins: old validated targets for new anti-cancer drug development.* Mol Cancer, 2017. **16**(1): p. 153.

85. Zhang, X.L., et al., *Interleukin-6 regulation of transforming growth factor (TGF)-beta receptor compartmentalization and turnover enhances TGF-beta1 signaling.* J Biol Chem, 2005. **280**(13): p. 12239-45.

86. Mosser, D.M. and X. Zhang, *Interleukin-10: new perspectives on an old cytokine.* Immunological Reviews, 2008. **226**: p. 205-218.

87. Giri, D., M. Ozen, and M. Ittmann, *Interleukin-6 is an autocrine growth factor in human prostate cancer.* Am J Pathol, 2001. **159**(6): p. 2159-65.

88. Yanagawa, H., et al., *Serum levels of interleukin 6 in patients with lung cancer.* Br J Cancer, 1995. **71**(5): p. 1095-8.

89. Kozlowski, L., et al., *Concentration of interleukin-6 (IL-6), interleukin-8 (IL-8) and interleukin-10 (IL-10) in blood serum of breast cancer patients.* Rocz Akad Med Bialymst, 2003. **48**: p. 82-4.

90. Elias, J.A. and R.J. Zitnik, *Cytokine-cytokine interactions in the context of cytokine networking.* Am J Respir Cell Mol Biol, 1992. **7**(4): p. 365-7.

91. Mierke, C.T., *Role of the endothelium during tumor cell metastasis: is the endothelium a barrier or a promoter for cell invasion and metastasis?* J Biophys, 2008. **2008**: p. 183516.

92. Ugen, K.E., et al., *Inhibition of tumor cell-induced platelet aggregation and experimental tumor metastasis by the synthetic Gly-Arg-Gly-Asp-Ser peptide.* J Natl Cancer Inst, 1988. **80**(18): p. 1461-6.

93. Saiki, I., et al., *Anti-metastatic and anti-invasive effects of polymeric Arg-Gly-Asp (RGD) peptide, poly(RGD), and its analogues.* Jpn J Cancer Res, 1990. **81**(6-7): p. 660-7.

94. Saiki, I., et al., *Antimetastatic effect by anti-adhesion therapy with cell-adhesive peptide of fibronectin in combination with anticancer drugs.* Jpn J Cancer Res, 1993. **84**(3): p. 326-35.

95. Johnson, J.P., et al., *De novo expression of intercellular-adhesion molecule 1 in melanoma correlates with increased risk of metastasis.* Proc Natl Acad Sci U S A, 1989. **86**(2): p. 641-4.

96. Saiki, I., *Cell adhesion molecules and cancer metastasis.* Jpn J Pharmacol, 1997. **75**(3): p. 215-42.

97. Orr, F.W., et al., *Interactions between cancer cells and the endothelium in metastasis.* Journal of Pathology, 2000. **190**(3): p. 310-329.

98. Steinbach, F., et al., *The influence of cytokines on the adhesion of renal cancer cells to*

endothelium. J Urol, 1996. **155**(2): p. 743-8.

99. Bevilacqua, M.P., et al., *Interleukin-1 (Il-1) Induces Biosynthesis and Cell-Surface Expression of Procoagulant Activity in Human Vascular Endothelial-Cells.* Journal of Experimental Medicine, 1984. **160**(2): p. 618-623.

100. Kolanus, W., et al., *Alpha L beta 2 integrin/LFA-1 binding to ICAM-1 induced by cytohesin-1, a cytoplasmic regulatory molecule.* Cell, 1996. **86**(2): p. 233-42.

101. Raz, A. and R. Lotan, *Endogenous galactoside-binding lectins: a new class of functional tumor cell surface molecules related to metastasis.* Cancer Metastasis Rev, 1987. **6**(3): p. 433-52.

102. Naisbett, B. and J. Woodley, *The Potential Use of Tomato Lectin for Oral-Drug Delivery .4. Immunological Consequences.* International Journal of Pharmaceutics, 1995. **120**(2): p. 247-254.

103. Ho, W.L., et al., *Protein glycosylation in cancers and its potential therapeutic applications in neuroblastoma.* Journal of Hematology & Oncology, 2016. **9**.

104. Bies, C., C.M. Lehr, and J.F. Woodley, *Lectin-mediated drug targeting: history and applications.* Advanced Drug Delivery Reviews, 2004. **56**(4): p. 425-435.

105. Yu, L.G., *The oncofetal Thomsen-Friedenreich carbohydrate antigen in cancer progression.* Glycoconjugate Journal, 2007. **24**(8): p. 411-420.

106. Lee, Y.C. and R.T. Lee, *Carbohydrate-Protein Interactions - Basis of Glycobiology.* Accounts of Chemical Research, 1995. **28**(8): p. 321-327.

107. Hakomori, S., *Aberrant Glycosylation in Cancer Cell-Membranes as Focused on Glycolipids - Overview and Perspectives.* Cancer Research, 1985. **45**(6): p. 2405-2414.

108. Peumans, W.J. and E.J.M. Vandamme, *Lectins as Plant Defense Proteins.* Plant Physiology, 1995. **109**(2): p. 347-352.

109. Lis, H. and N. Sharon, *Lectins: Carbohydrate-specific proteins that mediate cellular recognition.* Chemical Reviews, 1998. **98**(2): p. 637-674.

110. Fu, L.L., et al., *Plant lectins: Targeting programmed cell death pathways as antitumor agents.* International Journal of Biochemistry & Cell Biology, 2011. **43**(10): p. 1442-1449.

111. Ghazarian, H., B. Idoni, and S.B. Oppenheimer, *A glycobiology review: carbohydrates, lectins and implications in cancer therapeutics.* Acta Histochem, 2011. **113**(3): p. 236-47.

112. Kaltner, H. and H.J. Gabius, *Animal lectins: from initial description to elaborated structural and functional classification.* Adv Exp Med Biol, 2001. **491**: p. 79-94.

113. Wurzburg, B.A., S.S. Tarchevskaya, and T.S. Jardetzky, *Structural changes in the lectin domain of CD23, the low-affinity IgE receptor, upon calcium binding.* Structure, 2006. **14**(6): p. 1049-

58.

114. Dumic, J., S. Dabelic, and M. Flogel, *Galectin-3: an open-ended story.* Biochim Biophys Acta, 2006. **1760**(4): p. 616-35.

115. Endo, K., et al., *Galectin-3 expression is a potent prognostic marker in colorectal cancer.* Anticancer Research, 2005. **25**(4): p. 3117-3121.

116. Vijayan, M. and N. Chandra, *Lectins.* Curr Opin Struct Biol, 1999. **9**(6): p. 707-14.

117. Shi, Z., et al., *Antitumor effects of concanavalin A and Sophora flavescens lectin in vitro and in vivo.* Acta Pharmacol Sin, 2014. **35**(2): p. 248-56.

118. Knejzlikova, H., et al., *Cell reaction to concanavalin A in cancer patients.* Acta Univ Palacki Olomuc Fac Med, 1989. **123**: p. 123-30.

119. Yu, L.G., et al., *Opposite effects on human colon cancer cell proliferation of two dietary Thomsen-Friedenreich antigen-binding lectins.* J Cell Physiol, 2001. **186**(2): p. 282-7.

120. Geraldino, T.H., et al., *Jacalin Has Chemopreventive Effects on Colon Cancer Development.* Biomed Res Int, 2017. **2017**: p. 4614357.

121. Bogoeva, V., et al., *Interaction of Wheat Germ Agglutinin with Porphyrin Compounds—Potential Anticancer Agents.* Biotechnology & Biotechnological Equipment, 2011. **25**(3): p. 2519-2521.

122. Koyama, S., et al., *Wheat germ agglutinin binding is a useful prognostic indicator in stomach cancer.* International Journal of Clinical Oncology, 1999. **4**(2): p. 96-100.

123. Schneider, E.M. and A. Sievers, *Concanavalin A binds to the endoplasmic reticulum and the starch grain surface of root statocytes.* Planta, 1981. **152**(3): p. 177-80.

124. Banerjee, R., et al., *Crystal-Structure of Peanut Lectin, a Protein with an Unusual Quaternary Structure.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(1): p. 227-231.

125. Natchiar, S.K., et al., *Peanut agglutinin, a lectin with an unusual quaternary structure and interesting ligand binding properties.* Crystallography Reviews, 2007. **13**(1): p. 3-28.

126. Wu, A.M., et al., *Differential contributions of recognition factors of two plant lectins - Amaranthus caudatus lectin and Arachis hypogea agglutinin, reacting with Thomsen-Friedenreich disaccharide (Galbeta1-3GalNAcalpha1-Ser/Thr).* Biochimie, 2008. **90**(11-12): p. 1769-80.

127. Wright, C.S., *Crystal structure of a wheat germ agglutinin/glycophorin-sialoglycopeptide receptor complex. Structural basis for cooperative lectin-cell binding.* J Biol Chem, 1992. **267**(20): p. 14345-52.

128. Rhodes, J.M., B.J. Campbell, and L.G. Yu, *Lectin-epithelial interactions in the human colon.* Biochem Soc Trans, 2008. **36**(Pt 6): p. 1482-6.

129. Pinho, S.S. and C.A. Reis, *Glycosylation in cancer: mechanisms and clinical implications.* Nature Reviews Cancer, 2015. **15**(9): p. 540-555.

130. Dewald, J.H., et al., *Role of Cytokine-Induced Glycosylation Changes in Regulating Cell Interactions and Cell Signaling in Inflammatory Diseases and Cancer.* Cells, 2016. **5**(4).

