CELLULAR INTERACTIONS IN THE TUMOUR MICROENVIRONMENT DURING INFLAMMATION-ASSOCIATED CARCINOGENESIS

A thesis submitted to the National University of Ireland in fulfilment of the requirements for the degree of

Doctor of Philosophy

By

Grace A. O'Malley B.Sc

Immunology and Transplant Biology Group,

Regenerative Medicine Institute

Discipline of Pharmacology and Therapeutics

National University of Ireland, Galway

Thesis supervisors: Dr. Aideen E. Ryan, Professor Laurence J. Egan

August 2017

Table of Contents

Declaration

I declare that the work presented in this thesis is work that was performed personally for the purpose of this thesis. Where other individuals have aided has been stated appropriately. I certify that I have not obtained a degree in this university, or elsewhere on the basis of any of this work

Abstract

The colon tumour microenvironment is highly stromal in composition and a greater stromal cell density correlates with a poor prognosis for patients. The majority of these stromal cells are of mesenchymal origin (MSCs) and are known contributors to tumour angiogenesis and invasiveness. Little is known about the role of their immunosuppressive potential in the TME. We investigated the molecular regulation of the induced immunosuppressive, tumour-promoting phenotype of tumour-associated MSCs, and the effect of inflammation on this process. Balb/c bone marrow derived MSCs were treated with conditioned medium from untreated CT26 tumour cells (MSC^{TCM}) or TNF-α treated CT26 cells ($MSC^{TNF\text{-}TCM}$), resulting in increased expression of TCR ligands MHC-I, MHC-II and PD-L1 compared to $MSC^{Control}$. This was significantly enhanced by TNF-α induced tumour cell inflammation. MSC^{TCM} co-cultured with syngeneic activated T cells displayed an enhanced ability to suppress CD8+ T cell proliferation, which was further potentiated by inflammatory activation of CT26 ($MSC^{TNF\text{-}TCM}$). This effect was dependent on induced PD-L1 expression on MSCs as PD-1 blockade restored $CD8^+$ T cell proliferation and granzyme B secretion. In an immunocompetent Balb/c syngeneic model, we assessed tumour growth and anti-tumour immune responses following sub-cutaneous injection of CT26 cells alone or co-injection with $MSC^{Control}$ or $MSC^{TNF\text{-}TCM}$. Co-injection of $MSC^{Control}$ significantly promoted tumour growth, and this was further potentiated by the co-injection of $MSC^{TNF\text{-}TCM}$. We showed that this stromal cell mediated tumour promotion could be reversed by administration of a PD-1 blocking antibody, via restoration of granzyme B secreting CD8+ T cells. Furthermore, we validated our findings by microarray profiling of clinical samples from human colon cancer patients, and we show that the phenomenon of stromal cell PD-L1 induction in response to the inflammatory tumour secretome holds true in the human system. Together, this data shows for the first time that stromal cells in the tumour microenvironment directly modulate anti-tumour immune responses via PD-L1. This data will lead to better

stratification of patients for immunotherapeutic regimens resulting in more targeted and durable responses.

Acknowledgements

Firstly I would like to extend my gratitude to my supervisors – Prof. Laurence Egan and Dr. Aideen Ryan. It was in Larry's lab during my final year undergraduate project that I discovered a love for immunology.

To Aideen, without whom this thesis would not exist! I will be forever grateful for your insight, mentorship and advice –both professional and personal. Your passion for what you do is inspirational.

A special mention to Dr. Thomas Ritter for your helpful input over the years, welcome suggestions, and permission to place orders on your grant every now and then!

I would like to thank Prof. Matt Griffin and Prof. Rhodri Ceredig for their valuable contributions and insights over the course of my project.

To Kevin Lynch, I'll be eternally grateful for all the help, "collaboration", billions of MSCs, friendship and plenty of discussions around the merits of mixed martial arts. All equally important.

To Paul Lohan and Serika Naicker, from both of whom I've learned and gotten invaluable help. Thanks for letting me following you around!

To the other members of the immunology group – Nick Murphy, Ollie Treacy, Joana Cabral, Tomás Griffin, Nahidul Islam, Irene Calvo and Andreia Ribeiro, you've all made my experience in Galway unforgettable and enjoyable.

I would also like to extend my gratitude to Dr. Declan McKernan, Conor Hennessy and Claire Feerick for advice and discussion at the bright and early GI meetings

To my oldest and closest friends (Rosemary, Stephanie, Janine, Martina, Elizabeth, Eimear, Katie), thanks for all the fun, and for not forgetting me despite all the times I was "unavailable due to work"!

To Philip – for putting up with me. Although, to be fair I put up with you too, and that's an achievement worthy of more than a PhD.

Finally, to my family, without whom I would not be where I am. To my mum for support, guidance, advice, friendship, really really long phone calls, a lot of complaining and a lot of tea! Those early mornings up colouring paid off. Ciarán – for inspiration to get away. Sarah – also inspiration to get away! I'm joking, you can visit. You've all been there for me in ways I cannot describe, and I will be eternally grateful.

