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Classical monocytes from patients with pancreatic ductal adenocarcinoma exhibit a significantly altered transcriptome profile compared with healthy volunteers.

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Thesis submitted for the degree of

Doctor of Philosophy

Queen Mary University of London

June 2014

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Abstract

Pancreatic Ductal Adenocarcinoma (PDAC) affects approximately 8000 people every year in the UK and is the fifth leading cause of cancer related death. At a molecular level PDAC is characterized by a significant immune infiltrate. Tumour-associated macrophages (TAMs) infiltrate the tumour and contribute to a worse prognosis by promoting growth, metastasis and resistance to chemotherapy. TAMs are derived from circulating 'classical' $CD14^{++}$ $CD16^{-}$ monocytes in the peripheral blood. Current work in murine models suggests targeting monocyte recruitment in PDAC can reduce TAM infiltration and disease burden therefore improving survival. This project aims to identify markers specific to monocytes from PDAC patients and to investigate their biological relevance and potential for therapeutic intervention.

Gene expression and metabolomics analysis was carried out on classical $CD14^{++}$ $CD16^{-}$ monocytes from locally advanced PDAC patients and age matched healthy donors. Transcriptomic profiling revealed a significantly altered gene expression profile in classical monocytes from patients and genes with the highest fold change difference were chosen for validation using qPCR. Validated gene targets were investigated further *in vitro* and large-scale gene expression analysis from pancreatic tumours assessed.

The results from my work demonstrate that the gene expression profile of classical monocytes from PDAC patients is significantly different compared to healthy volunteers. Identification and validation of up-regulated genes and their biological relevance may represent a relevant ~~novel~~ novel biomarker or therapeutic strategy~~ies~~ to target monocytes and myeloid recruitment in cancer.

Acknowledgements

I would like to thank my supervisor and mentor Thorsten for his endless support and every opportunity he has given me to learn an incredible amount. I would like to say a huge thank to Viia for her copious guidance and my visits to MedImmune for discussion. I would also like to extend a warm thank you to John Marshall for believing in me and putting me forward for this studentship.

I would like to offer a warm thank you to Cristina, Juliana, Maryam, Eleni and Fiona for their advice in the lab and realistic feedback during meetings. I would like to give a special Thank you to Raphael for his lessons in data analysis and statistics also for helping me to improve my clarity and communication skills.

I would like to thank all the members of Thorsten's group past and present for their instruction and counsel as well as friendship. I would like also to thank all the other members of Cancer and Inflammation for making my time at Barts Cancer Institute memorable and enjoyable.

I offer a very big thank you to everyone at the hospital who helped me with sample collection, the ward nurses in the chemotherapy unit, the clinicians for taking consent and the phlebotomists for taking the blood. I would also like to thank Guglielmo and Will for working out of hours to help process samples as soon as possible.

I have to offer a huge thank you to all the patients who were included in this project as well as everyone in the lab and their friends or families who donated blood as healthy volunteers.

Lastly, I would like to give a glass of wine and a pizza to my patient proof-reading friends, exceptional thanks to my family for their unending love, kindness and support and an immense thank you to Alex, for everything.

Abbreviations

BSA	Bovine Serum Albumin
CA19-9	Carbohydrate antigen 19-9
CCL2	Chemokine (C-C motif) ligand 2
CCR2	Chemokine (C-C motif) receptor 2
CD	Cluster of differentiation
cDNA	Complementary DNA
CEA	Carcino-embyronic antigen
CEBP	Ccaat-enhance binding protein
ChIP	Chromatin Immunoprecipitation
CMP	Ccommon myeloid progenitor
CP	Chronic pancreatitis
CSF1R	Colony stimulating Factor 1 receptor
DAPI	4',6-diamidino-2-phenylindole
DC	dendritic cells
DEG	Differentially expressed gene
EGFP	Enhanced green fluorescence mice
EGR2	Early Growth Response 2
EGR3	Early Growth Response 3
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial mesenchymal transition
ERK	E26 transformation-specific
EUS	Electronic Ultasound
FACS	Fluorescence Assisted cell sorting
Fc	Fragment, crystallizable
FCS	Fetal calf serum
FIRE	Fms intronic regulatory region
FNA	Fine needle aspirate
FOLFIRINOX	Fluorouracil, leucovorin, irinotecan and oxaliplatin
FOLR1	Folate receptor 1
GMP	Ggranulocyte-macrophage progenitor
GPI	Glycosyl-phosphatidyl inositol

GSEA	Gene set enrichment analysis
GTP	Guanosine triphosphate
HA	Hyaluronan
hAB	Human AB
Hif	Hypoxia inducible factors
HLA	Human Leukocyte Antigen
HLA-DR	Human Leukocyte antigen
ICSPB	Iinterferon consensus sequence binding protein
IFN	Interferon
IgG	Immunoglobulin
KRAS	Kirsten rat sarcoma viral oncogene homolog
LPS	Lipopolysaccharide
M-CSF	Macrophage – Colony stimulating factor
M1	Classically activated macrophage
M2	Alternativelyactivated macrophage
MARCO	Macrophage Receptor Collagenous Structure
MDP	~~macrophage~~Macrophage-dendritic cell progenitor
MEK	Mitogen-activated protein kinase kinase
MHC	Membrane histocompatibility Complex
mRNA	Messenger RNA
MSD	Mesoscale discovery
NADPH	Nnicotinamide adenine dinucleotide phosphate-oxidase
NfKB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram
PanIN	Pancreatic Intraepithelial neoplasia
PanIN	Pancreatic intraepithelial neoplasia in situ
PBMC	Peripheral blood mononuclear cell
PD	Programmed Death
PDAC	Pancreatic Ductal Adenocarcinoma
PDGFR	Platelet derived growth factor receptor

PLX	Plexxicon
PTX	Paclitaxel
qPCR	Quantitative Polymerase Chain Reaction
RANTES	Regulated on activation, normal T cell expressed and secreted
RASGEF1B	RAS
Rho	Ras homolog
RHOC	Ras homolog gene family, member C
RHOF	Ras homolog gene family, member F
RNA	Ribonucleic Acid
RNA-Seq	RNA- sequencing
ROI/ROS	Reactive oxygen intermediates/species
RPM	Revolutions per minute
rTK	Receptor tyrosine kinase
SMA	Smooth muscle actin
STAT	Signal transducer and activator of transcription
TAM	Tumour-associated macrophage
TGF	Transforming growth factor
TNF	Tumour Necrosis Factor
TNF	Tumour necrosis factor
TREM	Triggering receptor expressed on myeloid cells
µg	Micro gram
µl	Micro litre
VEGF	Vascular endothelial growth factor

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Statement of Originality

I, Jenny Anne Cook confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

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Date: 20th June 2014

Details of collaboration and publications:
This work is funded by MedImmune (Grant number CIF1RA3R)

Chapter 1. Introduction

1.1 Myeloid cells

Myeloid cells represent the major leukocyte population in the peripheral blood. Composed of circulating monocytes and granulocytes, these cells are derived from common progenitors in the bone marrow. Commitment to the monocyte or granulocyte lineage is under the control of distinct transcription factors and occurs before release into the peripheral ~~blood .~~ blood. Monocytes and granulocytes are continuously delivered to many tissues types and play an important role in innate immunity as a first line of defence against pathogen invasion and responses to tissue injury (Miranda et al., 2005).

Upon activation monocytes are rapidly recruited to local tissues by chemokine signals where they are able to phagocytose pathogens and produce pro-inflammatory cytokines (Dolcetti et al., 2010, Chioda et al., 2011). Once they have reached the tissue, monocytes are able to differentiate into macrophages or dendritic cells as required (Sica and Mantovani, 2012, Gabrilovich et al., 2012).

Geissman *et al*., recently defined two waves of haematopoietic expansion; a 'primitive' wave during embryonic development and 'definitive' wave later on (Gomez Perdiguero and Geissmann, 2013). During the primitive wave of embryonic development, several types of resident macrophages originate in the yolk sac and form populations in privileged locations; these macrophages are named according to the tissue they reside in. For example, microglia reside in the brain (Hoeffel et al., 2012, Ginhoux et al., 2013) or the Langerhans cells within the skin (Chopin and Nutt, 2014), the Kupffer cells in the liver (Kawamoto and Minato, 2004) or osteoclasts in the bones (Rogers and Holen, 2011). These populations are self-renewing, maintained locally under normal homeostatic conditions.

Monocytes provide a pluripotent circulating pool of progenitor cells for the ‘definitive’ wave of re-population of tissue macrophages and dendritic cells as required under inflammatory conditions (Bird, 2012, Schulz et al., 2012).

1.1.1 Origins of monocytes

Monocytes originate from haematopoietic stem cells in the bone marrow through a stepwise process of lineage differentiation and commitment, collectively called monopoesis (Yona and Jung, 2010). These advances in differentiation are tightly irreversible, regulated by environmental signals (Auffray et al., 2009, Swirski et al., 2009, Cortez-Retamozo et al., 2012). As shown in Figure 1.1 below. The early stages are very distinctive; from a common myeloid progenitor (CMP) and granulocyte-macrophage progenitor (GMP) (Akashi et al., 2000) these cells differentiate into the macrophage-dendritic cell progenitor (MDP) (Fogg et al., 2006) which serves as a common precursor for monocytes, macrophages and dendritic cells (DCs) (Kawamoto and Minato, 2004). As the MDPs give rise to monocytes they are thought to lose their proliferative capabilities and can be subdivided into classified subsets (Geissmann et al., 2010b) as discussed later in chapter 1.2.1.

Haematopoetic stem cells are produced in the bone marrow to provide progenitor populations of all lymphoid, myeloid and erythroid cells. They give rise to a population of daughter stem cells that have a loss of self-renewal capacity and progressive restriction of lineage options. Figure 1.1 below indicates the origins of cells derived from myeloid and lymphoid lineages

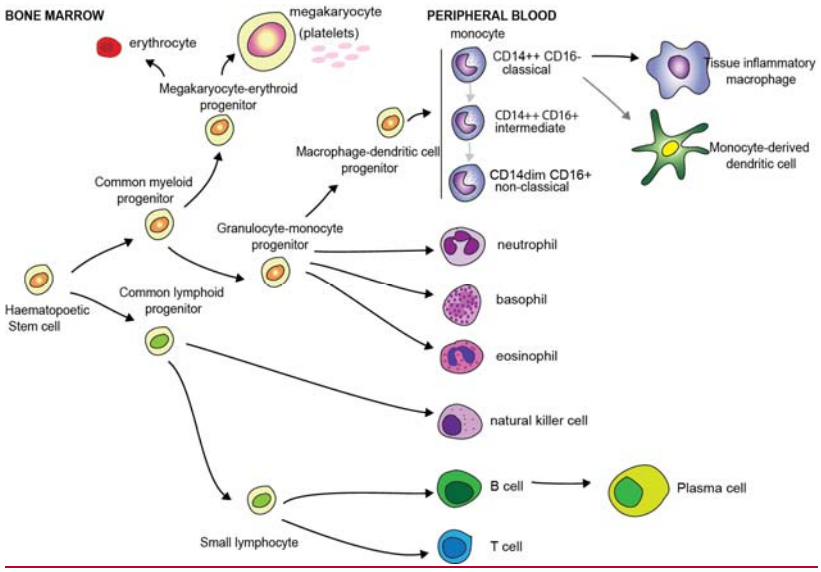

Figure 1.1 Haematopoetic cell origins

Stages leading to the development of myeloid and lymphoid subsets in the peripheral blood. Adapted from (Ardavin et al., 2001, Auffray et al., 2009). Haematopoietic stem cells in the bone marrow give rise to multipotent myeloid and lymphoid progenitor. ~~Haematopoetic stem cells are produced in the bone marrow to provide progenitor populations of all lymphoid, myeloid and erythroid cells. They give rise to a population of daughter stem cells that have a loss of self-renewal capacity and progressive restriction of lineage options.~~ Common lymphoid progenitors (CMP) to produce cells of the lymphocyte lineage including B cells, T cells and natural killer cells. Common myeloid progenitors (CMP) form the megakaryocyte and erythrocyte progenitor to provide red blood cell and platelet populations. The CMP also provides granulocyte-monocyte progenitors (GMP). These have the ability to differentiate into neutrophils, basophils and eosinophils as well as providing the macrophage dendritic cell progenitors (MDP). MDPs are progenitors for the population of circulating monocytes. The intermediate and non-classical monocyte populations are thought to be sequentially derived from the classical monocyte populations. The classical monocytes give rise to inflammatory tissue macrophages and tissue monocyte derived dendritic cells.

Transcription factors play an essential role during lineage commitment. PU.1 is a member of the E26 transformation-specific (ETS) transcription factors. Over expression of PU.1 has been shown to increase myeloid cell

development (Rosa et al., 2007). PU.1 inhibits GATA-1 activity very early in myelopoeisis, thereby promoting differentiation away from the erythroid lineage (Zhang et al., 2000). Egr1, Maf-B, and c-MAF are transcription factors critical in promoting myeloid differentiation away from the granulocyte lineage (DeKoter and Singh, 2000). PU.1 enhances expression of ICSPB (interferon consensus sequence binding protein), which promotes monocyte development (Friedman, 2002). This tightly regulated internal cellular cascade of transcriptional control can be modified by external stimuli in the microenvironment (Varol et al., 2009).

1.2 Monocyte functions

Monocytes form part of the mononuclear phagocyte system, a subset of white blood cells that circulate in the peripheral blood until receiving signals to differentiate (Auffray et al., 2009, Shi and Pamer, 2011). Monocytes are remarkably multipotent and play a critical role in inflammation, wound healing and resolution (Ziegler-Heitbrock, 2007).

The ability of monocytes to mobilise from the bone marrow, circulate in the peripheral blood and traffic to distant tissue sites as required is a central factor in immune defences (Gordon and Taylor, 2005). One of the main functions of bloodstream monocytes is the capacity to mediate host anti-microbial defences due to their ability to phagocytose pathogens. This is mediated by various cell surface scavenger receptor interactions with lipoproteins and signalling through immunoglobulin and complement receptors (Yarovinsky, 2014). Toll-like receptors are essential for myeloid cell activation and can recognise bacterial products (Takeda and Akira, 2007). Monocytes are also able to present antigens and secrete pro-inflammatory cytokines in particular IL-10, IL-6, IL-1, Tumour Necrosis Factor-alpha (TNF-alpha) and IL-12 (Serbina et al., 2008).

This notable multipotency however can act as a double edged sword when monocyte recruitment and differentiation promote inflammation where it is not required (Murray and Wynn, 2011). This is the case in cardiovascular disease when monocytes are known to contribute to atherosclerotic plaques (Saha et al., 2009, Woollard and Geissmann, 2010, Shalhoub et al., 2011, Gratchev et al., 2012), and in the case of several solid tumour types where monocytes are recruited to be polarised towards tumour promoting macrophage populations (Sica et al., 2008, Moses et al., 2009, Chioda et al., 2011, Denardo et al., 2011, Shiao et al., 2011, Mahmoud et al., 2012, Tang et al., 2012).

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1.2.1 Monocyte subsets and classification

There is still no unifying consensus surrounding monocyte classification. The highly responsive and plastic nature of monocytes is advantageous in their reactivity; however, it presents challenges when trying to define phenotype and function. Investigations into monocyte heterogeneity have been hindered in the past by ambiguity over cell surface markers. Recently the classification of three circulating monocyte subtypes (Table 1.1) has been accepted (Ziegler-Heitbrock, 2007, Geissmann et al., 2010a, Zawada et al., 2011, Wong et al., 2012). In 1989, Ziegler Heitbrock first described the use of CD14 (a lipopolysaccharide receptor) and CD16 (Fragment crystallisable (Fc) Gamma receptor III) to characterise monocytes.

The three monocyte subsets have been associated with differential expression of cell surface markers and functional abilities as I will further outline below.

1.2.1.1 Classical ($CD14^{++}$ $CD16^{-}$) monocytes

The 'classical' $CD14^{++}$ $CD16^{-}$ monocyte population subset is the most abundant, comprising around 80% of the monocytes found circulating in the peripheral blood in humans (Gordon and Taylor, 2005).

Classical monocytes play an important part in mediating host immunity to invading pathogens. They are, in the first instance, defined by their cell surface expression of CD14. CD14 is a glycosyl-phosphatidyl inositol (GPI) phospholipid anchored membrane co-receptor that acts with Toll Like Receptor 4 and MD-2 to form a complex that detects pathogen-associated molecular patterns (PAMPS) (Simmons et al., 1989). The main identified ligand is bacterial lipopolysaccharide (LPS) (Kitchens, 2000, Moreno et al., 2004). CD14 also recognises peptidoglycans, lipoteichonic acid and phospholipids on gram-positive bacteria (Scherberich and Nockher, 2000).

Carman *et al*., implicated classical monocytes in regulation of leukocyte trafficking in the blood due to their selective expression of CD62L, a homing receptor for ligands expressed on endothelial cells (Mishra et al., 2012), as well as CD64, an Fc receptor required for binding IgG-type antibodies (Carman, 2008). Other markers to characterise classical monocyte have been described and are reviewed by Martinez *et al*., and summarised in Figure 1.2 (Martinez et al., 2009)

Upon activation, classical monocytes are able to phagocytose invading pathogens (Flego et al., 2013). Classical monocytes are able to increase production of pro-inflammatory cytokines, in particular IL-10, TNF-alpha, ~~NFkappaB (NfkB)~~ and IL-6. The expression of pro-inflammatory cytokines occurs via the Raf-1/MEK1-MEK2/ERK1-ERK2 pathway (Viriyakosol and Kirkland, 1995, van der Bruggen et al., 1999, Skrzeczynska-Moncznik et al., 2008). In pancreatic cancer, monocyte expression of cytokines, including IL-6 and IL-10, has been linked with the development of cachexia and a poorer prognosis (Carter and Tourtellotte, 2007, Moses et al., 2009, Steele et al., 2013).

Classical monocytes selectively express CCR2 (chemokine C-C receptor 2), the receptor for CCL2 (chemokine C-C ligand 2, also known as monocyte chemoattractant protein (MCP-1)), and are recruited to the site of solid malignancies to contribute to the tumour promoting macrophage populations (Beatty et al., 2011, Denardo et al., 2011, Mitchem et al., 2012, Sanford et al., 2013).

Interestingly, the total number of classical monocytes has been used as a biomarker in patients with stroke. An increase in $CD14^{++}CD16^{-}$ classical monocytes in the peripheral blood correlates positively with the onset of stroke (Urra et al., 2009) and cardiovascular disease (Olivares et al., 1993, Heine et al., 2008).

1.2.1.2 Non-classical ($CD14^{dim}$ $CD16^{++}$) monocytes

The non-classical monocytes express lower levels of CD14, but are strongly positive for CD16. The CD16 antigen is an anchored polypeptide expressed on the cell surface and is a low affinity receptor consisting of the Fc ~~(Fragment, crystallizable)~~ gamma RIIIA and Fc gamma RIIIb part. They form the receptor for the Fc portion for the immunoglobulin IgG. Upon IgG binding to the Fc receptor complex of CD16, downstream signalling cascades are activated that increase production of pro-inflammatory cytokines such as interleukin-1 and tumour necrosis factor alpha as well as increasing phagocytic capabilities (Scherberich, 2003).

Non-classical monocytes are highly ~~reactive~~motile. Cros *et al*. (2010) observed, after adoptive transfer of non-classical monocytes, these cells 'crawled' along the endothelium in contrast to their classical counterparts. The group used intravital microscopy to record the 'patrolling' the luminal side of endothelial blood vessels to rapidly extravasate and migrate to the site of immune reaction (Cros et al., 2010). Ras homolog gene family, members C and F (RHOC and RHOF), Rho GTPases are significantly over-expressed in the $CD14^{dim}CD16^{++}$ subset linking them to the increased motility and rapid mobilisation into the bloodstream after exercise and infection (Ancuta *et al*., 2006, Frankenberger *et al*., 2012).

1.2.1.3 Intermediate ($CD14^{+}CD16^{++}$) monocytes

The intermediate population of monocytes expresses both CD14 and CD16. This population has only been relatively recently identified as a subset (Ziegler-Heitbrock et al., 2010, Zawada et al., 2011). Intermediate monocytes have been shown to be pro-angiogenic by expressing Tie-2 the receptor for vascular growth factors angiopoetin-1 and -2 (Mazzieri et al., 2011).

An increased intermediate monocyte population has been identified in patients with breast (Feng et al., 2011) and colorectal cancer (Schauer et al., 2012). However, the reasons for this increase are not well understood. This $CD14^{++}CD16^{+}$ cell population expansion has been correlated with a positive outcome in cardiovascular diseases and other pathological settings, including sarcoidosis and asthma (Ancuta et al., 2004, Tacke et al., 2007, Saha et al., 2009, Subimerb et al., 2010, Rogacev et al., 2011).

A recent investigation into human monocyte kinetics showed that, over time, an increase in intermediate monocytes occurs before an increase in non-classical monocyte populations in the blood during and after pulmonary infection. This may suggest a sequential developmental relationship (Ziegler-Heitbrock and Hofer, 2013, Frankenberger et al., 2013).

The monocyte subsets have been classified by their differential cell surface expression of functional markers, transcriptomic profile and functional abilities *in vitro*. However, it is still under debate whether each subset represents a distinct cell type derived from the monocyte-dendritic cell precursor, or the same cells at different stages of differentiation. In a bid to elucidate the mechanisms behind monocyte to macrophage differentiation and where the key to this responsiveness lies, several approaches are currently being used which are outlined in more detail below.

1.2.1.4 Fate mapping

To define the origin and fate of monocytes during maturation and differentiation, fate mapping has been carried out in murine models (Schulz et al., 2012, Gomez Perdiguero and Geissmann, 2013).

In humans, there are thought to be three monocyte subsets due to the recent discovery of the intermediate population. This has also been shown in the three subsets defined in mice (Auffray et al., 2009). The mouse equivalent of a classical monocyte can be defined by positive expression for Gr1+ and Ly6C+ (Auffray et al., 2009). Expression of Gr1+ and Ly6G+ can be used to define the equivalent non-classical population. These are defined as equivalent to the human classical and non-classical populations due to the conservation of expression of CCR2 and CX3CR1 across species. Work by Sunderkotter *et al*., characterised the Ly6C^{med} monocytes and, based on the similarities in phenotype and function between man and mouse, classified them also as intermediate monocytes (Sunderkotter et al., 2004). This avoids confusion generated by dated nomenclature and classification of monocyte subsets as 'inflammatory' and 'pro-inflammatory' as reciprocal populations (Ziegler-Heitbrock, 2014). The corresponding human and mouse subsets are shown in Table 1.1 below.

Human	**Mouse**
Classical (CD14^{++} CD16^{-}) CCR2^{hi} CX3CR1^{lo} CD163^{+}	**CD11b^{+} Gr1^{+} Ly6C^{++}** CD43^{+} CCR2^{hi} CX3CR1^{lo}
Intermediate (CD14^{++}CD16^{+}) CX3CR1^{hi} CCR2^{lo} CD163^{+}	**CD11b^{+} Gr1^{+} Ly6C^{++}** CD43^{++}
Non-classical (CD14^{dim} CD16^{++}) CX3CR1^{hi} CCR2^{lo} CD163^{-}	**CD11b^{+} Gr1^{+} Ly6G^{+}** CD43$^{+\,+}$ CX3CR1^{hi} CCR2^{lo}

Table 1.1 Markers used to classify monocyte subsets in humans and mice

Table adapted from (Shi and Pamer, 2011, Ziegler-Heitbrock, 2014)

Selective expression of CCR2 on the Ly6C$^+$ classical monocytes links their phenotype closely to their function. The CCR2-CCL2 interaction is important for monocytes to exit the bone marrow into the blood circulation (Qian et al., 2011). CCR2-/- mice have significantly lower numbers of circulating Ly6C$^+$ monocytes as a result of this exit block with respective accumulation in the bone marrow and spleen (Yona and Jung, 2010). It is suggested that this Ly6C$^+$ population, similar to the classical monocytes forms a pool of short-lived circulating progenitor cells supplying the Ly6C$^-$ population.

To explore further whether the monocyte dendritic cell precursor (MDP) forms separate monocyte subsets, or monocytes at different stages of differentiation, a study was carried out by Yona *et al.*, in 2013. In this study, mice were used that harbour a GFP reporter in the CX3CR1 locus. Constitutively expressing CX3CR1$^+$ GFP$^+$ positive Ly6C$^+$ positive monocytes from the splenic reservoir were adoptively transferred into congenic wild type mice. After day 1, flow cytometry was used to show that the GFP$^+$ cells in the blood were Ly6C$^+$ CX3CR1 intermediate and, after day 3, the GFP$^+$ CX3CR1 expressing cells in the blood were Ly6C$^-$. This data contributes to the hypothesis that Ly6C$^+$ monocytes may form part of a developmental sequence in mice. In humans the corresponding non-classical monocytes might be formed from the precursor classical monocyte population (Yona et al., 2013). To further elucidate the differences and similarities between the monocyte subsets, one approach commonly used is transcriptome profiling.

1.3 Transcriptomic Profiling

Transcriptomics refers to study of the transcriptome: 'The complete complement of mRNA molecules generated by a cell or population of cells' as defined in 1997 by Charles Auffray (Pietu et al., 1999). Transcriptomic networks are incredibly complex and the advent of novel technologies is leading to more 'data-driven' approaches to derive hypotheses.

Gene expression microarrays are a reliable, robust and cost-effective way to gain a biological snapshot of gene expression. Microarray technology is based on a library collection of DNA probes attached to a solid surface, which hybridise to cDNA reverse transcribed from RNA samples. The levels of hybridisation are measured using fluorescence and converted to expression values (Martinez, 2009).

1.3.1 Transcriptomic profiling as a reflection of immune system activation

Evaluation of immune cells in disease might include absolute numbers and relative subset proportions of cells and their distribution, as well as transcriptional state, secretory functions and proliferative ability (Chattopadhyay et al., 2014). Immune cells in the blood harbour a wealth of information after activation *ex vivo* and in their homeostatic state (Tuomela et al., 2013, Dolcino et al., 2014). A major limitation in studying immune mediated human disease is restricted access to the relevant tissue or cell type.

Transcriptomic profiling of immune cells is expected to reflect the influences of environmental, genetic and cellular factors (Chaussabel et al., 2010). Easily accessible from blood samples they are valuable investigational tools. Past work analysing transcriptomic profiles in the peripheral blood immune cells from cancer patients used isolated PBMCs. In Pancreatic cancer an eight-gene signature was identified in circulating PBMCs from PC patients. These included *SSBP2, Ub2b-RS, CASB, TBC1D8, ANX3A, ARG1 and ADAMTS20*. These were shown to be efficacious in a small cohort of patients with a sensitivity of 83% and a specificity of 75% in a blinded subset of samples. The genes identified ~~and genes~~ were not shown to overlap with PBMCs from ~~other inflammatory conditions~~different stimuli or pathogens suggesting they were PC specific (Baine et al., 2011). Gene expression signatures derived from profiling PBMCs can be linked to certain cell types but are not considered particularly accurate due to heterogeneous cell populations.

1.3.2 Transcriptomic profiling of human monocytes

In 2007, qPCR was used to assess differential expression of known monocyte associated genes in both the classical ($CD14^{++}$ and $CD16^{-}$) and non-classical ($CD14^{dim}$ $CD16^{+}$) population of human monocytes. The chosen gene list examined adhesion molecules, such as CD11b and CCR2, as well as scavenger receptors, such as CD163, CD14, macrophage receptor with collagenous structure (MARCO)~~,~~ which are now used as typical monocyte markers (Mobley et al., 2007).

In 2009, three groups published data on transcriptomic profiles of human classical monocytes compared with non-classical monocytes. The first was (Zhao et al., 2009), the second (Ancuta et al., 2009) and the third merged all three data sets to produce overlapping validated gene lists (Martinez, 2009).

Figure 1.2 below, indicates the top differentially expressed genes comparing monocyte subsets from available transcriptomic data.

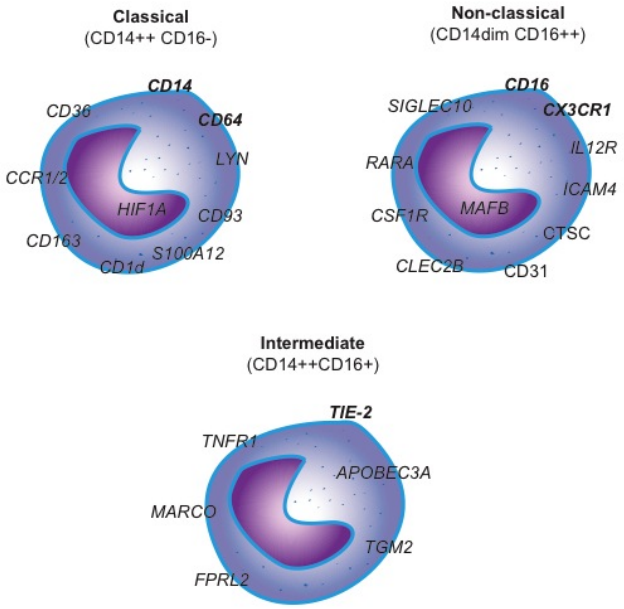

Figure 1.2~~1.2~~ Monocyte subset transcriptomic profiling reveals differentially expressed genes

Identified genes that are more highly expressed in one monocyte subset compared with the others. A selection of validated top differentially expressed genes adapted from (Martinez, 2009, Zhao et al., 2009, Ancuta et al., 2009, Zawada et al., 2011).

The three combined data sets confirm the distinct differences between these subsets. The results also suggest that the non-classical ($CD14^{dim}$ $CD16^{++}$) monocytes appear to have a more advanced stage of differentiation due to their closer resemblance to more dendritic cell or macrophage transcriptomic profiles (Ancuta et al., 2009). It was only in 2011 that the presence of the intermediate population ($CD14^{++}$ $CD16^{+}$) was confirmed using gene expression analysis, contradicting the dichotomised view held over the previous twenty years (Zawada et al., 2011).

Building upon this data, work by Schmidl *et al.* (Schmidl et al., 2014) profiled all three monocyte subsets to provide novel insights into metabolic profiles

and functional biological differences. As well as identifying the known subset specific features, they also identified differential promoter and enhancer regulatory elements corresponding to transcription factor activity of PU.1 and C/EBP beta. Gene ontology (GO) terms were enriched to show striking differences between the subsets for metabolic pathway genes. A significant number of genes involved in carbohydrate metabolism, especially the glycolytic pathway was up-regulated in the classical monocytes.

The intermediate monocytes could be characterised by an increase in genes associated with antigen processing and presentation. The non-classical monocytes were more associated with the oxidative phosphorylation pathway, in particular the components of the mitochondrial respiratory chain complexes. These results were confirmed at protein level and suggest there is a metabolic bias across the monocyte subsets (Schmidl et al., 2014).

When assessing the monocyte transcriptome under other conditions, there are three main approaches that are used. One approach, to reduce variables, is using a monocyte cell line known as THP-1. This human cell line derives from a 1-year-old infant with acute monocytic leukaemia. Although the cell line can be differentiated into macrophage-like cells using phorbol 12-myristate 13-acetate (PMA), the cell line does not accurately represent primary human monocytes or macrophages or correspond to a particular monocyte subset (Qin, 2012).

A common way to assess gene expression and monocyte function is to isolate healthy monocytes and treat them with different stimuli or pathogen, which represents a more closed system, to identify core genes and phenotypic changes (Tuomela et al., 2013). This has been utilised mainly in assessing the effects of bacterial or viral infections (Barker et al., 2005, Harun et al., 2013, Tan and Chu, 2013, Marangoni et al., 2014).

Transcriptome profiling of human primary monocytes has been investigated in several inflammatory conditions, in particular cardiovascular disease, due

to their essential role in promoting atherosclerosis (Sivapalaratnam et al., 2012, Maiwald et al., 2013).

Gene expression profiling of peripheral blood monocytes from patients with ~~Crohn's~~ coeliac disease identified a four-gene signature (*c-REL, LPP, TNFAIP3, KIAA1109*) that could be used to discriminate between patients with disease and healthy volunteers. This signature was even significant without the corresponding clinical data (Galatola et al., 2013). This result is promising in for the use of monocytes as indicators of disease presence or progression.