131. Vavasseur, F., et al., *Synthesis of O-Glycan Core-3 - Characterization of Udp-Glcnac-Galnac-R Beta-3-N-Acetyl-Glucosaminyltransferase Activity from Colonic Mucosal Tissues and Lack of the Activity in Human Cancer Cell-Lines.* Glycobiology, 1995. **5**(3): p. 351-357.

132. Brockhausen, I., *Pathways of O-glycan biosynthesis in cancer cells.* Biochimica Et Biophysica Acta-General Subjects, 1999. **1473**(1): p. 67-95.

133. Singh, R., et al., *Cell surface-expressed Thomsen-Friedenreich antigen in colon cancer is predominantly carried on high molecular weight splice variants of CD44.* Glycobiology, 2001. **11**(7): p. 587-92.

134. Reis, C.A., et al., *Alterations in glycosylation as biomarkers for cancer detection.* Journal of Clinical Pathology, 2010. **63**(4): p. 322-329.

135. Ghazarian, H., B. Idoni, and S.B. Oppenheimer, *A glycobiology review: Carbohydrates, lectins and implications in cancer therapeutics.* Acta Histochemica, 2011. **113**(3): p. 236-247.

136. Berger, R.P., et al., *Glycosylation and stem cells: Regulatory roles and application of iPSCs in the study of glycosylation-related disorders.* Bioessays, 2016. **38**(12): p. 1255-1265.

137. Lillehoj, E.P., et al., *Cellular and molecular biology of airway mucins.* Int Rev Cell Mol Biol, 2013. **303**: p. 139-202.

138. Byrd, J.C. and R.S. Bresalier, *Mucins and mucin binding proteins in colorectal cancer.* Cancer Metastasis Rev, 2004. **23**(1-2): p. 77-99.

139. Leclaire, C., et al., *Molecular basis for intestinal mucin recognition by galectin-3 and C-type lectins.* FASEB J, 2018. **32**(6): p. 3301-3320.

140. Corfield, A.P., et al., *Mucins and mucosal protection in the gastrointestinal tract: new prospects for mucins in the pathology of gastrointestinal disease.* Gut, 2000. **47**(4): p. 589-94.

141. Sato, M., et al., *Functional analysis of the carbohydrate recognition domains and a linker peptide of galectin-9 as to eosinophil chemoattractant activity.* Glycobiology, 2002. **12**(3): p. 191-7.

142. Diaz-Alvarez, L. and E. Ortega, *The Many Roles of Galectin-3, a Multifaceted Molecule, in Innate Immune Responses against Pathogens.* Mediators Inflamm, 2017. **2017**: p. 9247574.

143. Vasta, G.R., *Galectins as Pattern Recognition Receptors: Structure, Function, and Evolution.* Current Topics in Innate Immunity Ii, 2012. **946**: p. 21-36.

144. Yang, R.Y., G.A. Rabinovich, and F.T. Liu, *Galectins: structure, function and therapeutic potential.* Expert Rev Mol Med, 2008. **10**: p. e17.

145. Newlaczyl, A.U. and L.G. Yu, *Galectin-3-A jack-of-all-trades in cancer.* Cancer Letters, 2011. **313**(2): p. 123-128.

146. Ingrassia, L., et al., *Anti-galectin compounds as potential anti-cancer drugs.* Current Medicinal Chemistry, 2006. **13**(29): p. 3513-3527.

147. Fidler, I.J., *Timeline - The pathogenesis of cancer metastasis: the 'seed and soil' hypothesis revisited.* Nature Reviews Cancer, 2003. **3**(6): p. 453-458.

148. Kufe, D.W., *Mucins in cancer: function, prognosis and therapy.* Nature Reviews Cancer, 2009. **9**(12): p. 874-885.

149. Chen, C., et al., *Increased circulation of galectin-3 in cancer induces secretion of metastasis-promoting cytokines from blood vascular endothelium.* Clin Cancer Res, 2013. **19**(7): p. 1693-704.

150. Yu, L.G., et al., *Galectin-3 interaction with Thomsen-Friedenreich disaccharide on cancer-associated MUC1 causes increased cancer cell endothelial adhesion.* Journal of Biological Chemistry, 2007. **282**(1): p. 773-781.

151. Zhao, Q., et al., *Interaction between circulating galectin-3 and cancer-associated MUC1 enhances tumour cell homotypic aggregation and prevents anoikis.* Mol Cancer, 2010. **9**: p. 154.

152. Yu, L.G., *The oncofetal Thomsen-Friedenreich carbohydrate antigen in cancer progression.* Glycoconj J, 2007. **24**(8): p. 411-20.

153. Chen, C., et al., *Increased Circulation of Galectin-3 in Cancer Induces Secretion of Metastasis-Promoting Cytokines from Blood Vascular Endothelium.* Clinical Cancer Research, 2013. **19**(7): p. 1693-1704.

154. Mukhopadhyay, S., et al., *In vitro and in vivo antitumor effects of Peanut agglutinin through induction of apoptotic and autophagic cell death.* Food Chem Toxicol, 2014. **64**: p. 369-77.

155. Ryder, S.D., et al., *Peanut lectin stimulates proliferation in colonic explants from patients with inflammatory bowel disease and colon polyps.* Gastroenterology, 1994. **106**(1): p. 117-24.

156. Wang, Q., et al., *Identification of intact peanut lectin in peripheral venous blood.* Lancet, 1998. **352**(9143): p. 1831-2.

157. Yu, L.G., D.G. Fernig, and J.M. Rhodes, *Intracellular trafficking and release of intact edible mushroom lectin from HT29 human colon cancer cells.* Eur J Biochem, 2000. **267**(7): p. 2122-6.

158. Burke, D.J., et al., *Glutamine-supplemented total parenteral nutrition improves gut immune function.* Arch Surg, 1989. **124**(12): p. 1396-9.

159. Jordinson, M., et al., *Gastrointestinal responses to a panel of lectins in rats maintained on total parenteral nutrition.* Am J Physiol, 1999. **276**(5): p. G1235-42.

160. Ryder, S.D., et al., *Peanut ingestion increases rectal proliferation in individuals with mucosal expression of peanut lectin receptor.* Gastroenterology, 1998. **114**(1): p. 44-9.

161. Rhodes, J.M., R.R. Black, and A. Savage, *Glycoprotein Abnormalities in Colonic Carcinomas, Adenomata, and Hyperplastic Polyps Shown by Lectin Peroxidase Histochemistry.* Journal of Clinical Pathology, 1986. **39**(12): p. 1331-1334.

162. Dore, J.F., et al., *Selective Expression of Pna-Binding Glycoconjugates by Invasive Human Melanomas - a New Marker of Metastatic Potential.* Pigment Cell Research, 1994. **7**(6): p. 461-464.

163. Rhodes, J.M., B.J. Campbell, and L.G. Yu, *Lectin-epithelial interactions in the human colon.* Biochemical Society Transactions, 2008. **36**: p. 1482-1486.

164. Sharma, V. and A. Surolia, *Analyses of carbohydrate recognition by legume lectins: size of the combining site loops and their primary specificity.* J Mol Biol, 1997. **267**(2): p. 433-45.

165. Ryder, S.D., J.A. Smith, and J.M. Rhodes, *Peanut Lectin - a Mitogen for Normal Human Colonic Epithelium and Human Ht29 Colorectal-Cancer Cells.* Jnci-Journal of the National Cancer Institute, 1992. **84**(18): p. 1410-1416.

166. Zhao, Q.H., et al., *Peanut agglutinin appearance in the blood circulation after peanut ingestion mimics the action of endogenous galectin-3 to promote metastasis by interaction with cancer-associated MUC1.* Carcinogenesis, 2014. **35**(12): p. 2815-2821.

167. Lorea, P., et al., *In vitro characterization of lectin-induced alterations on the proliferative activity of three human melanoma cell lines.* Melanoma Research, 1997. **7**(5): p. 353-363.

168. Kiss, R., et al., *In vitro influence of Phaseolus vulgaris, Griffonia simplicifolia, concanavalin A, wheat germ, and peanut agglutinins on HCT-15, LoVo, and SW837 human colorectal cancer cell growth.* Gut, 1997. **40**(2): p. 253-261.

169. Burks, A.W., et al., *Identification of Peanut Agglutinin and Soybean Trypsin-Inhibitor as Minor Legume Allergens.* International Archives of Allergy and Immunology, 1994. **105**(2): p. 143-149.

170. Stiefel, G., et al., *BSACI guideline for the diagnosis and management of peanut and tree nut allergy.* Clinical and Experimental Allergy, 2017. **47**(6): p. 719-739.

171. Astwood, J.D., J.N. Leach, and R.L. Fuchs, *Stability of food allergens to digestion in vitro.* Nature Biotechnology, 1996. **14**(10): p. 1269-1273.

172. Dorrington, K.J. and H.H. Bennich, *Structure-Function-Relationships in Human Immunoglobulin-E.* Immunological Reviews, 1978. **41**: p. 3-25.

173. Bjorklund, J.E.M., T. Karlsson, and C.G.M. Magnusson, *N-glycosylation influences epitope expression and receptor binding structures in human IgE.* Molecular Immunology, 1999. **36**(3): p. 213-221.

174. Gould, H.J. and B.J. Sutton, *IgE in allergy and asthma today.* Nature Reviews Immunology, 2008. **8**(3): p. 205-217.

175. Yamaguchi, M., et al., *IgE enhances mouse mast cell Fc epsilon RI expression in vitro and in vivo: Evidence for a novel amplification mechanism in IgE-dependent reactions.* Journal of Experimental Medicine, 1997. **185**(4): p. 663-672.

176. Wedemeyer, J., M. Tsai, and S.J. Galli, *Roles of mast cells and basophils in innate and acquired immunity.* Current Opinion in Immunology, 2000. **12**(6): p. 624-631.