Abbreviations

Akt	Protein kinase 3
APS	Ammonium persulfate
ATCC	American type culture collection
BM-MSC	Bone marrow derived mesenchymal stromal cell
BSA	Bovine serum albumin
CAF	Cancer-associated fribroblast
CCR2	C-C motif chemokine receptor 2
CD--	Cluster of differentiation--
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CO_2	Carbon dioxide
COX2	Cyclooxygenase 2
CRC	Colorectal cancer
CT26/EV	CT26/Empty Vector
CT26/IκB-α SR	CT26/IκB-α super-repressor
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCL12	C-X-C motif Chemokine ligand 12
CXCR4	C-X-C motif chemokine receptor 4
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's medium
DMMB	1,9 Dimethylmethylene blue
DNA	Deoxyribonuclease acid
ELISA	Enzyme-linked immunosorbent assay
Ep-CAM	Epithelial cell adhesion molecule
FACS	Fluorescence-activated cell sorting
FAP	Fibroblast-activating protein
FasL	Fas Ligand
FBS	Foetal bovine serum
FSP	Fibroblast-stimulating protein
GAG	Glycosaminoglycan
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GvHD	Graft versus host disease
HBSS	Hanks' balanced salt solution
HCl	Hydrochloric acid
HER2/HER3	Human epidermal growth factor receptor 2/3
HGF	Hepatocyte growth factor
HIF-1α	Hypoxia inducible factor 1 alpha
HRP	Horse radish peroxidase
IDO	Indoleamine 2, 3-Dioxgenase
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL-10	Interleukin 10

IL-10	Interleukin 10
IL-12	Interleukin 12
IL-1β	Interleukin 1 beta
IL-4	Interleukin 4
IL-6	Interleukin 6
iNOS	Inducible nitric oxide synthase
MCP-1	Monocyte chemotactic protein 1
M-CSF	Macrophage colony stimulating factor
MDSC	Myeloid derived suppressor cell
MEM-α	Minimum essential medium alpha
mg	Milligram
MHC	Major histocompatibility complex
MI	Myocardial infarction
MIP-α	Macrophage inflammatory protein-1α
MIX	Methylisobutylxanthine
ml	Millilitre
mm	millimtre
MSC	Mesenchymal stromal cell
NF-κB	Nuclear factor kappa B
NK cell	Natural killer cell
NLR	Nod like receptor
nm	Nanometre
NO	Nitric oxide
NRG1	Neuregulin 1
NSAID	Non-steroidal anti-inflammatory
PAGE	Polyacrylamide gel electrophoresis
PAI-1	Plasminogen activator inhibitor type 1
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PD-1	Programmed death receptor 1
PDA	Pancreatic ductal adenocarcinoma
PDGFFR-α	Platelet-derived growth factor alpha
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
pg	Picogram
PGE_2	Prostaglandin E2
PHA	Phytohemagglutinin
PHA	Phytohemagglutinin
PI	Propidium iodide
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
p-STAT 1	Phosphorylated signal transducer and activator of transcription 1
p-STAT 3	Phosphorylated signal transducer and activator of transcription 3
p-STAT 6	Phosphorylated signal transducer and activator of transcription 6

RPMI	Roswell Park Memorial Institute medium
Sca-1	Stem cells antigen-1
SDS	Sodium dodecyl sulfate
STAT 1	Signal transducer and activator of transcription 1
STAT 3	Signal transducer and activator of transcription 3
STAT 6	Signal transducer and activator of transcription 6
TAM	Tumour-associated macrophages
TEMED	Tetramethylethylenediamine
TGF-β	Transforming growth factor beta
Th1	Type 1 T helper cell
Th2	Type 2 T helper cell
TLR	Toll like receptor
TME	Tumour microenvironment
TNF-α	Tumour necrosis factor alpha
T_{reg}	Regulatory T cell
VEGF	Vascular endothelial growth factor
VEGF	Volts
x g	x Gravitational force
α-SMA	Alpha smooth muscle actin
µg	Microgram
µM	Micromolar

List of Figures

Chapter One

Chapter Two

Chapter Three

List of Tables

Chapter One

Chapter Two

CHAPTER ONE

Introduction

INTRODUCTION

1.1 Colorectal Tumour Microenvironment

Worldwide colorectal cancer (CRC) is the third most common cancer diagnosed in men, and the second most common in women (Ferlay J). In 2014 in the US alone, an estimated 130,000 people were diagnosed with colorectal cancer, with 50,000 deaths from the disease (Siegel et al., 2014). This case is similar in Ireland with 2,500 people diagnosed with colorectal cancer each year here (IrishCancerSociety, 2017). Colorectal cancer develops slowly, beginning with adenoma and progressing over several years to carcinoma (Brenner et al., 2014). Although the stages of colorectal cancer may be defined (Compton and Greene, 2004), the origins of the disease are numerous and multifactorial. Microsatellite instability is detected in about 15% of colorectal cases, meaning that these tumours have defective DNA mismatch repair. It is thought that these tumours are more immunogenic than their microsatellite stable counterparts due to the generation of large numbers of abnormal peptides (Deschoolmeester et al., 2011). As such, these tumours area characterised by a larger lymphocyte infiltrate and better prognosis for the patient (Deschoolmeester et al., 2011, Boland and Goel, 2010). In fact, it has been demonstrated in colon cancer patients, that the type, density and location of immune cells within the colorectal tumour is predictive of clinical outcome (Galon et al., 2006). Colon cancer patients without tumour recurrence were shown to have higher immune cell densities ($CD3^+$, $CD8^+$, granzyme B^+) than those patients whose tumours recurred after treatment (Galon et al., 2006). These observations highlight the need to better understand the mechanisms that drive an immunosuppressive microenvironment in colorectal cancer.