The results of these studies give an outline of genes associated with the inflammatory response and differentiation, however there is no current published data expressing monocyte transcriptomes from patients with cancer.

1.3.3 Transcriptomic profiling and transcriptional regulation of monocyte to macrophage differentiation.

Transcriptome profiling has supported the identification of differential monocyte subsets and provided useful insight into responses and biological processes. It has also been useful in understanding transcriptional regulation in monocyte to macrophage differentiation under normal conditions.

In the past, a simplified model system of macrophage biology separated macrophage polarisation into two phenotypes, M1 and M2, in analogy to the Th1 / Th2 dichotomy of T cells (Gordon and Taylor, 2005). These two phenotypes were linked to the two polarising stimuli: LPS, or IL-4 and IL-13. Martinez *et al*. (and others) carried out transcriptomic profiling of this process, in 2006 and again in 2009, and expanded it to include moderately differentiated cells at an earlier time point of three days post-differentiation (Martinez et al., 2006, Martinez et al., 2009). This work indicated genes that were involved in early differentiation ~~steps, that~~steps that had not been previously recognised. Pelegrin *et al*., built upon this by showing elegantly the plasticity of the macrophage phenotype and how macrophages can switch from one to the other phenotype (Pelegrin and Surprenant, 2009).

In 2012, Beyer *et al*., similarly polarised macrophages and carried out RNA-sequencing (RNA-Seq) analysis (Beyer et al., 2012). RNA-Seq technology offers a wider dynamic range and, in contrast to hybridisation microarray methods, RNA–Seq directly determines the cDNA sequence, as well as having the ability to distinguish between different isoforms. This means it is not limited to detecting transcripts corresponding to existing known genomic sequences (Wang et al., 2009). The study supported previous findings, and built upon these results. Due to the higher resolution given by RNA-seq data, novel M1-associated (TLR2, SLAMF7, CD120b) and M2-associated (CD93, CD226, CD1a and CD1b) cell surface markers were identified.

Transcriptional regulatory network profiling of monocyte to macrophage differentiation takes this analysis one step further to identify multiple transcription factors linked with gene expression. Although these networks are very complex, a recent study by Huber et al., (Huber et al., 2014) identified a chain of transcription factors in differentiation that appear to be key in modulation and drive downstream gene expression. This study discovered 4 core inducers of monocyte specific regulatory pathways SP11, CEBPA, IFR8 and MDNA. These inducers were shown to positively affect the expression of other key genes in monocyte development such as *JUNB*, *FOS*, *MAF* and *EGR2.*

These models however only account for two extreme polarised macrophage phenotypic states, which we now know is an over-simplified model. Observations from macrophages in chronic inflammation, infections or cancer suggest a much broader repertoire of phenotypic regulation depending on environmental signals. A study published this year used 28 different conditions to profile the macrophage transcriptome and extend the current model of macrophage polarisation and identify key genes associated with distinct stimuli (Xue et al., 2014). This work proves that monocyte to macrophage differentiation is not as simple as previously thought.

The studies mentioned above derive macrophages from $CD14^+$ cells. Contributing to this field, macrophages were derived from classical and non-classical monocyte populations. Frankenberger *et al*., (2012) reported significant differences between derived macrophage population transcriptomic profiles and functions. The macrophages derived from the non-classical monocytes had a significantly increased ability to phagocytose opsonised *E.coli.* In terms of developmental biology this is an important finding as depletion of non-classical monocytes leads to a depletion of Kupffer cells in the liver, but not in other tissues suggesting there are differential progenitor subset population pools (Frankenberger et al., 2012).

The genes and associated phenotypes have been used as a basis of much of the understanding of macrophage functions and their role in inflammation and disease. Cell surface markers, for example, can be associated with ~~tumour associated macrophages~~TAMs. In pancreatic cancer, expression of CD163 and CD204 in the tumour microenvironment can be linked to poorer survival (Kurahara et al., 2011). This expression correlates with infiltrating tumour-associated macrophages.

1.4 Circulating immune cells as biomarkers

Using circulating immune cells is attractive for early detection of disease as they are accessible in the blood (Misek et al., 2007, Baine et al., 2011). The table below shows published changes in monocyte distribution in several inflammatory conditions.

Disease	Classical	Intermediate	Non-classical	Reference
Tuberculosis	10% +	9% +	13% +	(Castano et al., 2011)
Sepsis	9.5% +	12% +	6% +	(Skrzeczynska et al., 2002)
Stroke		3% +	3% -	(Urra et al., 2009)
HIV	2.5% -	3% +	3%+	(Tippett et al., 2011)
Rheumatoid arthritis		5% +		(Rossol et al., 2012)
Abdominal aortic aneurysm		2.2% +	1.9% +	(Ghigliotti et al., 2013)
Dengue Fever	12-18% -	9% +		(Azeredo et al., 2010)

Table 1.2 Monocyte subset distribution in disease

Monocyte subset percentage changes as indicated in each disease. +/- indicate direction of change increase or decrease as compared with healthy donors. Table adapted from (Yang et al., 2014)

Transcriptomic profiling of PBMCs in chronic pancreatitis and PDAC revealed an eight-gene predictor signature with sensitivity and specificity of 83% and 75%, respectively, that was significantly associated with increased CA19-9 expression (Baine et al., 2011). PBMC profiling has also been used as a predictor of cancer presence in renal (Burczynski et al., 2005) and rectal cancer (Palma et al., 2013). Other studies indicate their reflection of immune responses by being able to predict drug-induced toxicity (Todorova et al., 2012).

These studies, however, represent transcriptomic profiling of a mixed cell population. Mononuclear cells in the blood encompass monocytes, lymphocytes, dendritic cells and natural killer cells. Therefore the results gained are not cell type specific and differential distribution of each cell type could bias results. Circulating monocytes represent a biomarker and monocytosis can be used as a clinicopathological factor in several inflammatory conditions. Elevated counts are seen in cardiovascular disease (Waterhouse et al., 2008), acute myocardial infarctions (Khan et al., 2012), chronic kidney disease (Heine et al., 2008) and coronary arterial disease (Afiune Neto et al., 2006).

A recent study examining gene expression profiles in $CD14^+$ peripheral blood monocytes identified a four-gene signature that could be used to differentiate patients with ~~Crohn's~~ coeliac disease from healthy volunteers, without additional clinical data. Their sensitivity was higher than 95% in validation set (Galatola et al., 2013).

Metabolite profiling of circulating immune cells could serve as a less invasive and more direct alternative to tissue biopsies. Recent work highlights the interactions between metabolism and inflammation. 'Metabolomic' profiling has been used for biomarker discovery in urine, plasma, metabolic and haematological disorders as well as endocrinopathies (Russell et al., 2013). A recent study in monocytes revealed marked differences between the monocyte subsets comparing metabolic gene signatures. The classical monocytes had a much higher level of genes associated with carbohydrate metabolism compared with the non-classical (Schmidl et al., 2014)

Metabolomic analysis of urine can be used to identify prognostic markers in lung and bladder cancer (Jin et al., 2014, Mathe et al., 2014) and in serum of pancreatic cancer patients (Kobayashi et al., 2013). Metabolomic analysis can also be carried out on cells, and in pancreatic cancer has been used to profile responses of human cell lines to gemcitabine and other chemotherapy

agents (Spratlin et al., 2011, Ohmine et al., 2012). Current literature suggests metabolomic profiling of monocytes from humans with cancer has not been carried out and may represent an effective method to identify novel pancreatic cancer associated biomarkers in the peripheral blood.

1.5 Pancreatic ductal adenocarcinoma

Pancreatic cancer is the 9th most common cancer in the United Kingdom, with an incidence of approximately 8,000 patients per year. Lack of symptoms until disease has advanced can often account for a delayed diagnosis (Dabizzi et al., 2011, Tokar and Walia, 2013). Due to late presentation with advanced disease and lack of therapeutic options, the incidence nearly matches the mortality of the disease; the 5-year survival rate in the United Kingdom is less than 3%. These statistics have not improved in the last 30 years (Coupland et al., 2012) highlighting the huge unmet clinical need in this disease.

Current knowledge suggests that smoking, family history, diabetes and chronic pancreatitis are associated risk factors for the development of pancreatic adenocarcinoma (Elena et al., 2013). It is suggested that due to the links between chronic inflammation and neoplastic development, tumour growth and progression are likely to be influenced and contributed to by inflammatory cytokines and cells (Dafforn et al., 2004, Vermeulen et al., 2009). However, the incidence for acute and chronic pancreatitis is much higher and inflammation does not solely explain development of the disease.

Clinical presentation of pancreatic cancer can vary due to disease stage and tumour location (Huggett and Pereira, 2011). Patients with pancreatic cancer often present with common symptoms including jaundice, weight loss and epigastric pain. Blood tests can be used to determine the presence of tumour markers, such as carcino-embyronic antigen (CEA) or carbohydrate antigen 19.9 (CA19.9) (Greer and Brand, 2011, Ballehaninna and Chamberlain, 2013).

A computed tomography (CT) scan is often the first test to visualise the disease (Rustagi and Farrell, 2014). The CT provides an estimation of tumour size and spread. To complement this approach endoscopic ultra-sound is also utilised to guide fine needle aspirate (EUS-FNA) biopsies for

diagnostic cytology. Endoscopic retrograde cholangiopancreatography (ERCP) combines the use of an endoscope with fluorescent dye using x-ray to examine the biliary tract and pancreas (Dhir et al., 2014, Nikolaidis et al., 2014). EUS-FNA together with ERCP has the advantage of gaining diagnostic material and being able to therapeutically intervene at the same time, for example insertion of a stent (De Angelis et al., 2014).

Pancreatic cancer can be considered for surgical resection with curative intent if the tumour has not spread to distant metastatic sites or involved local structures such as coeliac axis or the superior mesenteric artery (Lall et al., 2007). Once the tumour has progressed to become locally advanced or metastatic, palliative chemotherapy will be considered. Current first line regimens for locally advanced or metastatic pancreatic cancer are either single agent gemcitabine or combination therapies such as with Paclitaxel (Von Hoff et al., 2013) or Abraxane (Wang et al., 2009). Alternatively, FOLFIRINOX, a multi-drug combination of leucovorin, fluorouracil, irinotecan and oxaliplatin (Labianca et al., 2012), offers an improved median survival compared with single agent gemcitabine, however the toxicity is comparably increased. Therefore FOLFIRINOX is reserved for patients with a better performance status (Conroy et al., 2011, Gunturu et al., 2013).

1.5.1 Pancreatic cancer development

Pancreatic cancer progresses through a series of lesions known as pancreatic intraepithelial neoplasia in situ (PanIN). Arising in the pancreatic ducts, PanINs are characterised by columnar to cuboidal cells with architectural atypia that advances over the development from panIN-1 to panIN-3. PanIN-3 stage is characterised by severe cellular atypia and can be considered 'carcinoma in-situ' at this stage (Scarlett et al., 2011). These PanIN lesions have been integrated into a model of tumour progression that links cytological changes with the genetic mutations (Zamboni et al., 2013, Saiki and Horii, 2014). Early PanIN lesion formation is associated with telomere shortening and KRAS activation and progression associated with subsequent intermediate mutations, as shown in the figure below adapted from (Bardeesy and DePinho, 2002).

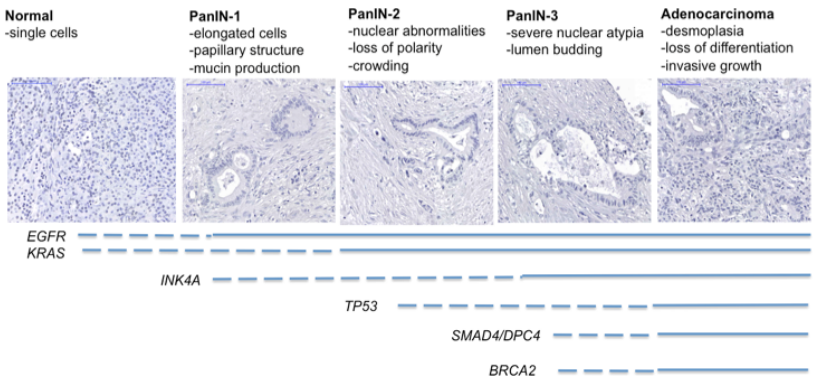

Figure 1.3 Pancreatic cancer progression through PanIN lesions identification morphologically and associated genetic mutations.

Images representative of PanIN lesion development from a healthy region in the normal pancreas. PanIN formation and development has been associated with gene expression mutations in the pancreatic cancer ductal cells. Images of human formalin fixed paraffin embedded tissue stained with haematoxylin. Figure adapted from (Bardeesy and DePinho, 2002).

The earliest identified oncogene in pancreatic cancer is KRAS, identified almost 25 years ago, in its mutated form $Kras^{G12D}$ (Smit et al., 1988). However, the presence of mutated KRAS alone is insufficient for progression, and further genetic alterations are required to drive the formation of PanIN lesions. Global genomic analyses recognise hundreds of differentially expressed genes in pancreatic cancer compared with healthy tissue (Jones et al., 2008, Biankin et al., 2012).

Genetic mutation acquisition studies are unveiling a vast heterogeneity of genes. A large scale Australian consortium, as part of the International Genome Consortium is leading the way in identifying gene signatures associated with mutation processes and development. Using whole exome sequencing they identified some novel and some confirmed significantly mutated genes such as: *KRAS*, *CDKN2A*, *TP53*, *BRCA2*, *SMAD4/DPCA* and *MLL3* (Biankin et al., 2012, Alexandrov et al., 2013).

1.5.2 The inflammatory tumour microenvironment in pancreatic cancer

Relatively recently, the components of the inflammatory tumour microenvironment have been acknowledged as major players in neoplastic promotion and maintenance (Hanahan and Weinberg, 2011). Infiltrating immune cells are an abundant component of solid tumours (Martinez et al., 2009, Qian and Pollard, 2010, Brower, 2012) and are reported to influence the growth of tumours, neoangiogenesis and resistance to therapy (Bronte et al., 2006, Denardo et al., 2011). The complex balance between pro- and anti-tumoral effects of immune cell infiltration is thought to create a chronic inflammatory microenvironment essential for tumour growth, progression and invasion (Hanahan and Weinberg, 2011, Balkwill and Mantovani, 2012, Candido and Hagemann, 2012). It is becoming increasingly important to be able to dissect these different cell types and examine their roles in the inflammatory microenvironment.

Oncogene activation causes activation of transcription factors such as Nuclear Factor kappa Beta (NFκB), Hypoxia Inducible Factor 1-alpha (HIF-1alpha) and STAT3 activation in tumour cells, which leads to production of inflammatory chemokines and cytokines. These signals recruit inflammatory immune cells to the tumour site (Mantovani et al., 2008, Balkwill and Mantovani, 2012). This creates a positive feedback promoting the development of cancer related inflammation and subsequent effects on invasion, metastasis, adaptive immunity and chemoresistance (Coussens and Werb, 2002, Allavena et al., 2011, Candido and Hagemann, 2012).
The subsequent recruitment of certain immune cells to the site of the tumour, has been shown to promote tumour growth, metastases and resistance to therapy (Nosho et al., 2010, Hanahan and Weinberg, 2011, Mahmoud et al., 2011, Tjomsland et al., 2011, Ruffell et al., 2012).

1.6 Peripheral blood monocytes in cancer progression

Monocytes have been implicated in a diverse range of responses to solid tumours. In several solid cancer types, the presence of cancer has been shown to affect monocyte phenotype and is thought to 'deactivate' their usual functions (Pardoll, 2003, Mytar et al., 2008). In ovarian and gastric cancer, cell surface expression of co-stimulatory factors such as PD-L1 ~~or PD-L2 were affected~~were increased (Brooks et al., 2012, Zheng et al., 2014) leading to ~~altered~~ reduced adaptive immune activation by affecting cytotoxic T cell priming and activation. Increased expression of IL-10 by monocytes in cancer has also been linked with promoting immunosuppression, alongside a concurrent reduction in IL-12, IFN-gamma and TNF-alpha production (Sica et al., 2006). Deactivation of monocytes in the tumour microenvironment has also been linked to tumour-derived hyaluronan (HA), an important component of the extracellular matrix. HA has the ability to ligate CD44 and negatively regulate monocyte adhesive abilities (Mytar et al., 2003).

Monocytes also play a role in angiogenesis. Depletion of monocytes in a murine model of glioma reduced tumour vascularity and therefore growth (De Palma et al., 2005). Tie-2 expressing monocytes are selectively recruited to spontaneous and orthotopic tumours and are reported to promote angiogenesis in a paracrine manner (Lewis et al., 2007). Evidence also exists for monocytes facilitating breast tumour primary pulmonary metastasis, due to the monocyte's ability to promote extravasation of tumour cells in a vascular endothelial growth factor (VEGF) dependent manner (Qian et al., 2011).

Monocytes in culture have been shown to increase the invasive capability of tumour cells in a tumour necrosis factor alpha (TNF-alpha) dependent manner (Baran *et al*., 2009). Since movement of tumour cells is crucial for invasion and the formation of metastasis, it was hypothesized that infiltration and subsequent production of TNF-alpha by monocytes may play a role in epithelial mesenchymal transition (EMT) dependent cancer progression.

1.6.1 Monocyte recruitment in cancer

Certain chemokines and growth factors have been found at high levels in tumours and can influence survival, differentiation and recruitment of monocytes to the tumour site. Elevation of ~~vascular endothelial growth factor (~~VEGF~~)~~ and RANTES (CCL-5) is associated with monocyte recruitment in breast cancer (Elbarghati *et al*., 2008).

Colony stimulating factor 1 (CSF1) and CCL2 (MCP-1) have been identified as major drivers in myeloid cell recruitment. Both can be produced by tumour cells, often as a result of oncogene activation (Mantovani et al., 2008). CSF1op/op mice deficient in the CSF1 gene show reduced neuroendocrine tumour development and decreased mammary metastases (Lin et al., 2002, Pyonteck et al., 2012).

CCL2 can be produced by most nucleated cell types, in response to a range of microbial products or activation by pro-inflammatory cytokines (Conductier et al., 2010). CCL2 in humans has been found at higher levels in serum of pancreatic cancer patients in comparison to healthy donors (Monti et al., 2003, Ancuta et al., 2006). There is evidence in mouse models that increased expression facilitates metastasis and increased monocyte infiltration, and therefore ~~tumour-associated macrophage (~~TAM~~)~~ recruitment in a murine mammary carcinoma model (Qian *et al*., 2011).

The translational relevance of monocyte population abundance in PDAC patients was revealed in study of a cohort of chemotherapy naïve surgically resected patients (Sanford et al., 2013). This study found that decreased presence of classical monocytes in the peripheral blood was associated with a better survival in patients. The patient group with higher monocytes counts had a higher incidence of lymph-node positive tumours. The ratio of bone marrow to blood monocytes was also decreased in the PDAC patients. It is hypothesised that the monocytes are mobilised from the storage reservoir in the bone marrow in response to recruitment signals present in PDAC

patients, in particular CCL2. This interaction has been identified previously in several pre-clinical models (Beatty et al., 2011, Denardo et al., 2011, Leuschner et al., 2011, Mitchem et al., 2012).

1.6.2 Monocytes and their precursors provide tumour-promoting cell types

The mononuclear phagocyte system has been implicated in several stages of tumour development, promotion and maintenance.

1.6.2.1 Myeloid derived suppressor cells

The early stages of myeloid cell development can be affected in chronic conditions like cancer or persisting inflammation. This can lead to the accumulation of stunted immature progenitor cells that have been suggested to have a myeloid bias (Wesolowski et al., 2013). Immature myeloid cells are continually generated in the bone marrow of healthy people. However, in some pathologies and cancer, this process is halted, and immature myeloid cells, also known as myeloid derived suppressor cells (MDSCs) are generated. These are a heterogenous population of cells and it is hypothesised that they are created to avoid an over-reaction by the immune system in cases of chronic inflammation. In humans MDSCs are characterised by positive expression of CD11b, CD15, CD45 and CD33 but they do not express MHC-Class II HLA-DR or CD14 (Bronte, 2009, Ostrand-Rosenberg, 2010).

In cancer the impact of MDSCs might be described in two stages; the first being abnormal myelopoeisis. A rapid accumulation of MDSCs in the blood in patients with pancreatic cancer (Marigo et al., 2010, Verschoor et al., 2013). MDSCs are recruited to the tumour microenvironment and the second stage is characterised by active cell-cell interactions and cytokine production. MDSCs infiltrating the PDAC tumour and are thought to promote tumour growth by production of reactive oxygen species and inducible nitric oxide synthase (Wormann et al., 2013). MDSCs have also been shown to suppress anti-tumour responses in particular effector T cell priming and activation (Gabrilovich et al., 2012, Steele et al., 2013). MDSCs isolated from PDAC patients express higher levels of inhibitory co-stimulatory molecule

Programmed Death Ligand 1 (PD-L1) and reducing expression of CTLA4, thereby causing a marked reduction T- cell proliferation contributing to suppression of anti-tumour host responses (Basso et al., 2013).

1.6.2.2 Macrophages

An inflammatory reaction can range from a limited leukocyte infiltrate to an intense desmoplasia. The stromal element often surrounding pancreatic cancer contains a significant proportion of macrophages recruited from classical monocytes that can act as a barrier for anti-tumoural immune cell infiltration (Kraman et al., 2010, Watt and Kocher, 2013, Feig et al., 2013) and contribute to cancerous growth by expression of cytokines and angiogenic growth factors (Korc, 2007, Matsuo et al., 2012, Poggi et al., 2014).

Macrophage infiltration in pancreatic cancer negatively influences survival and prognosis (Beatty et al., 2011, Mielgo and Schmid, 2013, Steele et al., 2013). Patients with $CD204^{+}$ infiltrating macrophages are reported to have a higher incidence of peritoneal metastases and recurrence (Sugimoto et al., 2014). Macrophages can promote chemoresistance by up-regulating production of cytidine deaminase, the enzyme that catabolizes gemcitabine (Amit and Gil, 2013). Inhibition of macrophage recruitment in pancreatic cancer reduces tumour volume, enhances responses to chemotherapy and decreases metastasis (Mitchem et al., 2012).

One of the challenges faced in evaluation of human tumour specimens is a lack of clear markers for mature macrophages. CD68, one of the most commonly used markers has shown to also be expressed on fibroblasts (Ruffell et al., 2012). It is therefore vital to further elucidate the phenotype of macrophage subsets in order to target the tumour promoting populations and avoid subsets with essential immune function.

1.6.2.3 Dendritic cells

Monocyte derived dendritic cells (MoDCs) play a pivotal role in initiation and regulation of tumour specific immune responses (Varol et al., 2007). The number of infiltrating MoDCs is reported to be associated with a better prognosis in breast cancer (Pinzon-Charry et al., 2006) and it hypothesized that their ability to effectively present antigens to cytotoxic T cells is beneficial.

In pancreatic cancer, literature focuses mainly on the role of DCs in immunotherapy. Pulsing with tumour antigens *ex-vivo* and infusing back into the patients has been shown to increase~~d~~ percentages of tumour infiltrating functional CD4 and CD8 cells thereby improving therapeutic effects (Tan et al., 2011, Lewinski et al., 2014).

1.7 Targeting monocytes in cancer

Current pre-clinical investigations into monocyte abrogation as an anti-cancer strategy have shown promising results in murine models of breast and pancreatic cancer (Denardo et al., 2011, Mitchem et al., 2012). Clodronate has been previously used in many tumour studies to dissect the impact of macrophages on tumour progression (Zeisberger et al., 2006). An elegant study by Ahn (Ahn et al., 2010) showed that using a neutralising antibody to CD11b inhibited disease recurrence in xenograft models, after animals received radiotherapy for tumour control.

The potentially simplest way to prevent tumour associated macrophage build up within solid tumours is targeting their recruitment. The recruitment of myeloid cells may not only be induced by the presence of the tumour but also by subsequent chemotherapy treatment. Administration of paclitaxel (PTX), a standard chemotherapy drug in patients with breast cancer, to mammary epithelial cells *in vitro* induced production of macrophage recruitment factors including CSF1 associated with TAM infiltration (Denardo et al., 2011).

Monocyte recruitment often depends on CCR2/CCL2 interactions and, accordingly, genetic deletion of either has been shown to reduce inflammation in a number of models (Abdi et al., 2004, Dewald et al., 2005, Lu and Kang, 2009). Extending these findings, Leuschner et al devised an elegant system interrupting this interaction, using lipid nanoparticles containing short interfering RNA against CCR2. The results showed efficient degradation of monocyte CCR2 mRNA and subsequently reduced TAM infiltration in an implanted lymphoma model (Leavy, 2011, Shantsila et al., 2011, Leuschner et al., 2011). In parallel, using a CCR2 inhibitor significantly reduc<u>ed</u>~~ing~~ TAM recruitment in a murine model of pancreatic cancer (Mitchem et al., 2012).

In a murine syngeneic orthotopic model of pancreatic cancer Mitchem *et al*., also used inhibitors against CCR2 (Pfizer's ~~(~~PF-04136309) and CSF1R (Plexxicon's PLX-3397~~, a bispecific inhibitor of c-FMS and c-KIT rTKs, and CSF1Ri PLS6134 containing GW2850~~) both individually and in combination. This model is relevant to human PDAC as it recapitulates the hepatic and peritoneal metastases successfully. Individually, monocyte recruitment was reduced within 4 days. Inhibition of CSF1R in combination with gemcitabine reduced peritoneal metastases and increased survival. CCR2 inhibition also normalised tumour vasculature and improved delivery of chemotherapeutic agents (Mitchem et al., 2012).

Compared with chemotherapy alone, Denardo showed, in a spontaneous murine mammary carcinoma model, that the Plexxicon (PLX-3397) inhibitor, ~~which targets CSFR1, cKit and PDGFR~~ improved chemosensitivity and overall survival. Early pre-clinical results suggest the compound reduced the volume of primary tumours and the number of circulating tumour cells and~~,~~ ~~primary tumours and~~ metastases (Denardo et al., 2011, Hume and MacDonald, 2012). These findings support the beneficial effects of combining current treatment regimes with agents that inhibit key TAM recruitment factors. Plexxicon currently investigates PLX-3397~~8~~ in early phase clinical studies in several solid tumour types including metastatic breast cancer (NCT01004861 and NCT01596751). The results from these studies are eagerly awaited.

The challenges faced in classification and understanding differentiation do not undermine the role of the monocytes. Strategically located in the peripheral blood to reach any destination, monocytes complement and provide dendritic cells and macrophage populations. The ability of monocytes and their descendants to read local cues and control the initiation and resolution of inflammation, play a vital role in cancer development and promotion. Manipulation of monocytes and their favourable plasticity has considerable potential for therapeutic intervention in inflammation and cancer.

As a first step, however, characterization of monocytes in pancreatic cancer is expected to yield information about how they may phenotypically differ from those in healthy donors. Comparison of monocytes with tumour-primed monocytes or macrophages may identify potentially useful peripheral biomarkers or therapeutic targets.

Chapter 2. Aims

The aims of this project wereare:

- To investigate whether there are differences in distribution and gene expression and metabolic profiles between classical monocytes from patients with pancreatic ductal adenocarcinoma compared with healthy volunteers

- To identify and examine whether these are suitable as potential therapeutic strategies in targeting monocytes in PDACTo validate identified targets in a larger cohort and publicly available gene expression databases

Chapter 3. Materials and Methods

3.1 Blood sample preparation and monocyte isolation

3.1.1 Patient clinical characteristics

This study was performed with ethical approval (REC05/Q0408/65) and written informed consent was obtained from all patients (n=28). Patients were assigned unique identifiers for the purposes of anonymisation.

Patients were selected that had a confirmed diagnosis of unresectable locally advanced or metastatic stage III-IV pancreatic ductal adenocarcinoma. Patients with stage III show cancer spread into the local major blood vessels, such as the superior mesenteric artery, the portal vein, the celiac axis or the common hepatic artery. The cancer may also have spread into the local lymph nodes. Patients with stage IV have a pancreatic tumour of any size that has spread to distant organs, such as the liver or lungs or invaded into the peritoneal cavity. Patients were excluded retrospectively if they were subsequently found to have an alternative diagnosis. Blood was taken before the first cycle of the chosen chemotherapy regime, so all included patients were previously chemotherapy untreated. Healthy volunteers were selected with no major health concerns (n=28). Healthy volunteers for gene expression analysis were age matched to be 60 years (+/-10 years).

3.1.2 Sample handling and storage

3.1.3 Plasma

Blood was collected in anti-coagulant treated EDTA vacutainer tubes (*Beckon Dickinson Cat #K2E*), which prevent clotting. These were preferential over heparinized tubes due to the potential contamination by endotoxins, which may stimulate cytokine release in white blood cells.

Whole blood in vacutainer tubes was transferred to 50ml falcon tubes and centrifuged at 1600rpm (440xg) for 6 minutes at 4°C. All subsequent centrifugation steps were also at 4°C and 10,000rpm. The plasma supernatant was collected and aliquot into 1.5ml Eppendorf tubes. The plasma was centrifuged for 5 minutes and supernatant was transferred to a new Eppendorf, avoiding the cell pellet. The plasma supernatant was centrifuged again for 3 minutes twice more and the supernatant removed each time, to be aliquot for storage at -80°C avoiding freeze thaw cycles.

3.2 Monocyte isolation

3.2.1 Ficoll isolation peripheral blood mononuclear cells (PBMCs)

Ficoll Hypaque (*GE Healthcare Cat #17-1440*) solution was used to isolate PBMCs. This solution is designed to produce a density gradient for cell type separation from whole blood during centrifugation. This technique is simple, has been widely used for the last 30 years and results in a high yield and good purity population of mononuclear cells.

Fresh blood was diluted with room temperature PBS. 25ml of the diluted blood was slowly pipetted at a regular speed on top of 15ml of Ficoll Hypaque solution in a 50ml falcon tube without perturbing the blood-Ficoll interface. The sample tubes were centrifuged at 2600 rpm at 19°C for 30 minutes without hard braking.

After removing the supernatant, the layer containing PBMC was isolated by using a pipette to slowly and gently collect the cells, which were resuspended in cold PBS up to a volume of 50ml. The PBMCs were centrifuged at 1600rpm for 10 minutes at 4°C to remove any remaining Ficoll solution, the supernatant was discarded and PBMCs resuspended in 50ml PBS for centrifugation at 1000rpm for 10 minutes. The pellet was resuspended in monocyte culture medium RPMI-1640 (*PAA Cat #E15840*) supplemented with 0.01% β-mercaptoethanol (*Invitrogen Cat #31350*), 10% fetal bovine serum (FBS) (*Gibco Cat #16500*), 100u/ml penicillin and 100µg/ml streptomycin (*PAA Cat #P11010*) at 37C in 5% CO_2 or FACS buffer (PBS with 0.05% BSA (*Sigma Aldrich Cat #A4503*) and 2mM EDTA (*Life Technologies Cat #AM9262*)).

3.2.2 $CD14^+$ magnetic bead isolation

Monocytes were isolated using anti-CD14 antibody labelled with magnetic MicroBeads (*Miltenyi Biotec Cat #130-050-201*) for positive selection. The principle is based on cell isolation from a mixed population using specific binding of antibody bound magnetic particles to a particular cell surface antigen. In this experiment, the PBMCs were incubated with anti-CD14 conjugated beads and passed through a column placed in a magnetic stand. Positively labelled $CD14^+$ cells were retained in the column as the unlabelled fraction was washed out. The $CD14^+$ monocytes were then eluted from the column by removing it from the magnetic stand and flushing through with buffer. The purity of the resulting population was checked using flow cytometry.

This technique is simple and rapid and results in cell suspensions with a good viability (>95%) and purity (>96%). The magnetic particles are small and non-toxic so they do not saturate or activate epitopes.

After Ficoll separation, PBMCs were counted and resuspended in the appropriate amount of assay buffer (PBS pH 7.2, containing 0.5% BSA and 2mM EDTA at 80µl /1x10^7 cells according to manufacturers instructions). PBMCs were incubated at 4°C for 15 minutes in the presence of the CD14 MicroBeads. PBMCs were washed with assay buffer and centrifuged for 10 minutes at 1500rpm. 1000µl of assay buffer was added to the MS column (*Miltenyi Biotec Cat #130-042-201*) connected to the magnetic stand.