177. Wang, B., et al., *Epidermal Langerhans Cells from Normal Human Skin Bind Monomeric Ige Via Fc-Epsilon-Ri.* Journal of Experimental Medicine, 1992. **175**(5): p. 1353-1365.

178. Presta, L., et al., *The Binding-Site on Human-Immunoglobulin-E for Its High-Affinity Receptor.* Journal of Biological Chemistry, 1994. **269**(42): p. 26368-26373.

179. Dombrowicz, D., et al., *Allergy-associated FcR beta is a molecular amplifier of IgE- and IgG-mediated in vivo responses.* Immunity, 1998. **8**(4): p. 517-529.

180. Stone, K.D., C. Prussin, and D.D. Metcalfe, *IgE, mast cells, basophils, and eosinophils.* J Allergy Clin Immunol, 2010. **125**(2 Suppl 2): p. S73-80.

181. Bettler, B., et al., *Binding site for IgE of the human lymphocyte low-affinity Fc epsilon receptor (Fc epsilon RII/CD23) is confined to the domain homologous with animal lectins.* Proc Natl Acad Sci U S A, 1989. **86**(18): p. 7118-22.

182. Rosenberg, M., et al., *Detection and enumeration of monocytes in human blood with peanut agglutinin.* J Immunol Methods, 1985. **81**(1): p. 7-13.

183. Howard, D.R. and J.G. Batsakis, *Peanut Agglutinin - a New Marker for Tissue Histiocytes.* American Journal of Clinical Pathology, 1982. **77**(4): p. 401-408.

184. McMichael, A.J., *Food, nutrition, physical activity and cancer prevention. Authoritative report from World Cancer Research Fund provides global update.* Public Health Nutr, 2008. **11**(7): p. 762-3.

185. Dore, J.F., et al., *Selective expression of PNA-binding glycoconjugates by invasive human melanomas: a new marker of metastatic potential.* Pigment Cell Res, 1994. **7**(6): p. 461-4.

186. Zhao, Q., et al., *Peanut agglutinin appearance in the blood circulation after peanut ingestion*

mimics the action of endogenous galectin-3 to promote metastasis by interaction with cancer-associated MUC1. Carcinogenesis, 2014. **35**(12): p. 2815-21.

187. Orntoft, T.F., N. Harving, and N.C. Langkilde, *O-linked mucin-type glycoproteins in normal and malignant colon mucosa: lack of T-antigen expression and accumulation of Tn and sialosyl-Tn antigens in carcinomas.* Int J Cancer, 1990. **45**(4): p. 666-72.

188. Berthier-Vergnes, O., et al., *Expression of cell surface sialic acid and galactose by normal adult human melanocytes in culture.* Pigment Cell Res, 1990. **3**(2): p. 55-60.

189. Novogrodsky, A., K.H. Stenzel, and A.L. Rubin, *Stimulation of human peripheral blood lymphocytes by periodate, galactose oxidase, soybean agglutinin, and peanut agglutinin: differential effects of adherent cells.* J Immunol, 1977. **118**(3): p. 852-7.

190. Springer, G.F., P.R. Desai, and E.F. Scanlon, *Blood group MN precursors as human breast carcinoma-associated antigens and "naturally" occurring human cytotoxins against them.* Cancer, 1976. **37**(1): p. 169-76.

191. Langkilde, N.C. and T.F. Orntoft, *A comparative study of peanut agglutinin and amaranthin binding to human urinary bladder tumor glycoproteins.* Scand J Urol Nephrol Suppl, 1995. **172**: p. 57-64.

192. Saulsbury, F.T., *Alterations in the O-linked glycosylation of IgA1 in children with Henoch-Schonlein purpura.* J Rheumatol, 1997. **24**(11): p. 2246-9.

193. Chacko, B.K. and P.S. Appukuttan, *Peanut (Arachis hypogaea) lectin recognizes alpha-linked galactose, but not N-acetyl lactosamine in N-linked oligosaccharide terminals.* Int J Biol Macromol, 2001. **28**(5): p. 365-71.

194. Carsons, S., *Enhanced expression of a peanut agglutinin reactive O linked oligosaccharide on Fibronectins from the synovial fluid of patients with rheumatic disease: Quantitation, domain localization, and functional significance.* Journal of Rheumatology, 2002. **29**(5): p. 896-902.

195. London, J., S. Berrih, and J.F. Bach, *Peanut agglutinin. I. A new tool for studying T lymphocyte subpopulations.* J Immunol, 1978. **121**(2): p. 438-43.

196. Newman, R.A. and M.A. Boss, *Expression of Binding-Sites for Peanut Agglutinin during Murine Lymphocyte-B Differentiation.* Immunology, 1980. **40**(2): p. 193-200.

197. Brustein, V.P., et al., *Chemiluminescent Detection of Carbohydrates in the Tumoral Breast Diseases.* Applied Biochemistry and Biotechnology, 2012. **166**(2): p. 268-275.

198. Favoriti, P., et al., *Worldwide burden of colorectal cancer: a review.* Updates Surg, 2016. **68**(1): p. 7-11.

199. Doll, R. and R. Peto, *The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today.* J Natl Cancer Inst, 1981. **66**(6): p. 1191-308.

200. Alexandratos, N., *The Mediterranean diet in a world context.* Public Health Nutr, 2006. **9**(1A): p. 111-7.

201. Kearney, J., *Food consumption trends and drivers.* Philos Trans R Soc Lond B Biol Sci, 2010. **365**(1554): p. 2793-807.

202. Steyn, K., et al., *Urbanization and the risk for chronic diseases of lifestyle in the black population of the Cape Peninsula, South Africa.* J Cardiovasc Risk, 1997. **4**(2): p. 135-42.

203. Gunewardene, A., G.F. Huon, and R. Zheng, *Exposure to westernization and dieting: a cross-cultural study.* Int J Eat Disord, 2001. **29**(3): p. 289-93.

204. Waxman, A. and A. World Health, *WHO global strategy on diet, physical activity and health.* Food Nutr Bull, 2004. **25**(3): p. 292-302.

205. Armstrong, B. and R. Doll, *Environmental factors and cancer incidence and mortality in different countries, with special reference to dietary practices.* Int J Cancer, 1975. **15**(4): p. 617-31.

206. Key, T.J., et al., *The effect of diet on risk of cancer.* Lancet, 2002. **360**(9336): p. 861-8.

207. *Nutritional and toxicological aspects of food safety.* Adv Exp Med Biol, 1984. **177**: p. 1-584.

208. Dungal, N. and J. Sigurjonsson, *Gastric cancer and diet. A pilot study on dietary habits in two districts differing markedly in respect of mortality from gastric cancer.* Br J Cancer, 1967. **21**(2): p. 270-6.

209. Wang, H., et al., *Plants vs. cancer: a review on natural phytochemicals in preventing and treating cancers and their druggability.* Anticancer Agents Med Chem, 2012. **12**(10): p. 1281-305.

210. Wang, L.S. and G.D. Stoner, *Anthocyanins and their role in cancer prevention.* Cancer Lett, 2008. **269**(2): p. 281-90.

211. Mazewski, C., M.S. Kim, and E. Gonzalez de Mejia, *Anthocyanins, delphinidin-3-O-glucoside and cyanidin-3-O-glucoside, inhibit immune checkpoints in human colorectal cancer cells in vitro and in silico.* Sci Rep, 2019. **9**(1): p. 11560.

212. Mazewski, C., K. Liang, and E. Gonzalez de Mejia, *Comparison of the effect of chemical composition of anthocyanin-rich plant extracts on colon cancer cell proliferation and their potential mechanism of action using in vitro, in silico, and biochemical assays.* Food Chemistry, 2018. **242**: p. 378-388.

213. Ros, E., *Health benefits of nut consumption.* Nutrients, 2010. **2**(7): p. 652-82.

214. Aune, D., et al., *Nut consumption and risk of cardiovascular disease, total cancer, all-cause and cause-specific mortality: a systematic review and dose-response meta-analysis of prospective studies.* BMC Med, 2016. **14**(1): p. 207.

215. Lee, J., et al., *The relationship between nut intake and risk of colorectal cancer: A case control study.* Nutrition Journal, 2018. **17**.

216. Jenab, M., et al., *Association of nut and seed intake with colorectal cancer risk in the European Prospective Investigation into Cancer and Nutrition.* Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology, 2004. **13**: p. 1595-603.

217. Wesseling, J., S.W. van der Valk, and J. Hilkens, *A mechanism for inhibition of E-cadherin-mediated cell-cell adhesion by the membrane-associated mucin episialin/MUC1.* Mol Biol Cell, 1996. **7**(4): p. 565-77.

218. Colomb, F., et al., *Galectin-3 interacts with the cell-surface glycoprotein CD146 (MCAM, MUC18) and induces secretion of metastasis-promoting cytokines from vascular endothelial cells.* J Biol Chem, 2017. **292**(20): p. 8381-8389.

219. Ryder, S.D., J.A. Smith, and J.M. Rhodes, *Peanut lectin: a mitogen for normal human colonic epithelium and human HT29 colorectal cancer cells.* J Natl Cancer Inst, 1992. **84**(18): p. 1410-6.

220. Samuel, J., et al., *Analysis of human tumor associated Thomsen-Friedenreich antigen.* Cancer Res, 1990. **50**(15): p. 4801-8.

221. Yu, L.G., et al., *Galectin-3 interaction with Thomsen-Friedenreich disaccharide on cancer-associated MUC1 causes increased cancer cell endothelial adhesion.* J Biol Chem, 2007. **282**(1): p. 773-81.

222. Balkwill, F. and A. Mantovani, *Inflammation and cancer: back to Virchow?* Lancet, 2001. **357**(9255): p. 539-45.

223. Multhoff, G., M. Molls, and J. Radons, *Chronic inflammation in cancer development.* Front Immunol, 2011. **2**: p. 98.

224. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.

225. Korkaya, H., S. Liu, and M.S. Wicha, *Regulation of cancer stem cells by cytokine networks: attacking cancer's inflammatory roots.* Clin Cancer Res, 2011. **17**(19): p. 6125-9.

226. Yasukawa, K., et al., *Structure and expression of human B cell stimulatory factor-2 (BSF-2/IL-6) gene.* EMBO J, 1987. **6**(10): p. 2939-45.

227. Gobert, A.P., et al., *Helicobacter pylori heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like receptor (TLR)-2-, TLR-4-, and myeloid differentiation factor 88-independent mechanism.* J Biol Chem, 2004. **279**(1): p. 245-50.

228. Lotz, M., et al., *B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes.* J Exp Med, 1988. **167**(3): p. 1253-8.

229. Houssiau, F.A., P.G. Coulie, and J. Van Snick, *Distinct roles of IL-1 and IL-6 in human T cell activation.* J Immunol, 1989. **143**(8): p. 2520-4.

230. Castell, J.V., et al., *Recombinant human interleukin-6 (IL-6/BSF-2/HSF) regulates the synthesis of acute phase proteins in human hepatocytes.* FEBS Lett, 1988. **232**(2): p. 347-50.

231. Udagawa, N., et al., *Interleukin (IL)-6 induction of osteoclast differentiation depends on IL-6 receptors expressed on osteoblastic cells but not on osteoclast progenitors.* J Exp Med, 1995. **182**(5): p. 1461-8.

232. Bleier, J.I., et al., *Increased and long-term generation of dendritic cells with reduced function from IL-6-deficient bone marrow.* J Immunol, 2004. **172**(12): p. 7408-16.

233. Hodge, D.R., E.M. Hurt, and W.L. Farrar, *The role of IL-6 and STAT3 in inflammation and cancer.* Eur J Cancer, 2005. **41**(16): p. 2502-12.

234. Tanaka, T., M. Narazaki, and T. Kishimoto, *IL-6 in inflammation, immunity, and disease.* Cold Spring Harb Perspect Biol, 2014. **6**(10): p. a016295.

235. Dankbar, B., et al., *Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma.* Blood, 2000. **95**(8): p. 2630-6.

236. Bharadwaj, U., et al., *Elevated interleukin-6 and G-CSF in human pancreatic cancer cell conditioned medium suppress dendritic cell differentiation and activation.* Cancer Res, 2007. **67**(11): p. 5479-88.

237. Wei, L.H., et al., *Interleukin-6 in cervical cancer: the relationship with vascular endothelial growth factor.* Gynecol Oncol, 2001. **82**(1): p. 49-56.

238. Watson, J.M., et al., *Constitutive production of interleukin 6 by ovarian cancer cell lines and by primary ovarian tumor cultures.* Cancer Res, 1990. **50**(21): p. 6959-65.

239. Kim, H.K., et al., *Elevated levels of circulating platelet microparticles, VEGF, IL-6 and RANTES in patients with gastric cancer: possible role of a metastasis predictor.* Eur J Cancer, 2003. **39**(2): p. 184-91.

240. Lahm, H., et al., *Growth stimulation of a human colorectal carcinoma cell line by interleukin-1 and -6 and antagonistic effects of transforming growth factor beta 1.* Eur J Cancer, 1992. **28A**(11): p. 1894-9.

241. Podor, T.J., et al., *Human endothelial cells produce IL-6. Lack of responses to exogenous IL-6.* Ann N Y Acad Sci, 1989. **557**: p. 374-85; discussion 386-7.

242. Deshmane, S.L., et al., *Monocyte chemoattractant protein-1 (MCP-1): an overview.* J Interferon Cytokine Res, 2009. **29**(6): p. 313-26.

243. Boring, L., et al., *Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in*

the initiation of atherosclerosis. Nature, 1998. **394**(6696): p. 894-7.

244. Dawson, T.C., et al., *Absence of CC chemokine receptor-2 reduces atherosclerosis in apolipoprotein E-deficient mice.* Atherosclerosis, 1999. **143**(1): p. 205-11.

245. Hokimoto, S., et al., *Increased expression of monocyte chemoattractant protein-1 in atherectomy specimens from patients with restenosis after percutaneous transluminal coronary angioplasty.* Circ J, 2002. **66**(1): p. 114-6.

246. Szalai, C., et al., *Involvement of polymorphisms in the chemokine system in the susceptibility for coronary artery disease (CAD). Coincidence of elevated Lp(a) and MCP-1 -2518 G/G genotype in CAD patients.* Atherosclerosis, 2001. **158**(1): p. 233-9.

247. Nelken, N.A., et al., *Monocyte chemoattractant protein-1 in human atheromatous plaques.* J Clin Invest, 1991. **88**(4): p. 1121-7.

248. Cushing, S.D., et al., *Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells.* Proc Natl Acad Sci U S A, 1990. **87**(13): p. 5134-8.

249. Grewal, I.S., et al., *Transgenic monocyte chemoattractant protein-1 (MCP-1) in pancreatic islets produces monocyte-rich insulitis without diabetes: abrogation by a second transgene expressing systemic MCP-1.* J Immunol, 1997. **159**(1): p. 401-8.

250. Khan, W.I., et al., *Critical role of MCP-1 in the pathogenesis of experimental colitis in the context of immune and enterochromaffin cells.* Am J Physiol Gastrointest Liver Physiol, 2006. **291**(5): p. G803-11.

251. Grimm, M.C., et al., *Enhanced expression and production of monocyte chemoattractant protein-1 in inflammatory bowel disease mucosa.* J Leukoc Biol, 1996. **59**(6): p. 804-12.

252. Bhatia, M., et al., *Treatment with bindarit, an inhibitor of MCP-1 synthesis, protects mice against trinitrobenzene sulfonic acid-induced colitis.* Inflamm Res, 2008. **57**(10): p. 464-71.

253. Conti, I. and B.J. Rollins, *CCL2 (monocyte chemoattractant protein-1) and cancer.* Semin Cancer Biol, 2004. **14**(3): p. 149-54.

254. Salcedo, R., et al., *Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression.* Blood, 2000. **96**(1): p. 34-40.

255. Saji, H., et al., *Significant correlation of monocyte chemoattractant protein-1 expression with neovascularization and progression of breast carcinoma.* Cancer, 2001. **92**(5): p. 1085-91.

256. Hefler, L., et al., *Monocyte chemoattractant protein-1 serum levels in ovarian cancer patients.* Br J Cancer, 1999. **81**(5): p. 855-9.

257. Negus, R.P., et al., *The detection and localization of monocyte chemoattractant protein-1 (MCP-*

1) in human ovarian cancer. J Clin Invest, 1995. **95**(5): p. 2391-6.

258. Monti, P., et al., *The CC chemokine MCP-1/CCL2 in pancreatic cancer progression: regulation of expression and potential mechanisms of antimalignant activity.* Cancer Res, 2003. **63**(21): p. 7451-61.

259. Kuroda, T., et al., *Monocyte chemoattractant protein-1 transfection induces angiogenesis and tumorigenesis of gastric carcinoma in nude mice via macrophage recruitment.* Clin Cancer Res, 2005. **11**(21): p. 7629-36.

260. Lu, Y., et al., *Monocyte chemotactic protein-1 (MCP-1) acts as a paracrine and autocrine factor for prostate cancer growth and invasion.* Prostate, 2006. **66**(12): p. 1311-8.

261. Huang, M., et al., *A high-fat diet enhances proliferation of prostate cancer cells and activates MCP-1/CCR2 signaling.* Prostate, 2012. **72**(16): p. 1779-88.

262. Kushi, L.H., et al., *American Cancer Society Guidelines on nutrition and physical activity for cancer prevention: reducing the risk of cancer with healthy food choices and physical activity.* CA Cancer J Clin, 2012. **62**(1): p. 30-67.

263. Brown, J.K., et al., *Nutrition and physical activity during and after cancer treatment: an American Cancer Society guide for informed choices.* CA Cancer J Clin, 2003. **53**(5): p. 268-91.

264. Salgado, R., et al., *Circulating interleukin-6 predicts survival in patients with metastatic breast cancer.* Int J Cancer, 2003. **103**(5): p. 642-6.

265. Eustace, D., et al., *Interleukin-6 (IL-6) functions as an autocrine growth factor in cervical carcinomas in vitro.* Gynecol Oncol, 1993. **50**(1): p. 15-9.

266. Miki, S., et al., *Interleukin-6 (IL-6) functions as an in vitro autocrine growth factor in renal cell carcinomas.* FEBS Lett, 1989. **250**(2): p. 607-10.

267. Gangopadhyay, A., D.A. Lazure, and P. Thomas, *Adhesion of colorectal carcinoma cells to the endothelium is mediated by cytokines from CEA stimulated Kupffer cells.* Clin Exp Metastasis, 1998. **16**(8): p. 703-12.

268. Scholz, D., et al., *Ultrastructure and molecular histology of rabbit hind-limb collateral artery growth (arteriogenesis).* Virchows Arch, 2000. **436**(3): p. 257-70.

269. Goede, V., et al., *Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1.* Int J Cancer, 1999. **82**(5): p. 765-70.

270. Viedt, C., et al., *MCP-1 induces inflammatory activation of human tubular epithelial cells: involvement of the transcription factors, nuclear factor-kappaB and activating protein-1.* J Am Soc Nephrol, 2002. **13**(6): p. 1534-47.

271. Saiki, I., et al., *Characterization of the invasive and metastatic phenotype in human renal cell*

carcinoma. Clin Exp Metastasis, 1991. **9**(6): p. 551-66.