Most deaths in colorectal cancer are due to the presence of metastatic disease (Riihimaki et al., 2016). Tumour growth and metastasis are promoted by factors in the vicinity of the tumour, known as the tumour

microenvironment. The “seed and soil” hypothesis was proposed as far back as 1889 by Stephen Paget when he noticed that a cancer cell (the “seed”) would only grow if the environment (the “soil”) was suitable (Paget, 1989). It is now widely accepted that the microenvironment within which a tumour grows is critical to its survival and progression (Hanahan and Coussens, 2012). The tumour microenvironment is composed of many cell types including endothelial cells, immune cells, fibroblasts and more (Liotta and Kohn, 2001, Gout and Huot, 2008, Weber and Kuo, 2012, Mbeunkui and Johann, 2009). These constituents serve in many ways to aid the survival and growth of the tumour through activities including secreting factors necessary for angiogenesis, evasion of tumour cell apoptosis, or enabling tumours to evade the immune system detection and elimination (Gout and Huot, 2008, Kalluri and Zeisberg, 2006, Condeelis and Pollard, 2006). More recently, a newer accomplice to the crime has been identified - the mesenchymal stromal cell (MSC). In fact, both the healthy colon, and colon tumour microenvironment are highly stromal in nature, with a majority of this stromal compartment being of mesenchymal origin (Owens, 2015). For this reason, the stromal compartment is now beginning to be investigated in order to better understand its role in promoting tumour growth and metastasis. In fact, significant evidence now exists to show that the stromal compartment plays a significant role in promoting colon cancer progression (Isella et al., 2015). It also been reliably shown that a higher stromal cell density correlates with the development of a more aggressive and invasive tumour, with the net result being a much poorer prognosis for patients (Vellinga et al., 2016, Chen et al., 2015, West et al., 2010, Calon et al., 2015). Furthermore, stromal cells are potent modulators of the various components that make up the anti-tumour immune response, and have the capacity to skew this response in a direction that favours tumour growth and progression (Turley et al., 2015). Considering the high density of stromal cells in the healthy colon and colon tumour microenvironment, it is likely that these stromal cells have a central and important role in modulating anti-tumour immune responses and aiding tumour promotion. However, the

mechanisms underlying this process are poorly understood at present and so opportunities for therapeutic targeting of this critical compartment of the tumour microenvironment remain limited. (Owens et al., 2013, West et al., 2010). A better understanding of the precise mechanism of immunomodulation by the stromal compartment in this microenvironment will undoubtedly lead to the development of better and more targeted immunotherapies and improved outcomes for patients.

1.1.1 The stromal compartment as a physical and functional barrier for the anti-tumour immune response

The stromal compartment in a solid tumour such as that of the colon is located adjacent to both the cancerous epithelium and the colonic vasculature and lymphatic network, and so represents a physical barrier to entry for immune cells in response to inflammation or epithelial transformation (Turley et al., 2015). In addition to their structural role, these cells have been shown to produce soluble mediators and express proteins that can influence immune and epithelial cells in diseased tissue (Han et al., 2011). The interactions between these stromal cells and various components of the innate and adaptive immune system will be discussed in detail further on in this chapter. However, while some progress has been made in beginning to understand the mechanisms underpinning the interactions between MSCs and immune cells, the role of these stromal cells in modulating the immune response in the tumour microenvironment, and the influence of the tumour secretome on this capability is unknown. *The research described in this thesis will address this knowledge gap and demonstrate an important, previously undescribed role for tumour-conditioned stromal cells in modulating the T cell mediated anti-tumour immune response.*