The cell suspension was added to the column and unlabelled cells were collected as flow through into a 15ml falcon tube on ice below. The column was washed 3 times with 500µl assay buffer to remove any unbound cells. The remaining $CD14^+$ bound fraction was flushed out of the column by adding 1000µl assay buffer then forcing the cells from the column using the provided syringe plunger. The number of $CD14^+$ monocytes was counted and the purity checked by measurement of CD14 expression by flow cytometry (Fig 5.1).

3.2.3 Fluorescence activated cell sorting (FACS)

Flow cytometry is commonly used for immunophenotyping of cells in the peripheral blood as it has the advantage of being able to rapidly measure several cellular characteristics using light emitted from specific fluorescent-conjugated antibodies bound to cells and cellular parameters of size and granularity using light scattering.

When the labelled cells pass the light source, the antibody-conjugated fluorochomes are excited to a higher energy state. As they return to resting state, the fluorochromes emit energy as light at a higher wavelength, which is detected by photomultiplier tubes. Using multiple antibody-conjugated fluorochromes that are excited by a similar wavelength, but with different emission wavelengths, allows detection of several cellular markers in the same sample.

Using the Aria II, a combination of up to 12 colours can be used, allowing a high number of antibody variations to be tested. This flow cytometer can also be used to electrostatically sort cell populations as they form droplets in pressurized sheath fluid as they break away from the solid stream immediately after the moment of analysis. The principle is based on applying to each droplet a positive or negative charge. As the droplets pass between two charged plates they are attracted to the plate of opposite polarity and deflected into separate collection tubes. The Aria can collect up to 4 different cell population collection tubes.

FACS is rapid and can be carried out in a closed aseptic environment to allow sorted cells to be kept sterile and used in culture. The purity and viability are high and the yields consistent. The use of CD14 and CD16 to define monocytes is well established and supported in the literature to define the three sub-populations (Cros et al., 2010, Ziegler-Heitbrock et al., 2010, Heimbeck et al., 2010). This gating strategy was chosen at the start of this

project, as defined by Heimbeck *et al.*, in 2010, to be a simple standardized method for analysis.

Samples used in this investigation were collected fresh on the same day to avoid an overnight incubation. Comparison studies by Affymetrix show that overnight storage of blood significantly alters the transcriptome compared to fresh PBMCs (according to the Affymetrix technical data sheet).

Prior to FACS, the blood was treated with a red blood cell lysis buffer. This reduces the time spent by sorting to remove unwanted cells from the sort. The treatment with RBC lysis buffer has not been shown to detrimentally affect monocyte viability or gene expression (Mallone et al., 2011). This is also preferable to Ficoll treatment, which has also been shown to affect gene expression (Beyan et al., 2010, Martinez, 2012).

FACS was chosen to isolate monocyte subsets for gene expression analysis. FACS was shown to have the highest purity and viability (>96% and >91% respectively) compared with positive or negative selection using magnetic beads. This is of extra importance when evaluating gene expression, as the presence of other leukocytes can affect the relative gene expression, introducing bias.

To isolate monocytes using flow cytometry, peripheral venous blood samples (15-20ml) were taken from patients or age-matched healthy volunteers and processed within 24 hours. Blood was transferred to 50ml falcon tubes and centrifuged at 1500 rpm for 3 minutes to remove plasma, which was then stored at -80°C (section 3.1.3). Erythrocytes were lysed using red blood cell lysis buffer (*Beckton Dickinson Cat #555899*). This buffer contains NH_4Cl, and selectively affects the erythrocytes to take up excess water due to osmotic pressure, until the cell membrane becomes stretched to capacity and bursts. The whole blood was treated for 5 minutes in this buffer, centrifuged and the remaining cell pellet washed with 50ml PBS. The resulting cell suspension, containing mononuclear and lymphocytic cells, granulocytes and platelets, was centrifuged (5 minutes at 1600rpm/461xg)

and re-suspended in ~~fluorescence activated cell sorting (~~FACS~~)~~ buffer (PBS with 0.05% BSA (*Sigma Aldrich Cat #A4503*) and 2mM EDTA (*Life Technologies Cat #AM9262*). Cells were treated with Fc receptor blocking agent (*eBioscience Cat #16-0161-86)* 1:100 in FACS buffer for 10 minutes at 4°C.

Antibodies were diluted as shown below in Table 3.1 and incubated with cells at 4°C for 30 minutes before centrifugation (5 minutes at 1600rpm/461xg) to wash the cells and re-suspension in flow cytometric buffer for acquisition.

Antibody *(Cat#)*	**Fluorochrome**	**Dilution**	**Isotype Control**	**Expressed on**
CD14 *(558121)*	Pacific Blue	1:50	Mouse IgG2a, κ	Monocytes Interfollicular macrophages, resident dendritic cells
CD16 *(557744)*	PeCy7	1:100	Mouse IgG1 κ	Natural Killer Cells Monocytes Macrophages Granulocytes
HLA-DR Class II *(560896)*	FITC	1:100	Mouse IgG2b κ	B cells, activated T cealls and antigen presenting cells.
CD19 *(555413)*	PE	1:25	Mouse IgG1 κ	Expressed at all stages of B cell differentiation
CD15 *(555402)*	PE	1:50	Mouse IgM κ	Granulocytes.
CD56 *(555516)*	PE	1:50	Mouse IgG1 κ	Expressed on large granular lymphocytes and natural killer cells.
NKp46 *(557991)*	PE	1:50	Mouse IgG1 κ	Resting and activated natural killer cells.
CD2 *(555327)*	PE	1:50	Mouse IgG1 κ	T cells NK cells
CCR2 *(558406)*	Alexa 647	1:40	Mouse IgG2b κ	Classical monocytes

Table 3.1 Multi-colour flow cytometry panel of antibodies used to sort monocytes from peripheral blood.

All antibodies were ordered from Beckton Dickinson. The table shows the catalogue number, dilution, appropriate Isotype and expression on cell surface.

The gating strategy used to isolate Monocyte populations using FACS can be seen in Figure 4.1. Flow cytometric acquisition was performed using FACS Aria II and the same operator carried out all sorting. Analysis was carried out using FlowJo 8.8.6 software. Sorted monocyte populations were collected into FACS buffer and centrifuged for 20 minutes at 13,000rpm

(15682xg). Cell pellets were resuspended in 350µl of RLT buffer (*Qiagen micro kit component Cat #74004*) for storage at -80°C.

Monocyte population purity was verified using flow cytometry. Purity checks are carried out by re-acquiring 1000 cells to check expression on every sorted cell population and purity is expected to be above 95% (as shown in Figure 5.1).

3.3 Gene expression analysis

3.3.1 RNA isolation

An on-column solid phase RNA isolation kit using centrifugation and filter columns from the Qiagen RNeasy Micro kit (*Qiagen Cat #74004)* was used in this project. After lysing samples in the presence of guanidine salts, samples were passed through the filter using centrifugation and nucleic acids bind due to low pH and high salt concentration. At this stage, an on-column DNase treatment can be carried out to remove genomic DNA. The column was washed with ethanol to dry the membrane, and RNA eluted using water. This method is very quick and convenient, however must be optimised to prevent over-loading due to the fixed binding capacity and to prevent filters clogging with particulate material.

RNA was isolated from monocytes according to manufacturer's instructions. 350µl of fresh 70% ethanol was added to the lysed cells in RLT solution and mixed well by pipetting. The sample mix was transferred to an RNeasy MinElute spin column and placed in a 2ml collection tubes to be centrifuged for 15 seconds at 10,000rpm (9279xg). The flow-through was discarded and 350µl of RW1 buffer was added to the RNeasy MinElute column to desalt the filter prior to centrifuging again at 13,000rpm (15682xg) for 15 seconds. An additional on-column DNase I treatment step was included to eliminate potential genomic DNA contamination. The spin column was washed and dried with 80% ethanol, placed in a new 1.5ml labelled collection tube, and 14µl of RNase-free water added directly to the centre of the membrane. The column was left to sit for 1 minute before centrifuging at 13,000rpm (15682xg) for 1 minute to collect the RNA eluate.

RNA concentration was measured using a Nanodrop Spectrometer (*Thermo Scientific Cat #ND1000*). The ratio between the absorbance at 260 and 280nm gives an indication about the purity of the nucleic acids. This ratio should be around 2 and if lower means there is protein contamination of the sample.

The RNA integrity was assessed with Agilent RNA 6000 Pico Kit (*Agilent Technologies Cat #5067-1513*) using the Agilent Bioanalyser 2100 (*Agilent Technologies*) as per manufacturer's instruction. The RNA integrity number (RIN) is calculated by the software to assign an integrity value to evaluate the degree of fragmentation. RNA is thermodynamically stable, however it is readily fragmented by the presence of RNase. Using electrophoretic separation, these shorter fragments can be detected by the software as signal between the 5S and 18S bands.

Due to the limited RNA quantities isolated from monocytes from individual donors, the Nugen Ovation whole transcriptome amplification kit (*Cat No #3302* Version 2) was used to increase cDNA yield suitable for hybridization on Affymetrix GeneChip as indicated in section 3.3.5.

3.3.2 Quantitative polymerase chain reaction (qPCR)

3.3.3 cDNA synthesis

Complementary DNA is a DNA copy synthesised from mRNA. cDNA is easier to work with in PCR reactions, as RNA ~~is~~ has a short ~~half-life~~half-life and is very easily degraded.

An in house cDNA synthesis protocol was used to generate cDNA. The reaction master mix was created using first strand buffer (1X *Life Technologies Cat* # 18067017), dithiothreitol (10mM *Life Technologies Cat* #D1532), deoxynucleotide triphosphates (dNTPs) (0.5mM *Promega Cat #U1240*), random hexamers (12.5ng/µl *Promega Cat #C1181*), oligo dT 15mers (6.25ng/µl *Promega Cat #C1101*) RNasin (1U/µl *Promega Cat #N2111*) and SuperScript (5U/µl *Invitrogen Cat #18064-014*). Required concentrations were given per sample reaction. 8.75µl of master mix was distributed per sample.

RNA concentrations were normalised across samples and the appropriate volume was added to the master mix to ensure equivalent concentration in each sample. Distilled RNase free water was added to make up the volume to 20µl per sample reaction. Reactions were run in a thermal cycler for 10 minutes at 25°C, 60 minutes at 42°C, 5 minutes at 90°C and cooled to 4°C. cDNA product was stored at -20°C until further use.

3.3.4 qPCR

~~Quantitative Polymerase chain reaction~~ qPCR was~~is~~ used to determine the relative amounts of targeted genes by primer-driven amplification and subsequent quantification. The relative gene expression ~~is~~ was calculated by the threshold value determined by the number of cycles required to reach a defined DNA concentration in real time.

For each qPCR reaction, diluted cDNA corresponding to 5ng of total RNA was made up to 9µl using RNase free water. 10µl of 2X qPCR mix (*Life Technologies Cat #4369016*) and 1µl of Fam labelled target gene primer (Table 3.2) were added to each sample to create a final volume of 20µl.

Reference:	Symbol	Name	Amplicon	Design
Hs00327243_m1	RASGEF1B	RasGEF domain family, member 1B	67	E4-E5
Hs00166165_m1	EGR2	Early growth response 2	66	E1-E2
Hs00231780_m1	EGR3	Early growth response 3	91	E1-E2
Hs00188486_m1	CD83	CD83 molecule	104	E3-E4
Hs00198935_m1	MARCO	Macrophage receptor with collagenous structure	122	E4-E5
Hs01124179_g1	FOLR1	Folate receptor 1 (adult)	116	E4-E5
Hs03044361_m1	CYBA	Cytochrome b-245, alpha polypeptide	159	E4-E5

Table 3.2 Primers for gene expression validation

Primers were purchased from Applied Biosystems. The table shows the reference, gene symbol, gene name, amplicon and design.

3.3.5 Transcriptome amplification

The Ovation Pico Whole Transcriptome Amplification kit (Nugen *Cat #3302*) was used to create cDNA for single primer isothermal amplification (SPIA). This type of amplification uses sensitive strand displacement methods to amplify the whole genome. This type of robust amplification can be carried out on samples with limited quantity RNA.

Single stranded cDNA was generated using 50ng total input RNA using a primer mix containing a unique mixture of random and oligo dT primers across the whole transcript. The amplification step uses RNaseH to remove the RNA portion of the SPIA tag sequence, which reveals the binding site for the primer. DNA polymerase synthesizes cDNA in the 5' direction, which displaces the existing strand and this process was repeated thus creating double stranded cDNA.

The cDNA was purified using Agencourt RNAClean XP beads (*Beckman Coulter Cat #A63987*) and subsequent product was purified again for hybridisation using Qiaquick PCR purification (*Qiagen Cat #28104*) protocol, as recommended by Nugen. The purified, amplified cDNA product can be stored at -20°C at this stage.

3.3.6 Gene Chip hybridisation

Fragmentation of the created cDNA and biotin labelling was carried out using labelling master mix from the Encore Biotin Labelling module kit (*Nugen Cat #4200*).

Array hybridisation was carried out using the Affymetrix Hybridisation Wash and Stain kit (*Affymetrix Cat #900720*). Affymetrix Human U133 ~~GeneChips~~Gene Chips 2.0 plus were run at Barts Cancer Institute platform, with support from Tracy Chaplin-Perkins.

Affymetrix Gene Chips are designed to measure the expression of particular genomic sequences using probes. Probes matching the target mRNA sequence exactly consist of hundred of short oligonucleotide strands (25-mer). Once the RNA samples have been transformed and amplified into complementary DNA, it is fragmented and labelled with biotin, and these fragments are washed over the gene chip during hybridisation. These fragments bind to their specific complementary oligonucleotide sequences on the probes. A fluorescent dye is then washed over the chip, which binds to the biotin labels creating a 'map' of probe specific fluorescence intensity data, which can be used to infer ~~to~~ the expression levels of the relative abundance of specific mRNA sequences in the transcriptome.

3.3.7 Data Normalisation

Scanned images and raw data were normalised using the GeneChip Robust Multi-Array average (GC-RMA) algorithm implemented in the Partek 'Genomics Suite' analysis software (*Partek Incorporated*). The result was a spreadsheet with 54,000 lines (probe sets) and 8 columns. The quantitative expression values are shown in log base 2 format.

Gene Chip normalization was required to compare the relative levels of mRNA between samples. Robust Multi-array Average (RMA) is a normalisation procedure that corrects the background level of fluorescence intensity and normalises the probe level information. However, it does not take into account an inbuilt control on these gene chips designed to measure non-specific binding. Affymetrix gene chips contain between 11-20 probes at different locations across the gene chip, known as a probe set. Each probe is designed to have a matched probe known as a 'mismatch probe' (MM probe) these differ to their paired 'perfect match probe' (PM probe) by one nucleotide at position 13 which should be incorrect for the target sequence. This allows the signal produced by the mismatch probe to be subtracted from the perfect match probe to define the true signal and therefore takes into account background noise produced by non-specific binding.

RMA normalisation alone does not take the fluorescence intensity of the mis-match probes into account. Therefore GeneChip RMA (GC-RMA) is used to account for the information provided by the mismatch probes for more accurate gene expression values.

3.3.8 Data analysis

Data analysis was carried out using R labs at Barts Cancer Institute with support from Dr Raphael Zollinger.

An independent filtering step was performed to remove probe sets below detection level (Affymetrix $\log_2$ fluorescence intensity 3.5) in all samples. Genes that were not considered to be differentially expressed have a lower than 2-fold change in mean expression between PDAC and healthy, were considered irrelevant and removed. A gene was classified as differentially expressed if the fold change between healthy and cancer was greater than or equal to 2 and the p-value of the t-test lower than or equal to 0.05.

3.4 *In vitro* cell culture

3.4.1 Monocyte culture conditions

Monocytes isolated from peripheral blood were cultured immediately in monocyte culture medium RPMI-1640 medium (*PAA Cat #E15840*) supplemented with 0.01% β-mercaptoethanol (*Invitrogen Cat #31350*), 10% fetal bovine serum (FBS) (*Gibco Cat #16500*), 100u/ml penicillin and 100µg/ml streptomycin (*PAA Cat #P11010*) at a concentration of 1x10^6/ml. Monocytes were cultured at 37°C in 5% CO_2.

3.4.2 Monocyte stimulation conditions

Monocytes in culture were plated at 1x10^6/ml. For whole cell lysates monocytes were plated in 1ml in a 6 well plate (9.5m^2 growth area) and, for gene expression analysis, monocytes were plated in 200µl of monocyte culture medium.

~~3.4.3 M-CSF and MEK inhibitor U0126~~

Fresh Monocytes were plated and stimulated with human recombinant M-CSF *(*100ng/ml *Peprotech Cat #300-25)* and treated with or without the presence of a MEK-1,-2 specific inhibitor (10µM *Sigma Aldrich Cat #U012).* Stimuli were added simultaneously and incubated 37°C in 5% CO_2.

3.5 Immunoassays

3.5.1 Mesoscale discovery

Mesoscale Discovery Human Pro-Inflammatory 9-Plex Ultrasensitive Kit (*Mesoscale Discovery Multi-spot assay system Cat #K15007C2*) was used to determine cytokines in human plasma.

Mesoscale discovery (MSD) multiplex arrays were used to profile these samples instead of traditional enzyme linked immunosorbent assay techniques (ELISA). The volume loaded into each well is reduced compared with ELISA as less sample is required to coat the bottom of the well, the sensitivity is much higher and multiple cytokines can be measured in the same sample.

Plasma was prepared as above in section 3.1.3 and used straight from storage in -80°C avoiding freeze thawing.

IL-2	IL-8	IL-12p70	IL-1β	TNF-α
IFN-ϒ	IL-6	IL-6	GM-CSF	

Table 3.3 Pro-inflammatory cytokines multiplex analysis Mesoscale discovery plate.

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MSD is a single-plex assay that allows the detection of multiple protein targets using specific spatially defined electrodes within a well. The Figure 3.1 below indicates these principles.

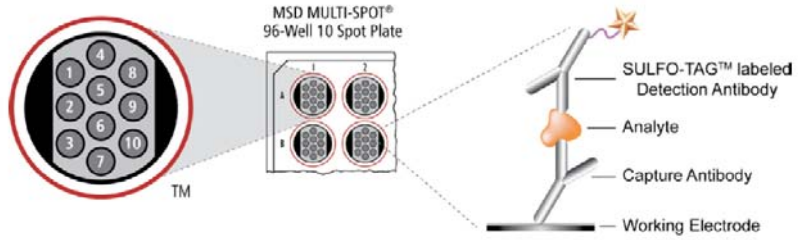

Figure 3.1 Principles behind the MSD assay

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Samples are added as well as specific labelled detection antibodies. Sample analytes bind to capture antibody electrode spots immobilised on the bottom of the well and labelled detection antibodies are recruited and bind to form a sandwich. The MSD buffer then provides the appropriate environment for electrochemiluminescence and the MSD plate is read inside an MSD Sector plate reading instrument.

All reagents were brought to room temperature and calibrator solution was thawed on ice. Prior to the assay, the following solutions and standards were prepared. The diluted stock calibrator solution was prepared using 10µl of Pro-inflammatory 9-plex-calibrator blend in 990µl of Diluent 2. The highest calibrator point as shown below (STD-01) was prepared by adding 50µl of the previously diluted stock calibrator to 150µl of diluent 2 and subsequent 4-fold serial dilutions were created to cover a range from 2500pg/ml to 0.61pg/ml. Detection antibody solution was diluted 50X to create a 1X solution known as Diluent 3. Read buffer was diluted 2-fold in deionized water.

Duplicate calibrator samples were run to create a standard curve with a wide dynamic range. The standard curve model uses the least squares fitting algorithm to calculate the concentration of analyte in the sample.

Standard	Calibrator blend concentration [pg/ml]
Stock	1000000
Diluted Stock	10000
1	2500
2	625
3	156
4	39
5	9.8
6	2.4
7	0.61
8	0

Table 3.4 Standard dilutions required to determine cytokine concentrations using MSD multiplex kit.

The assay protocol was followed as per the manufacturer's instruction at room temperature and 25µl of Diluent 2 was added to each well, the plate sealed and incubated for 30 minutes with vigorous shaking (300-1000rpm). 25µl of undiluted plasma or calibrator solution was added into separate wells of the MSD plate, which was sealed and incubated for 2 hours with vigorous shaking. The plate was washed 3 times using PBS-T (PBS-Tween) before addition of 25µl of 1X Diluent 3 detection antibody to each well. The plate was sealed and incubated for 2 hours with vigorous shaking. The plate was washed 3 times using PBS-T and 150µl of read buffer added to each well before the plate was read immediately using the Sector Imager (*Mesoscale Discovery*).

3.5.2 Tissue immunofluorescence

The principles behind immunofluorescent staining are similar to immunohistochemistry. The staining procedure used in this investigation was indirect as secondary antibodies are conjugated to fluorochromes. These are excited by laser and emit light at different wavelengths, which can be measured using a confocal microscope. This provides the advantage of measuring multiple markers simultaneously due to differing emission wavelengths enabling co-localisation of specific antigens. Counterstaining can also be used to identify cell nucleus using nuclear stain such as DAPI.

Human pancreas samples were fixed in formalin and embedded into paraffin at 5μm thick. Slides were deparaffinised using Xylene (*Fisher Scientific Cat #H/1800/15*) and dehydrated through ethanol (*Sigma Aldrich Cat #E7023*).

Xylene	2 x 5 minutes
100% ethanol	2 x 5 minutes
95% ethanol	2 x 2 minutes
70% ethanol	2 x 2 minutes
50% ethanol	1 x 2 minutes
Distilled water	2 x 2 minutes

Slides were immersed in 1X antigen unmasking solution (*Vector Cat #H-3300*) in distilled water and placed in a slide container with a loose fitting lid. This chamber was transferred into a large beaker containing water to jacket the slide container. The slides were heated in a microwave for 9 minutes at full power, before the slide container was removed and allowed to cool for 15 minutes at room temperature. The slides were washed three times in PBS and a hydrophilic marker pen (*Vector Cat #H4000*) was used to mark a barrier around the sections. Sections were permeablised using 0.1% Triton-X100 (*Sigma Aldrich Cat #X100*) in PBS for 5 minutes at room temperature

before being washed in PBS. Sections were quenched using 50nM ammonium chloride (NH_4Cl) (*Sigma Aldrich CAT #A4934*) in PBS for 15 minutes at room temperature.

After quenching with NH_4Cl as above, wash slides in PBS for three minutes and block for 30 minutes at room temperature in 2% BSA in PBS. Aspirate the blocking buffer and add the uncoupled primary antibody solution.

Antibody	Cat #	Concentration	Isotype
CD68	Abcam AB63896	1:80	Rabbit IgG
EGR2	Thermo Scientific PA1-46019	1:2500	Rabbit IgG
EGR3	Cell Signalling 2559	1:750	Rabbit IgG

Table 3.5 Primary and secondary antibodies used for immunohistochemical analysis in pancreatic tissue.

Primary antibody solution was aspirated and the slides washed three times in PBS-T before a 1-hour incubation at room temperature with fluorescence labelled secondary antibody. The antibody solution was aspirated and the slides washed 3 times for 5 minutes and once in water before dehydration in 100% isobutanol. The slides were then mounting in DPX containing DAPI (*Life Technologies Cat #P-36931).*

Chapter 4. Monocytes, subset distribution and pro-inflammatory cytokine profiling in the peripheral blood.

4.1 Introduction

The peripheral blood is an easily accessible non-invasive source for analysis of biomarkers of disease such as cells or molecules. Blood is taken routinely from patients with PDAC, assessing a number of parameters to evaluate patient suitability for systemic treatment and well being.

Clinical blood test samples are subjected to automatic analysis using a flow cytometer. Monocytes make up 1-9% of blood leukocytes (Hubl *et al*., 1995). The monocyte concentration in the blood can be used as a helpful indicator of various inflammatory conditions or infection (Brew *et al*., 2004, Sasaki *et al*., 2006). A marked increase of monocytes in the peripheral blood is known as monocytosis. Monocytosis (>800 monocytes per microliter) has been shown to be associated with cardiovascular disease, stroke and sepsis as well as long standing chronic inflammatory conditions like rheumatoid arthritis and kidney disease (Maekawa *et al*., 2002, Waterhouse *et al*., 2008, Heine *et al*., 2008, Rogacev *et al*., 2011). A marked reduction in monocyte numbers is known as monocytopenia, which can point towards an immunodeficiency (Calvo *et al*., 2011).

Acute monocytic leukaemia is a progressive malignant disease, where proliferation and production of monocytes is distorted and increased in the bone marrow, leading to more monocytes in the blood (Moore et al., 2013). Myelomonocytic leukaemia is a chronic malignant condition of the myelocytes and monocytes and also contributes to a higher number of circulating monocytes (Koike and Matsuda, 2013, Lachenaud et al., 2014).

Pancreatic cancer is associated with significant inflammation (Steele *et al*., 2013). It is known that monocyte counts, levels of CCL2 (monocyte chemoattractant protein 1) and CRP levels are correlated in patients with acute pancreatitis (Rahman *et al*., 2004, Regner *et al*., 2008, Fu *et al*., 2012) and pancreatic cancer (Mitchem *et al*., 2012). Neoplastic tissue damage through hypoxia or necrosis combined with oncogene activation and pro-

inflammatory cytokine signalling recruits monocytes to the tumour microenvironment. Once exposed to the tumour microenvironment they contribute to ~~tumour-associated macrophage~~TAM infiltration and promote PDAC development as discussed previously. A recent study showed that an increase in the number of classical monocytes in the peripheral blood of pancreatic cancer patients is thought to be due to mobilisation of monocytes from the bone marrow in response to increased myeloid recruitment factor CCL2 signalling (Sanford *et al*., 2013).

The identification of three distinct subsets of monocyte in the peripheral blood and the use of multi-colour flow cytometry allows the distribution to be examined. Extracellular expression of CD14 and CD16 are measured to differentiate between subsets. This study aims to investigate whether the presence of PDAC and its associated inflammatory response affects monocyte distribution in the peripheral blood of patients compared with healthy.

4.2 Aims:

The aims of this chapter are to:

- Examine absolute monocyte counts relative to the clinical characteristics of PDAC patients compared to normal levels
- Identify whether pro-inflammatory cytokines in the plasma are affected by the presence of PDAC compared with healthy volunteers
- Define and compare monocyte subset distribution in the peripheral blood of PDAC patients compared with healthy donors

4.3 Results

4.3.1 Clinical Characteristics

The numbers of healthy volunteers and patients with PDAC included in this project are summarised below in Table 4.1. ~~.~~

Total numbers	Healthy	PDAC
~~Healthy donors~~	n=28	n=28
Age	**Mean**	**SD**
Healthy volunteers	61.8	3.6
PDAC patients	65.7	7.9
Sex	**F**	**M**
Healthy volunteers	15	23
PDAC patients	12	19
Diabetes	**Y**	**N**
Healthy volunteers	0	28
PDAC patients	6	11
Smokers	**Y**	**N**
Healthy volunteers	1	27
PDAC patients	12	5

Table 4.1 Healthy volunteer and patient sample numbers and characteristics

Results are expressed as the number of individuals in each category. The age matched healthy volunteers are represented for gene expression analysis only (n=11). Healthy volunteers for flow cytometry age range from 22-65. PDAC patients with unknown diabetes status (n=11~~0~~) unknown smoking status (n=11~~0~~).

The patient sample cohort represents similar sample size. The PDAC patient cohort however consisted of more smokers and patients with diabetes compared with the healthy volunteer group.

Blood test results were taken when each patient had their initial consultation after diagnosis, at the same time samples were taken for this project. Blood was taken in advance of any chemotherapy treatment so all included patients were previously untreated. The results are shown in Table 4.2

	Normal	PDAC
CA19.9 (units/mL)	0-35	1510.12 ± 1831.86
Bilirubin (μmolM/L)	1-21	18.85 ± 20.6
Alkaline phosphatase (x10^9/L)	30-300	251.52 ± 217.68
Monocyte count (x10^9/L)	0.3-0.9	0.85 ± 0.73
WBC count (x10^9/L)	4-11	9.46 ± 6.36

Table 4.2 Differential blood count test results comparing PDAC patients with healthy range

Differential blood counts for CA19.9, Bilirubin, alkaline phosphatase, monocyte counts and white blood cell counts. Normal ranges as used at Barts Hospital. PDAC patients (n=28) values expressed as mean ± SD.

The range of CA19.9 in the blood of the PDAC patients was increased compared with the normal range and the patient values were spread over a large range (from 30 – 5678, mean ± SD 1510.12 ± 1831.86). The normal range for bilirubin presence in the blood is 1-21 μM/L in the blood and mean value in the PDAC patients was at the higher end of the normal range (mean ± SD, 18.85mg/dL ± 20.6). The alkaline phosphatase mean expression level in the PDAC patients was also towards the top end of the normal range (mean ± SD 251.52 ± 217.68).

Monocyte counts were within the normal range but towards the higher end (mean ± SD 0.85 ±0.73) as were white blood cell counts (mean ± SD 9.46 ± 6.36).

Each parameter was plotted against another to determine if any correlation between them could be identified. The table below shows the r-squared values as determined by this analysis.

X-axis	Y-axis	r-squared	p-value
Alkaline phosphatase	White blood cell count	0.2621	0.025
Alkaline phosphatase	Monocyte count	0.07351	0.2765
Monocyte count	White blood cell count	0.1097	0.3197
CA19.9	Alkaline phosphatase	0.04382	0.3897
CA19.9	White blood cell count	0.01823	0.5815
Bilirubin	White blood cell count	0.01823	0.5815
CA19.9	Monocyte count	0.006079	0.751
Bilirubin	Monocyte count	0.006079	0.751
Bilirubin	Alkaline phosphatase	0.004438	0.7928
CA19.9	Bilirubin	0.0007397	0.912

Table 4.3 Clinical parameter goodness of fit in PDAC patients

Table expressing r-squared correlation co-efficient values as determined using GraphPad Prism. *P-values <0.05* considered significant. Correlations ranked using *p-value.*

These results show there is no statistical correlation between clinical blood test parameters in PDAC patients apart from a statistically significant increase in white blood cell count occurring at the same time as a rise in alkaline phosphatase levels ($p<0.05$).

4.3.2 Monocyte distribution is affected in the peripheral blood of PDAC patients compared to healthy donors.

Monocyte counts for patients are assessed in the clinic routinely as part of their initial consultation and diagnosis. Although this is also assessed using a basic flow cytometer, the differential subsets are not quantified in the clinical tests. This can be done using a simple multi-colour panel to examine HLA-DR, CD14 and CD16 expression whilst excluding other cell types present in the blood sample like B cells, T cells or natural killer cells. This type of investigation has not been published currently in pancreatic cancer patients.

To examine monocyte subset distribution in PDAC patients compared with healthy donors, multi-colour flow cytometric analysis was used to define monocyte subset using cell surface expression of CD14 and CD16. Total monocytes in fresh peripheral blood were selected as HLA-DR positive and negative for a cocktail of lineage markers to exclude B and T cells as well as granulocytes, natural killer and dendritic cells. Classical monocytes were defined as expressing $CD14^{++}$ $CD16^{-}$, intermediate express $CD14^{++}$ $CD16^{+}$ and non-classical express $CD14^{dim}$ $CD16^{++}$. The gating strategy used to sort the monocyte populations is shown below.