272. Meromsky, L., R. Lotan, and A. Raz, *Implications of endogenous tumor cell surface lectins as mediators of cellular interactions and lung colonization.* Cancer Res, 1986. **46**(10): p. 5270-5.

273. Updyke, T.V. and G.L. Nicolson, *Malignant melanoma cell lines selected in vitro for increased homotypic adhesion properties have increased experimental metastatic potential.* Clin Exp Metastasis, 1986. **4**(4): p. 273-84.

274. Glinsky, V.V., et al., *Intravascular metastatic cancer cell homotypic aggregation at the sites of primary attachment to the endothelium.* Cancer Res, 2003. **63**(13): p. 3805-11.

275. Mierke, C.T., et al., *Breakdown of the endothelial barrier function in tumor cell transmigration.* Biophys J, 2008. **94**(7): p. 2832-46.

276. Eichbaum, C., et al., *Breast cancer cell-derived cytokines, macrophages and cell adhesion: implications for metastasis.* Anticancer Res, 2011. **31**(10): p. 3219-27.

277. DeCicco-Skinner, K.L., et al., *Endothelial cell tube formation assay for the in vitro study of angiogenesis.* J Vis Exp, 2014(91): p. e51312.

278. Li, A., et al., *IL-8 directly enhanced endothelial cell survival, proliferation, and matrix metalloproteinases production and regulated angiogenesis.* J Immunol, 2003. **170**(6): p. 3369-76.

279. Bao, S., et al., *Stem cell-like glioma cells promote tumor angiogenesis through vascular endothelial growth factor.* Cancer Res, 2006. **66**(16): p. 7843-8.

280. Kerbel, R.S., *Tumor angiogenesis: past, present and the near future.* Carcinogenesis, 2000. **21**(3): p. 505-15.

281. van der Schaft, D.W., et al., *Effects of angiogenesis inhibitors on vascular network formation by human endothelial and melanoma cells.* J Natl Cancer Inst, 2004. **96**(19): p. 1473-7.

282. Knupfer, H. and R. Preiss, *Significance of interleukin-6 (IL-6) in breast cancer (review).* Breast Cancer Res Treat, 2007. **102**(2): p. 129-35.

283. Tawara, K., J.T. Oxford, and C.L. Jorcyk, *Clinical significance of interleukin (IL)-6 in cancer metastasis to bone: potential of anti-IL-6 therapies.* Cancer Manag Res, 2011. **3**: p. 177-89.

284. Motro, B., et al., *Pattern of interleukin 6 gene expression in vivo suggests a role for this cytokine in angiogenesis.* Proc Natl Acad Sci U S A, 1990. **87**(8): p. 3092-6.

285. Giraudo, E., et al., *IL-6 is an in vitro and in vivo autocrine growth factor for middle T antigen-transformed endothelial cells.* J Immunol, 1996. **157**(6): p. 2618-23.

286. Lin, C., et al., *CGI-99 promotes breast cancer metastasis via autocrine interleukin-6 signaling.* Oncogene, 2017. **36**(26): p. 3695-3705.

287. Lira, S.A. and G.C. Furtado, *The biology of chemokines and their receptors.* Immunol Res, 2012. **54**(1-3): p. 111-20.

288. Ueno, T., et al., *Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer.* Clin Cancer Res, 2000. **6**(8): p. 3282-9.

289. Saeidnia, S. and M. Abdollahi, *Antioxidants: friends or foe in prevention or treatment of cancer: the debate of the century.* Toxicol Appl Pharmacol, 2013. **271**(1): p. 49-63.

290. Nikkola, J., et al., *Integrin chains beta1 and alphav as prognostic factors in human metastatic melanoma.* Melanoma Res, 2004. **14**(1): p. 29-37.

291. Danen, E.H., et al., *Emergence of alpha 5 beta 1 fibronectin- and alpha v beta 3 vitronectin-receptor expression in melanocytic tumour progression.* Histopathology, 1994. **24**(3): p. 249-56.

292. Brooks, P.C., et al., *Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels.* Cell, 1994. **79**(7): p. 1157-64.

293. Geng, Y., et al., *Phenotypic switch in blood: effects of pro-inflammatory cytokines on breast cancer cell aggregation and adhesion.* PLoS One, 2013. **8**(1): p. e54959.

294. ten Kate, M., et al., *Influence of proinflammatory cytokines on the adhesion of human colon carcinoma cells to lung microvascular endothelium.* Int J Cancer, 2004. **112**(6): p. 943-50.

295. Maruo, Y., et al., *ICAM-1 expression and the soluble ICAM-1 level for evaluating the metastatic potential of gastric cancer.* Int J Cancer, 2002. **100**(4): p. 486-90.

296. Alexiou, D., et al., *Serum levels of E-selectin, ICAM-1 and VCAM-1 in colorectal cancer patients: correlations with clinicopathological features, patient survival and tumour surgery.* Eur J Cancer, 2001. **37**(18): p. 2392-7.

297. O'Hanlon, D.M., et al., *Soluble adhesion molecules (E-selectin, ICAM-1 and VCAM-1) in breast carcinoma.* Eur J Cancer, 2002. **38**(17): p. 2252-7.

298. Raz, A. and R. Lotan, *Lectin-like activities associated with human and murine neoplastic cells.* Cancer Res, 1981. **41**(9 Pt 1): p. 3642-7.

299. Tieu, B.C., et al., *An adventitial IL-6/MCP1 amplification loop accelerates macrophage-mediated vascular inflammation leading to aortic dissection in mice.* J Clin Invest, 2009. **119**(12): p. 3637-51.

300. Gabor, F., et al., *The lectin-cell interaction and its implications to intestinal lectin-mediated drug delivery.* Adv Drug Deliv Rev, 2004. **56**(4): p. 459-80.

301. Kim, M., et al., *Lectin-induced apoptosis of tumour cells.* Glycobiology, 1993. **3**(5): p. 447-53.

302. Lorea, P., et al., *In vitro characterization of lectin-induced alterations on the proliferative*

activity of three human melanoma cell lines. Melanoma Res, 1997. **7**(5): p. 353-63.

303. Lotan, R., et al., *The purification, composition, and specificity of the anti-T lectin from peanut (Arachis hypogaea).* J Biol Chem, 1975. **250**(21): p. 8518-23.

304. Swamy, M.J., et al., *Further characterization of the saccharide specificity of peanut (Arachis hypogaea) agglutinin.* Carbohydr Res, 1991. **213**: p. 59-67.

305. Petrossian, K., L.R. Banner, and S.B. Oppenheimer, *Lectin binding and effects in culture on human cancer and non-cancer cell lines: examination of issues of interest in drug design strategies.* Acta Histochem, 2007. **109**(6): p. 491-500.

306. Singh, R., et al., *Peanut lectin stimulates proliferation of colon cancer cells by interaction with glycosylated CD44v6 isoforms and consequential activation of c-Met and MAPK: functional implications for disease-associated glycosylation changes.* Glycobiology, 2006. **16**(7): p. 594-601.

307. Amado, M., et al., *Peanut agglutinin high phenotype of activated CD8+ T cells results from de novo synthesis of CD45 glycans.* J Biol Chem, 2004. **279**(35): p. 36689-97.

308. Manjunath, N., et al., *Negative regulation of T-cell adhesion and activation by CD43.* Nature, 1995. **377**(6549): p. 535-8.

309. Wu, W., et al., *Identification of CD8 as a peanut agglutinin (PNA) receptor molecule on immature thymocytes.* J Exp Med, 1996. **184**(2): p. 759-64.

310. Sindrewicz, P., L.Y. Lian, and L.G. Yu, *Interaction of the Oncofetal Thomsen-Friedenreich Antigen with Galectins in Cancer Progression and Metastasis.* Front Oncol, 2016. **6**: p. 79.

311. Newlaczyl, A.U. and L.G. Yu, *Galectin-3--a jack-of-all-trades in cancer.* Cancer Lett, 2011. **313**(2): p. 123-8.

312. Lagana, A., et al., *Galectin binding to Mgat5-modified N-glycans regulates fibronectin matrix remodeling in tumor cells.* Mol Cell Biol, 2006. **26**(8): p. 3181-93.

313. Shih, I.M., *The role of CD146 (Mel-CAM) in biology and pathology.* J Pathol, 1999. **189**(1): p. 4-11.

314. Vainio, O., et al., *HEMCAM, an adhesion molecule expressed by c-kit+ hemopoietic progenitors.* J Cell Biol, 1996. **135**(6 Pt 1): p. 1655-68.

315. Lehmann, J.M., G. Riethmuller, and J.P. Johnson, *MUC18, a marker of tumor progression in human melanoma, shows sequence similarity to the neural cell adhesion molecules of the immunoglobulin superfamily.* Proc Natl Acad Sci U S A, 1989. **86**(24): p. 9891-5.

316. Erdbruegger, U., et al., *Circulating endothelial cells: markers and mediators of vascular damage.* Curr Stem Cell Res Ther, 2010. **5**(4): p. 294-302.

317. Ruma, I.M., et al., *MCAM, as a novel receptor for S100A8/A9, mediates progression of malignant melanoma through prominent activation of NF-kappaB and ROS formation upon ligand binding.* Clin Exp Metastasis, 2016. **33**(6): p. 609-27.

318. Jouve, N., et al., *CD146 mediates VEGF-induced melanoma cell extravasation through FAK activation.* Int J Cancer, 2015. **137**(1): p. 50-60.

319. Horl, S., et al., *CD146 (MCAM) in human cs-DLK1(-)/cs-CD34(+) adipose stromal/progenitor cells.* Stem Cell Res, 2017. **22**: p. 1-12.

320. Tu, T., et al., *CD146 acts as a novel receptor for netrin-1 in promoting angiogenesis and vascular development.* Cell Res, 2015. **25**(3): p. 275-87.