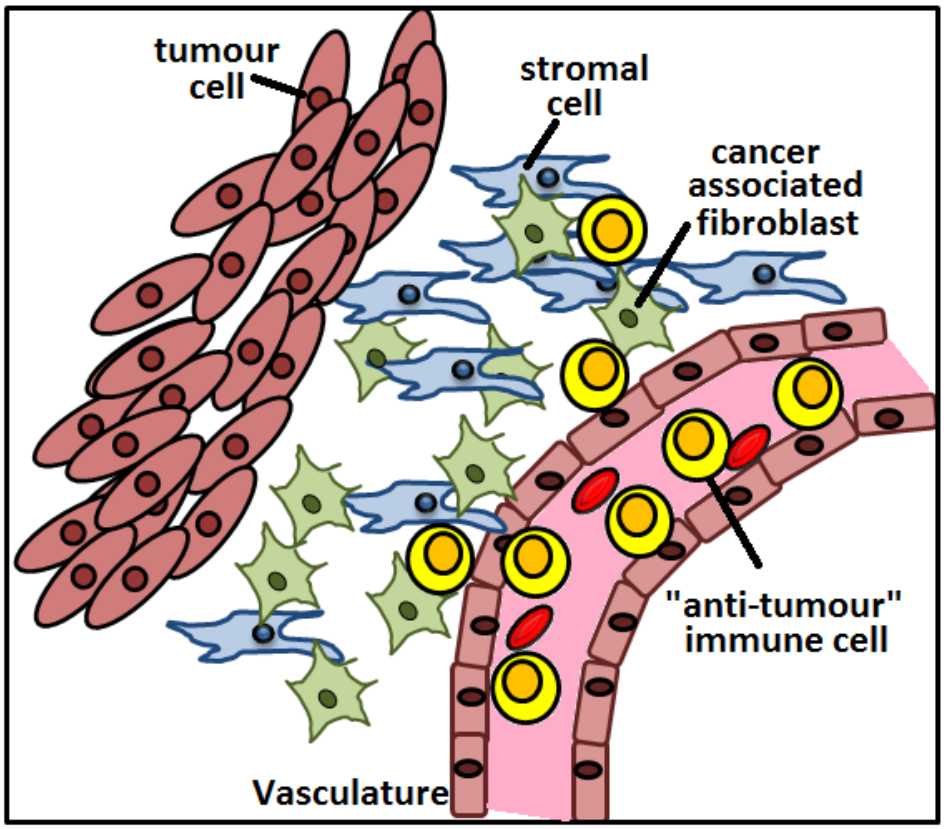


Figure 1.1. The tumour stromal compartment *The stromal compartment acts as both a physical and functional barrier to entry for anti-tumour immune cells given (1) Its positioning between the vasculature and malignant tumour cells and (2) the potent immunomodulatory potential of stromal cells and cancer-associated fibroblasts*

1.1.2 Mesenchymal stromal cells and Cancer-associated fibroblasts – one and the same

Mesenchymal stromal cells are non-haematopoietic multipotent adult stromal cells which display a fibroblast-like morphology. Mesenchymal stromal cells reside in the bone marrow, but are also found in adipose tissue, umbilical cord blood, and dental pulp (Erices et al., 2000, Zuk et al., 2002, Alge et al., 2010), and support homeostasis in healthy tissue during regeneration and wound healing. Mesenchymal stromal cells are defined *ex*

vivo by the following basal cell surface protein expression: $CD45^-$ $CD31^-$ $CD34^-$ $CD14^-$ $CD11b^-$ $DC79\alpha^-$ $CD19^-$ $MHC\text{-}II^-$ and $MHC\text{-}I^+$ $CD105^+$, $CD90^+$ and $CD73^+$, as well as their ability of tri-lineage differentiation i.e. differentiation to osteoblasts, adipocytes or chondrocytes (Dominici et al., 2006, Ryan et al., 2005). While MSCs from other species share the characteristics of tissue culture plastic and tri-lineage differentiation, their cell surface characterisation is more complex and varies greatly between species. For example, Peister et al., found mouse MSCs to express varying levels of CD34 and stem cell antigen-1 (Sca-1) (Peister et al., 2004). In addition to these characteristics, MSCs have potent immunomodulatory capacity and are being investigated as a cellular therapy for use in a broad range of inflammatory diseases, including osteoarthritis, graft-versus-host disease (GvHD), and myocardial infarction (MI) (NIH, 2014). Attributes that make MSCs attractive as an immunomodulatory therapy include their ability of MSCs to home to the sites of inflammation and injury and release growth factors or cytokines, to promote healing (Tögel et al., 2005, Hofstetter et al., 2002), to dampen inflammation (Bernardo and Fibbe, 2013) or to differentiate into various types of damaged tissues (Horwitz et al., 1999). The anti-inflammatory properties of MSCs are dependent on the ability of MSCs to respond to their environment and become "activated". Pro-inflammatory stimuli, including TNF-α, IL-1β, IL-6 or IFN-γ can enhance the immunosuppressive capabilities of MSCs (English et al., 2007, Sheng et al., 2008) . This enhanced immunosuppressive ability in response to inflammation is obviously attractive in diseases such as GvHD or MI. In the context of the tumour microenvironment, however, in the presence of a high level of pro-inflammatory signalling, these potent immunomodulatory properties can potentially influence the anti-tumour immune response and angiogenesis (Peddareddigari et al., 2010)

1.1.3 The tumour stromal compartment

The stromal cell compartment of the tumour microenvironment has recently been shown to have important prognostic and diagnostic relevance to patient outcomes in colon cancer. The stromal cell compartment includes cancer-associated fibroblasts (CAF), myofibroblasts, myeloid cells, endothelial cells and MSCs (Hanahan and Coussens, 2012). Recently, it has been demonstrated that MSCs are precursors to cancer-associated fibroblasts **(Figure 1)** and the two cell types have been shown to express similar cell surface markers (Mishra et al., 2008, Cirri and Chiarugi, 2011, Spaeth et al., 2009).