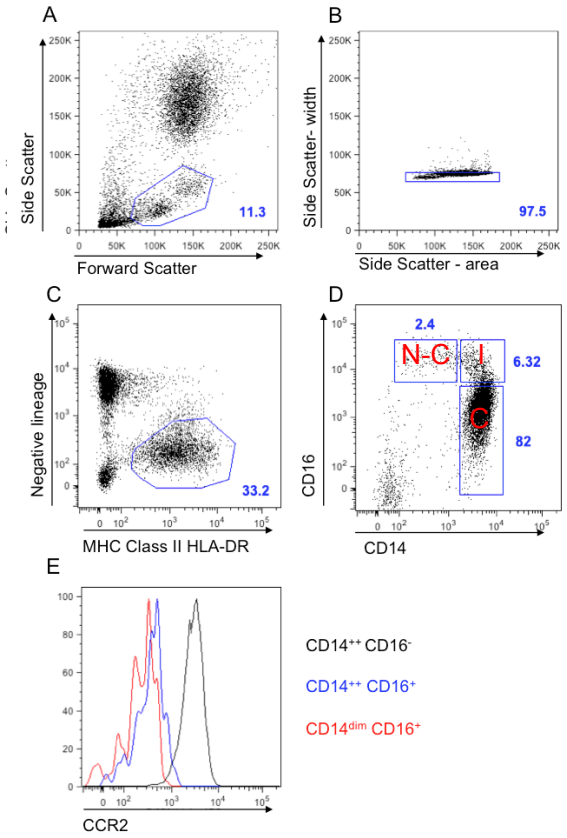

Figure 4.1~~4.1~~ Monocyte subset definition using flow cytometry

Monocyte and leukocyte populations were selected from this classic blood profile (~~4.~~1.A) using side and forward scatter properties measuring granularity and size respectively in a healthy volunteer. A gate was created around lymphocytes and monocytes avoiding granulocytes and cell debris. Figure ~~4.~~1.B was used to exclude cell doublets by plotting area versus width. In Figure ~~4.~~1.C, a negative lineage selection cocktail in the phycoerythrin (PE) channel against CD2, CD15, CD19, CD56 and NKp46 (Table 3.1) was used to exclude other peripheral blood cells. ~~(as indicated in table X above).~~ The gate was placed around the Human Leukocyte Antigen class II (HLA-DR) positive, PE negative population. Figure ~~4.~~1.D plots CD14 versus CD16 to define the three populations as mentioned above (N-C non-classical, I intermediate, C classical). Figure ~~4.~~1.E shows representative CCR2 expression exclusively on the classical monocyte population, and was used to define the interface between the subsets.

~~Subsets were gated as shown in the methods.~~ The three sub-populations were defined using CD14 and CD16 expression and positive expression for CCR2, exclusively in the classical $CD14^{++}$ $CD16^{-}$ population. These gates are well characterised and published as standard strategy to identify monocyte sub-populations (Heimbeck *et al*., 2010, Pandzic Jaksic *et al*., 2010, Shantsila *et al*., 2011, Ferrer *et al*., 2014).

Monocyte subset definition is defined by the gated percentage of monocyte populations in gate from Figure 4.1.D as shown above of the $HLA\text{-}DR^{+}$ lineage cocktail negative cells. Figure 4.2 shows a representative flow cytometric plot of a PDAC patient compared with a healthy volunteer. It can be seen that the distinctive shape of the dot plot is altered by the presence of cancer in these representative images.

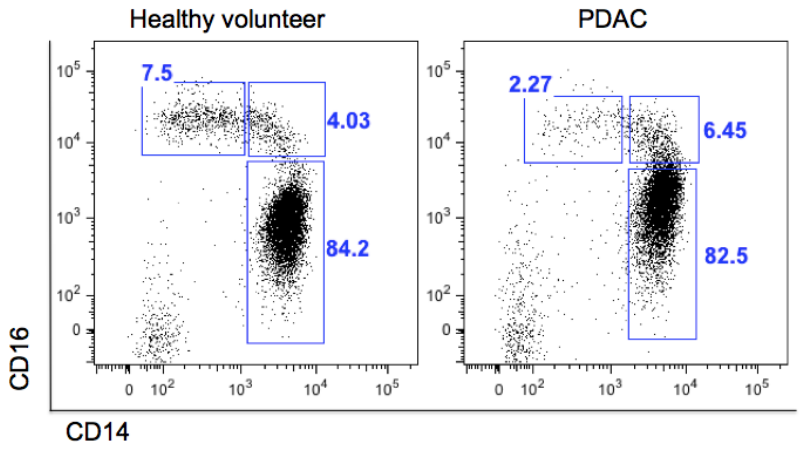

Figure 4.2 Representative image of the change in Monocyte distribution comparing healthy donor blood with blood from a PDAC patient.

Data shown as a representative flow cytometric plot (gated as such in Figure 4.4.1.D) using CD14 and CD16 expression on HLA-DR positive cells and lineage cocktail negative population. Fresh blood sample after red blood cell lysis from one healthy volunteer (left) and one PDAC patient (right).

The calculated percentages of MHC Class II positive monocytes were plotted for 28 healthy donors and 20 PDAC patients. The results are expressed in Figure 4.3 below.

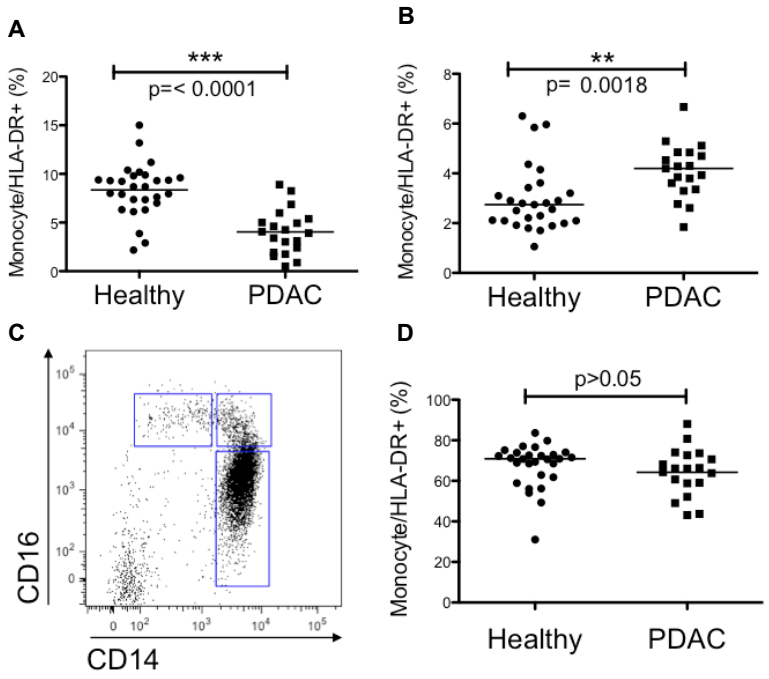

Figure 4.3 Monocyte subset distribution in PDAC patients compared to healthy volunteers

Quantification of the percentage of cells in each defined subset of the number of MHC-Class II HLA-DR positive cells. Figure A shows non-classical monocyte population $CD14^{dim}$ $CD16^{++}$. Figure 4.3.B shows the intermediate population $CD14^{++}$ $CD16^{+}$ monocytes. Figure 4.3.D shows the classical monocytes $CD14^{++}$ $CD16^{-}$. Figure 4.3.C shows the flow cytometric distribution from FlowJo analysis. X-axis - CD14 expression, Y-axis – CD16 expression of HLA-DR positive cell populations in whole blood. Dot plot to delineate distribution of cellular populations per individual (median = horizontal line), Mann Whitney test was used to calculate *p values)*.

Results

The classical monocyte population is the most abundant in the peripheral blood making up approximately 80-90% of total monocytes (Hubl *et al*., 1995, Brew *et al*., 2004, Rogacev *et al*., 2011). In this patient cohort the median value does not appear to be affected by the presence of PDAC compared to healthy volunteers (~~mean~~median ± SD: PDAC 66% ± 11.2%; HV 70.85% ± 10.7% respectively, using Mann-Whitney statistical test $p<0.05$). The intermediate population is increased in the peripheral blood of the PDAC patients compared to healthy (PDAC 4.2% ± 1.09%; HV 2.7% ± 1.32% respectively; $p=0.0018$) and the non-classical reduced compared to healthy volunteers (3.99% ± 2.30 and 8.7% ± 2.27 respectively $p<0.0001$).

4.3.3 Monocyte distribution and clinical characteristics

Monocyte distribution in humans can be affected by not only the presence of cancer, but also by gender (Heimbeck et al., 2010). To examine whether this might affect the distribution of results for the pancreatic cancer patients, the monocyte percentage distribution profiles for the PDAC patients were plotted according to gender.

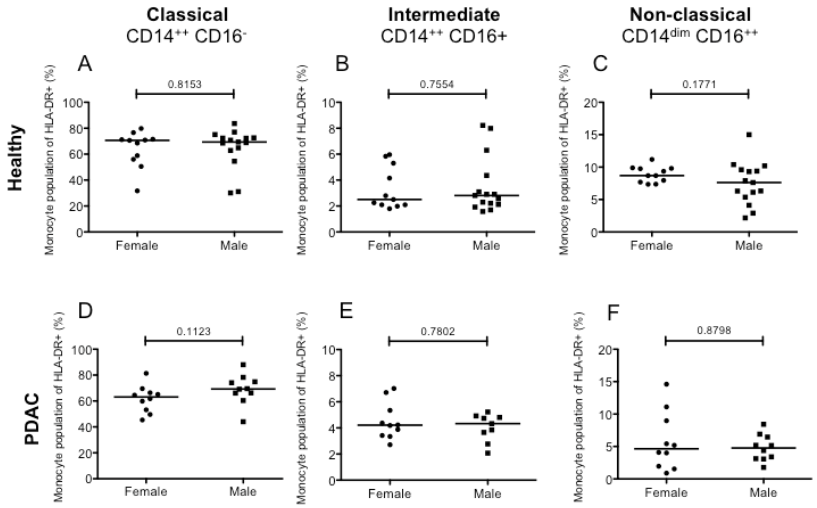

Figure 4.4~~4.4~~ Monocyte distribution profiling of patients according to gender

Quantification of the percentage of cells in each defined subset of the number of MHC-Class II HLA-DR positive cells (~~4.4.~~A-C) Healthy volunteers males n=15) females (n=11)). (~~4.4.~~D-F) PDAC patients (males (n=10) females (n=10)) monocyte distribution of non-classical monocytes $CD14^{dim}$ $CD16^{++}$, Intermediate population $CD14^{++}$ $CD16^{+}$ monocytes, classical monocytes $CD14^{++}$ $CD16^{-}$. Dot plot to delineate distribution of cellular populations per individual (median = horizontal line), Mann Whitney test was used to calculate *p values*.

The results show no significant difference between monocyte subsets and gender in patients with PDAC or healthy volunteers. For other factors such as smoking and diabetes the numbers of patient we have this information for are small ~~for~~ and statistical significance cannot be reliably concluded.

4.3.4 Inflammatory cytokine profiling in the peripheral blood of PDAC patients compared to healthy shows significant increases in IL-2, IL-8 and TNF-alpha

Blood was taken from patients with pancreatic ductal adenocarcinoma, collected into EDTA coated vacutainers to prevent clotting and the centrifuged to collect the plasma fraction. The Mesoscale Discovery human pro-inflammatory multiplex array was used to simultaneously measure 9 pro-inflammatory cytokines present circulating in the peripheral blood.

The table below shows the median, mean and standard deviation calculated from the results of the 9 pro-inflammatory cytokines ~~profiling~~ circulating in the blood of PDAC patients compared to healthy volunteers.

Cytokine	Healthy Median Mean/SD	PDAC Median Mean/SD	*p-value*
IL-6	0.321 0.341±0.177	1.270 7.797±11.57	0.1079
IL-8	2.71 2.98±1.502	12.9 30.39±34.06	0.0353 (*)
TNF-alpha	3 3.19±1.178	6.212 7.630±5.6	0.0424 (*)
IL-2	0.188 0.216±0.07	0.483 0.559±0.4269	0.050 (*)
IL-1beta	0.358 0.383 ±0.3	0.239 0.234±0.38	0.2925
IFN-gamma	0.684 0.637±0.18	1.018 1.41±1.007	0.0612
GM-CSF	0.225 0.301±0.156	0.5 1.083±1.129	0.0869
IL-12p70	0.658 0.718±0.478	0.76 21.78±36.98	0.1261
IL-10	0.798 1.292±1.28	3.33 62.95±128.4	0.1923

Table 4.4 MSD profiling serum from healthy volunteers and PDAC patients.

Human pro-inflammatory cytokine profiling array from Mesoscale Discovery. Healthy (n=7) and PDAC patients (n=15) *p*-value is calculated using unpaired t-test. Values are expressed as mean and median.

The levels of pro-inflammatory cytokines IL-8, IL-2 and TNF-alpha were statistically significantly increased in the plasma of patients with PDAC compared to plasma from healthy volunteers.

Figure 4.5 below shows the individual data points for each volunteer or patient. The x-axis indicates healthy volunteer or PDAC patients, and the y-axis shows measured cytokine concentrations in the plasma as measured in picograms per millilitre.

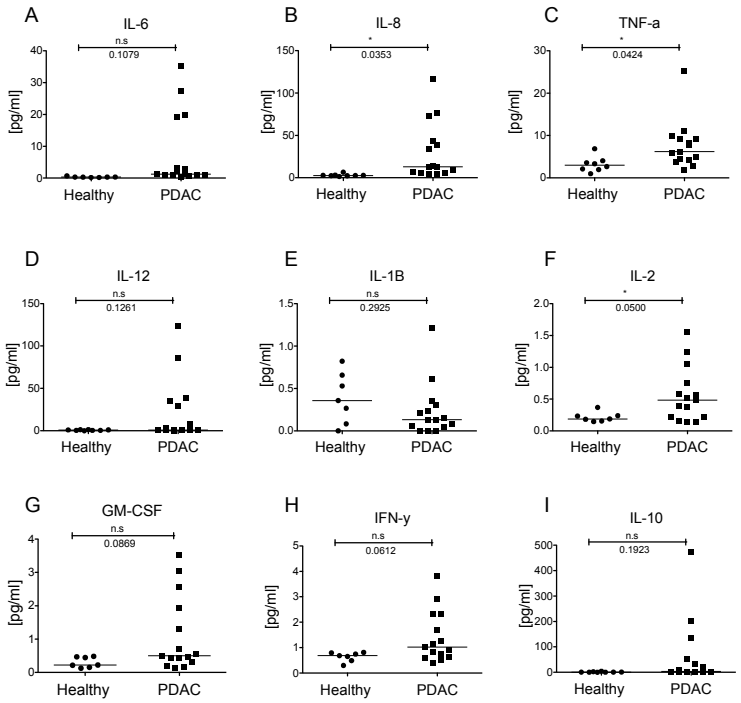

Figure 4.54.5 Pro-inflammatory cytokine profiling of plasma from peripheral blood of PDAC patients and healthy volunteers

A multiplex array was used to profile pro-inflammatory cytokines in plasma from PDAC patients and healthy donor blood. Panel shows IL-6, IL-8, TNF-alpha, IL-12, IL-1β, IL-2, GM-CSF, IFN- γ and IL-10. Each square represents a single blood donor or patient (7 healthy and 15 PDAC patients). Statistical significance is calculated using an unpaired t-test, * = significant values ~~(*)~~ *p<0.05; n.s* = non-significant.

These results show that the pro-inflammatory cytokine profile is affected in PDAC patients compared to healthy volunteers when comparing IL-2, IL-8 and TNF-alpha.

4.4 Summary

- The percentage distribution of classical monocytes does not appear to change in this cohort of PDAC patients compared with healthy volunteers.
- The percentage distribution of non-classical monocytes in the peripheral blood of PDAC patients is significantly reduced compared with healthy volunteers and the intermediate monocyte population is significantly increased. ~~These changes reflect monocyte distribution in other inflammatory conditions.~~
- The levels of pro-inflammatory cytokines IL-2, IL-8 and TNF-alpha are statistically significantly increased in the serum of PDAC patients compared with healthy donors.

4.5 Discussion

The intention of this chapter is to examine whether the presence of the tumour and associated inflammation has an effect on the distribution of monocyte subsets. To address this, available data derived from the differential blood counts was taken at the same time as samples from the hospital. The distribution of monocyte subsets in the patients compared with healthy volunteers was assessed and the presence of pro-inflammatory cytokines in the peripheral blood.

CA19.9 is a tumour marker used in routine blood tests in pancreatic cancer patients and other benign biliary stricture diseases (Lin et al., 2014). CA19.9 is increased in the PDAC patients compared with the normal range. A recent meta-analysis summarised the importance of CA19.9 in the diagnosis of pancreatic cancer as they pooled available data to determine sensitivity (0.80 95%CI 0.77-0.82) and specificity (0.80 95% CI 0.78-0.82) (Huang and Liu, 2014). CA19.9 levels could also serve as prognostic indicators in patients treated with radiotherapy due to correlating disease progression and expression (Shultz et al., 2014, Tzeng et al., 2014).

Alkaline phosphatase (AP) is an enzyme often increased in hepatobiliary diseases. Blood tests for AP are used to detect liver damage and bile duct obstructions (Carr and Guerra, 2013, Casale et al., 2013). AP levels in the PDAC patients were at the higher end of the normal range. A higher level of expression in the blood is associated with a reduced median survival time in patients (Storniolo et al., 1999, Matsubara et al., 2010, Dua et al., 2013).

Bilirubin is a substance found in bile, a breakdown product of haem catabolism. Blood tests for bilirubin are recommended in case of liver damage, bile duct blockage or jaundice, often a presenting symptom in patients with PDAC (Wang et al., 2013a). The mean level of bilirubin detected in these patients was at the top end of the normal range. Elevated bilirubin levels represent a significant prognostic factor in PDAC (Haas et al., 2013). Using combination of CAa19.9 with raised bilirubin levels and CRP

can be a better predictor with higher sensitivity and specificity than individual markers (La Greca et al., 2012, Dumitra et al., 2013).

Total white blood cell count (WBC) mean value in the PDAC patients was slightly raised compared to the normal range. WBC has not shown to be a significant prognostic indicator (Hamed et al., 2013, Pabinger et al., 2013). However patients with pancreatic carcinoma have been reported to have higher WBC than other periampullary cancers (Liang et al., 2013).

The monocyte counts in PDAC patients were at the higher end of the normal range compared with healthy volunteers. Sanford et al., suggested monocyte counts in the blood of patients were higher than the healthy donors due to mobilisation from the bone marrow and showed this was associated with a poorer outcome (Sanford et al., 2013). This may be due to the small sample cohort in this investigation, it could also be linked to the patient selection as Sanford et al., used a mixed group of early and late stage pancreatic cancer and patients who had received chemotherapy.

4.5.1 Monocyte distribution changes in PDAC patients

Flow cytometry is commonly used for immunophenotyping cells in the peripheral blood as it has the advantage of being able to identify numerous markers in the same sample. The use of CD14 and CD16 to define monocyte nomenclature is well established and supported in literature to define the three sub-populations (Cros *et al*., 2010, Ziegler-Heitbrock *et al*., 2010, Heimbeck *et al*., 2010). This gating strategy was chosen at the start of this project as defined by Heimbeck *et al*., in 2010 to be a simple standardized method for analysis.

Using percentage changes in monocyte distribution is a useful method to examine population skewing but bias may be introduced by the presence of other cell types. This is why the percentage of monocyte is worked out from the HLA-DR positive cell populations after excluding a number of other cell types using a negative lineage cocktail to remove B, T, NK cells and granulocytes. This ensures that the cells being included in this analysis are monocytes and change in distribution of other cell types does not affect subset percentages.

It is argued that defining monocytes into individual subsets may be misleading as it is still unknown as to whether these are distinct cell populations or cells in transitional phases of maturation or differentiation. However due to their differential characterised abilities, analysis and enumeration in healthy and disease is still warranted.

Classical monocytes have been shown to decrease in percentage compared with other monocyte populations in sepsis (Poehlmann *et al*., 2009), Hepatitis B (Zhang *et al*., 2011), Dengue fever (Azeredo *et al*., 2010) and tuberculosis (Castano *et al*., 2011). The intermediate population has been shown to increase in all the above inflammatory conditions as well as heart failure (Barisione *et al*., 2010), rheumatoid arthritis (Rossol *et al*., 2012) and stroke (Urra *et al*., 2009). However in these studies the effects on the non-

classical monocytes is variable, increasing in hepatitis, sepsis and dengue fever, but decreasing after stroke.

In common with the inflammatory conditions, in patients with breast cancer, the intermediate monocyte population was reported to expand compared with healthy donors and this was suggested to correlate with the amount of CCR2 (MCP1) in the blood (Feng *et al*., 2011). In patients with colorectal carcinoma (CRC), the intermediate ($CD14^{++}$ $CD16^{+}$) monocyte population was also shown to expand compared to healthy donors (Schauer et al., 2012).

In human pancreatic cancer, monocyte counts were determined in a cohort of patients after surgical resection (Sanford *et al*., 2013). The number of classical monocytes in the peripheral blood was reported to increase in resected PC patients compared to healthy volunteers and this increase was inversely correlated with patient survival. It was hypothesised that the monocytes are being mobilized from the bone marrow to the blood in response to CCL2 signalling. Previous data in murine models indicated that inhibition or interruption of this CCR2-CCL2 interaction reduces the infiltration of monocytes into the tumours, therefore relieving immunosuppression and improving responses to chemotherapy (Mitchem *et al*., 2012). This approach may merit further investigation to evaluate potential clinical benefit.

The results in this chapter showed that there appeared to be no significant differences in classical monocyte population percentage, a significant increase in the intermediate monocyte population and a concurrent decrease in the non-classical population in patients with PDAC relative to healthy controls.

This data does not reflect the increase in classical monocytes as shown by Sanford *et al*. This could be due to several factors. First, the patients in this investigation have unresectable advanced disease, often with local invasion.

The cohort used in the Sanford study had resectable disease suitable for surgical intervention and therefore may have been detected at an earlier stage. Second, the patients in the Sanford study were not selected at the same stage of treatment or stratified before and after surgery, which may increase the patient numbers but does not provide a consistent cohort. Whereas the cohort used in this investigation was exclusively locally advanced non-resectable patients that have not undergone any therapy.

This evidence suggests that either we cannot directly compare the results of both investigations; or it may suggest that monocyte mobilisation to the periphery might be an early event and may not be maintained as the PDAC progresses. Alternatively, it could suggest that the classical monocytes may form a pool of precursor cells for the other monocyte subsets as debated by Yona *et al*., in 2013 and their recent fate mapping experiments in murine models (Yona et al., 2013).

There is also evidence in the literature that monocyte distribution can be affected by other factors. For example, exercise is reported to affect the distribution profile as the non-classical monocytes were hypothesized to be mobilized out of the blood stream after short bursts of activity without showing changes in gene expression (Frankenberger *et al*., 2012). The number of non-classical monocytes is also shown to be lower in females than males, for reasons that are unknown (Heimbeck *et al*., 2010). However, in that study, absolute counts rather than percentages of non-classical monocytes were reported. In contrast, monocyte distribution was not significantly altered by gender in healthy volunteers, or the PDAC patient cohort in the study reported here.

To further this part of the investigation, single pro-inflammatory cytokines could be used to stimulate isolated peripheral blood mononuclear cells *in vitro* to identify if any of the above cytokines can modulate CD14 or CD16 expression. However, this is a very narrow and simplistic view of the complex signalling mechanisms activated in PDAC.

Schauer *et al.* used an *in vitro* co-culture method to identify cell surface expression changes in CD14 and CD16 on healthy primary monocytes cultured with primary or metastatic CRC cell lines. This data showed that induction of intermediate monocyte was enhanced by culturing them with primary tumour cells rather than those from metastatic sites, which may relate back to the immunosuppressive milieu (Schauer *et al*., 2012).

To build upon this, plasma from PDAC patients could be used to stimulate isolated PBMCs to determine whether this causes any effects on monocyte distribution. Using PBMCs instead of monocytes may provoke cells other than monocytes to release signalling molecules and may create a more 'real' reflection of the peripheral blood. As a further extension, the flow cytometric panel could be expanded to include other markers of monocyte differentiation such as CX3CR1 and CCR2,

4.5.2 Cytokine changes in the peripheral blood of PDAC patients compared with healthy volunteers

Cytokine measurement is used routinely to assess the inflammatory responses and immune signalling capabilities in subjects. The Mesoscale Discovery platform multiplex array was used to assess the presence of cytokines in the plasma of PDAC patients compared with healthy volunteers. This assay was preferred to ELISA methods as MSD can measure multiple cytokines in the same sample. Also the MSD platform has a significantly higher sensitivity (0.6pg/ml compared to 6pg/ml for ELISA).

In PDAC there are several published studies examining circulating factors in the plasma or serum of patients compared to healthy donors. The results gained by these investigations are variable, potentially due to discrepancies in sample sizes and patient groups containing untreated and treated patients. A large study carried out mass spectrometry using serum samples from a total of 333 PDAC patients, 144 patients with benign pancreatic conditions and 277 healthy controls. This study identified three biomarkers in the serum (CA19.9, CEA and osteoprotegrin (OPG) a member of the tumour necrosis receptor superfamily) with high sensitivity, specificity and the ability to distinguish pancreatic cancer from other solid tumours in the breast, lung or colon (Brand et al., 2011).

Inflammation is strongly implicated in pancreatic cancer and for this reason the MSD pro-inflammatory cytokine panel was chosen, which measures 9 of the most common pro-inflammatory cytokines in an individual sample. Although the measure of cytokine using this method gives biological snapshot of certain cytokines, it may provide a useful indication of pre-treatment systemic inflammation For a more complete analysis mass spectrometry based quantitative proteomics would provide a broader approach. Identification of prognostic useful biomarkers however would require a much larger sample size (Tonack et al., 2013, Wang et al., 2013b).

Alternative approaches to measure large numbers of analytes or cytokines in the blood, using bead based arrays on an 'inflammation map' from Myriad.

The data in this chapter shows several cytokines are significantly increased in these PDAC patient plasma compared to healthy volunteers. These are IL-2 (0.55±0.42 versus 0.21±0.07 respectively), IL-8 (2.98±1.5 versus 30.39±34.06) and TNF-a (3.19±1.17 versus 7.63±5.6).

IL-2 is an important cytokine in the development of T effector and regulatory cells. It plays an important role in the adaptive immune system to fight autoimmunity develop immunological memory (Cantrell and Smith, 1984, Grigorian *et al*., 2012). IL-2 in the plasma is increased in untreated PDAC patients in this cohort and is also reported in a previous study (Plate *et al*., 1999). It is also increased in NSCLC cancer, which is associated with poorer outcome (Orditura *et al*., 2000). IL-2 concentration values extrapolated from the standards in my cohort suggest the levels are below the limit of detection for this assay.

IL-8 is a chemotactic cytokine with roles in neutrophil activation. In pancreatic cancer, IL-8 has been shown to promote tumour cell migration and the over-expression of associated receptors CXCR1 and CXCR2 in PDAC cell lines and human tissue microarrays (Kuwada *et al*., 2003).

TNF-alpha levels were increased in patients with post resection pancreatitis (Kilciler *et al*., 2008), breast cancer (Berberoglu *et al*., 2004) and gastrointestinal cancers (Bossola *et al*., 2000) compared with normal levels. This is also true for this dataset. TNF-alpha is an adipokine involved in immune regulation and systemic inflammation.

IL-12, GM-CSF, IL-10, IL-6, IL-1 and IFN-γ are not significantly increased in this cohort of patients. Published data in PDAC suggests there is a strong link between IL-6 and related inflammation (Okada *et al*., 1998, Dhillon *et al*., 2008, Gabitass *et al*., 2011, Wormann *et al*., 2013). Increased expression in

the blood has been linked with the development of cachexia (Martignoni et al., 2005) and correlated with poorer outcome (Waugh and Wilson, 2008). A recent study published in 2013 highlighted a clear link between increased IL-6 and IL-1 expression and reduced PFS in patients undergoing gemcitabine therapy (Mitsunaga et al., 2013).

Circulating levels of IFN-γ have been used previously to predict effects of adoptive T cell therapy in PDAC patients. In 2013, a group identified that levels correlated over time with therapy response (Ishikawa *et al*., 2013).

The lack of GM-CSF was also surprising as several recent studies in murine models of PDAC have associated elevated levels with myeloid regulation through tumour derived and oncogenic Kras induced GM-CSF (Pylayeva-Gupta *et al*., 2012) and subsequent promotion of tumour growth (Bayne *et al*., 2012). There ~~were~~was however some patients with high levels of GM-CSF, which indicates there may be heterogeneity in human populations compared with mouse.

Pro-inflammatory cytokine expression can be affected by other factors, including medications. An example of this is the changes in TNF-a expression after administration of non–steroidal inflammatory agents (NSAIDs) (Page *et al*., 2010).

A study comparing pro-inflammatory responses of PBMCs in advanced PDAC patients to healthy showed that stimulated monocytes from cachectic pancreatic cancer patients were primed to produce significantly higher levels of IL-6 when stimulated (10ug/ml LPS for 24h)(Moses *et al*., 2009). Another study showed that plasma cytokine levels and the ability of monocytes to produce pro-inflammatory cytokines such as TNF-alpha were affected by the presence of obstructive jaundice, a common symptom of pancreatic cancer (Puntis and Jiang, 1996). IL-6 and IL-8 levels in the blood are implicated as prognostic biomarkers (Chen et al., 2012), and are thought to have the ability to predict gemcitabine efficacy (Mitsunaga et al., 2013) in patients.

Targeting inflammation in PDAC is an attractive target in re-education of the immune system to improve immunosurveillance (Steele *et al*., 2013). However the presence of inflammatory cytokines in the blood is not a predictor of pancreatic cancer risk as shown in a large retrospective meta-study compiled of several large-scale prospective cohort studies (Bao *et al*., 2013).

To build upon these results it would be interesting to increase sample numbers and to also include patients with acute and chronic pancreatitis. This might allow us to further dissect the inflammation or cancer related changes occurring in the peripheral blood. It would also be interesting to measure levels of monocyte chemoattractant molecules such as CCL2 and M-CSF determine if these correlate with monocyte distribution or counts in the PDAC patients. The confounding ability of a higher number of PDAC patients with a history of smoking or the presence of diabetes also warrants further investigation.

Chapter 5. Gene expression profiling classical monocytes from patients with PDAC compared with healthy volunteers.

5.1 Introduction

In the previous chapter significant changes in the distribution of monocytes in the peripheral blood of PDAC patients compared to healthy volunteers were identified. It is known that monocytes in the peripheral blood are incredibly responsive to exogenous signals they receive and this determines their function and phenotype (Gordon and Taylor, 2005, Auffray et al., 2009, Yona and Jung, 2010, Ma et al., 2014, Xue et al., 2014).

The aim of this chapter is to examine the transcriptome of classical monocytes isolated from the peripheral blood of PDAC patients in comparison to monocytes from healthy volunteers. We hypothesised that changes in the classical monocyte subset potentially mirror those in the tumour microenvironment. The classical monocyte subset might – as outlined above – represent a cellular sensor of stress. The transcriptome holds a wealth of potential information about the inflammatory response, maturation or differentiation state of monocytes. The genomic stability of monocytes (Jie et al., 2012) compared to tumour cells makes them attractive for biomarker read-outs as the transcriptional changes may be more consistent in response to a particular stressor. Also sampling blood cells provides an easier source of cells compared with the tumour cells, which are much less abundant in the peripheral blood. I would like to investigate initially whether monocytes in the blood of PDAC patients are 'primed' by the presence of the tumour and its associated inflammation.

Classical monocytes ($CD14^{++}$ $CD16^{-}$) were chosen for this investigation, over the non-classical or intermediate populations, due to their prevalence in the peripheral blood and selective expression of chemokine receptor 2 (CCR2). CCR2 is the receptor for chemokine ligand 2 (CCL2 also referred to as Monocyte Chemoattractant Protein 1 (MCP1)), which is reported to be essential for the recruitment of classical monocytes in PDAC from the bone marrow into the peripheral blood (Sanford et al., 2013). Several recent studies have shown that targeting only this population of monocytes reduced ~~tumour-associated macrophage~~TAM recruitment, tumour volume and

metastasis in murine models of solid cancer as mentioned in more detail in the ~~i~~Introduction (Section 1.6.2.2) (Leavy, 2011, Leuschner et al., 2011, Mitchem et al., 2012, Ino et al., 2014).