321. Filshie, R.J., et al., *MUC18, a member of the immunoglobulin superfamily, is expressed on bone marrow fibroblasts and a subset of hematological malignancies.* Leukemia, 1998. **12**(3): p. 414-21.

322. Jiang, T., et al., *CD146 is a coreceptor for VEGFR-2 in tumor angiogenesis.* Blood, 2012. **120**(11): p. 2330-9.

323. Flanagan, K., et al., *Laminin-411 is a vascular ligand for MCAM and facilitates TH17 cell entry into the CNS.* PLoS One, 2012. **7**(7): p. e40443.

324. Jouve, N., et al., *The involvement of CD146 and its novel ligand Galectin-1 in apoptotic regulation of endothelial cells.* J Biol Chem, 2013. **288**(4): p. 2571-9.

325. Newman, P.J., *Switched at birth: a new family for PECAM-1.* J Clin Invest, 1999. **103**(1): p. 5-9.

326. Zehnder, J.L., et al., *The cell adhesion molecule CD31 is phosphorylated after cell activation. Down-regulation of CD31 in activated T lymphocytes.* J Biol Chem, 1992. **267**(8): p. 5243-9.

327. Ashman, L.K., et al., *Different epitopes of the CD31 antigen identified by monoclonal antibodies: cell type-specific patterns of expression.* Tissue Antigens, 1991. **38**(5): p. 199-207.

328. Berman, M.E., Y. Xie, and W.A. Muller, *Roles of platelet/endothelial cell adhesion molecule-1 (PECAM-1, CD31) in natural killer cell transendothelial migration and beta 2 integrin activation.* J Immunol, 1996. **156**(4): p. 1515-24.

329. Muller, W.A., et al., *PECAM-1 is required for transendothelial migration of leukocytes.* J Exp Med, 1993. **178**(2): p. 449-60.

330. Newman, P.J., et al., *PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily.* Science, 1990. **247**(4947): p. 1219-22.

331. Paddock, C., et al., *Structural basis for PECAM-1 homophilic binding.* Blood, 2016. **127**(8): p. 1052-61.

332. Cardin, A.D. and H.J. Weintraub, *Molecular modeling of protein-glycosaminoglycan*

interactions. Arteriosclerosis, 1989. **9**(1): p. 21-32.

333. Vaporciyan, A.A., et al., *Involvement of platelet-endothelial cell adhesion molecule-1 in neutrophil recruitment in vivo.* Science, 1993. **262**(5139): p. 1580-2.

334. Gratzinger, D., M. Barreuther, and J.A. Madri, *Platelet-endothelial cell adhesion molecule-1 modulates endothelial migration through its immunoreceptor tyrosine-based inhibitory motif.* Biochem Biophys Res Commun, 2003. **301**(1): p. 243-9.

335. Park, S., et al., *PECAM-1 regulates proangiogenic properties of endothelial cells through modulation of cell-cell and cell-matrix interactions.* Am J Physiol Cell Physiol, 2010. **299**(6): p. C1468-84.

336. Brenner, A.K., T.H. Andersson Tvedt, and O. Bruserud, *The Complexity of Targeting PI3K-Akt-mTOR Signalling in Human Acute Myeloid Leukaemia: The Importance of Leukemic Cell Heterogeneity, Neighbouring Mesenchymal Stem Cells and Immunocompetent Cells.* Molecules, 2016. **21**(11).

337. Kishimoto, T., et al., *Interleukin-6 family of cytokines and gp130.* Blood, 1995. **86**(4): p. 1243-54.

338. Heinrich, P.C., et al., *Interleukin-6-type cytokine signalling through the gp130/Jak/STAT pathway.* Biochem J, 1998. **334 (Pt 2)**: p. 297-314.

339. Cokic, V.P., et al., *Proinflammatory Cytokine IL-6 and JAK-STAT Signaling Pathway in Myeloproliferative Neoplasms.* Mediators Inflamm, 2015. **2015**: p. 453020.

340. Nyati, K.K., et al., *TLR4-induced NF-kappaB and MAPK signaling regulate the IL-6 mRNA stabilizing protein Arid5a.* Nucleic Acids Res, 2017. **45**(5): p. 2687-2703.

341. Cambien, B., et al., *Signal transduction involved in MCP-1-mediated monocytic transendothelial migration.* Blood, 2001. **97**(2): p. 359-66.

342. Ouhtit, A., et al., *Towards understanding the mode of action of the multifaceted cell adhesion receptor CD146.* Biochim Biophys Acta, 2009. **1795**(2): p. 130-6.

343. Ouhtit, A., et al., *CD146, a novel target of CD44-signaling, suppresses breast tumor cell invasion.* Cell Commun Signal, 2017. **15**(1): p. 45.

344. Li, G., et al., *Reciprocal regulation of MelCAM and AKT in human melanoma.* Oncogene, 2003. **22**(44): p. 6891-9.

345. Zhang, Y.Y., et al., *CD31 regulates metastasis by inducing epithelial-mesenchymal transition in hepatocellular carcinoma via the ITGB1-FAK-Akt signaling pathway.* Cancer Lett, 2018. **429**: p. 29-40.

346. Carrithers, M., et al., *Enhanced susceptibility to endotoxic shock and impaired STAT3 signaling*

in CD31-deficient mice. Am J Pathol, 2005. **166**(1): p. 185-96.

347. Whiteman, E.L., H. Cho, and M.J. Birnbaum, *Role of Akt/protein kinase B in metabolism.* Trends Endocrinol Metab, 2002. **13**(10): p. 444-51.

348. Song, G., G. Ouyang, and S. Bao, *The activation of Akt/PKB signaling pathway and cell survival.* J Cell Mol Med, 2005. **9**(1): p. 59-71.

349. Lawlor, M.A. and D.R. Alessi, *PKB/Akt: a key mediator of cell proliferation, survival and insulin responses?* J Cell Sci, 2001. **114**(Pt 16): p. 2903-10.

350. Wang, S. and M.D. Basson, *Identification of functional domains in AKT responsible for distinct roles of AKT isoforms in pressure-stimulated cancer cell adhesion.* Exp Cell Res, 2008. **314**(2): p. 286-96.

351. Fresno Vara, J.A., et al., *PI3K/Akt signalling pathway and cancer.* Cancer Treat Rev, 2004. **30**(2): p. 193-204.

352. Cantley, L.C., *The phosphoinositide 3-kinase pathway.* Science, 2002. **296**(5573): p. 1655-7.

353. Thomas, D., et al., *Protein kinase activity of phosphoinositide 3-kinase regulates cytokine-dependent cell survival.* PLoS Biol, 2013. **11**(3): p. e1001515.

354. Luo, J., B.D. Manning, and L.C. Cantley, *Targeting the PI3K-Akt pathway in human cancer: rationale and promise.* Cancer Cell, 2003. **4**(4): p. 257-62.

355. Hawkins, P.T. and L.R. Stephens, *PI3K signalling in inflammation.* Biochim Biophys Acta, 2015. **1851**(6): p. 882-97.

356. Fruman, D.A. and C. Rommel, *PI3K and cancer: lessons, challenges and opportunities.* Nature Reviews Drug Discovery, 2014. **13**(2): p. 140-156.

357. Koorella, C., et al., *Novel regulation of CD80/CD86-induced phosphatidylinositol 3-kinase signaling by NOTCH1 protein in interleukin-6 and indoleamine 2,3-dioxygenase production by dendritic cells.* J Biol Chem, 2014. **289**(11): p. 7747-62.

358. Du, L., et al., *Role of phosphatidylinositol-3-kinase pathway in head and neck squamous cell carcinoma.* J Oncol, 2012. **2012**: p. 450179.

359. Isaacsson Velho, P.H., G. Castro, Jr., and C.H. Chung, *Targeting the PI3K Pathway in Head and Neck Squamous Cell Carcinoma.* Am Soc Clin Oncol Educ Book, 2015: p. 123-8.

360. Wiza, C., E.B. Nascimento, and D.M. Ouwens, *Role of PRAS40 in Akt and mTOR signaling in health and disease.* Am J Physiol Endocrinol Metab, 2012. **302**(12): p. E1453-60.

361. Wang, L., et al., *PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding.* J Biol Chem, 2007. **282**(27): p. 20036-44.

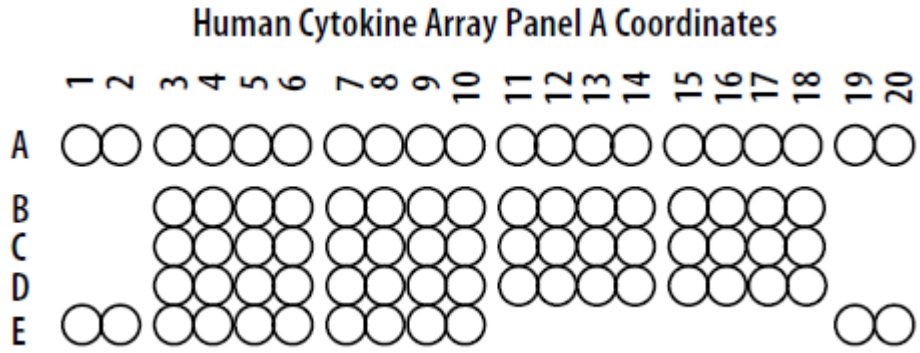
362. Magnuson, B., B. Ekim, and D.C. Fingar, *Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks.* Biochem J, 2012. **441**(1): p. 1-21.

363. Dann, S.G., A. Selvaraj, and G. Thomas, *mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer.* Trends Mol Med, 2007. **13**(6): p. 252-9.