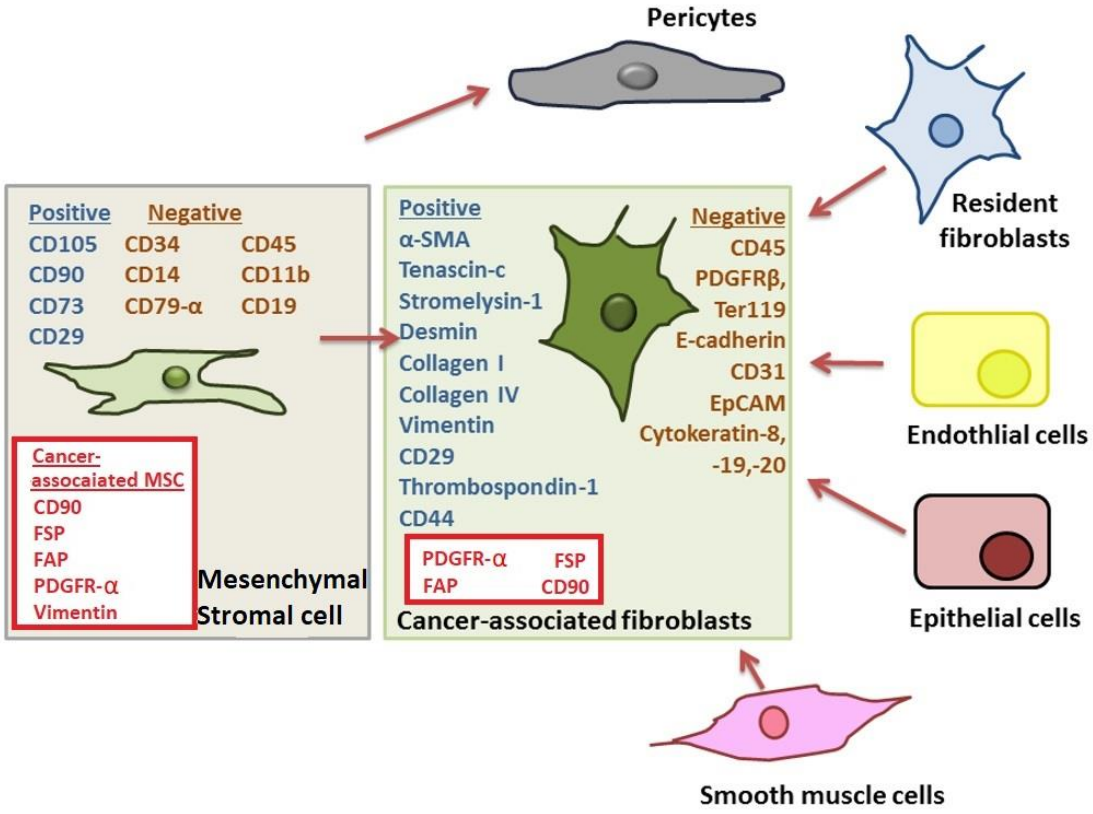


Figure 1.2 Mesenchymal stromal cells share cell surface markers with cancer-associated fibroblasts and represent a precursor population for these cells. *CAFs share a number of characteristics with mesenchymal stromal cells (MSCs) (highlighted in red text and box) including the expression of platelet derived growth receptor (PDGFR)-α, and, upon isolation from a tumour MSCs have been shown to*

express fibroblast-activating protein (FAP) and fibroblast-specific protein (FSP) the reported hallmarks of CAFs

Fibroblasts are a population of non-vascular, non-epithelial, non-inflammatory cells that form part of and help synthesise connective tissue (Kalluri and Zeisberg, 2006). Fibroblasts are known to play an active role in wound healing, and the activate fibroblasts found in the tumour microenvironment, CAFs, are believed to be of a similar "wound-heal-promoting" phenotype (Kalluri and Zeisberg, 2006). Like MSCs, CAFs originate in the bone marrow (Mishra et al., 2008, Direkze and Alison, 2006, Direkze et al., 2004). In terms of cell surface marker expression, there are many similarities between MSCs and CAFs. MSCs present in the tumour microenvironment have been found to express fibroblast-activating protein (FAP) and fibroblast-specific protein (FSP), CAF-defining markers (Spaeth et al., 2009). Additionally, platelet-derived growth factor receptor-alpha (PDGFR)-α has been used to identify CAFs, based on reports demonstrating PDGFR-α expression on up to 90% of stromal fibroblasts in solid tumours (Erez et al., 2010, Micke and Ostman, 2004). This marker however, is not unique to fibroblasts, and is commonly used to purify murine bone marrow-derived MSCs (Houlihan et al., 2012). **(Table 1)**

In fact, no specific marker or group of markers or gene promoters are currently agreed upon to definitively identify tumour-associated mesenchymal stromal cells or cancer-associated fibroblasts. The lack of cell specific markers or promoters has even led to conflicting results in models of colitis associated cancer. To elucidate the role of IKKβ signalling in "intestinal mesenchymal cells" or "CAFs" and where one study found IKKβ in these cells to be tumour-promoting, the second found it to be anti-tumourigenic (Koliaraki et al., 2015, Pallangyo et al., 2015) . The major difference between these two studies was the use of two different Cre drivers to target the cell in which to delete IKKβ – ColVCre where tumour

progression was seen and Col1a2Cre-ER where an anti-tumour effect was observed. In fact, many of the markers used to define MSCs, e.g. FSP1 (S100A4) are also expressed by macrophages in the stromal compartment of tumours. The lack of specificity in cell surface markers used to ascribe different populations of stromal cells in the tumour microenvironment has led to many disparities in assigning cellular functions to these populations. The disparity in these studies highlights the heterogeneity that exists in an environment like the intestine and demonstrates the importance in elucidating the role of each of these different populations in promoting tumourigenesis.