Monocyte distribution in PDAC patients showed significant differences in the non-classical and intermediate subsets versus healthy controls. In contrast, the proportion of classical monocytes was unchanged in the PDAC patients compared with healthy volunteers. Transcriptome profiling of the classical population may allow the detection of differentially expressed genes involved in regulation of monocyte activation and differentiation in PDAC. In addition, by looking at the transcriptome of the classical and more stable subset I may reduce bias that is introduced by shifts in the population dynamics. Previous fate mapping data in murine models suggests that the intermediate and non-classical monocytes may be derived from the classical monocytes, which acts as a pool of circulating progenitor cells. Profiling this population may reveal characteristics that help characterise the transition into non-classical and intermediate subsets.

RNA was isolated from classical monocytes purified from the peripheral blood by FACS~~fluorescence assisted cell sorting (FACS)~~. The monocyte transcriptome was evaluated in eight individual volunteers (three healthy and five PDAC patients) using Affymetrix Human Genome U133 2.0 plus arrays. This is a whole genome profiling technique that has been used in the past to reveal novel molecular targets and pathways. Over the last decade, this type of gene expression profiling has become a standard for biomarker and pathway discovery as well as pathological subtype differentiation.

Outputs from the gene chip array analysis are reported as fluorescence intensities for each individual probe. Comparison of intensities between samples allows the relative gene expression changes to be quantified after gene chip normalisation and annotation. Fold changes for each individual gene are calculated; comparing healthy and PDAC can be used to rank the statistically significant differentially expressed genes. The created gene lists

can then be used to determine whether genes from particular pathways are enriched, increasing confidence in the biological relevance.

5.2 Aims

The aims of this chapter are:

- To evaluate the purity gained from sorting classical monocytes from peripheral blood for RNA isolation
- To obtain and assess the quality of RNA isolated from the classical monocytes
- To use Affymetrix Gene Chips analysis to identify differentially expressed genes comparing classical monocytes from PDAC patients with healthy volunteers.

5.3 Results

5.3.1 High quality RNA can be isolated from classical monocytes obtained from sorting

Classical $CD14^{++}$ $CD16^{-}$ monocyte populations from healthy volunteers and PDAC patients were sorted from the peripheral blood for transcriptome analysis. For this type of analysis, it is essential that the RNA isolated is of good quality, to increase confidence of accurately representing the whole transcriptome. Classical monocytes were directly collected into FACS buffer after sorting, centrifuged and resuspended immediately in lysis buffer (RLT buffer from the Qiagen RNA isolation kit).

Using the Aria flow cytometer and markers for HLA-DR, CD14 and CD16, the purity of the sorted cell populations was approximately 98%. Figure 5.1 below is representative of the purity for each sorted monocyte subset.

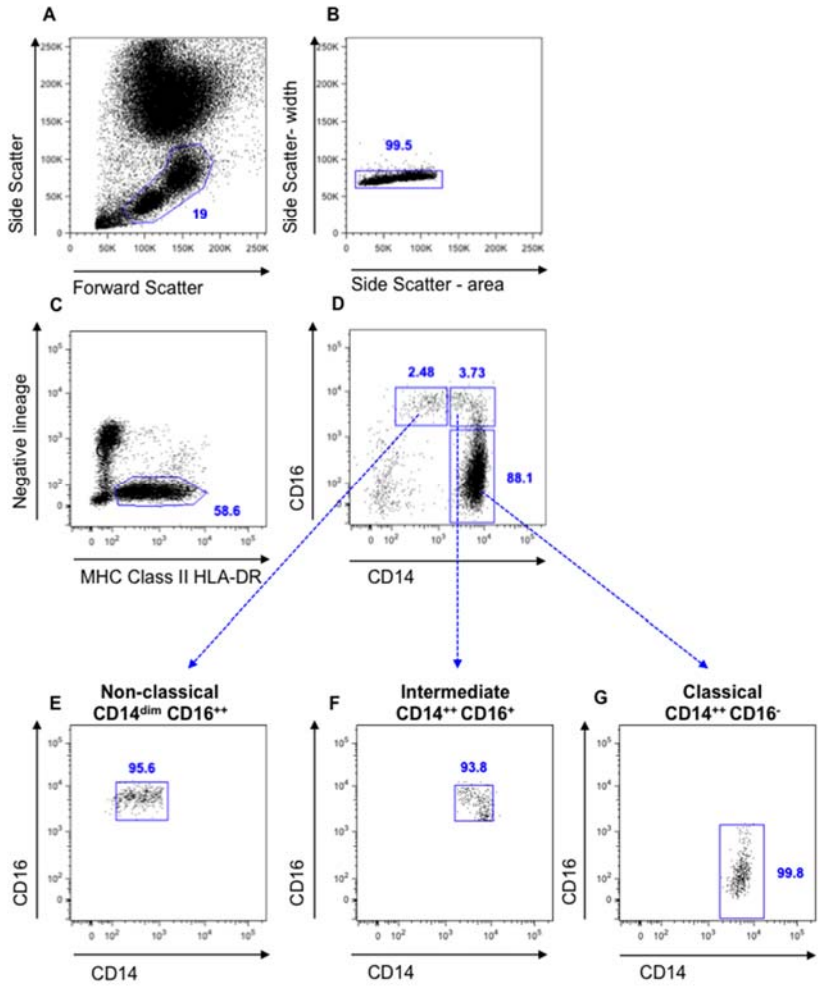

Figure 5.15.1 Classical monocytes sorted using the Aria II purity check.

Representative FlowJo cytometric plots showing the purity of classical monocytes isolated by FACS sorting. Gating strategy in Figures 5.1A-D as described in results section 4.3.2. Figure 5.1 E-G show the percentage purity of the sorted HLA-DR positive cells using the Aria II flow cytometer. Viability was assessed using the Beckman Coulter cell counter (89-97%). Representative shown of all samples used for gene expression.

RNA isolation was carried out using the Qiagen RNeasy Micro Kit as per manufacturer's instructions with an on-column DNase treatment to reduce genomic DNA contamination. Figure 5.2 shows a representative image of the RNA quality for samples chosen for subsequent gene expression analysis by Affymetrix or quantitative PCR. This image (Figure 5.2) is generated by microcapillary electrophoresis using the Agilent Bioanalyser to calculate the RNA integrity values (RIN) to assess quality and fragmentation.

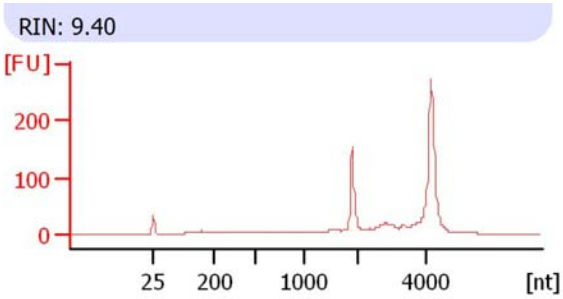

Figure 5.25.2 Agilent Bioanalyser: Unfragmented RNA sample with calculated RNA integrity value of 9.4.

Representative image (patient 515) showing the histogram produced by the Agilent Bioanalyser indicative of RNA with a calculated RIN number of 9.40. This is deemed good quality RNA for further analysis (RIN cut off >8.5). X-axis – nucleotides (nt). Y-axis-fluorescent units (FU).

Only samples with a RIN >8.5 were selected for Affymetrix analysis. RNA was amplified using the Nugen whole transcriptome amplification kit (as described in Materials and Methods 3.3.5). The Encore module was used for Biotin labelling and fragmentation before hybridisation was carried out with support from Tracy Chaplin-Perkins (Centre for Haematology-Oncology, Barts Cancer Institute).

5.3.2 Gene Chip normalization

RNA samples were converted to cDNA for hybridisation onto Affymetrix Human U133 2.0 plus Gene Chips. These chips represent up to 54,000 transcripts from the human genome. The data output from this type of array is reported as fluorescence intensity per probe set. The figure below is a box plot showing the distribution of the fluorescence intensity for each gene array chip.

To enable comparisons between different gene array chips, the fluorescence intensity values were normalised using Gene Chip Robust Multi-array Average (GC-RMA) using R-Lab software. The latter data was exported and converted to $\log_2$ values.

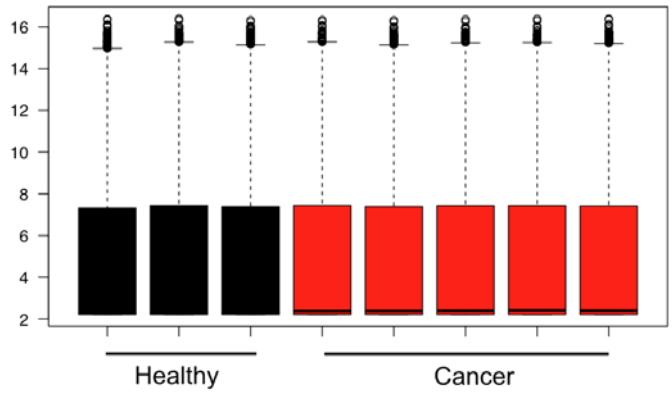

Figure 5.35.3 Distribution of GC-RMA-normalised $\log_2$ values of Affymetrix Gene Chip U133 2.0 plus array.

Fluorescence intensity values are normalised using gene chip robust multi-array averaging using R-lab.

After normalisation of this type in Figure 5.3, the $\log_2$ fluorescence intensity signals are observed to be equivalent with no distinctive outliers that need to be removed from this dataset.

Each gene chip represents transcriptomic analysis of a single patient sample and fluorescence intensity output values are given for each probe set on the gene chip. A probe set is the collection of probes present on the gene array that bind to and identify the presence of a single gene sequence. Each gene is represented by several probe sets. The relative fluorescence intensity comparing healthy samples with cancer samples is used to determine which genes (as represented by probe sets) increase or decrease in expression in patients. The change in expression intensity is calculated by taking the mean fluorescence value of one group of samples and dividing it by the other to give the fold change of that probe set. An unpaired t-test is used to test if the fold changes are significant between healthy and cancer samples. This is carried out for approximately 54,000 probes and the results can initially be visualised using a volcano plot.

5.3.3 Gene expression data visualisation

5.3.3.1 Volcano plot

Probe set expression changes can be visualised by plotting the fold change versus the p-value as a volcano plot to show whether there is a bias in expression towards one group of samples compared to the other. The statistically significant probe sets (*p<0.05)* with a fold change higher than 2 are included in Figure 5.4 below.

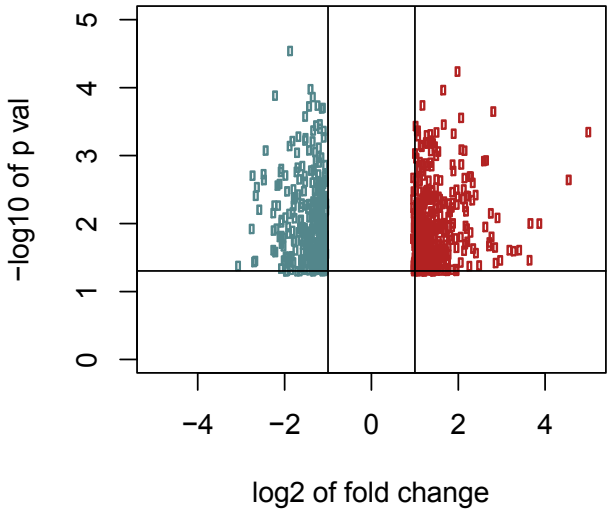

Figure 5.4~~5.4~~ Volcano plot showing the probe set expression profiles of classical monocytes from PDAC patients compared to healthy.

Fold change is plotted against the x-axis and the *p*-value for statistical difference (unpaired t-test) on the y-axis. The solid black lines represent the ~~cutoff~~cut-off values of a fold change >2 and *p*-value <0.05. Points shown in red are probe sets that are significantly increased in classical monocytes from PDAC compared to healthy volunteers. Points shown in blue are significantly decreased in classical monocytes from PDAC compared to healthy volunteers.

The results of this output are shown below in Table 5.1. Using a greater than 2 fold change there were 412 probesets showing increased expression in classical monocytes from PDAC patients compared to healthy volunteers.

There were 361 probesets that are decreased in PDAC patients (increased in healthy volunteers).

Number of differentially expressed probe sets (p<0.05)	Increase in classical monocytes from which group
412	Increase in PDAC
361	Increase in Healthy

Table 5.1 Differentially expressed probe sets with a fold change higher than 2 comparing fluorescence intensity values between healthy volunteers and PDAC patients.

As each gene is represented by more than one probe set, the probe sets were consolidated to remove all redundant probe sets that were not differentially expressed or do not represent a known gene. Table 5.2 gives the list of statistically significant differentially expressed genes (DEGs).

Number of differentially expressed genes (p-<0.05)	Increase in classical monocytes from which group
242	Increase in PDAC
280	Increase in Healthy

Table 5.2 Differentially expressed genes with a fold change higher than 2 comparing fluorescence intensity values between healthy volunteers and PDAC patients.

In this dataset there were 242 genes whose expression was increased in classical monocytes from PDAC patients compared to healthy and 280 genes that showed decreased expression.

These results show a number of significant differentially expressed genes when comparing the transcriptional profile of classical monocytes from PDAC patients with those of classical monocytes from healthy volunteers.

5.3.3.2 Classical monocytes from PDAC patients have a significantly altered gene expression profile compared to healthy volunteers

The top differentially expressed genes (DEGs) can be ranked using their fold change difference comparing healthy to PDAC patients. This is carried out to see which genes are the most up or down regulated. Figure 5.5 below uses a heat map to visualise the expression values for the top 25 DEGs in each group. Each column represents an individual donor and each row a gene.

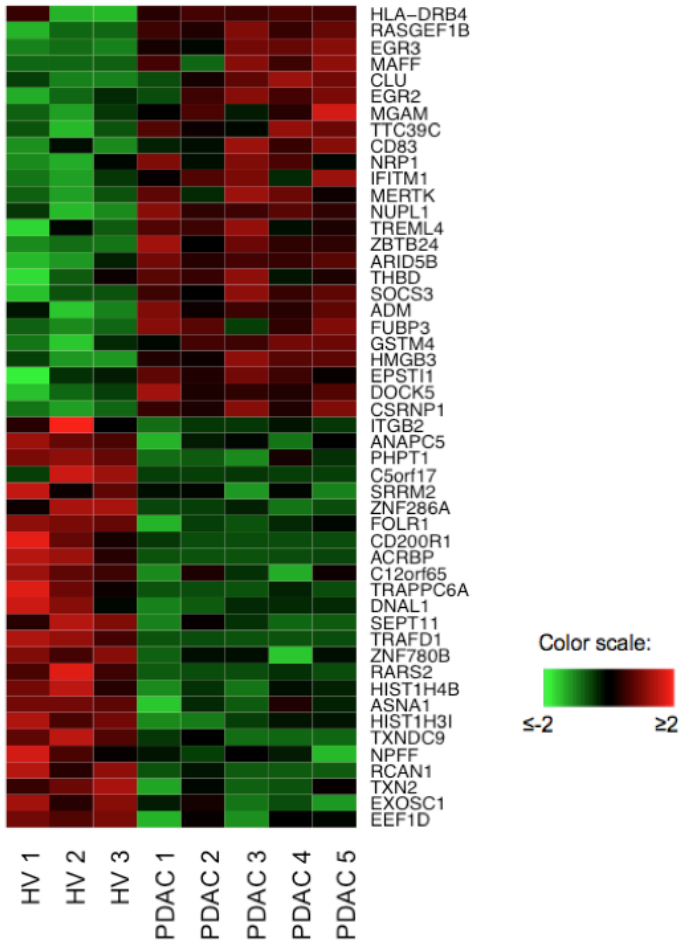

Figure 5.5~~5.5~~ Heat map showing the top 25 differentially expressed genes ranked by fold change that are increased and decreased comparing classical monocytes from PDAC patients with healthy volunteers.

Heat map expression using top 25 statistically significant DEGS (p<0.05, FC>2) ranked by fold change comparing classical monocytes isolated from PDAC patients compared to healthy volunteers using fluorescence intensity expression derived from Affymetrix Human Gene Chip U133 2.0 plus arrays. The rows represent genes and the columns individual healthy volunteers (n=3) or PDAC patients (n=5). The differences between the groups are centred using the mean fluorescence intensity value and standardised by

dividing the samples by their standard deviation to create a uniform plot. Heat map created using R-Lab.

The differentially expressed genes identified in this cohort of patients are very intriguing, however, particularly due to the small sample size, the biological relevance (or potential use as a PDAC monocyte signature) cannot be inferred without further validation. In the next chapter the top ranked differentially expressed genes will be examined using qPCR in an independent cohort of patients to confirm expression in a larger data set and therefore improve significance.

The gene lists created from this investigation can be processed using software to identify whether genes associated with particular biological pathways are affected.

5.3.4 Ingenuity Pathway analysis

Gene expression data were analysed using Ingenuity Pathway Analysis (IPA) software to elucidate whether the differentially expressed genes are reported to be involved in any particular known biological pathways. IPA is based on the Ingenuity Knowledge Base made up of functional annotations and biological or chemical interactions based on reported primary experimental data.

This can be very useful tool for interpretation of gene lists by comparing them with the current literature base and providing information on the potential biological relevance of the genes in a gene list. Statistical significance can also be improved if a set of genes in a pathway is affected compared to individual genes. Individual genes can also be found in more than one pathway.

IPA was used to define annotations for differentially expressed genes (FC>2, p<0.05) also including the fold change. Table 5.3 shows the functional groups of genes that are enriched in this dataset comparing classical monocytes from PDAC patients with healthy volunteers.

Diseases or Functions Annotation	p-Value	Molecules
Transcription	2.0E-07	ACVR1B,AP1G2,ARID1B,ARID5B,ATF6B,BCOR,BHLHE40,BMPR2,BTG1,BTRC,CALR,CAMKK2,CCNT2,CD44,CDKN1A,CEBPD,CIITA,CSRNP1,DAB2,E2F2,EEF1D,EGR2,ELL,ENO1,FLI1,FOXP1,FUBP3,HAS3,HEATR1,HGF,HIVEP2,HNRNPA1,HSF2,ID2,IFRD1,IKBKB,IL6R,IL6ST,IRF4,LRRFIP1,MAFF,MXD1,N4BP2L2,NAB1,NFIL3,NFKBIZ,NFRKB,NOTCH2,NRIP2,OTUB1,PABPN1,PATZ1,PIM2,PLSCR1,PML,POLR2E,PSEN2,PSMD10,PTGER2,PTGER4,RBBP6,RBM5,RCAN1,RNF10,RXRB,SEC61A1,SET,SH3KBP1,SIAH1,SIN3A,SMAD1,SMAD5,SMARCA2,SMURF2,SNIP1,SOCS3,SOS1,SP140,SP2,SP3,SRA1,SRSF6,STK16,SUPT3H,TLE3,TNFSF8,TRERF1,TRIM13,VDR,VPS72,ZBP1,ZNF398,ZXDC
Development of mononuclear leukocytes	4.3E-07	ADM,BHLHE40,CD83,CDKN1A,CIITA,E2F2,EEF1D,EGR2,EGR3,F5,FOXP1,FUS,HAVCR2,HIVEP2,ID2,IFNAR1,IKBKB,IL6ST,IRF4,ITGB2,JMJD6,MERTK,NAB1,NBR1,NF1,NFIL3,NOTCH2,PATZ1,PIM1,PLEKHA1,PLEKHA2,PRKCH,PTGER4,SLC19A1,SOCS3,SP3,SPN,TNFSF8
Development of lymphocytes	9.4E-07	BHLHE40,CD83,CDKN1A,CIITA,E2F2,EEF1D,EGR2,EGR3,F5,FOXP1,FUS,HAVCR2,HIVEP2,ID2,IFNAR1,IKBKB,IL6ST,IRF4,ITGB2,JMJD6,MERTK,NAB1,NBR1,NF1,NFIL3,NOTCH2,PATZ1,PIM1,PLEKHA1,PLEKHA2,PRKCH,PTGER4,SLC19A1,SOCS3,SP3,SPN,TNFSF8
Cell death of blood cells	1.9E-06	BTG1,CALR,CD44,CDKN1A,CIITA,CLU,CSNK2A2,E2F2,EGR3,FLI1,FUS,HAVCR2,HGF,HLA-DRB4,ID2,IFNAR1,IKBKB,IL6R,IL6ST,IRF4,ITGB2,MERTK,MPO,MT1E,MXD1,NAMPT,NF1,NFIL3,NLRC4,OSCAR,PIM1,PIM2,PML,PSEN2,PTGER4,RCAN1,SH3KBP1,SLC2A1,SPN,TNFSF8,TPP2,VDR,XIAP
Viral Infection	3.8E-06	ADARB1,AGO3,AP1G2,AP1S1,APOBEC3F,ARF1,ARGLU1,BTRC,CAMK1,CAMKK2,CARD16,CD200R1,CD44,CEBPD,CIITA,CLU,COG3,CYSTM1,DNAL1,E2F2,EIF4EBP2,EXOSC5,F5,FGD6,G3BP1,H3F3A/H3F3B,HAVCR2,HEATR1,HGF,IFITM1,IFNAR1,IKBKB,IRF4,ITGB2,KATNB1,LILRA2,LRPAP1,MAGT1,MAN1A1,MAP4,MBTPS1,MERTK,MGAM,MPHOSPH6,MXD1,NDUFA10,NF2,NFIL3,NFKBIZ,NMT1,NRP1,PELI1,PLCB2,PML,PRKCH,PSEN2,PSMD12,RAB3D,RBM5,RNF10,RNH1,RRAGD,RSL1D1,RXRB,SF3A1,SLC2A1,SMARCA2,SOCS3,SPAST,SPN,SRRM2,SRRT,SRSF6,STXBP2,SUCLG2,THBD,TRERF1,TRPV2,TTC3,VDR,WIPF1,XIAP,ZBP1
Expression of RNA	4.7E-06	ACVR1B,ARID1B,ARID5B,ATF6B,BCOR,BHLHE40,BMPR2,BTG1,BTRC,CALR,CAMKK2,CCNT2,CD44,CDKN1A,CEBPD,CIITA,CSRNP1,DAB2,E2F2,EEF1D,EGR2,EIF4EBP2,EIF5,ELL,ENO1,FLI1,FOXP1,FUBP3,HAS3,HEATR1,HGF,HIVEP2,HMGB3,HNRNPA1,HSF2,ID2,IFRD1,IKBKB,IL6R,IL6ST,IREB2,IRF4,LRRFIP1,MAFF,MXD1,N4BP2L2,NAB1,NFIL3,NFKBIZ,NFRKB,NOTCH2,NRIP2,ODC1,OTUB1,PABPN1,PATZ1,PIM2,PLSCR1,PML,POLR2E,PRKAB1,PSEN2,PSMD10,PTGER2,PTGER4,RBBP6,RCAN1,RNF10,RXRB,SEC61A1,SET,SH3KBP1,SIAH1,SIN3A,SMAD1,SMAD5,SMARCA2,SMURF2,SNIP1,SOCS3,SOS1,SP140,SP2,SP3,SRA1,STK16,SUPT3H,TLE3,TNFSF8,TRERF1,TRIM13,VDR,VPS72,ZBP1,ZNF398,ZXDC
Lymphocyte homeostasis	8.0E-06	BHLHE40,CD83,CDKN1A,CIITA,E2F2,EEF1D,EGR2,EGR3,F5,FOXP1,HAVCR2,HGF,HIVEP2,ID2,IFNAR1,IKBKB,IL6ST,IRF4,ITGB2,JMJD6,MERTK,NAB1,NBR1,NF1,NFIL3,NOTCH2,PATZ1,PIM1,PRKCH,PTGER4,SOCS3,SP3,SPN,TNFSF8
Cell death of immune cells	8.5E-06	BTG1,CALR,CD44,CDKN1A,CIITA,CLU,CSNK2A2,E2F2,EGR3,FUS,HAVCR2,HGF,HLA-DRB4,ID2,IFNAR1,IKBKB,IL6R,IL6ST,IRF4,ITGB2,MERTK,MPO,MT1E,NAMPT,NF1,NFIL3,NLRC4,OSCAR,PIM1,PIM2,PSEN2,PTGER4,RCAN1,SH3KBP1,SLC2A1,SPN,TNFSF8,TPP2,VDR,XIAP
Differentiation of leukocytes	9.8E-06	BHLHE40,CD83,CDKN1A,CEBPD,CIITA,E2F2,EGR2,EGR3,FLI1,FOXP1,HAVCR2,HGF,HIVEP2,HMGB3,ID2,IFNAR1,IL6ST,IRF4,ITGB2,JMJD6,LILRA2,MERTK,N4BP2L2,NBR1,NF1,NFIL3,NLRC4,NOTCH2,NRP1,PATZ1,PLSCR1,PML,PRKCH,PTGER4,SOCS3,SP3,SPN,TNFSF8,VDR

Table 5.3 Ingenuity Pathway Analysis derived from classical monocytes from PDAC patients compared with healthy donors.

Molecules in each group are taken from the statistically significantly differentially expressed list of genes for classification (FC>2, *p* value<0.05). The DEGs were functionally annotated into the above pathways listed in the left column. The pathways are ranked by statistical significance in ascending order using *p*-values. *p*-values derived using Fishers exact test.

The results in Table 5.3 suggest that there is an enrichment of differentially expressing genes involved in transcription and the development (and death) of mononuclear lymphocytes, also viral infection and expression of RNA.

The identification of particular groups of genes can also be used to identify upstream regulators that may exert effects on that differentially expressed gene list. Table 5.4 shows stimuli that may induce expression of the differentially expressed genes as suggested by the literature based data in IPA comparing classical monocytes from PDAC patients with healthy volunteers.

Upstream Regulator	Molecule Type	Predicted Activation State	Activation z-score	p-value of overlap	Target molecules in dataset
IL3	cytokine	Activated	2.399	4.38E-07	CALR,CAMKK2,CD83,CDKN1A,CLEC4C,EGR2,EGR3,FUS,GART,HGF,ITGAX,ITGB7,MPO,MT1E,NRP1,ODC1,PIM1,PIM2,PLCB2,PSMB7,SLC2A1,SMAD5,SOCS3,TNFSF8
PDGF BB	complex	Activated	2.515	2.99E-06	ACTA2,ADARB1,ADM,ANXA11,BHLHE40,CD44,CDKN1A,DAB2,EGR2,EGR3,MT1E,NAMPT,NDUFB8,NFIL3,ODC1,PIM1,SLC25A11,SOCS3,SRSF7,THBD,TRIB1,UCK1
PURA	transcription regulator		1.067	9.47E-06	ACTA2,CDKN1A,DHFR,ITGAX,SPN
lipopolysaccharide	chemical drug	Activated	3.172	1.16E-05	ABCF2,ACTA2,ADM,APOBEC3F,ARF1,BTG1,CALR,CD44,CD83,CDKN1A,CEBPD,CIITA,CYBA,EGR2,EGR3,ENO1,FLI1,GLCCI1,HGF,HIVEP2,ID2,IFITM1,IFRD1,IL6R,IL6ST,IREB2,IRF4,ITGAX,ITGB2,ITGB7,KYNU,LRBA,LRRFIP1,MAFF,MARCO,MERTK,MPO,MT1E,MXD1,NAMPT,NFIL3,NFKBIZ,NOTCH2,NRP1,ODC1,PELI1,PIM1,PIM2,PLEKHA1,PLSCR1,PML,PTGER2,PTGER4,RCAN1,RXRB,SIRT3,SLC10A1,SLC2A1,SOCS3,STXBP2,THBD,TRIB1,VDR
5-N-ethylcarboxamido adenosine	chemical reagent	Activated	2.172	1.53E-05	ADM,BHLHE40,CEBPD,CIITA,EGR2,NFIL3,NRP1,ODC1,SOCS3,THBD,VDR
TNF	cytokine	Activated	3.117	1.70E-05	ADM,ANXA11,B4GALT1,BHLHE40,BTG1,BTRC,CALR,CARD16,CD44,CD83,CDKN1A,CEBPD,CIITA,CLU,CYBA,EGR2,GNL1,GSPT1,HGF,HLA-DRB4,IFITM1,IRF4,ITGB2,ITGB7,KCNJ2,KYNU,MAFF,MPO,MT1E,NAMPT,NFKBIZ,NFRKB,NOTCH2,NRP1,ODC1,PAFAH2,PIM1,PIM2,PLSCR1,PML,RANBP9,RCAN1,RGS14,RRM1,RXRB,SCO2,SIRT3,SLC10A1,SLC2A1,SMAD1,SMURF2,SOCS3,SP3,THBD,TNFSF8,TPP2,TXN2,UCK1,VDR,XIAP
NFATC2	transcription regulator		1.667	2.29E-05	ACPP,CDKN1A,DAB2,EGR2,EGR3,IRF4,MERTK,NFKBIZ,PELI1,PML,RCAN1,SOCS3,TNFSF8
MTOR	kinase		0.816	2.95E-05	ADM,CDKN1A,CEBPD,DHFR,ENO1,OTUB1,PFKP,PIM2,PPP2CA,RBM5,RXRB,SLC2A1,SOCS3,TPP2
TNFSF11	cytokine	Activated	2.879	3.66E-05	CD44,CD83,CDK14,CDKN1A,DAB2,EGR2,GRB10,IRF4,NFIL3,NFKBIZ,OSCAR,PIM1,PTGER2,PTGER4,RCAN1,SLC2A1,SOCS3

Table 5.4 Upstream regulators ~~identified which may affect expression of~~identified, which may affect expression of, identified differentially expressed genes in classical monocytes from PDAC patients compared with healthy donors.

Absolute z-scores are defined as significantly increased or decreased when <2 or >2 respectively. Z-score calculated by assessment of literature-based effect the transcriptional regulator (TR) has on downstream genes. Predicted activation state of the TR is given a positive value of 1 when downstream gene activated and -1 when the gene is inhibited. Upstream regulators are also ranked in order of significance by their ascending p-values,

Suggested upstream regulators identified in this cohort are based on experimental published data. The target molecules in this dataset had previously been shown in the literature to be downstream of the identified upstream regulators. The upstream regulators IL-3 and platelet derived growth factor (PDGF) were identified along with PURA, a transcription factor; lipopolysacccharide (LPS), expressed on bacterial cells walls; as well as Tumour Necrosis Factor (TNF); Nuclear Factor of activated T cells (NFAT); Mammalian target of rapamycin (MTOR) and TNF superfamily member 11 (TNFSF11, RANKL).

This is a diverse list of upstream regulators that may potentially affect gene expression in classical monocytes in PDAC. To understand this further, the top differentially expressed genes will be validated in a larger cohort of patients using qPCR. The validated genes will then be tested in experiments to determine whether soluble factors present in the blood of patients or produced by the tumour cells may affect the expression of these genes.

5.4 Summary

- Good quality RNA can be isolated from classical monocytes sorted from fresh peripheral blood.
- Transcriptome profiling reveals significantly differentially expressed genes in classical monocytes from patients with PDAC compared with those from healthy volunteers.
- Ingenuity Pathway Analysis points to functional enrichment of several pathways in classical monocytes from PDAC patients compared with healthy donors

5.5 Discussion

Microarray hybridisation of mRNAs is widely used to examine transcriptomes across many cell types, diseases and physiological conditions. The data obtained using this method gives a snapshot of the biological processes taking place at the time the samples are taken. The intention of this part of the investigation was to determine whether classical monocytes from healthy volunteers and PDAC patients could be differentiated by their gene expression profiles. Although there are no gross changes in classical monocyte abundance in the peripheral blood, the results show that there are a significant number of differentially expressed genes in classical monocytes from PDAC patients compared to healthy volunteers. Pathway analysis shows several pathways are affected in these monocytes, which suggests these differentially expressed genes may have some importance in monocyte differentiation and transcriptional regulation.

Careful consideration was given to the collection and processing of these samples at all stages of the process to ensure the sample quality and this will be discussed below.

5.5.1 Cell sorting and RNA isolation

FACS was chosen preferentially over the CD14 magnetic bead isolation for sorting of monocytes for gene expression analysis. This was due to the lack of subset definition using only $CD14^+$ as a marker, and the presence of magnetic particles on positively selected cells. These particles, although they are small, and do not activate the epitope, bind to the monocytes, and may interfere with magnetic purification steps in the whole transcriptome amplification procedure. To improve the cell viability of sorted cells a marker such as DAPI or propidium iodide could be used to remove dead cells.