364. Wang, L., T.E. Harris, and J.C. Lawrence, Jr., *Regulation of proline-rich Akt substrate of 40 kDa (PRAS40) function by mammalian target of rapamycin complex 1 (mTORC1)-mediated phosphorylation.* J Biol Chem, 2008. **283**(23): p. 15619-27.

Appendix 1

The human cytokine array was purchased from the R&D systems. (Catalog Number ARY005)



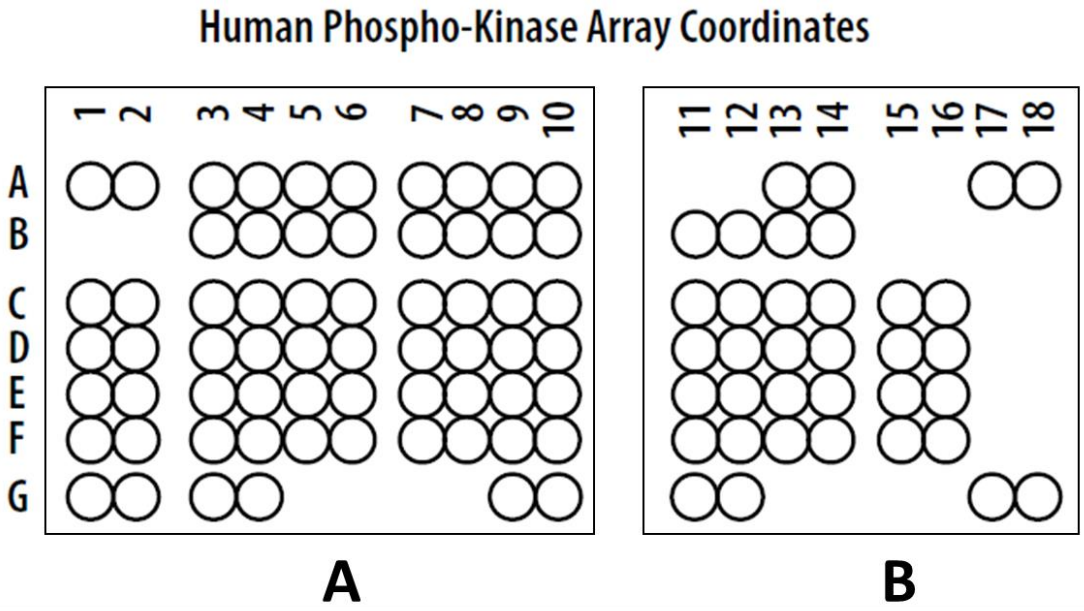
Coordinate	Target/Control	Alternate Nomenclature
A1,A2	Reference Spot	_____
A3,A4	C5/C5a	Complement Component 5/5a
A5,A6	CD40 ligand	CD154
A7,A8	G-CSF	CSFβ,CSF-3
A9,A10	GM-CSF	CSFα,CSF-2
A11,A12	GROα	CSCL1
A12,A13	I-309	CCL1
A13,A14	sICAM-1	CD54
A15,A16	IFN-γ	Type II IFN
A17,A18	Reference Spot	_____
A19,A20	IL-1α	IL-1F1
B3,B4	IL-1β	IL-1F2
B5,B6	IL-1ra	IL-1F3
B7,B8	IL-2	_____

B9,B10	IL-4	_____
B11,B12	IL-5	_____
B13,B14	IL-6	_____
B15,B16	IL-8	CXCL8
B17,B18	IL-10	_____
C3,C4	IL-12 p70	_____
C5,C6	IL-13	_____
C7,C8	IL-16	LCF
C9,C10	IL-17	_____
C11,C12	IL-17E	_____
C13,C14	IL-23	_____
C15,C16	IL-27	_____
C17,C18	IL-32α	_____
D3,D4	IP-10	CXCL10
D5,D6	I-TAC	CXCL11
D7,D8	MCP-1	CCL2
D9,D10	MIF	GIF, DER6
D11,D12	MIP-1α	CCL3
D13,D14	MIP-1β	CCL4
D15,D16	Serpin E1	PAI-1
D17,D18	Reference Spot	_____
E1,E2	RANTES	CCL5
E3,E4	SDF-1	CXCL12
E5,E6	TNF-α	TNFSF1A
E7,E8	sTREM-1	_____

E9,E10	Negative Control	_____

Appendix 2

The human phospho-kinase array was purchased from the R&D systems. (Catalog Number ARY003B)



Membrane/Coordinate	Target/Control	Phosphorylation Site
A-A1, A2	Reference Spot	___
A-A3, A4	p38α	T180/Y182
A-A5, A6	ERK1/2	T202/Y204,Y187
A-A7, A8	JNK 1/2/3	T183/Y185,Y223
A-A9, A10	GSK-3α/β	S21/S9
B-A13, A14	p53	S392
B-A17, A18	Reference Spot	___
A-B3, B4	EGF R	Y1086
A-B5, B6	MSK1/2	S376/S360
A-B7, B8	AMPKα1	T183
A-B9, B10	Akt 1/2/3	S473
B-B11, B12	Akt 1/2/3	T308
B-B13, B14	p53	S46
A-C1, C2	TOR	S2448

A-C3, C4	CREB	S133
A-C5, C6	HSP27	S78/S82
A-C7, C8	AMPKα2	T172
A-C9, C10	β-Catenin	___
B-C11, C12	p70 S6 Kinase	T389
B-C13, C14	p53	S15
B-C15, C16	c-Jun	S63
A-D1, D2	Src	Y419
A-D3, D4	Lyn	Y397
A-D5, D6	Lck	Y394
A-D7, D8	STAT2	Y689
A-D9, D10	STAT5a	Y694
B-D11, D12	p70 S6 Kinase	T421/S424
B-D13, D14	RSK1/2/3	S380/S386/S377
B-D15, D16	eNOS	S1177
A-E1, E2	Fyn	Y420
A-E3, E4	Yes	Y426
A-E5, E6	Fgr	Y412
A-E7, E8	STAT6	Y641
A-E9, E10	STAT5b	Y699
B-E11, E12	STAT3	Y705
B-E13, E14	p27	T198
B-E15, E16	PLC-γ1	Y783
A-F1, F2	Hck	Y411
A-F3, F4	Chk-2	T68
A-F5, F6	FAK	Y397
A-F7, F8	PDGF Rβ	Y751
A-F9, F10	STAT5a/b	Y694/Y699
B-F11, F12	STAT3	S727
B-F13, F14	WNK1	T60

B-F15, F16	PYK2	Y402
A-G1, G2	Reference Spot	___
A-G3, G4	PRAS40	T246
A-G9, G10	PBS (Negative Control)	___
B-G11, G12	HSP60	___
B-G17, G18	PBS (Negative Control)	___