Study	Cancer type	Source of fibroblasts/ CAFs	CAF marker(s)	Effect on tumour	Effect on immune response
Nakagawa	Metastatic colon cancer	Fibroblasts isolated from 3patients with liver metastasis	Lack of epithelium specific markers cytokeratin-19 and -20. Positive expression of vimentin (RT-PCR) and α-SMA (immunofluorescence)	Increased HCT116 proliferation *in vivo*	-
Nagasaki	Colon cancer	Fibroblasts isolated from 64 year old patient	Lack of cytokeratin, positive for CD90 and vimentin (immunostaining) α-SMA (immunofluorescence)	Blocking stromal IL-6 decreased tumour growth and angiogenesis in mouse xenograft model	-
Zhang	Epithelial Ovarian Carcinoma (EOC)	Fibroblasts isolated from 61 patients with EOC	Negative for cytokeratin-8, positive for FAP and vimentin (immunohistochemistry)	Increased α-SMA staining in advanced disease and in cases with lymph node and omentum metastasis. Positive correlation between α-SMA and lymphatic and microvessel densities *In vitro* fibroblasts promoted invasion and migration of ovarian cancer	-
Olumi	Prostate cancer	Healthy fibroblasts from normal	Negative for cytokeratin and positive for α-SMA and	CAFs promoted tumour progression when grafted as	-

		human prostatic tissue, CAFs from 3 prostate cancer patients	vimentin (immunofluorescence)	tissue recombinants into nude mice. Tissue recombinants + CAFs appeared metastatic, recombinants + normal fibroblasts appeared benign	
Direkze	Pancreatic insulinoma	Fibroblasts isolated from mouse bone marrow	α-SMA for myofibroblasts vimentin for fibroblasts (immunostaining)	RIPTag mice administered GFP+ bone marrow via tail vain following whole body irradiation. In pancreatic tumours that developed, 25% of myofibroblats found to be bone marrow derived	-
Mishra	Breast Cancer	*In vitro* expansion of human bone marrow MSCs.	α-SMA, vimentin, FSA (immunofluorescence)	Tumour-conditioned MSCs co-cultured with tumour cell line increased tumour cell growth and proliferation	-
Spaeth	Ovarian cancer	Human bone marrow MSCs	FSP, FAP, tenascin-c, thrombospondin-1, stromelysin-1, α-SMA, desmin, VEGF (immunohistochemistry)	Co-injection of MSCs with tumour cells resulted in significantly larger tumours	-
Erez	Squamous skin carcinoma	CAFs isolated from mouse	PDGFR- α (flow cytometry)	Tumours co-injected with CAFs demonstrated enhanced growth and vascularisation	Co-injection of CAFs resulted in increased recruitment of

		dysplastic skin			macrophages which supported increased tumour vascularisation
Shainagawa	Colon cancer	Human bone marrow MSCs expanded *in vitro*	α-SMA, PDGFR-β, desmin, FSP, FAP (Immunofluorescence)	Tail vein injection of MSCs into tumour bearing mice. MSCs detected in primary tumour site and liver metastasis. Co-injection of MSCs and tumour cells resulted in enhanced tumour growth, increased PCNA-LI, increased MVA and decreased AI	-
Koliaraki	Colon cancer	Intestinal tissue	CD45, Ter119, CD31 E-cadherin negative, CD29, CD44, CD104- α positive (Flow cytometry) α-SMA, vimentin, collagen IV (immunohistochemistry) Vimentin, collagen IV (intracellular flow cytometry)	Deletion of Ikkβ in intestinal mesenchymal cells protected against inflammation associated carcinogenesis	Ikkβ in intestinal mesenchymal cells regulated immune cell infiltrate and cytokine production
Pallangyo	Colon cancer	Intestinal tissue	PDGFRα, CD29, CD44 positive, PDGFRβ CD45, Ter119, CD31 EpCAM negative (flow cytometry) Vimentin, FSP, α-SMA (immunofluorescence)	Lack of Ikkβ in intestinal fibroblasts increases tumour size	-

Kraman	Lewis lung carcinoma and pancreatic ductal adenocarcinoma	Mouse tumour tissue	FAP, α-SMA, Col I (Immunostaining)		LLC – depletion of FAP^+ cells induced necrosis of tumour cells PDA – depletion of FAP+ cells allowed immunogenic control of tumour growth
Feig	Pancreatic ductal adenocarcinoma	Mouse tumour tissue	FAP, α-SMA (Immunostaining) PDGFR-α positive, CD45 negative (Flow cytometry)		Inhibiting CXCR4, a receptor for FAP^+ stromal cell CXCL12 promoted T cell accumulation and synergised with checkpoint antagonists resulting in tumour regression
Calon	Colon cancer	Human colon adenoma and carcinoma tissue	FAP	Pharmacological inhibition of stromal cell TGF-β signalling blocked initiation of metastasis	-

Table 1.1 Outline of specific cellular markers used to define the role of stromal cells in various cancers, and the observed effects of these cells

1.1.4 Cancer associated fibroblasts have direct tumour promoting abilities

As well as sharing cell surface markers with MSCs, CAFs have also been shown to have tumour promoting properties in the tumour microenvironment. CAFs have been shown to influence the growth and progression of tumours (Nakagawa et al., 2004, Cirri and Chiarugi, 2011, Nagasaki et al., 2014, Zhang et al., 2011). Activated fibroblasts, or myofibroblasts, identified by their expression of α-smooth muscle actin (α-SMA) – are important forces driving tumour progression not least in the case of colorectal cancer (De Wever et al., 2008, Olumi et al., 1999). Conditioned medium from CAFs isolated from patients with metastatic colon cancer promote colon cancer cell proliferation to a greater extent thanj normal fibroblasts from the same organ (Nakagawa et al., 2004). MSCs exposed to the conditioned medium from tumours have also been found to exhibit a similar gene expression profile to CAFs (Mishra et al., 2008, Peng et al., 2014, Shinagawa et al., 2010, Spaeth et al., 2009). In colorectal cancer, the same effect has been demonstrated i.e. that MSCs co-injected with CRC cells expressed CAF-defining markers via TGF-β/SMAD signalling, an important cancer-promoting signalling pathway, and enhanced tumour growth (Peng et al., 2014, Shinagawa et al., 2010).