The extraction of good quality RNA is crucial for downstream applications. Conventional methods used in the past isolate RNA by lysing the cells and separating nucleic acid from the debris. In the past this was carried out using guanidinium isothiocyanate-phenol-chloroform, as a single step technique where RNA is separated from DNA in aqueous phases. It was rapid but

laborious and generated waste chlorinated reagents. Advances in technology have led to simplified methods; an example is the use of magnetic particle separation, where nucleic acid in lysed samples incubated with magnetized cellulose particles is then separated using a magnetic field. However, this technique can also potentially result in contamination with magnetic particles.

An on-column solid phase method was used for isolation in this investigation, as it is reliable and rapid. The micro kit from Qiagen was chosen due to the expected cell numbers and RNA yield.

A cell number threshold was applied to ensure adequate amounts of RNA could be isolated from 50,000 sorted monocytes. All samples were amplified before hybridization using the Ovation whole transcriptome amplification (WTA) kit from Nugen. There are several published large-scale comparison studies to show that the Ovation whole transcriptome amplification procedure produces a consistent yield of cDNA regardless of RNA input. Early comparison studies identified correlation co-efficient comparing amplified and non-amplified material from as little as 25ng of RNA to be *r=0.97* from as little as 1ng (Dafforn et al., 2004, Vermeulen et al., 2009). Another study compared four methods using picograms of RNA. The Nugen Ovation whole transcriptome amplification protocol was shown to preserve differentially expressed genes without introducing any substantial bias, did not co-amplify any contaminating genomic DNA if it was present and the most reproducible across operators and labs (Clement-Ziza et al., 2009).

However, the above results are indicative only for RNA that is uncontaminated and has not been degraded. Consideration was given to the potential knowledge that contamination of samples with cellular proteins or lipids affects purity. Also, amplification of samples with poorer quality may not represent the sample accurately if fragmented. RNA integrity was calculated using the Agilent Bioanalyser and samples with a RIN higher than 8.5 were used for Gene Chips. This is preferable over the 18S to 28S ratio as it is based on the interpretation of an algorithm of the electropherogram

measuring nucleotides to account for the presence or absence of RNA degradation.

5.5.2 Gene expression analysis

These results show there are significant differences in the transcriptome profiles of classical monocytes isolated from PDAC patients compared to healthy volunteers. The gene array technique is widely accepted for the determination of differentially expressed genes and the resulting list can be used for subsequent pathway analysis. The gene expression results showed a total of 242 genes to be significantly increased ($p<0.05$ using unpaired t-test) in classical monocytes in pancreatic cancer compared with healthy volunteers and 280 to be significantly decreased by a fold change of more than $\log_2$.

Gene Chips are the most widely used and accepted method for large-scale gene expression profiling (Poukoulidou et al., 2011). However, they are limited to the genes represented on the array. The probes present on the chip can differ in their hybridisation properties and low abundance transcripts are potentially under-represented. This is not a major concern in this investigation as our interest is in known genes that are differentially regulated.

The Affymetrix Gene Chip U133 Plus 2.0 was chosen due its representation of a large number of genes and widespread use with consideration for cost, its reliability. The initial results gained in this project generated a large amount of data so careful consideration was given to data handling. With results from approximately 54,000 probes, the potential for detection of false positives is high. For this reason it was important to be very stringent in our analysis of differentially expressed genes. Dr Raphael Zollinger at Barts Cancer Institute and bioinformatician Dr John Prime at MedImmune were consulted to ensure appropriate analysis approaches were used.

A simple control in this type of analysis was to scramble the samples across groups and carry out the same investigation. Our analysis revealed no statistically significant differentially expressed genes, indicating that the identified DEGs were caused by the presence of the cancer.

Factors affecting gene expression in human cells have been widely discussed over the last few years. Specifically, for human peripheral blood cells, inter-subject variation can be affected by age, gender, ethnic background, nutritional status, metabolism and medical history (Fan and Hegde, 2005, Heimbeck et al., 2010). Intra-subject variation has also been investigated and can be affected by circadian rhythm and hormonal or diurnal variation or even exercise throughout the day (Whitney et al., 2003, Frankenberger et al., 2012). However, if genes demonstrate strong statistical regulation comparing PDAC patients with healthy then this supports further investigation into their biological relevance.

5.5.3 Developments in gene expression analysis

RNA-Seq is a sequence-based method used to gain a quantitative snapshot of the transcriptome. It has advantages over gene expression microarrays in that it can also detect levels of transcripts and their isoforms to glean information on post-transcriptional modifications and individual splice variants. Due to its higher sensitivity, RNA-Seq offers wide dynamic detection range and a better detection of low abundance transcripts. As it does not rely on probe set detection methods, there are no issues with hybridisation to contend with and the technical reproducibility has been shown to be high.

This technique, however, is relatively new and therefore still incurs a high cost, although this is reducing rapidly. There still remain logistical challenges in large amounts of data transfer, and technical challenges and the expertise required to analyse and interpret this type of data (Sokolova et al., 2014).

To compare and contrast the technologies, Beyer et al., used a monocyte to macrophage differentiation model and ran Affymetrix gene chips and RNA-Seq on the same samples. Their results showed high levels of correlation between microarray and RNA-Seq data for monocyte-derived macrophages. However they identified that the higher coverage provided by RNA-Seq was beneficial due to a better identification of transcripts identified as differentially expressed at a lower expression level (Beyer et al., 2012). Nevertheless this shows gene chip technology is still a valid approach for analysis.

5.5.4 Pathway analysis

Ingenuity Pathway Analysis (IPA) is an established knowledge base derived from several trustworthy and large sources. Annotations and functions are curated by scientists and combined from sources including EntrezGene, OMIM, GWAS, RefSeq and Gene Ontology making it one of the most comprehensive and widely used databases available.

The two most significant ($p<0.05$) pathways enriched in PDAC patients compared with healthy donors are transcription and the development of mononuclear leukocytes. This ~~is may~~ suggests that the presence of malignant disease may be inducing monocyte maturation or differentiation. This result could support the distribution changes observed in the flow cytometric profiling results demonstrated in the first results chapter.

Another enriched functional annotated pathway, identified by the IPA analysis, was the cell death of blood cell~~;~~, this is interesting as it may suggest there are factors produced in PDAC patients affecting monocyte apoptosis or it could point to the processing method inducing cell death. Viral infection was also noted as significant in the IPA analysis. Access to the clinical data for these patients suggests there was no other viral or bacterial infection at the time blood was taken. Monocytes are incredibly responsive and this may potentially suggest that monocytes may have been responding to a minor infection. This could also be the result of a general inflammatory response in PDAC patients, which is in line with data showing mainly inflammatory molecules as top hits in biomarker studies (Okada et al., 1998, Farrow and Evers, 2002, Groblewska et al., 2007, Moses et al., 2009).

IPA also can be utilised in identification of genes that have been identified to drive expression of certain genes or biological pathways. IL-3 is a haematopoietic cytokine with important roles in stem cell differentiation (Mroczko et al., 2005b) and is used *ex vivo* to expand enriched progenitor cells to generate sufficient numbers for transplant and abrogation of cytopenia (Filip et al., 2000). There is no current data on levels of IL-3 in PDAC patients.

Platelet derived growth factor (PDGF) plays a significant role in blood vessel formation. PDGF is increased in pancreatic tumours, cholangiocarcinomas and ampullary adenocarcinomas (Su et al., 2001, Fjallskog et al., 2007). PDGF circulates in the blood and may be of more relevance to investigate further in this patient cohort.

The data input into this IPA analysis for each gene was connected to its fold change comparing healthy versus cancer to improve functional annotations. However, when using IPA, it is important to consider that the pathways identified are based on experimental data that may not be relevant to the same cell type or condition. IPA also takes into account data from Affymetrix Gene Chip U133 2.0 plus as a standard reference set, which may bias statistical significance due to irrelevant genes that were not identified in my data set. It is reassuring however, that pathways associated with immune system were identified in immune cells under different conditions.

Other techniques available include Gene ontology, Biocarta, KEGG and Ingenuity Pathway analysis programmes that can be used to confirm these findings. For GSEA however, unlike other pathway analysis, the entire probe set list with all values is used, instead of only inputting the differentially expressed genes. This reduces the bias that may be introduced by the original method of differential gene expression determination.

Gene expression analysis inferences cannot be reliably made with a cohort of this size. To improve the statistical analysis it might be helpful to run Affymetrix gene chips for another batch of healthy volunteers and patients as a validation set to identify whether the top ranked differentially expressed genes are reproduced. The next chapter aims to validate the top differentially expressed genes in this cohort to gain further insight into the biological relevance.

Chapter 6. Validation of identified differentially expressed genes.

6.1 Introduction

Top statistically significant differentially expressed genes (DEGs) and pathways were identified in the previous chapter by comparing transcriptomic profiles from classical monocytes isolated from healthy volunteers compared with PDAC patients. These were defined as the genes with the highest ranked fold change comparing mean log fluorescence intensity.

To confirm the significance of the identified genes, targeted gene expression analysis was carried out in the same samples to compare results across both platforms, and also in a validation cohort of new healthy volunteers and PDAC patients.

Classical monocytes were sorted and RNA isolated from a further 9 healthy volunteers and 13 PDAC patients to validate targeted gene expression using quantitative polymerase chain reaction (qPCR). This technique is used for gene expression validation and will help determine whether the genes identified as significantly differentially expressed are upheld in a larger sample cohort, which will also increase statistical power.

The top DEG from the Affymetrix data set was RAS guanine-nucleotide exchange factor 1B (*RASGEF1B*). This family of nucleotide exchange factors stimulates intrinsic activity of GDP/GTP exchange to promote the formation of active Ras-GTP. *RASGEF1B* is a responsive gene to bacterial infections via interactions via toll like receptors 2,3 and 4 (Ferreira *et al*., 2002).

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EGR2 and *EGR3* are Zinc finger nuclear transcription factors with various roles in development and immune regulation (Li et al., 2012). Early growth response family members are essential for development of many cell types, particularly neural development (Svaren and Meijer, 2008) and activation of B and T cells, (Kishore et al., 2002, Shi et al., 2002, Droin et al., 2003, Park et al., 2007, Kearney et al., 2013)

CD83 is a cell surface Ig family member glycoprotein, expressed on B and T cells although it is better known for being a marker of mature monocyte derived dendritic cells. In pancreatic cancer, culturing human healthy dendritic cells with plasma from PDAC patients was shown to induce CD83 gene expression. This was linked to a more mature antigen-presenting phenotype, with a better ability to generate specific T cells against the pancreatic cancer CEA antigen (Kalady et al., 2004). The number of $CD83^+$ cells in the stromal compartment of pancreatic cancer is significantly increased compared to healthy pancreas tissue (Tjomsland et al., 2011). The presence of this gene could potentially be indicative of dendritic cell contamination or potential evidence of skewing towards a more dendritic like maturation phenotype of the monocytes in pancreatic cancer.

~~Another interesting gene identified is~~ Macrophage receptor collagenous structure gene (*MARCO*), codes for a macrophage scavenger receptor as identified to be increased in monocytes after challenge with endotoxins (Talwar et al., 2006). ~~It~~ MARCO is highly homologous with Scavenger receptor A (SR-A), a deficiency in which on macrophages in co-culture models has been shown to inhibit tumour cell migration and ~~has been~~is recognised as a potential target for novel therapy in cancer treatment (Neyen et al., 2013, Getts et al., 2014)

Folate Receptor 1 (*FOLR1*) gene is a member of the folate receptor family that binds folic acid and its derivatives. It has been identified alongside *FOLR2* and *FOLR3* as down regulated in monocytes from patients with early onset coronary artery disease (Sivapalaratnam *et al*., 2012). Expression of folate receptor 2 on myeloid cells is a common characteristic on 'M2' alternatively activated macrophage phenotype. Folate receptor 1 has been identified as a target to reduce macrophage infiltration in gliblastoma multiforme, but there is no current evidence for FOLR1 in alternative activation (Puig-Kroger *et al*., 2009).

Cytochrome B-245 alpha polypeptide (*CYBA*) has again been implicated in monocytes in cardiovascular disease. A negative association between this gene and superoxide producing NADPH oxidase release from peripheral blood mononuclear cells is thought to impact on leukocyte survival and adhesion ability (Macias-Reyes et al., 2008, Moreno et al., 2011).

The top differentially expressed genes that are confirmed in this manner will then be investigated further for protein expression in the respective monocyte subsets by ~~immunohistochemistry.~~qPCR.

6.2 Aims

The aim~~s~~ of this chapter is~~are~~:

- To confirm significantly differentially expressed genes by quantitative PCR in classical monocytes from PDAC patients and healthy volunteers.
- ~~To test qPCR validated targets further at protein level using immunohistochemistry of primary classical monocytes from PDAC patients or healthy volunteers.~~

6.3 Results

6.3.1 qPCR validation of top differentially genes defined by Affymetrix analysis.

In the previous chapter, gene expression analysis was carried out on classical monocytes from healthy donors and PDAC patients and the top DEGs in each group were identified. Table 6.1 displays the genes probed for validation using qPCR. Interestingly, some of these genes have been previously implicated in myeloid cells function and biology, as discussed later in this chapter.

Fold change in expression is calculated using the mean value for PDAC patients divided by the mean value of the healthy volunteers for each gene. Statistical significance is calculated using an unpaired students t-test ($p<0.05$).

Gene symbol	Gene name	Log_2 of Fold change	*p-value*
RASGEF1B	Guanine nucleotide exchange factor for Ras family proteins	5.0368	0.004
EGR3	Early Growth Response-3	4.5812	0.0022
EGR2	Early Growth Response-2	3.2138	0.0240
CD83	Cell surface marker expressed on monocyte derived dendritic cells	2.8984	0.0374
MARCO	Macrophage Receptor with Collagenous Structure	1.7149	0.0288
FOLR1	Folate receptor 1	-2.7	0.00581
CYBA	Cytochrome B-245	-2.397	0.0071

Table 6.1 Top differentially expressed genes as ranked using fold change increase or decrease in PDAC patients compared with healthy volunteers.

The top ranked statistically significant DEGs that were chosen for validation using qPCR in the same samples and in a larger cohort.

The top DEGs were assessed using qPCR in the original samples that were used for the Affymetrix analysis. Prior to the Affymetrix analysis, a small aliquot of RNA was reserved from the sample for this subsequent qPCR analysis. This aliquoted RNA sample was stored at -80°C and was not subject to the pre-amplification procedure required for Affymetrix hybridization onto the gene chips. The intention behind maintaining un-amplified samples was to compare gene expression platforms, and to ensure differential gene expression is not affected by the amplification procedure.

Figure 6.1 below, the black bars represent the Affymetrix fluorescence expression values (log_2) and the white bars represent Delta Ct values for each gene as calculated by subtracting the Ct value of the sample gene from the Ct value of the reference gene. Figure 6.1 below shows the genes that

were significantly increased in classical monocytes from PDAC patients compared with healthy volunteers.

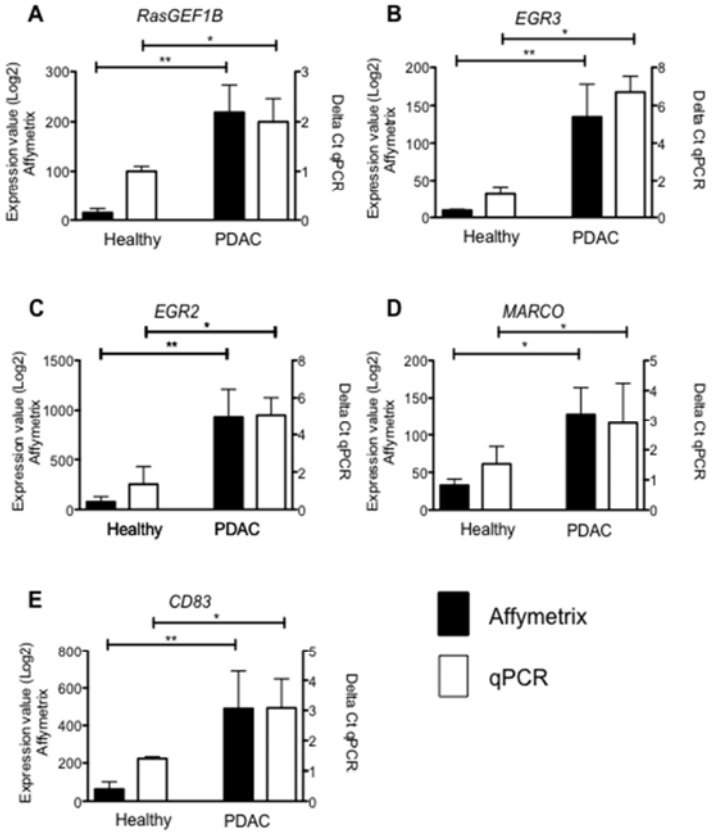

Figure 6.16.1 Comparison between Affymetrix fluorescence intensity values with Delta Ct values from qPCR in statistically significant DEGs that are increased in PDAC patients compared with healthy volunteers.

Expression values for Affymetrix log_2 probe fluorescence intensities are plotted as black bars (mean +/- SD) to be read on the left y-axis; Delta Ct qPCR values (Ct RPL34 reference gene minus Ct gene of interest) are plotted as white bars (mean +/- SD) to be read on the y-axis on the right side. Data represent the same number of healthy volunteers (n=3) and PDAC patients (n=5) for both gene expression platforms. *p< 0.05 (*), p<0.01 (**).*

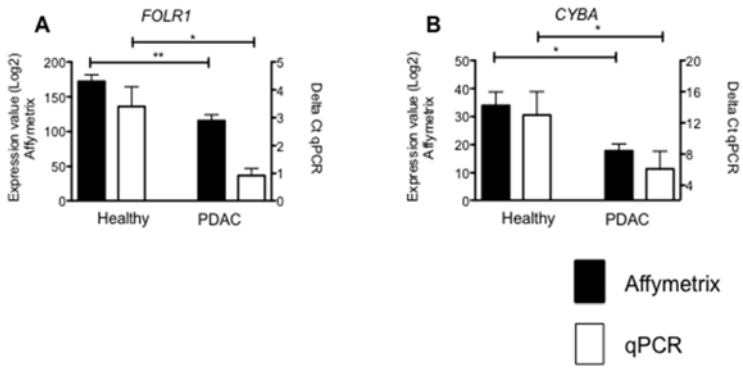

Figure 6.26.2 Figure Comparison between Affymetrix fluorescence intensity values with Delta Ct values from qPCR in statistically significant DEGs that decrease in PDAC patients compared with healthy volunteers.

Expression values for Affymetrix log_2 probe fluorescence intensities are plotted as black bars (mean +/- SD) referring to the left y-axis; Delta Ct qPCR values (Ct RPL34 reference gene minus Ct gene of interest) are plotted as white bars on the y-axis on the right side. Data represent the same number of healthy volunteers (n=3) and PDAC patients (n=5) for both gene expression platforms. *p< 0.05 (*), p<0.01 (**).*

Analysis using qPCR validated and confirmed DEG expression determined by Affymetrix in this cohort. This indicates the gene expression results across the platforms are comparable and the whole transcriptome amplification procedure used in the gene chip preparation has had no adverse effects on these differentially expressed genes.

6.3.2 Gene expression of the top differentially expressed genes in an independent validation cohort.

6.3.2.1 Classical monocytes

The aim of this validation experiment is to determine whether the statistically significant gene expression differences are maintained in a larger cohort of patients. This analysis will also increase subject numbers and therefore statistical power.

Top DEGs identified by Affymetrix gene expression analysis were tested using qPCR. The original sample set as analysed by Affymetrix (n=3 healthy, n=5 PDAC) was used, as well as an additional validation cohort (n= 5 healthy and n=8 PDAC). The results below in Figure 6.3 show the Delta Ct values of the new cohort of PDAC patients (n=8) and healthy (n=5) volunteers combined with the original Affymetrix samples.

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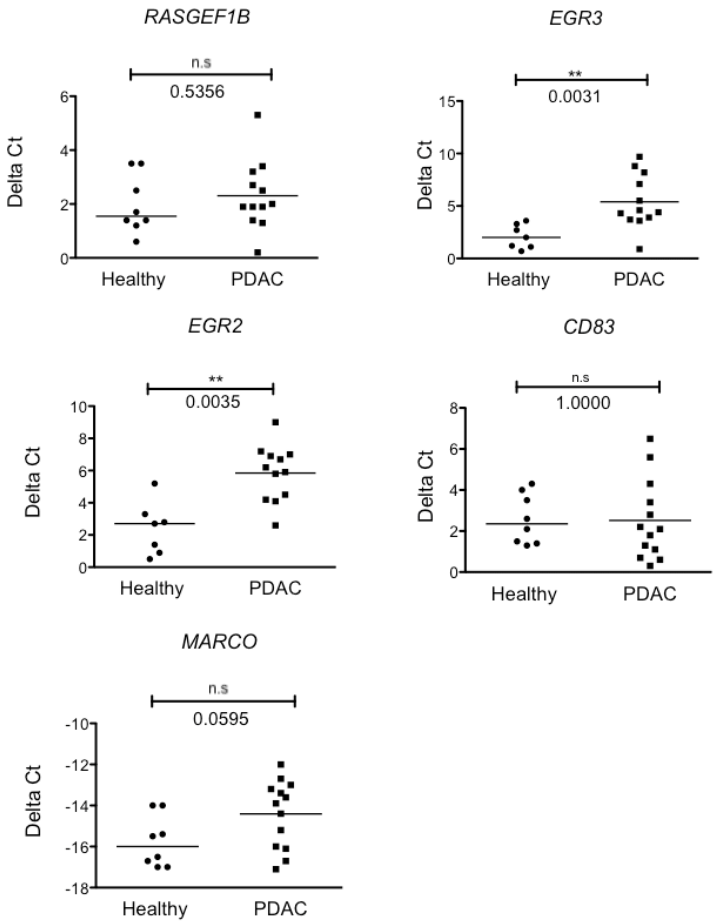

Figure 6.3~~6.3~~ Gene expression profiles of classical monocytes from PDAC compared to healthy monocytes using qPCR for identified differentially expressed genes that increase in PDAC.

RNA was isolated from sorted classical monocytes from the combined cohorts (n=8 healthy volunteers; n=13 PDAC patients). The analysis shows statistical significance is maintained for *EGR2* and *EGR3 gene expression* only. Delta Ct values calculated by (Ct *RPL34*)-(Ct gene of interest). Data shown as a dot plot to represent individual gene expression values for health volunteer versus PDAC sample. The horizontal line represents median value. Statistical significance was calculated using Mann Whitney *(p*-values are <0.05).

Next, I analysed the genes that showed a decrease in expression in the Affymetrix analysis. Figure 6.4 outlines the qPCR gene expression results of these genes. Neither *FOLR1* nor *CYBA* were differentially expressed between healthy volunteers and PDAC in this combined cohort.

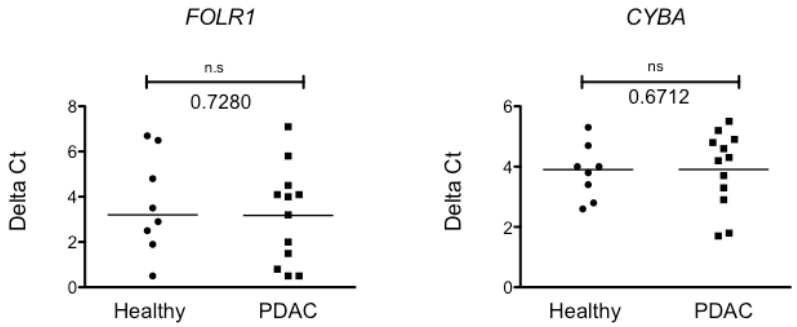

Figure 6.46.4 Gene expression profiles of classical monocytes from PDAC compared to monocytes from healthy volunteers using qPCR for identified differentially expressed genes that increase in PDAC.

RNA was isolated from sorted classical monocytes from healthy volunteers (n=8) and PDAC patients (n=13). Delta Ct values calculated by (Ct *RPL34*)-(Ct gene of interest). Data shown compares individual donors, horizontal line expresses median value and statistical significance calculated using Mann Whitney (significant *p*-values are <0.05).

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In summary, my validation results show that several of the top DEGs as identified by Affymetrix and validated by qPCR did not maintain significance in a larger cohort. The expression of Early Growth Response 2 gene *(EGR2)* and Early Growth Response 3 gene *(EGR3)* were increased in classical monocytes from PDAC patients compared with healthy donors and therefore warrant further investigation.

6.3.2.2 Non-classical monocytes

To identify whether the gene expression changes represent an exclusive event in the most prevalent classical monocyte population, or is a characteristic of other patient monocyte populations, I carried out a qPCR for *EGR2* and *EGR3* expression on the corresponding non-classical monocyte populations from the same individuals.

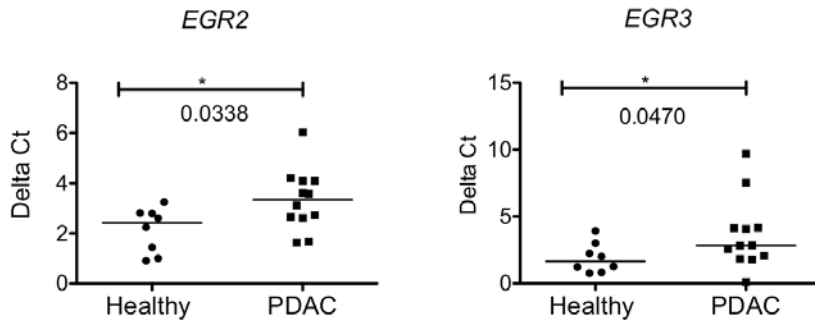

Figure 6.56.5 Gene expression measured using qPCR in the non-classical CD14^{dim} CD16^{++} monocyte population.

RNA was isolated from sorted non-classical monocytes (CD14^{dim} CD16^{++}). The cells were obtained from the same healthy volunteers and PDAC patients as the classical monocytes used above for qPCR analysis. Delta Ct values were calculated by (Ct reference gene *(RPL34)* minus Ct gene of interest). Data shown represents individual donor (n=8 healthy and n=13 PDAC), horizontal lines represent the median value and statistical significance calculated using Mann Whitney *(p-<0.05*).

Although the statistical significance is not as strong as in the classical monocyte subset, qPCR shows a statistically significant increase in EGR2 and EGR3 gene expression in the non-classical monocyte population. The data suggests the increase is not subset specific and potentially represents a general (systemic) myeloid response to PDAC.

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6.4 Summary

- Gene expression results are comparable for DEGs using qPCR and Affymetrix Gene Chip technologies
- Gene expression for Zinc finger transcription factor family members *EGR2* and *EGR3* were confirmed to be significantly differentially upregulated in a larger cohort of PDAC patients and healthy volunteers in classical $CD14^{++}$ $CD16^{-}$
- Gene expression for Zinc finger transcription factor family members *EGR2* and *EGR3 are also* significantly differentially upregulated in non-classical $CD14^{dim}$ $CD16^{++}$ monocytes

6.5 Discussion

The primary aims of this section were to validate the Affymetrix results by qPCR, compare expression amongst a larger cohort and finally use measures to identify if mRNA expression translates into protein expression.

Of the top DEGs determined using Affymetrix analysis in classical monocytes, only two genes *EGR2* and *EGR3* were confirmed as significantly differentially expressed in the validation sample cohort. In addition, the analysis also revealed that EGR2 and EGR3 are not exclusively up-regulated in classical monocytes but also in the non-classical $CD14^{dim}$ $CD16^{++}$ monocyte population.

However, the changes evaluated on gene expression level could be validated by immunohistochemistry. Whilst healthy volunteers do not show any EGR2 or EGR3 expression, increased expression of EGR2 and EGR3 in classical monocytes PDAC patients could be demonstrated. This is a novel finding that has not been identified in the literature in any myeloid cell type and cancer.

6.5.1 Gene expression validation

Genes chosen for validation using qPCR showed the highest ranked fold change in the original cohort of patients, consisting of 3 healthy volunteers and 5 PDAC patients. qPCR validation for target genes is widely used (Chen et al., 2010, Wong et al., 2011, Wu et al., 2013, Italiani et al., 2014). qPCR offers the advantage of a focused screen across a large cohort of samples.

Taqman offers microfluidics cards that can be customised with a limited number of targeted known genes to be run in the same sample. These are useful because the sample volume required is very small for a single experiment, the results are generated very quickly and the amplification system is efficient and sensitive.

Bridging the middle ground between qPCR and large-scale microarrays is the NanoString nCounter Gene expression array. This works using a colour bar-coded system to digitally detect target specific probe pairs. The expression level of the gene is assessed by counting the coloured bar codes (Kulkarni, 2011). Compared to gene expression microarrays, the NanoString technology might be favourable as it is highly reproducible, does not require pre-amplification and can be used for small amounts of RNA however it is less representative of the whole transcriptome due to the targeted nature of the assay restricting the number of genes (Ullal et al., 2014) .

6.5.2 Evaluation of identified targets

The results presented here showed that the gene expression trends for the top DEGs in the same samples were maintained across platforms and cDNA synthesis protocols, indicating they are comparable. However, all but two of the genes were not sustained as statistically significant in the independent cohort. This could be due to several factors, in particular the sample size and heterogeneity between patients. Gene expression as mentioned before can be affected by many variables within or inter patients. Gene expression in immune cells in the peripheral blood could be affected by a plethora of factors from minor infection (Wu et al., 2013), cardiovascular disease (Woollard and Geissmann, 2010, Sivapalaratnam et al., 2012) to exercise (Frankenberger et al., 2012) or diet and obesity (Gil et al., 2007).

These genes all have interesting roles in immunity and cancer however, apart from *EGR2* and *EGR3* they were not confirmed to be increased in the classical monocytes from PDAC patients compared with healthy donors in the combined cohort. qPCR primers were used from Applied Biosystems (Life Technologies) as they have the best coverage of the gene of interest. The company website provides for each primer the interrogated gene sequence to allow comparisons with the Affymetrix GeneChip probe sets.

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Protein expression is a more direct measure of biological processes and potentially more reflective of gene function than mRNA. However, transcriptional and post-transcriptional regulation, processing, differential splicing, stability and regulatory elements, translation and protein modifications all have the ability to modulate protein expression (Gautier et al., 2012).

In circulating monocytes, a study was carried out to determine the correlation between mRNA and protein expression from Affymetrix gene chips to Mass Spectrometry (Guo et al., 2008). The results showed an overall significant positive correlation between expression of genes and the relative protein on

average. Studying both mRNA and gene expression is important and complementary and in combination can provide a more comprehensive understanding on gene function. To improve this analysis, quantification of positive cells comparing PDAC patients with healthy volunteers would need to be carried out. To further this, intracellular flow cytometry could be considered for quantifying or potentially the Image Stream Platform to visualise fluorescent staining.

To investigate this further it would be helpful to examine whether the transcription factors are activated, this could be done by using nuclear translocation with immunofluorescence as a measure of activation, comparing monocytes from PDAC patients with healthy volunteers.

6.5.3 Relevance of early growth responses in myeloid cells in cancer

It is thought that EGRs may be important transcriptional regulators in myeloid cells. In early stage development of myeloid progenitors, *EGR2* was shown to repress genes associated with neutrophil differentiation and to promote expression of genes associated with macrophage development such as *PU.1* and *CEBP*. These are also able to induce expression of STAT3 and SOCS1, which are important to cytokine secretion and proliferation (Bradley et al., 2008). These genes were not statistically significantly increased in the PDAC cohort compared to healthy using Affymetrix.

Using a transcriptomic regulatory network (TRN) profiling map combined with gene expression data, Suzuki *et al*., identified EGR2 as a core transcription factor in human $CD14^{+}$ monocytes (Suzuki et al., 2012). EGR2 can positively regulated by SP1, CEBPA, and IRF8, key transcription factors in macrophage development and polarisation in physiological differentiation and disease (Lawrence and Natoli, 2011). Friedman also showed this in earlier work in 2002, hypothesising that EGR family members complex with the co-repressor Nab to maintain myeloid lineage fidelity (Friedman, 2002).