Appendix 3

Mass spectrometry results

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P35579	Myosin-9 OS=Homo sapiens GN=MYH9 PE=1 SV=4 - [MYH9_HUMAN]	1715.56	32.19%	10	45	54	61	1960	226.4	5.6
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	905.36	33.54%	4	15	15	19	644	66	8.12
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	829.75	43.50%	2	17	19	21	623	62	5.24
O43707	Alpha-actinin-4 OS=Homo sapiens GN=ACTN4 PE=1 SV=2 - [ACTN4_HUMAN]	811.42	32.71%	3	17	23	29	911	104.8	5.44
P21980	Protein-glutamine gamma-glutamyltransferase 2 OS=Homo sapiens GN=TGM2 PE=1 SV=2 - [TGM2_HUMAN]	684.08	23.73%	1	13	15	23	687	77.3	5.22
P08670	Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4 - [VIME_HUMAN]	673.83	44.21%	7	20	21	23	466	53.6	5.12
P12814	Alpha-actinin-1 OS=Homo sapiens GN=ACTN1 PE=1 SV=2 - [ACTN1_HUMAN]	644.18	25.78%	3	12	18	22	892	103	5.41
P02751	Fibronectin OS=Homo sapiens GN=FN1 PE=1 SV=4 - [FINC_HUMAN]	598.53	10.27%	1	19	19	20	2386	262.5	5.71
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	534.44	34.59%	11	12	14	15	639	65.4	8
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	505.04	26.54%	18	15	17	18	584	58.8	5.21
P09651	Heterogeneous nuclear ribonucleoprotein A1 OS=Homo sapiens GN=HNRNPA1 PE=1 SV=5 - [ROA1_HUMAN]	439.84	25.00%	2	7	8	9	372	38.7	9.13
Q07955	Serine/arginine-rich splicing factor 1 OS=Homo sapiens GN=SRSF1 PE=1 SV=2 - [SRSF1_HUMAN]	361.23	43.55%	9	13	13	16	248	27.7	10.36
P67936	Tropomyosin alpha-4 chain OS=Homo sapiens GN=TPM4 PE=1 SV=3 - [TPM4_HUMAN]	246.96	25.40%	4	6	6	7	248	28.5	4.69
P04275	von Willebrand factor OS=Homo sapiens GN=VWF PE=1 SV=4 - [VWF_HUMAN]	243.64	4.44%	1	7	8	9	2813	309.1	5.48
P08238	Heat shock protein HSP 90-beta OS=Homo sapiens GN=HSP90AB1 PE=1 SV=4 - [HS90B_HUMAN]	236.09	14.78%	5	5	9	9	724	83.2	5.03
P43121	Cell surface glycoprotein MUC18 OS=Homo sapiens GN=MCAM PE=1 SV=2 - [MUC18_HUMAN]	225.25	15.17%	4	7	7	7	646	71.6	5.76
P16284	Platelet endothelial cell adhesion molecule OS=Homo sapiens GN=PECAM1 PE=1 SV=1 - [PECA1_HUMAN]	172.94	8.67%	1	4	5	5	738	82.5	6.99
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	158.23	9.49%	8	3	6	7	590	62.3	7.74
P07900	Heat shock protein HSP 90-alpha OS=Homo sapiens GN=HSP90AA1 PE=1 SV=5 - [HS90A_HUMAN]	148.78	10.52%	3	2	7	7	732	84.6	5.02
P11021	78 kDa glucose-regulated protein OS=Homo sapiens GN=HSPA5 PE=1 SV=2 - [GRP78_HUMAN]	128.15	9.48%	1	3	5	5	654	72.3	5.16
Q14247	Src substrate cortactin OS=Homo sapiens GN=CTTN PE=1 SV=2 - [SRC8_HUMAN]	125.11	11.09%	1	4	5	5	550	61.5	5.4
P14866	Heterogeneous nuclear ribonucleoprotein L OS=Homo sapiens GN=HNRNPL PE=1 SV=2 - [HNRPL_HUMAN]	110.17	14.09%	1	6	6	6	589	64.1	8.22
P51991	Heterogeneous nuclear ribonucleoprotein A3 OS=Homo sapiens GN=HNRNPA3 PE=1 SV=2 - [ROA3_HUMAN]	110.15	18.78%	1	5	5	5	378	39.6	9.01
Q99729	Heterogeneous nuclear ribonucleoprotein A/B OS=Homo sapiens GN=HNRNPAB PE=1 SV=2 - [ROAA_HUMAN]	108.88	9.34%	1	2	3	3	332	36.2	8.21
P35580	Myosin-10 OS=Homo sapiens GN=MYH10 PE=1 SV=3 - [MYH10_HUMAN]	107.42	3.09%	8	1	6	7	1976	228.9	5.54
Q01130	Serine/arginine-rich splicing factor 2 OS=Homo sapiens GN=SRSF2 PE=1 SV=4 - [SRSF2_HUMAN]	100.44	11.31%	2	2	2	2	221	25.5	11.85
P11142	Heat shock cognate 71 kDa protein OS=Homo sapiens GN=HSPA8 PE=1 SV=1 - [HSP7C_HUMAN]	91.23	9.13%	6	4	5	5	646	70.9	5.52
Q14103	Heterogeneous nuclear ribonucleoprotein D0 OS=Homo sapiens GN=HNRNPD PE=1 SV=1 - [HNRPD_HUMAN]	89.92	11.27%	2	3	4	4	355	38.4	7.81
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	89.02	8.47%	14	2	4	4	472	51.5	5.16
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 OS=Homo sapiens GN=HNRNPA2B1 PE=1 SV=2 - [ROA2_HUMAN]	87.95	6.80%	1	2	2	2	353	37.4	8.95
P29692	Elongation factor 1-delta OS=Homo sapiens GN=EEF1D PE=1 SV=5 - [EF1D_HUMAN]	77.45	13.17%	1	3	3	3	281	31.1	5.01
P62995	Transformer-2 protein homolog beta OS=Homo sapiens GN=TRA2B PE=1 SV=1 - [TRA2B_HUMAN]	67.57	5.56%	1	1	1	1	288	33.6	11.25
Q9BQE3	Tubulin alpha-1C chain OS=Homo sapiens GN=TUBA1C PE=1 SV=1 - [TBA1C_HUMAN]	66.01	10.02%	5	2	3	3	449	49.9	5.1
P60660	Myosin light polypeptide 6 OS=Homo sapiens GN=MYL6 PE=1 SV=2 - [MYL6_HUMAN]	61.99	8.61%	2	1	1	1	151	16.9	4.65

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P21333	Filamin-A OS=Homo sapiens GN=FLNA PE=1 SV=4 - [FLNA_HUMAN]	61.35	0.87%	2	2	2	2	2647	280.6	6.06
P60900	Proteasome subunit alpha type-6 OS=Homo sapiens GN=PSMA6 PE=1 SV=1 - [PSA6_HUMAN]	58.99	4.07%	1	1	1	1	246	27.4	6.76
O43852	Calumenin OS=Homo sapiens GN=CALU PE=1 SV=2 - [CALU_HUMAN]	58.99	13.97%	1	4	4	4	315	37.1	4.64
Q15185	Prostaglandin E synthase 3 OS=Homo sapiens GN=PTGES3 PE=1 SV=1 - [TEBP_HUMAN]	58.62	8.13%	1	1	1	1	160	18.7	4.54
Q16643	Drebrin OS=Homo sapiens GN=DBN1 PE=1 SV=4 - [DREB_HUMAN]	58.08	6.32%	1	2	3	3	649	71.4	4.45
P25788	Proteasome subunit alpha type-3 OS=Homo sapiens GN=PSMA3 PE=1 SV=2 - [PSA3_HUMAN]	55.06	4.71%	1	1	1	1	255	28.4	5.33
P02768	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 - [ALBU_HUMAN]	49.43	4.27%	1	2	3	3	609	69.3	6.28
P07437	Tubulin beta chain OS=Homo sapiens GN=TUBB PE=1 SV=2 - [TBB5_HUMAN]	48.91	6.08%	1	2	2	2	444	49.6	4.89
Q16629	Serine/arginine-rich splicing factor 7 OS=Homo sapiens GN=SRSF7 PE=1 SV=1 - [SRSF7_HUMAN]	46.34	5.04%	1	1	1	1	238	27.4	11.82
P62306	Small nuclear ribonucleoprotein F OS=Homo sapiens GN=SNRPF PE=1 SV=1 - [RUXF_HUMAN]	44.95	15.12%	1	1	1	1	86	9.7	4.67
Q9H1E3	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1 OS=Homo sapiens GN=NUCKS1 PE=1 SV=1 - [NUCKS_HUMAN]	43.74	3.70%	1	1	1	1	243	27.3	5.08
Q99715	Collagen alpha-1(XII) chain OS=Homo sapiens GN=COL12A1 PE=1 SV=2 - [COCA1_HUMAN]	39.72	0.88%	1	2	2	2	3063	332.9	5.53
P28072	Proteasome subunit beta type-6 OS=Homo sapiens GN=PSMB6 PE=1 SV=4 - [PSB6_HUMAN]	39.06	4.18%	1	1	1	1	239	25.3	4.92
P17096	High mobility group protein HMG-I/HMG-Y OS=Homo sapiens GN=HMGA1 PE=1 SV=3 - [HMGA1_HUMAN]	38.93	23.36%	1	2	2	2	107	11.7	10.32
Q8NBS9	Thioredoxin domain-containing protein 5 OS=Homo sapiens GN=TXNDC5 PE=1 SV=2 - [TXND5_HUMAN]	38	2.31%	1	1	1	1	432	47.6	5.97
P25787	Proteasome subunit alpha type-2 OS=Homo sapiens GN=PSMA2 PE=1 SV=2 - [PSA2_HUMAN]	33.42	15.81%	1	3	3	3	234	25.9	7.43
P68104	Elongation factor 1-alpha 1 OS=Homo sapiens GN=EEF1A1 PE=1 SV=1 - [EF1A1_HUMAN]	32.73	3.25%	3	1	1	1	462	50.1	9.01
P20618	Proteasome subunit beta type-1 OS=Homo sapiens GN=PSMB1 PE=1 SV=2 - [PSB1_HUMAN]	29.66	4.15%	1	1	1	1	241	26.5	8.13
P12004	Proliferating cell nuclear antigen OS=Homo sapiens GN=PCNA PE=1 SV=1 - [PCNA_HUMAN]	29.35	4.98%	1	1	2	2	261	28.8	4.69
P19105	Myosin regulatory light chain 12A OS=Homo sapiens GN=MYL12A PE=1 SV=2 - [ML12A_HUMAN]	27.68	5.85%	2	1	1	1	171	19.8	4.81
P15144	Aminopeptidase N OS=Homo sapiens GN=ANPEP PE=1 SV=4 - [AMPN_HUMAN]	27.59	0.93%	1	1	1	2	967	109.5	5.48
P09382	Aminopeptidase N OS=Homo sapiens GN=ANPEP PE=1 SV=4 - [AMPN_HUMAN]	27.41	39.26%	1	3	4	4	135	14.7	5.5
P60709	Actin, cytoplasmic 1 OS=Homo sapiens GN=ACTB PE=1 SV=1 - [ACTB_HUMAN]	27.03	9.33%	3	2	2	2	375	41.7	5.48
O14818	Proteasome subunit alpha type-7 OS=Homo sapiens GN=PSMA7 PE=1 SV=1 - [PSA7_HUMAN]	25.01	3.63%	1	1	1	1	248	27.9	8.46
Q99436	Proteasome subunit beta type-7 OS=Homo sapiens GN=PSMB7 PE=1 SV=1 - [PSB7_HUMAN]	24.22	3.61%	1	1	1	1	277	29.9	7.68
P98179	Putative RNA-binding protein 3 OS=Homo sapiens GN=RBM3 PE=1 SV=1 - [RBM3_HUMAN]	23.39	6.37%	1	1	1	1	157	17.2	8.91
P81605	Dermcidin OS=Homo sapiens GN=DCD PE=1 SV=2 - [DCD_HUMAN]	22.85	10.00%	1	1	1	1	110	11.3	6.54
P17844	Probable ATP-dependent RNA helicase DDX5 OS=Homo sapiens GN=DDX5 PE=1 SV=1 - [DDX5_HUMAN]	21.8	1.95%	2	1	1	1	614	69.1	8.92
Q86YZ3	Hornerin OS=Homo sapiens GN=HRNR PE=1 SV=2 - [HORN_HUMAN]	21.59	1.68%	1	1	1	2	2850	282.2	10.04
P05556	Integrin beta-1 OS=Homo sapiens GN=ITGB1 PE=1 SV=2 - [ITB1_HUMAN]	20.15	3.76%	1	1	2	2	798	88.4	5.39