A recent review by Madar *et al*., put forward the notion that "CAF" is a cell "state" rather than a cell type (Madar et al., 2013), and this also fits with the evidence that MSCs are CAF-precursors. MSCs are a highly plastic cell type with robust differentiation capacity, are recruited to sites of inflammation and have a functional phenotype that is differentially regulated by inflammatory cytokines (Wang et al., 2014b). Furthermore, Calon *et al*. found TGF-β levels to be a robust predictor of disease relapse in a cohort of 345 CRC patients. This was an interesting finding in light of studies identifying TGF-β as an important factor in causing the differentiation of MSCs to a CAF-like phenotype (Calon et al., 2012). They also engineered CRC cell lines, which themselves display a mutational inactivation of the TGF-β

pathway, to secrete active TGF-β1. When injected *in vivo* these cells increased tumour formation compared with controls, and since the tumour cells were insensitive to TGF-β signalling, they concluded that the observed effects were due to TGF-β influencing stromal cells (Calon et al., 2012).

1.1.5 Immunomodulatory role of cancer-associated fibroblasts

Finally, MSCs have potent immunosuppressive capabilities and data shows that this property is maintained even as mesenchymal cells differentiate (Hoogduijn, 2015). As mentioned, CAFs, the product of tumour-induced MSC differentiation, have been found to be similarly immunosuppressive. Kraman *et al*., found that depleting fibroblast-activating protein-α $(FAP)^+$ cells in a murine model caused hypoxic necrosis and decreased tumour volumes (Kraman et al., 2010). This could be reversed by anti-TNF-α or anti-IFN-γ therapy, suggesting that FAP^+ cells attenuate cellular responses to these cytokines, thus protecting the tumour from cytokine-induced clearance. Feig *et al*., saw a similar result when they eliminated CAFs from a model of pancreatic ductal adenocarcinoma (PDA) (Feig et al., 2013). They observed significantly slowed tumour growth upon depletion of FAP^+ cells, but only in the presence of $CD4^+$ and $CD8^+$ T cells. This could be mimicked by administration of an inhibitor of CXCR4, the ligand for which, CXCL12, is present in CAFs and was suggested as being responsible for the tumour promoting effects of the CAFs. A rapid accumulation of T cells in the tumour was observed when CXCR4 was inhibited, thus restoring the ability of the immune system to eliminate the cancer cells. *While the limitations in defining exclusive cellular populations of mesenchymal origin has important implications in targeting these defined cell types in vivo, identification of common mechanisms of tumour promotion or immune modulation exerted by the stromal compartment in response to cues from the tumour, as investigated in this thesis, represents an alternative approach to understanding their individual cellular functions in the tumour*

microenvironment and will aid in the development immunotherapeutic treatment strategies for patients.

1.1.6 Recruitment of mesenchymal stromal cells to the tumour microenvironment

Inflammation is the body's response to tissue damage or injury (Hartnett and Egan, 2012, Wallach et al., 2014). The well-established link between chronic inflammation and the development of CRC gives weight to the notion that the tumour microenvironment is similar to that of a wound that does not heal (Dvorak, 1986, Ben-Neriah and Karin, 2011). In this sense, the factors that orchestrate recruitment of cells to the tumour microenvironment are likely to be similar to that of a wound, to counteract inflammation and promote wound healing. While desirable in a wound, this type of response is unfavourable in CRC and undoubtedly aids to progression of the disease.

Research on the molecular pathways associated with inflammation associated CRC have identified colon epithelial cell NF-κB, STAT3 and STAT6 activation with the progression of chronic intestinal inflammation to overt CRC (Foran et al., 2010, Ben-Neriah and Karin, 2011, Ryan et al., 2014, Colleran et al., 2011). With regards colorectal cancer, NF-κB is of particular interest, and is accepted as being a "critical link" between inflammation and cancer, and also found to feature in the wound healing process (Ben-Neriah and Karin, 2011, Gilbert et al., 2012, Karin, 2009). Research on the recruitment of MSCs to tumour cells has identified various signalling molecules as important to the process. Many, if not most of these, including CXCR4, MCP-1 and VCAM-1, have links to NF-κB (Wong et al., 2005, Rovin et al., 1995, Helbig et al., 2003, Maroni et al., 2007, Shi et al., 2007, Gao et al., 2009, Dwyer et al., 2007, Hu et al., 2012, Uchibori et al., 2013).

Shi *et al*., found the CXCR4/SDF1 signalling axis to be of importance in MSC homing to the bone marrow in irradiated NOD/SCID mice (Shi et al., 2007).

This was confirmed by Gao *et al*., who identified SDF1 to be an important stimulus for MSCs to migrate towards tumour conditioned medium (Gao et al., 2009). Other important factors implicated in this process are MCP-1, the blockade of which significantly reduced the migration of MSCs to breast tumour xenografts in mice, and VCAM-1 which has been shown to enhance MSC migration to glioma cells *in vitro* (Dwyer et al., 2007, Hu et al., 2012). With regards specifically to the setting of colorectal cancer, a study by Uchibori *et al*., in 2013 confirmed an important role for VCAM-1 in the process of migration. In addition to this important role for tumour cell NF-κB, recent research has shown that stromal cell NF-κB is also has a part to play in colorectal tumourigenesis, when a decreased tumour incidence was identified following intestinal stromal cell specific deletion of IKKβ (Koliaraki et al., 2015) In the context of inflammation associated and spontaneous colorectal cancer, it is likely that there are multiple mechanisms involved in MSC recruitment to the tumour microenvironment. However, while precise mechanisms of recruitment remain elusive, it is well established that MSCs are indeed recruited to the tumour microenvironment and once there, act to alter tumour biology and aid in immune evasion.