In parallel to my data Drexhage *et al*. demonstrated an increase in EGR3 expression in classical monocytes from patients with schizophrenia (Drexhage et al., 2010). This potentially indicates that under stress, such as disease or cancer, monocytes do respond locally and this can be assessed systemically.

The same group published the essential role of *EGR3* as a transcription factor due to its binding ability to the promoter region of Triggering Receptor Expressed on Myeloid cells -1 (*TREM-1*). This binding was confirmed using Chromatin Immunoprecipitation (ChIP) and is thought to play a role in the ability of the monocytes to produces pro-inflammatory cytokines (Weigelt et al., 2011). *TREM-1* activation induces expression of pro-inflammatory cytokines IL-8 and TNF-a (Golovkin et al., 2013), which were up-regulated in the blood of PDAC patients in the previous section 4.3.4. This expression has shown to be highest on the classical monocyte population compared to the intermediate and non-classical. (Poukoulidou et al., 2011)

A study was carried out by Xue *et al*., this ~~year which~~year that profiled the diversity of myeloid cell phenotypic responsiveness using a range of different stimuli, *EGR3* was identified as part of a core transcriptional network in IL-4 stimulated macrophages (Xue et al., 2014).

Increased expression of *EGR2* and *EGR3* was also observed in non – classical $CD14^{dim}$ $CD16^{++}$ monocytes from the same patients. According to recent fate mapping work by Geissmann *et al*., as discussed in section 1.2.1.4~~X~~, it is thought that the classical monocytes form a pool of circulating progenitor cells for the development of the more mature non-classical monocyte population. It is possible that the expression is maintained through development into non-classical monocytes, dendritic cells or macrophages. As a further experiment, these monocytes could be differentiated into macrophages in culture to assess whether the expression is maintained during differentiation.

The literature above mentions that *EGR2* and *EGR3* up-regulated under diverse and varied conditions, such as schizophrenia. As a consequence, the up-regulation may not be specific to cancer but may potentially be a general inflammatory reaction. It would be important to investigate whether the transcription factors are also increased in patients with chronic pancreatitis or other inflammatory conditions and infections.

EGR2 and *EGR3* gene ~~and protein~~ expression levels were significantly lower or non-existent in monocytes from healthy donors compared with PDAC patients. This suggests that the presence of PDAC and its associated inflammation is triggering an increase in expression of these transcription factors that may induce functional effects downstream, which will be examined in the next chapter.

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Chapter 7. *EGR2* and *EGR3* gene expression induction in classical monocytes from healthy volunteers.

7.1 Introduction

In the previous chapter, *EGR2* and *EGR3* were confirmed as showing significantly increased gene expression in classical monocytes from PDAC patients compared with those from healthy volunteers. The lack of expression of these transcription factors in healthy volunteers suggests they may play a role in monocyte responses to the presence of pancreatic cancer; or represent a general stress response. However without further investigation these roles cannot be explored. Chronic smouldering inflammation produced by or in response to pancreatic cancer is reported to produce cytokines that recruit monocytes and promote macrophage differentiation in the tumour microenvironment (Balkwill et al., 2005, Sanford et al., 2013).

A key regulator of monocyte to macrophage differentiation is macrophage colony simulating factor (also known as M-CSF or CSF1) (Lutter et al., 2008). The corresponding CSF1 receptor (CSF1R) is expressed on macrophages, and, when phosphorylated promotes survival and differentiation (Chitu and Stanley, 2006). M-CSF has also been shown to promote myeloid cell recruitment to the tumour microenvironment (Sweet and Hume, 2003, Geissmann et al., 2010a, MacDonald et al., 2010, Hume and MacDonald, 2012). Inhibition of M-CSF in mouse models of pancreatic cancer reduces tumour infiltrating macrophage populations and improves responses to chemotherapy (Mitchem et al., 2012). M-CSF is increased in the serum of PDAC patients compared with healthy donors (Mroczko et al., 2005a) and therefore represents a valid target for further investigation.

Although there is very little available literature on the role of the Early Growth Response (EGR) family in monocytes, it is suggested that in haematopoietic progenitors, these transcription factors are important in directing differentiation towards the myeloid lineage (Krishnaraju et al., 1995, Gibbs et al., 2008). Studies on these cells have shown early growth response gene expression to be very responsive to M-CSF. The addition of M-CSF to the progenitor cells increased expression of early growth response genes 1,2

and 3, this expression was associated with improved survival and promotion of differentiation (Bradley et al., 2008).

EGR3 was also identified in gene expression microarray data in patients with schizophrenia (Drexhage et al., 2010). Recognised as an inflammatory condition (Muller et al., 2012, Sommer et al., 2014). *EGR3* was also significantly increased in classical monocytes from these patients compared with healthy volunteers. Drexhage *et al*, went on to report that *EGR3* binds to the promoter region of Triggering Receptor Expressed on Myeloid cells (*TREM1*) using Chromatin Immunoprecipitation (Weigelt et al., 2011).

Egr2 in murine myeloid cells has been associated with binding to the FIRE promoter element of the *csf1r* gene, implicating it further in monocyte to macrophage differentiation (Sauter et al., 2013). Gene expression data published by Martinez et al.(2009) also showed *EGR2* to be significantly increased in human 'M2' like macrophages, indicating it may play a role in alternative macrophage activation (Martinez et al., 2009). ~~Tumour-associated macrophages~~ TAMs in PDAC are thought to represent macrophages with a more 'M2' like phenotype (Sica et al., 2006, Kurahara et al., 2011).

The aim of this chapter is to explore the signalling cascade that may lie behind increased expression of *EGR2* and *EGR3* in classical monocytes from PDAC patients.

7.2 Aims:

The aim of this chapter is:

- To explore the M-CSF signalling cascade and it's effect on gene expression of *EGR2* and *EGR3* in classical monocytes isolated from healthy donors

7.3 Results

7.3.1 Early growth response gene expression can be induced in healthy monocytes by the addition of human recombinant M-CSF in a dose and time dependent manner

Monocytes were isolated from healthy volunteer fresh peripheral blood using Miltenyi MACS separation columns and plated in serum free media. Monocytes were then stimulated with 100ng/ml recombinant M-CSF over a short time course 90 minutes before collection for RNA isolation and gene expression analysis using qPCR.

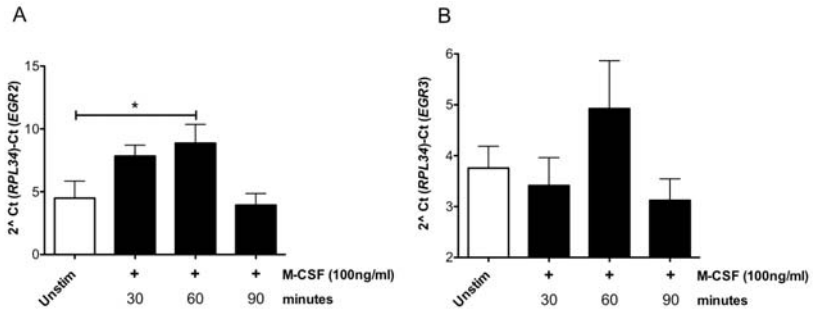

Figure 7.1~~7.1~~ qPCR gene expression of M-CSF treated classical monocytes over time.

RNA was isolated from 2x10^6 classical monocytes freshly isolated from MACS~~s~~ separation columns and ~~with~~ treatment with recombinant M-CSF (100ng/ml) over time. A) *EGR2* gene expression and B) *EGR3* gene expression relative to housekeeping gene *RPL34* using the Taqman system. Data is representative of four individual healthy donors in individual experiments and bars represent the mean and SEM. *p*-values calculated using students t-test and significant results $p<0.05$.

The addition of 100ng/ml M-CSF to healthy monocytes *in vitro* leads to statistically significant increase in *EGR2* and an increase *EGR3* expression

within 60 minutes. These results show the levels return to baseline however after 90 minutes.

7.3.2 Early growth response gene expression induction is dependent on MEK/ERK signalling at gene expression

It was also shown in haematopoietic progenitor cells that M-CSF induction was dependenting on MEK/ERK signalling. To test this in monocytes I used a specific MEK-1,2 inhibitor from Sigma (U0126).

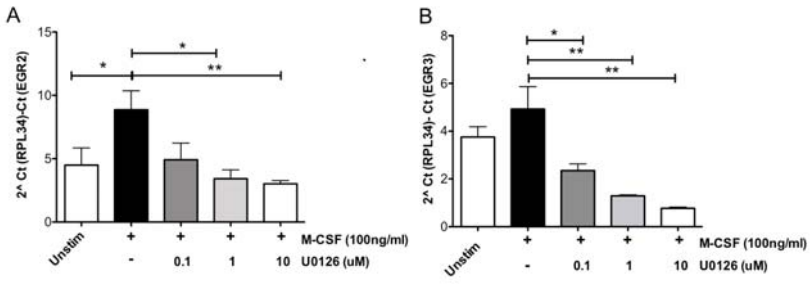

Figure 7.27.2 Specific MEK inhibition of M-CSF induced *EGR2* and *EGR3* after 60 minutes

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RNA was isolated from 2x10^6 classical monocytes freshly isolated from MACSs separation columns and ~~with~~ treatment with recombinant M-CSF (100ng/ml) alone or in combination with a MEK/ERK (Sigma) inhibitor U0126 over time. A) *EGR2* gene expression and B) *EGR3* gene expression relative to housekeeping gene *RPL34* was assessed using the Taqman system. Data is represented by three individual healthy donors in individual experiments and bars represent the mean and SEM. *p*-values calculated using students t-test and significant results $p<0.05$.

These results show a significant dose dependent inhibition of *EGR2* expression by MEK Inhibition of M-CSF induction in CD14$^+$ monocytes. *EGR3* is not significantly up-regulated by addition of M-CSF however constitutive expression can be reduced using the MEK-1,-2 inhibitor suggesting expression may also be downstream of the same pathway.

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7.3.3 Downstream targets of Early Growth Response genes 2 and 3

In order to determine whether activation of EGR2 and EGR3 affects downstream targets. Healthy monocytes were isolated and treated with recombinant M-CSF [100ng/ml] over 60 minutes with or without the presence of the MEK inhibitor U0126 (10μM). Primers were purchased to carry out qPCR for *TREM1*, thought to be a target of *EGR3,* and *CSF1R*, a known target of *EGR2* (Sauter et al., 2013).

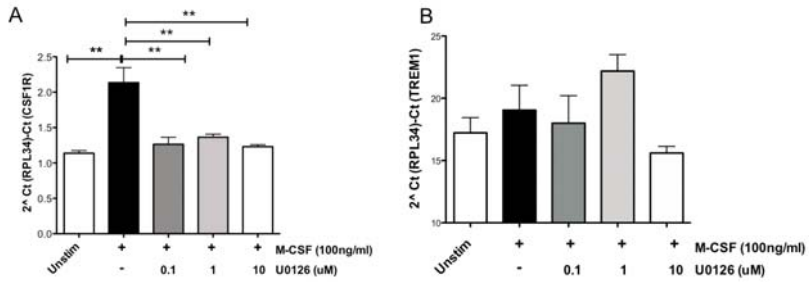

Figure 7.3~~7.3~~ Gene expression of *CSF1R* and *TREM1* in M-CSF stimulated healthy monocytes

RNA was isolated from 2x10^6 classical monocytes freshly isolated from MACS~~s~~ separation columns and ~~with~~ treatment with recombinant M-CSF (100ng/ml) alone or in combination treatment with Sigma U0126 MEK/ERK inhibitor after 60 minutes. A) *CSF1R* gene expression and B) *TREM1* gene expression relative to housekeeping gene *RPL34* using the Taqman system. Data is expressed as the mean value of three individual healthy donors in individual experiments and bars represent the mean and SEM. *p*-values calculated using students t-test and significant results $p<0.05$.

These results show increased expression of *CSF1R* with the addition of M-CSF and a decrease with the addition of the MEK inhibitor at all concentrations. This is not reflected in the results for TREM1 expression.

7.4 Summary

To summarise:

- Haematopoietic growth factor M-CSF can induce expression of *EGR2* and *EGR3* in healthy $CD14^+$ monocytes
- Induction of induced *EGR2* and constitutive *EGR3* expression can be inhibited by the use of a specific MEK-1,-2 inhibitor
- CSF1R could be downstream of M-CSF/MEK/EGR2 pathway

7.5 Discussion

This chapter aims to investigate the interaction between early growth response activation, upstream regulation and downstream targets. My first approach was to investigate the currently available literature on early growth responses and myeloid cells. Although there is very little published on the roles of EGR2 and EGR3 in monocytes, exploration of other myeloid cell literature suggests M-CSF-1 may be of interest.

Monocytes from four individual donors were plated in serum free media before treatment with M-CSF over time and at different concentrations. The results show an increase in expression of the early growth response genes, peaking at 60 minutes with a concentration of 100ng/ml of M-CSF. Previous work by Carter et al., and Bradley et al., suggest that induction of EGR expression using rM-CSF occurs at around 60 minutes and then expression levels drop and stay reduced over a longer time period of 24 hours (Carter and Tourtellotte, 2007, Bradley et al., 2008).

Early growth responses in myeloid cells can also be induced by GM-CSF and IL-3 and are thought to be maintained from monocyte to macrophage differentiation (Carter and Tourtellotte, 2007). IL-3 was also identified in results section 5.5.2 as an upstream regulator by Ingenuity Pathway Analysis for the top differentially expressed genes derived from the Affymetrix GeneChip arrays. There is no current data on IL-3 elevated expression in PDAC.

In haematopoietic progenitor cells, it was also reported that induction of *EGR2* and *EGR3* expression is potentially downstream of MEK/ERK signalling. Cytokine induced myeloid differentiation is also reported to be dependent on the MEK/ERK pathway (Miranda et al., 2005). To test this in monocytes, monocytes were incubated with 100ng M-CSF in the presence of a highly specific MEK-1,2 inhibitor (U0126 Sigma) in escalating doses. The results of this demonstrated that the increase in expression induced by M-

CSF could be inhibited. This was also shown by Bradley et al., over the same time course in haematopoietic progenitors (Bradley et al., 2008).

The link between MEK signalling and *EGR* expression was described only this year by Thiel *et al*. The *EGR* family promoters contain five individual serum response elements (SREs). These function as binding sites for the serum response factor and the ternary complex factor Elk-1. When Elk-1 is phosphorylated by MEK signalling, this leads to an increase in DNA binding activity of the ternary complex formation to the EGR family promoters and induces activation of the transcription factor (Thiel et al., 2014).

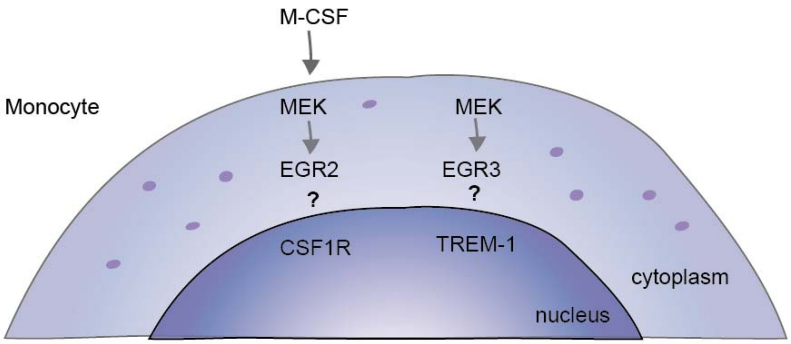

Figure 7.4~~7.4~~ Monocytes EGR2 and EGR3 expression is downstream of MEK signalling

Addition of ~~m-csf~~M-CSF to monocytes causes a significant increase in EGR2 but not EGR3 expression. Addition of a MEK inhibitor to monocytes with M-CSF abrogates the increase expression of EGR2 and reduces EGR3 expression below baseline. Studies suggest CSF1R gene is downstream of EGR2 activation and TREM-1 downstream of EGR3.

CSF1R and *TREM1* ~~are~~ were thought to be downstream targets of *EGR2* and *EGR3* respectively. These were examined at gene expression level by qPCR in the M-CSF and U0126 treated monocytes, CSF1R but not TREM-1 was ~~but were not~~ shown to be dependent on early growth responses with M-CSF stimulation. This may be due to the short time course used to measure

the increase in expression of the downstream effects and a longer time course should have been used. To determine over a range from 90 minutes up to 24 hours. Downstream target investigation in this project is hampered by the lack of a specific early growth response inhibitor; therefore any observed effects could not be linked directly to expression. To overcome this, small interfering RNA could be used to silence expression of EGRs in monocytes and the experiment repeated.

To improve these results it would be beneficial to look at M-CSF activation over a longer time course and it would be vital to confirm the expression of early growth responses with M-CSF stimulation and specific MEK inhibition at the protein level. Phosphorylation of ERK could also be investigated using western blot or flow cytometry.

Immunofluorescence or cellular fractionation western blotting would help to determine if the transcription factors have undergone nuclear translocation and are therefore activated. Chromatin Immunoprecipitation (ChIP) would be a useful tool to determine specific transcription factor binding to target genes.

One of the challenges faced in interpretation of this data, and the relevance to PDAC, is that stimulation of monocytes with a single cytokine does not recapitulate the plethora of growth factors and signals produced by the tumour and surrounding stroma.

Culturing monocytes in the presence of pancreatic cell line supernatant will allow investigation into whether the responses can be induced specifically in conjunction with soluble factors produced by the tumour cell lines themselves.

One has to choose the respective cell lines carefully as Miapaca 2 for example is well described in the literature to overexpress M-CSF (Fung et al., 1976). A range of cell lines representing primary and metastatic sites could be considered. Although this experimental design might provide a

simplified model of activation, it would not take into consideration the factors and signalling molecules produced by the numerous infiltrating immune cells in PDAC tumours. One possibility would be to incubate healthy and PDAC monocytes with plasma from healthy volunteers and PDAC patients.

Additionally, it would be insightful to measure functional aspects of monocyte activation or suppression under these circumstances by assessing factors such as pro-inflammatory cytokine production or phagocytic ability.

Chapter 8. Monocytes in the peripheral blood as a reflection of myeloid cells within the tumour microenvironment in PDAC.

8.1 Introduction

Monocytes are an attractive potential target for therapy due to their accessibility, relative plasticity and their potential contribution to development of tumour-associated macrophages. Once in the tumour, monocytes can further differentiate to macrophages, which can support development of an immunosuppressive microenvironment.

Macrophages are part of the mononuclear phagocyte system, recruited almost entirely from circulating classical monocytes in the peripheral blood (Gordon and Taylor, 2005). Macrophages are often found in the stromal compartment of solid tumours including breast, ovarian, pancreatic and hepatocellular carcinomas (McGettrick et al., 2012, Feig et al., 2012, Wu and Zheng, 2012, Ruffell et al., 2012). Unlike lymphocytes, they are reported to be less abundant on the periphery of the tumour microenvironment (Feig et al., 2013, Watt and Kocher, 2013). ~~Tumour associated macrophage~~TAM infiltration in PDAC has been reported to promote a poorer outcome by influencing invasion, suppression of immune responses and promotion of chemoresistance (Beatty et al., 2011, Mitchem et al., 2012, Sica and Mantovani, 2012, Sanford et al., 2013).

One of the challenges in targeting tumour associated myeloid cells is a lack of tumour specific markers that differentiate between macrophages in the tumour microenvironment and healthy functional macrophages in the rest of the body.

Carter and Tourtellotte (2007) investigating *Egr1-4* expression in differentiation in mice suggested that myeloid cells maintain expression of *Egr1, 2* and *3* into macrophage development (Carter and Tourtellotte, 2007). The aim of this chapter is to identify whether *EGR2* and *EGR3* expression in peripheral blood monocytes, as identified by the Affymetrix analysis, reflects the expression pattern in the myeloid compartment within the human PDAC tumour microenvironment.

8.2 Aims:

- To examine expression of EGR2 and EGR3 in normal healthy pancreas compared with PDAC tissue
- To identify whether this staining co-localises with a marker of tumour infiltrating macrophages
- To utilise available gene expression data to identify if there is differential expression of *EGR2* or *EGR3* in pancreatic ductal adenocarcinoma and whether expression can be correlated with survival.

8.3 Results

8.3.1 EGR expression on myeloid cells in the PDAC tumour microenvironment

To further investigate specific macrophage expression of the Early Growth Response (EGR) genes, qualitative expression of EGR2, EGR3 and CD68, a pan-macrophage marker, were assessed using fluorescence-conjugated antibodies on formalin fixed paraffin embedded PDAC tissue. Analysis was focused on the stromal compartment of the tumour as this is where most macrophage infiltrate can be expected.

Figure 8.1 below shows the appropriate isotype control antibodies for CD68 (mouse IgG) and for the EGR2 and EGR3 antibodies (rabbit IgG) in tissue from a patient with pancreatic ductal adenocarcinoma.

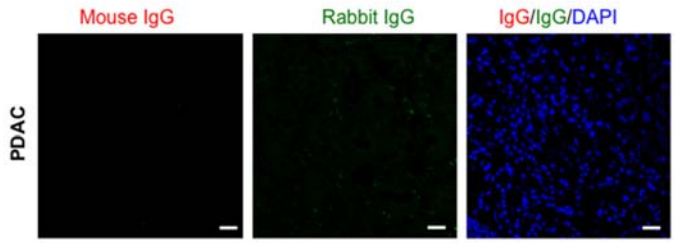

Figure 8.1 Isotype control staining in PDAC tissue

Appropriate isotype controls for rabbit IgG (green) and mouse IgG (red) were used to stain formalin fixed paraffin embedded tissue. DAPI was used for nuclear visualisation. Antigen retrieval was carried out for 9 minutes using Tris/EDTA buffer. Fluorescence conjugated antibodies. Anti-mouse IgG secondary conjugated 546 red, anti-rabbit IgG secondary conjugated 488 green. Magnification (X40). Scale bar represents 25μM.

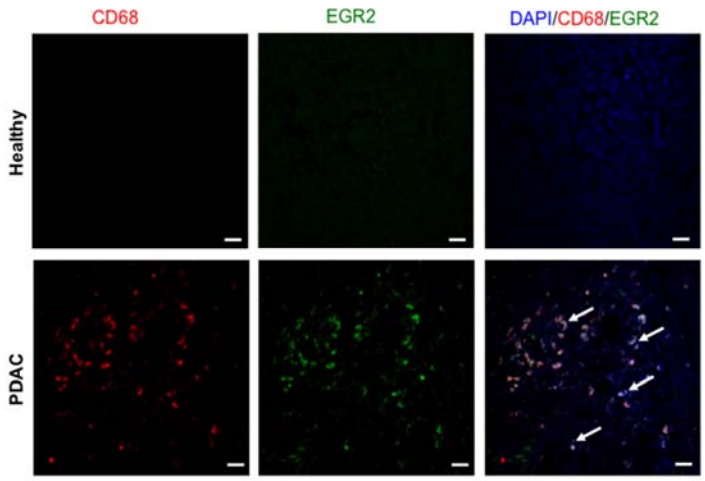

Figure 8.28.2 EGR2 co-localises with CD68 expression: confocal immunofluorescence staining of EGR2 (green) and CD68 (red) in PDAC tissue

FFPE embedded PDAC tissue was stained for CD68 (Abcam 1:40) and EGR2 (Thermo Pierce 1:2500) ~~in FFPE PDAC tissue~~. Tris/EDTA Antigen retrieval (9 minutes). Anti-mouse CD68 secondary conjugated 546 red, anti-rabbit EGR2 secondary conjugated 488 green. DAPI was used for nuclear visualisation. Image representative of PDAC patients (n=3) Magnification (X40) Scale bar represents 25μM. Images taken using LSM 510 confocal microscope. White arrows indicate several of the CD68+ cells that also express EGR2. Co-localisation appears as yellow. The picture shows that CD68 co-localises with EGR2 expression. There is no other positive staining for EGR2 detectable in the PDAC tissue.

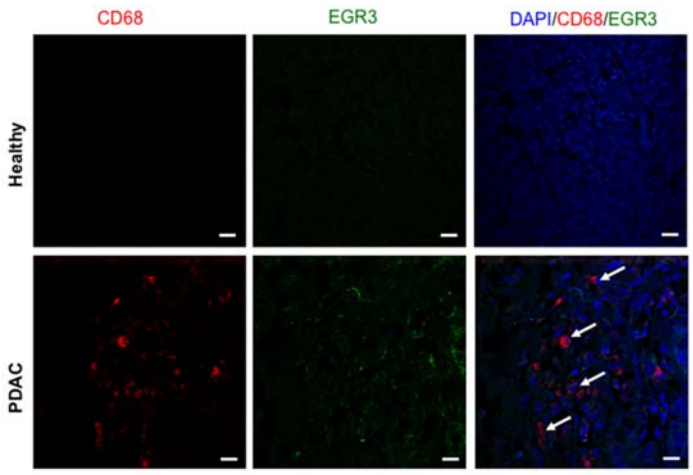

Figure 8.38.3 EGR3 does not co-localise with CD68: confocal immunofluorescence staining of EGR3 (green) and CD68 (red) in PDAC tissue

CD68 (Abcam 1:40) EGR3 (Thermo Pierce 1:2500) in FFPE PDAC tissue. Anti-mouse CD68 secondary conjugated 546 red, anti-rabbit EGR3 secondary conjugated 488 green. DAPI was used for nuclear visualisation. Tris/EDTA Antigen retrieval (9 minutes). Image representative of PDAC patients (n=3) Magnification (X40) Scale bar represents 25μM. Images taken using LSM 510 confocal microscope. White arrows indicate several of the CD68+ cells. It appears that few cells stain positive for EGR3 but this staining does not co-localise with CD68 expression.

These representative images in Figures 8.2 and 8.3 show positive expression and co-localization of EGR2 in $CD68^{+}$ cells in the PDAC tumour microenvironment from three patients and three ~~healthy donors~~tumour adjacent normal sections. The absence of colocalisation between CD68 and EGR3 suggests that EGR3 is not expressed on tumour-associated macrophages in PDAC. Staining in healthy pancreatic tissue revealed no CD68 positive ~~positive~~ cell infiltration.

8.3.2 Early growth response expression in human pancreatic cancer tissue using gene expression microarray data from the Pancreatic Expression Database

The aim of this section is to understand whether gene expression in monocytes can be used as a predictor for gene expression in the tumour microenvironment. The pancreatic expression database (PED) was created in 2007; at Barts Cancer Institute and is continuously updated. It is the largest collection of pancreatic data available for mining. Data is collected and stored from publicly available resources on pancreatic genomic, proteomic, microRNA, transcriptomic and methylomic profiles allowing users to search and compile their own analysis using the corresponding clinical data. The advantages of this type of data mining allow very specific research questions to be applied to the available data to obtain a focused, interpretable, annotated output.

Gene expression of *EGR2* and *EGR3* were examined in 644 samples from the Barts PED representing bulk tissue, micro-dissected tumour, adjacent healthy pancreas or pancreatic ductal adenocarcinoma.

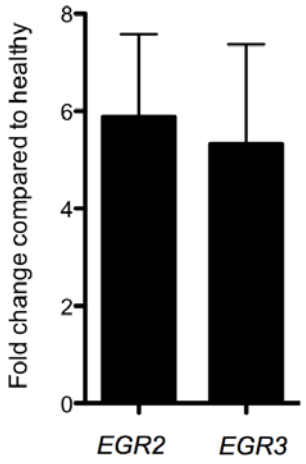

Figure 8.48.4 Gene expression results from 644 patients in the Pancreatic Expression Database

Gene expression results calculated as mean fold change PDAC tissue divided by gene expression in normal healthy resected pancreatic tissue (n=644). Error bars represent standard deviation.

The results showed a significant increase in both *EGR2* and *EGR3* expression in PDAC ($p<0.05$) with fold changes of 5.88 and 5.33 respectively.

In collaboration with Dr Jianmin Wu at the Kinghorn Cancer Centre, University of New South Wales, expression of *EGR2* and *EGR3* were assessed in Andrew Biankin's dataset published in Nature in 2012 (Biankin et al., 2012). This dataset accrued 159 clinical samples of pancreatic ductal adenocarcinoma without previous chemotherapy treatment. Biankin *et al*., carried out a detailed analysis identifying mutations, copy-number variations and core signalling pathways in that may be involved in pancreatic cancer development.

Figure 8.5 below represents the distribution of *EGR2* and *EGR3* gene expression over the 142 samples.

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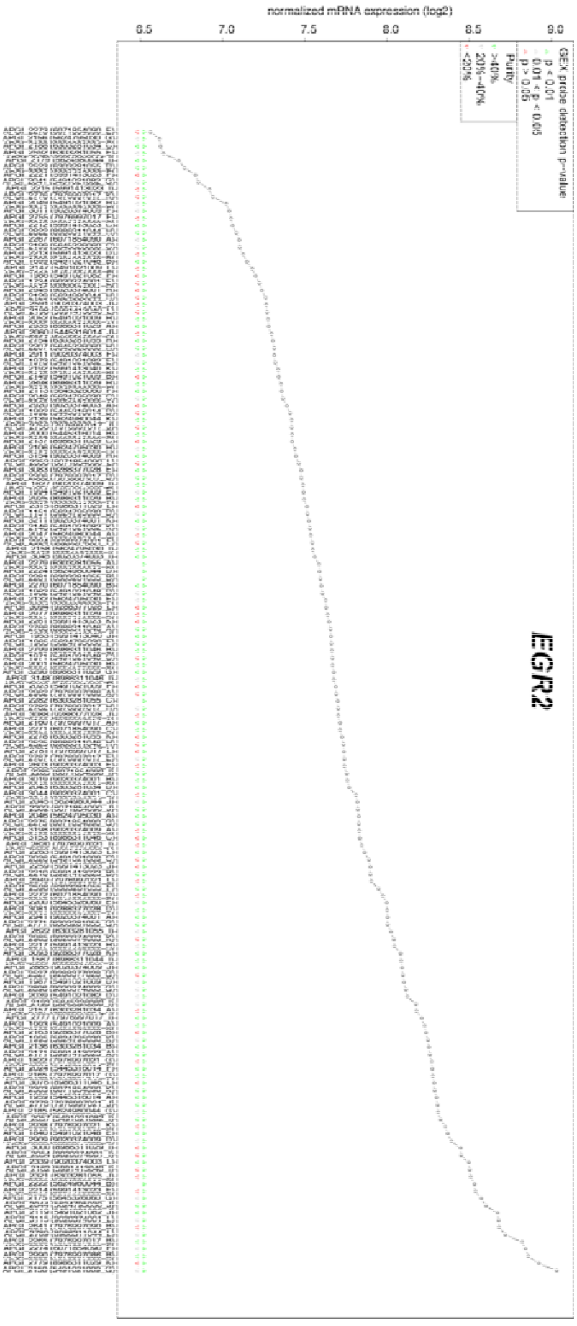

normalized mRNA expression (log2)
6.5
7.0
7.5
8.0
8.5
9.0
CEX probe deletion p-value
p < 0.01
0.01 < p < 0.05
p > 0.05
Purity
>40%
20%–40%
<20%
EGR2

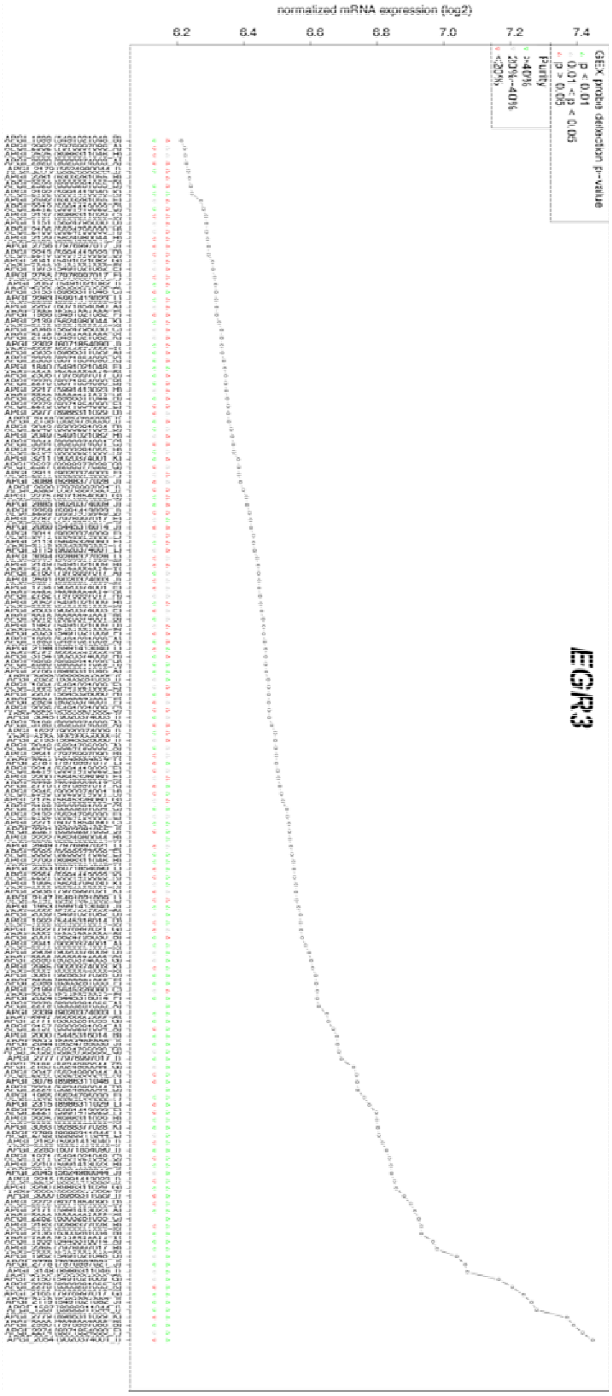
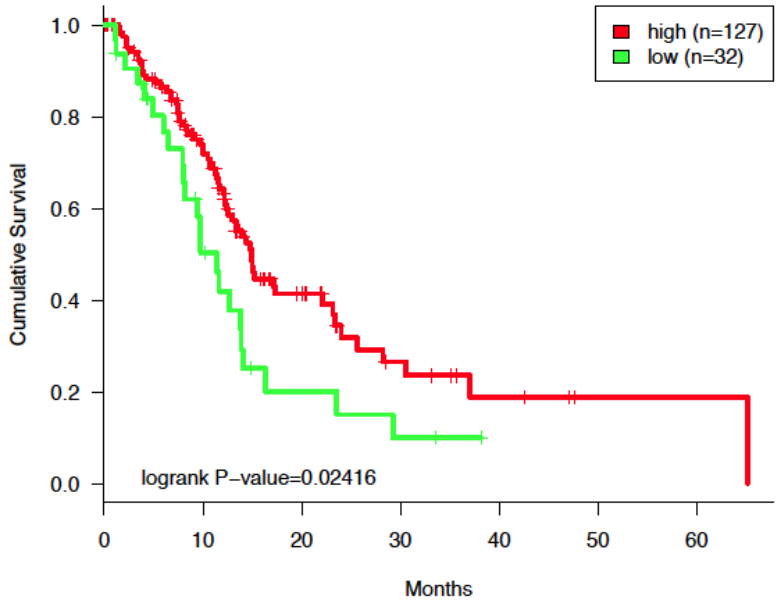

normalized mRNA expression (log2)
8.2
8.4
8.6
8.8
7.0
7.2
7.4
EGR3

Figure 8.58.5 Expression distribution of *EGR2* and *EGR3* expression in pancreatic cancer patients

Gene expression from whole tumour samples. GEX probe detection p-values were represented by coloured triangles in the row on the right. Green triangle indicates the p-values are lower than 0.01, the grey triangle indicates p-values are between 0.01 and 0.05. The red triangle indicates the p values are higher than 0.05 and therefore non-significant. To inform cellularity thresholds and define the impact of stromal DNA content, Biankin et al sequenced different mixtures of germline DNA and cancer cell line and used these to compare sample purity. The purity of the samples is also represented by triangles in the left column. Green triangle indicates the sample was >40%, the grey triangle represents 20-40% and the red triangle <20%.

The expression range for *EGR2* is broad and the log2 transformed intensities range from 6.5-9. In contrast, *EGR3* shows a smaller range of expression values. *EGR2* and *EGR3* were not identified as mutated genes in this cohort. A range of cut offs used were used to define high and low expression from 10% low and 90% high, 15% v. 85%, 20% v. 80%, 25% v. 80%, 30% v. 70%, 40% v. 60% and 50% v. 50% high and low respectively.

Three endpoints were tested: Disease free survival (DFS), the length of time after a primary treatment the patient is symptom free; Overall Survival (OS), the time to death from any cause; and Disease Specific Survival (DSS,) time to death caused by pancreatic cancer. No clear associations were seen between high and low expression of *EGR2* or *EGR3* and DSS or OS.


high (n=127)
low (n=32)
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
logrank P−value=0.02416
0
10
20
30
40
50
60
Months

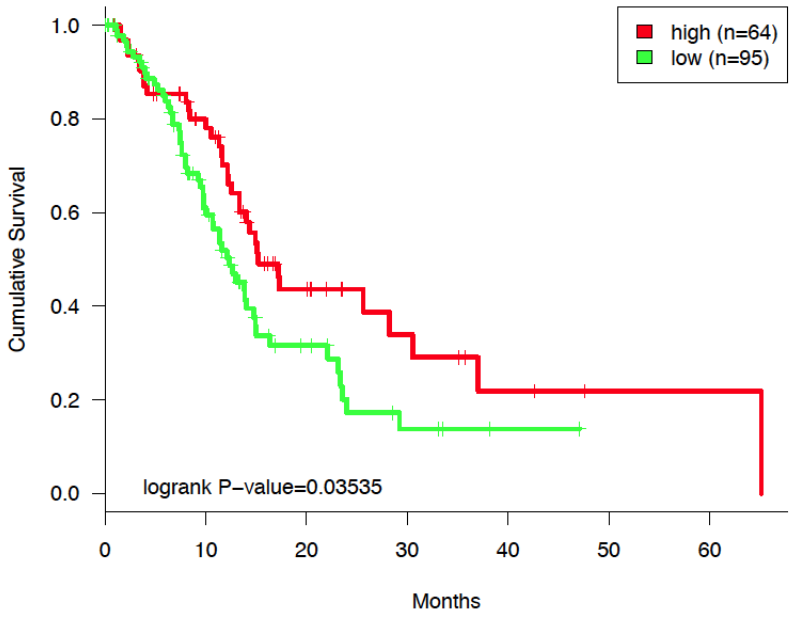

high (n=64)
low (n=95)
Cumulative Survival
1.0
0.8
0.6
0.4
0.2
0.0
logrank P−value=0.03535
0
10
20
30
40
50
60
Months

Figure 8.68.6 Kaplan-Meier curve *EGR2* expression and disease free survival.

Clinical data is derived from Biankin et al., (Biankin et al., 2012). The X-axis represents cumulative survival and the y-axis survival time in months. The red line represents patients expressing higher levels of *EGR2* compared with the green line representing lower expression. Logrank *p=<0.05* considered statistically significant. Figure representative of 159 patients.

EGR2 expression was significantly associated with DFS. The figure above shows the Kaplan Meier curve for *EGR2* expression and disease free survival using a cut off value of 40%20% low expression and 860% high expression, and demonstrates association between EGR2 expression and later relapse.

8.3.3 Early growth response genes 2 and 3 expression in murine model of PDAC

Colleagues in the Hagemann laboratory (Dr. Juliana Candido and Dr. Raphael Zollinger) are working on gene expression in a spontaneous well-characterised murine model of pancreatic cancer. The 'KPC' model is a genetically engineered murine model of pancreatic cancer, developed by Hingorani *et al*., to faithfully recapitulate the spontaneous development of pancreatic intraepithelial neoplastic lesions that progress into ductal adenocarcinoma (Hingorani et al., 2003).

The KPC model is derived by using a Cre recombinase under the control of the Pdx1 promoter, a gene only expressed in the pancreatic cells of endodermal origin. The mutations induced using a cre-lox system induce a mutated Kras (G12D) and P53 (R172H). This model is clinically relevant to human pancreatic cancer as the tumours have a dense stromal infiltrate and a moderately differentiated morphology.

'Clinical' symptoms observed in the KPC model are cachexia, peritoneal ascites and metastatic spread (Olive et al., 2009). This model is widely used in pancreatic cancer research in pre clinical models of drug development and has vastly improved the understanding of mechanisms behind disease pathogenesis (Courtin et al., 2013). Affymetrix GeneChip microarrays (Mouse 1.0) were used to profile gene expression.

8.3.3.1 PDAC tissue

To investigate whether *Egr2* and *Egr3* gene expression is affected in tumour lysates from KPC model compared with wild type. Affymetrix fluorescence intensity values were plotted for normal pancreas and untreated PDAC tissue~~. Figure X below indicated~~ as indicated below in Figure 8.7.

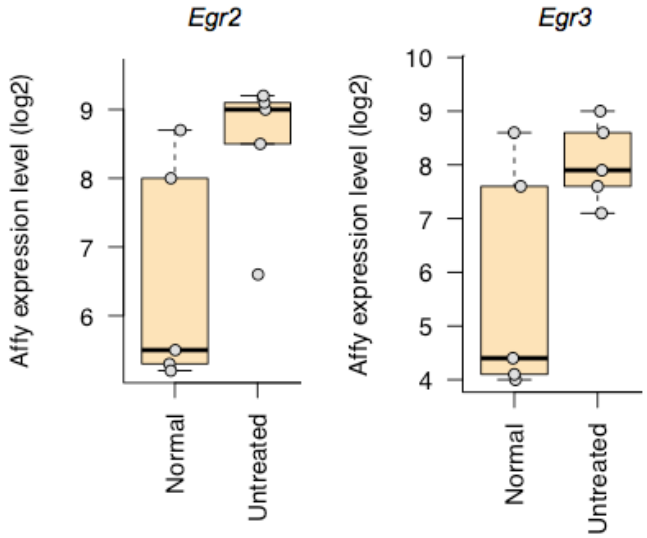


Figure 8.7~~8.7~~ *Egr2* and *Egr3* gene expression in normal murine pancreas compared with KPC tumour bearing pancreatic tissue.

Affymetrix fluorescence intensity values represent Gene expression of *Egr2* and *Egr3* in whole pancreas lysates from normal wild type mice and untreated KPC pancreatic tumours. Y-axis represents Affymetrix fluorescence intensity values and X-axis sample origin. Each circle represents an individual mouse

The results show an increase in gene expression of *Egr2* and *Egr3* in pancreatic cancer tumour tissue compared with healthy normal pancreas from wild type mouse.

8.3.3.2 Sorted cell populations

To identify the source of expression changes in the microenvironment the myeloid cells (CD11b positive), other leukocytes (CD45 positive) and tumour cells (EpCam positive) were sorted and microarray profiling used to examine gene expression.

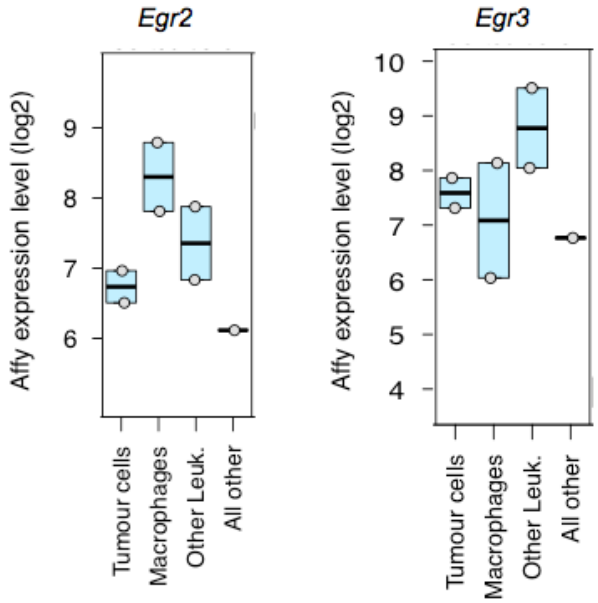


Figure 8.8~~8.8~~ Gene expression of *Egr2* and *Egr3* in sorted cell populations from KPC tumour bearing pancreas tissue lysates.

Gene expression of *Egr2* and *Egr3* in tumour cells (EpCam$^+$), macrophages (CD11b$^+$), other leukocytes (CD45$^+$) and all other cells sorted from whole pancreas tumours from KPC mice. Y-axis represents Affymetrix fluorescence intensity values and X-axis sample origin. Each circle represents an individual mouse.

~~The result showed that *Egr2* in the PDAC tumour microenvironment from KPC mice is more highly expressed in the macrophages compared to the tumour cells and other leukocytes. *Egr3* however, is expressed more highly in the other leukocytes.~~

8.4 Summary

- Expression of EGR2 but not EGR3 was observed on tumour infiltrating macrophages in pancreatic ductal adenocarcinoma
- Gene expression analysis from whole pancreatic tumour tissues identifies a significant correlation between *EGR2* expression in the tissue and improved disease free survival.

8.5 Discussion

The aims of this chapter are to determine whether the expression of *EGR2* and *EGR3* genes in monocytes are maintained in the tumour microenvironment in PDAC. Positive correlation of the expression patterns could strengthen the hypothesis that peripheral monocytes could potentially be used as a source of markers to assess the innate compartment in tumours.

It is known that macrophage infiltration into the tumour microenvironment increases in pancreatitis and pancreatic cancer (Amit and Gil, 2013, Liu et al., 2013, Puolakkainen et al., 2014). However, there are no published data on EGR2 or EGR3 immunostaining in the human pancreas. In order to optimise the staining protocols for the antibodies effectively, classical monocytes from healthy volunteers and pancreatic cancer patients were ~~used~~ employed as negative and positive controls, respectively. These were then fixed and embedded in the same manner as the tissue. The antibody concentration that showed differential staining of cancer to healthy monocytes was chosen.

The normal pancreatic tissue sections used in this project were isolated from patients undergoing surgical resection and therefore represent tumour–adjacent normal tissue. To identify whether macrophages in the PDAC tumour microenvironment express EGR2 or EGR3, fluorescence-conjugated secondary antibodies we employed to stain for EGR2 or EGR3 in combination with nuclear stain DAPI and pan-macrophage marker CD68. Lack of staining showed that EGR3 is not present in the tumour microenvironment. However, positive staining of EGR2 in the macrophage population suggests it is maintained during differentiation and within the tumour.

It is well known that ~~macrophages~~ TAMs infiltrate the tumour at an early stage in tumour development and this has been reported to correlate with

outcome (Yoshikawa et al., 2012, Sugimoto et al., 2014). To improve these results it would be beneficial to utilize a marker of alternatively activated macrophages, for example, CD163 or CD204, which are commonly used to define activation state (Kurahara et al., 2011), because it has been reported that CD68 can sometimes be expressed on fibroblast cells within the breast cancer microenvironment (Denardo et al., 2011).

To further investigate the relevance of EGR2 or EGR3 expression in pancreatic cancer I collaborated with Dr Jun Wang here at Barts Cancer Institute to utilise the Pancreatic Expression database. The results showed a significant increase in expression of EGR2 and EGR3 in PDAC patients compared with healthy volunteers.

In order to determine whether the increase in expression could be used in translational research, in collaboration with Dr Jianmin Wu at the University of New South Wales in Australia gene expression of *EGR2* and *EGR3* were assessed in the data set published by Andrew Biankin (Biankin et al., 2012). This set contains survival and gene expression data from 159 whole tumour lysates from untreated PDAC patient who underwent resection surgery with curative intent. The results showed a significant correlation with disease free survival with *EGR2* expression. Disease free survival is the length of time after the primary treatment that the patient survives without any symptoms and no disease or tumour progression can be detected.

The patients with ~~higher~~ increased *EGR2* expression have a longer disease free survival period before relapse, suggesting expression may have a protective effect. This is opposite of what we expected to see if the EGR2 expression correlated with increased tumour infiltrating macrophages that promote tumour progression.

There was, however, no significant association of expression of either *EGR2* or *EGR3* when assessing DSS or OS. These patients however, were

surgically resectable suggesting a lack of local invasion or metastases and therefore an earlier stage of detection.

This type of analysis used lysates from whole tissue tumour biopsies that represents a heterogeneous cell population; this may introduce bias in gene expression depending on leukocyte infiltration or the prevalence of stromal component in each biopsy.

I then went on to examine whether gene expression would be affected in a murine model of pancreatic cancer. Colleagues in my lab isolated samples from sorted cell populations within the tumour as well as whole tissue lysates. The aim of this investigation is to determine which cell types in the tumour have higher expression. From analysis of the gene expression data, it is clear that the expression of both *EGR2* and *EGR3* is higher in the pancreatic cancer group ($p<0.05$).

Additionally, tumours isolated from the KPC mice and cell populations sorted by flow cytometry provided tumour cells (as defined by their expression of EpCam), tumour associated myeloid cells (CD11b) and 'other' leukocyte (CD45) cell compartment. The results show that *Egr2* is higher expressed in tumour infiltrating myeloid cells and *Egr3* expression can be attributed to other leukocytes. This data supports my immunofluorescent staining (Figure 8.2) showing that EGR2 is expressed on the CD68 positive cells within the tumour microenvironment.

Prior to the current understanding that macrophage polarisation results in a spectrum of phenotypes, transcriptomic classification was based around two polar activation states known as classically activated 'M1' and alternatively activated 'M2'. Using these two classifications, it was reported that tumour associated macrophages bear a more similar phenotype to the alternatively activated or more 'M2' like macrophage (Sica et al., 2006). However it is now widely known that this classification is slightly over-simplified due to the plasticity in macrophage responses to environmental signals.

Martinez (Martinez, 2009) published data reporting gene expression changes in human monocyte to macrophage differentiation, which was a seminal article in identification of key regulatory genes in this process. $CD14^+$ monocytes were isolated and macrophage differentiation was polarized towards two well characterised macrophage phenotypes: classically activated 'M1' macrophages (stimulation with LPS and interferon gamma) or 'M2' (using IL-4 and IL-13). The results showed that they too had identified EGR2 in the 'M2' macrophage phenotype.

These results suggest that EGR2 expression associated with alternative macrophage activation is maintained in macrophages in the pancreatic tumour microenvironment. EGR3 staining was identified on few cells within the PDAC tissue, however this may be due to expression coming another cell type (Li et al., 2011).

Chapter 9. Discussion

Monocyte mobilisation from the peripheral blood has been reported to be associated increased tumour associated macrophage recruitment to the site of the primary tumour in pancreatic cancer and with a poorer outcome ~~and~~ (Mitchem et al., 2012, Steele et al., 2013, Sanford et al., 2013).

In several solid cancer types, including breast and pancreatic cancer, Monocytes have been shown to promote growth, facilitate metastasis and chemoresistance in pre clinical models of murine mammary and pancreatic cancer (Beatty et al., 2011, Brower, 2012, Amit and Gil, 2013, Deschenes-Simard et al., 2013) as well as humans (Watt and Kocher, 2013, Shibuya et al., 2014). Inhibition of myeloid recruitment to tumours is also a proposed therapeutic strategy in reducing TAM infiltration and tumour volume (Leuschner et al., 2011, Mitchem et al., 2012, Sanford et al., 2013).

My project aimed to investigate monocytes in pancreatic cancer by assessing the effects of the presence of pancreatic ductal adenocarcinoma on gene expression in classical monocytes. The hypothesis being tested was that systemic changes due to the presence of the tumour alter monocyte phenotype, which can be used as a marker of disease presence and potentially predict outcome or therapeutic response. As tumours show an enormous amount of genetic heterogeneity and plasticity, the more stable genome of stromal cells or immune cells would be far better suited for predictive analysis and potential target~~t~~ting.

The classical monocytes form the most abundant population in the peripheral blood and are selectively recruited to the tumour microenvironment by CCR2/CCL2 interactions. It is therefore of interest to determine if the monocytes are ‘primed’ by the presence of the tumour and whether any differences could be utilised as prognostic markers or valid targets for therapy.

Patients were carefully chosen, and all had locally advanced stage III/IV confirmed pancreatic ductal adenocarcinoma. In human studies, when

working with primary cells, patient heterogeneity cannot be avoided but can be reduced by careful matching and exclusion of patient characteristics. Patients can also be excluded from the study retrospectively if their initial diagnosis had changed. Inclusion of patients who were suitable for resection or patients undergoing chemotherapy was avoided, in order to remove as much variability as possible. It was also necessary to match healthy volunteers by age for gene expression, to ensure that there was no bias being introduced by this characteristic.

The first approach taken was to assess monocyte distribution in the peripheral blood of patients compared with healthy donors. Multi-colour flow cytometric analysis was used to assess cell surface expression of CD14 and CD16, markers used to classify monocyte subsets in the literature (Passlick et al., 1989, Ziegler-Heitbrock, 2007, Heimbeck et al., 2010, Frankenberger et al., 2012). This approach is widely used to profile monocyte responses under inflammatory conditions or infection (Sanchez-Torres et al., 2001, Nahrendorf et al., 2007, Pandzic Jaksic et al., 2010, Ozaki et al., 2012).

Distribution profiling of classical monocytes did not yield a significant difference between healthy and PDAC nor did the absolute monocyte counts from the clinical data. This suggests that replenishment and differentiation of classical monocytes may be unaffected by the presence of PDAC. This contrasts with data reported by Sanford *et al*., which showed an increase in classical monocyte number. However, the patient cohort selected in my study represented a consistent untreated sample set at a later stage of diagnosis as opposed to the mixed stages or treated patients used by Sanford.

The monocyte distribution shift observed in my work was similar to that reported in inflammatory conditions, such as rheumatoid arthritis and lupus (Scherberich, 2003, Zhang et al., 2010, Rossol et al., 2012). The concurrent increase in the intermediate monocyte population and decrease in the non-classical population has also been reported in breast and colorectal cancers

(Feng et al., 2011, Schauer et al., 2012). The non-classical monocyte population is thought to be the most mature and to be derived from the classical monocytes via a sequential differentiation through the intermediate monocytes (Ancuta et al., 2009, Beyer et al., 2012). The results seen here could suggest an expansion in the intermediate population due to the presence of cancer associated inflammation and soluble factors such as CSF1 or CCL2 being produced by the tumour and its surrounding environment. Alternatively there may also be a block preventing differentiation from the intermediate to the non-classical population. It would be interesting to measure these factors in the blood of patients to determine if the levels may correlate with the monocyte distribution or macrophage infiltrate in the corresponding tumour histological biopsy samples

Flow cytometric assessment of monocytes cultured under closed conditions in vitro, using plasma or tumour cell supernatants, would be beneficial here to further investigate which factors are producing these effects. To elucidate this metabolite or proteomic profiling from concurrent samples could be used to correlate expression with distribution changes or clinical counts.

Access to the clinical data showed no correlation between monocyte counts, distribution or tumour markers. This suggests that, in this small cohort, monocytes might not be a useful clinical biomarker for tumour presence. A recent paper by Steele *et al* however showed a correlation between CRP count and monocyte presence in the peripheral blood, which might be reflected in a larger cohort of patients (Steele et al., 2013).

With the advent of novel technologies, transcriptomic profiling is leading to more ‘data-driven’ approaches to derive hypotheses. This type of high throughput technology is becoming increasingly common and is helpful in understanding key regulators of biological function.

Classical monocytes were collected from 3 healthy and 5 PDAC patients for analysis. RNA was isolated and, to gain a sufficient amount for Affymetrix

GeneChip analysis the cDNA was amplified using the Nugen Ovation kit. This has proved to be a robust and reliable amplification procedure with a good coverage of the whole transcriptome from very small quantities of RNA. Monocytes are prevalent in the blood, but small sample sizes taken from patients at the hospital due to requirements for other blood tests or chemotherapy, as well as sample processing steps, lead to a small starting volume of RNA.

Affymetrix gene chips were chosen at the start of this project, as they are reliable, robust and cost-effective. The results of the gene expression microarrays yielded fluorescence intensity values for 54,000 probe sets on each array. Careful consideration was given to normalisation and data analysis methods, to ensure the top identified differentially expressed genes fit several criteria. Differentially expressed genes were increased by a fold change of more than 2 and were statistically significant ($p<0.05$). The resulting gene lists, as indicated in the second results chapter (Section 5.3), indicated there were 242 increased genes in PDAC and 280 genes increased in classical monocytes from healthy volunteers.

When analysing a large number of probes, it is vital to be stringent, considering a high background noise level. This helps avoiding the inclusion of genes that might give false positive results. However, it also may exclude genes that are present at low abundance, as it cannot be assumed that small changes in expression are not important or that large differences are always important.

Gene expression analysis of this kind presents several challenges. The results give an individual snapshot of expression, which is dynamic and transient (Fan and Hegde, 2005, Heimbeck et al., 2010). Homeostatic responses are not fixed or closed, increasing the time frame of experiments or repeated captures can improve this effect by accounting for these changes, although are not often financially feasible or practical.

To improve significance, the most straightforward method would be to increase the sample size. In this particular experimental design, the addition of another group of patients with pancreatitis would help identification of monocyte responses associated with inflammation without the presence of a tumour to extricate tumour-specific responses. This would be useful although not black and white due to the inextricable links between cancer- promoting inflammation and *vice versa*.

The top differentially expressed genes were then assessed using targeted qPCR in an expanded cohort of 9 healthy donors and 13 patients. These results showed significant increase in expression of two genes, interestingly from the same family of zinc finger nuclear transcription factors, early growth response genes 2 and 3 (*EGR2* and *EGR3*).

There is no published microarray data on monocytes from cancer patients, however, profiling results from pancreatic cancer patients identified an eight-gene signature from a mixed group of peripheral blood mononuclear cells in the presence of cancer. As well as monocytes, this population also includes lymphocytes (Baine et al., 2011). It can be argued that PBMCs represent a highly variable and heterogeneous cell population. On the other hand it could also be said that this population is more representative of the immune cell landscape in the peripheral blood and is also easier to isolate as a population, as there is less risk of contamination or effects or activation caused by sample processing. Recent studies analysing transcriptomic profiles of a single type of human immune blood cells, compared with single cell analysis, showed that, even within the subset of a defined cell type, cells can be considered heterogeneous due to a spectrum of phenotypic states and responses under homeostasis (Wu et al., 2014, Streets et al., 2014).

Data driven approaches generate large amounts of data however, when extrapolating genes of importance, it is important to consider that genes do not act alone, but participate in complex signalling pathways and networks. To determine whether differentially expressed genes could be associated

with a particular pathway or function, Ingenuity pathway analysis (IPA) was used. This analysis identified transcriptional regulation and development of mononuclear phagocytes as the top statistical pathways, which is consistent with assessment of monocytes. These results may also indicate that monocytes are encouraged to differentiate and that the transcriptional machinery for these processes is being switched on in patients with PDAC.

To further understand biological relevance of the up-regulated genes, investigation is required into what effects activation of these genes may have. In this instance, the identified genes are transcription factors. EGR2 and EGR3 were increased at gene expression level in a larger cohort of patients (n=13) compared with healthy donors. It would be helpful, however, to examine whether this increase is maintained at protein level using a western blot or intracellular flow cytometry.

To advance our understanding of the role of EGR2 and EGR3 in monocytes, molecules that may play a role in induction of EGR2 or EGR 3 expression were investigated~~opm~~. I showed that macrophage-colony stimulating factor could increase expression of EGR2. This expression could also be inhibited with the use of a specific MEK-1,-2 inhibitor suggesting potential upstream signalling pathway dependency of EGR2 in monocytes on MEK. To confirm this it would be useful to also check EGR2 expression at a protein level and assess phosphorylation of ERK after stimulation.

Additional downstream effects of M-CSF signalling in the monocytes were investigated, however, specific inhibitors of the EGRs would be required to confirm EGR-dependent responses. The time course used may also require extension to measure the changes in gene expression and downstream responses over a longer time. To further investigate the downstream activation targets of EGR2 and EGR3 as transcription factors Chromatin Immunoprecipitation (ChIP) would be helpful as a future experiment. However ChIP is limited by the need to know what the transcription factor is thought to bind to, or the ability to sequence the resulting target.

Currently in the literature there are no pharmacological inhibitors of EGR2 or EGR3. RNA interference technology would be helpful in selectively reducing expression of EGR2 or EGR3 in a bid to determine the effects this has on monocyte function. After confirmation of knock down, parameters such as cytokine production, phagocytosis or monocyte to macrophage differentiation could be investigated. This was carried out by ~~Zheng~~Zheng *et al*., in T cells and identified ~~of~~ EGR2 specific roles in suppression of cytokine production namely IL-2 and anergy (Zheng et al., 2012).

Macrophage-colony stimulating factor is a relevant upstream regulator in PDAC due to its known roles in myeloid differentiation and reported increased presence in pancreatic cancer (Bunger et al., 2011). However the use of one cytokine may not necessarily be representative of monocytes in the peripheral blood of cancer patients. To better exemplify the environment to which monocyte are exposed, it would be useful to culture the monocytes in the presence of plasma from PDAC patients compared with healthy donors or in the presence of pancreatic tumour cell line supernatants.

To investigate whether monocyte expression of EGR2 or EGR3 would be maintained in the tumour microenvironment, immunofluorescence microscopy was used to examine co-localisation with CD68, a marker expressed on macrophages. EGR2 and CD68 appeared to co-localise in the tumour, but not normal tissue, supporting the potential of monocytes to reflect the macrophage compartment in the tumour. The presence of EGR2 on the tumour-associated macrophages also supports the known roles of EGR2 in transcriptional regulation of monocyte to macrophage differentiation. EGR2 was also reported to be a marker of alternative macrophage activation by another transcriptomic profiling study (Martinez et al., 2009). Expression of EGR3 was seen on a few cells in the tumour microenvironment, however not on the cells expressing CD68, suggesting the EGR3 expression may not be maintained on tumour macrophages.

In collaboration with Dr Jianmin Wu at University of New South Wales ~~University~~, the distribution of *EGR2* and *EGR3* gene expression was examined in a cohort of 159 clinical samples from patients with surgically resectable confirmed PDAC. This dataset was collected by Biankin *et al*., and published in 2012 examining copy number variations, mutations and enriched pathways in PDAC. These results showed a significant correlation between EGR2 expression and disease free survival in patients. This result is interesting as it was assumed that expression on macrophages in the tumour-microenvironment would negatively affect DFS. However, as these patient tumours were resectable, it cannot be excluded that EGR2 expression may have different impact at different stages of disease.

Commented [JC4]: Early stages not the same?

If technically possible, and if annotated samples could be made available, it would be interesting to determine whether the expression in the Biankin dataset or the samples in this investigation correlate between blood and tissue. It might also be attractive to examine the desmoplastic responses and other infiltrating immune cells from the same patients.

To further this project, patient cohorts for Affymetrix microarray profiling should be expanded and also include patients with pancreatitis or other benign conditions of the pancreas. It would also be interesting to measure the concurrent effects on the intermediate and non-classical monocytes. It would also be desirable to include assays to address other changes between monocytes in PDAC patients compared with healthy. An example of this would be to measure cytokine production or phagocytic ability, to establish if monocyte function is changed by the presence of PDAC.

The transcriptome profile of classical monocytes from patients with pancreatic cancer is significantly altered compared with that of healthy volunteers. The lack of expression of EGR2 and EGR3 genes in healthy volunteers requires further investigation to examine their potential as circulating biomarkers in pancreatic cancer.

Further investigation into EGR2 and EGR3 in monocytes, showed that EGR gene expression is responsive to macrophage colony stimulating factor and, in combination with previous data on the roles of EGR2 in core transcription networking, implicates EGR2 in monocyte to macrophage differentiation. EGR2 is expressed on myeloid cells in the PDAC tumour compartment and gene expression in the tissue is associated with disease free survival. This could suggest that EGR2 is linked to a more alternatively activated tumour associated macrophage phenotype and that the monocytes in the peripheral blood reflect the myeloid cells within the tumour microenvironment.

To summarise, further investigation remains into the functional roles of monocytes in pancreatic cancer and the biological relevance of sustained EGR2 expression in monocyte to macrophage differentiation in the tumour microenvironment of patients with pancreatic ductal adenocarcinoma. However, the work carried out during my thesis demonstrated changes in the expression profile of PDAC monocytes. Larger cohort study analysis are warranted and need to be aligned with the functional readout to determine the potential value as a systemic marker of immune response to PDAC disease.

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