This activation of NF-κB can be induced by several ligands, most notably interleukin (IL)-1 and tumour necrosis factor(TNF)-α (Lawrence, 2009). TNF-α in particular is of critical importance in the setting of colorectal cancer (Al Obeed et al., 2014, Min et al., 2014). Studies of tissue samples from colorectal cancer patients show a higher TNF-α expression in cancerous tissue compared to adjacent normal colonic tissue, and that higher levels of TNF-α correlate with advanced stages of the disease (Al Obeed et al., 2014). TNF-α mediates several key aspects of tumour progression including oncogene activation, DNA damage and metastasis. Most tumour cells which express TNF-α display constitutive NF-κB expression, which is, as mentioned, a key driver of carcinogenesis (Aggarwal et al., 2012). However, in addition to NF-κB, TNF-α can signal via other molecular pathways to induce apoptosis, as opposed to the pro-survival signals elicited by NF-κB signaling.

The balance between these two opposing signals appears to be of critical importance, but how this is achieved is not clear. *The work described in this thesis will investigate the role of TNF-α in activating signaling pathways in colorectal cancer cells, and the effect of this signaling on the ability of these cancer cells to induce changes in stromal cell phenotype.*

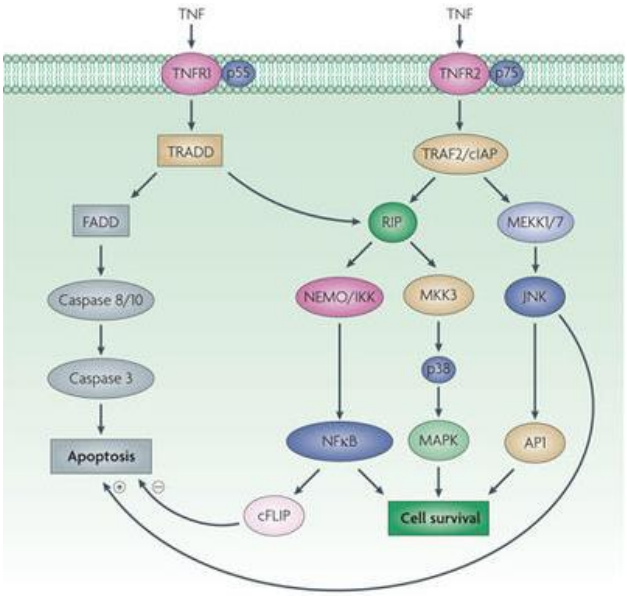


Figure 1.3 TNF-α is a potent activator of the NF-κB signaling pathway, leading to cell survival *Taken from (Faustman and Davis, 2010)*

1.1.7 Direct tumour promoting effects of mesenchymal stromal cells

Differentiation towards CAFs aside, MSCs have also been shown to be capable of modulating colon cancer cell activity through other mechanisms. In fact MSCs in CRC have been shown to directly influence at least three of the six seminal "Hallmarks of cancer" proposed by Hanahan and Weinberg, namely evasion of apoptosis (Shinagawa et al., 2010), sustained angiogenesis (Shinagawa et al., 2010, Liu et al., 2011, De Boeck et al., 2013) and tissue invasion and metastasis (Shinagawa et al., 2010, Hogan et al., 2013, De Boeck et al., 2013).

Numerous mechanisms are responsible for the observed effects of MSCs on colon tumour activity. Hogan *et al*., found bone marrow derived MSC (BM-

MSC)-secreted PAI-1 to be responsible for the increases they saw in HCT116 and HT29 colon tumour cell migration *in vitro* (Hogan et al., 2013). Liu *et al*., found pre-treating BM-MSCs with inflammatory cytokines induced VEGF expression via HIF-1α signalling in MSCs and resulted in increased angiogenesis in tumours following C26 colon cancer cell and MSC co-injection (Liu et al., 2011). Lin *et al*., showed that MSC-secreted IL-6 induced Notch1 and CD44 in HCT116 and increased the metastatic potential of tumours formed following co-injection of HCT116 and colon cancer-derived MSCs in Balb/c nu mice (Lin et al., 2013). De Boeck *et al*., found that BM-MSCs increased the invasion, survival and tumorigenesis of various colon cancer cell lines *in vitro* through the release of soluble NRG1 and subsequent activation of the HER2/HER3-dependent PI3K/Akt signalling cascade in colon tumour cells, and that co-administration of these BM-MSCs with the various cancer cell lines *in vivo* increased the percentage of animals presenting with tumours a number of weeks after injection (De Boeck et al., 2013). Similarly, Huang *et al*., found that MSCs enhanced angiogenesis and migration of tumours formed in athymic nude Balb/c mice following administration of HT29 colon cancer cells, and identified IL-6 secretion by MSCs as the putative mechanism responsible for these increases (Huang et al., 2013). A summary of these direct tumour promoting effects of stromal cells is outlined in **Figure 1.2**

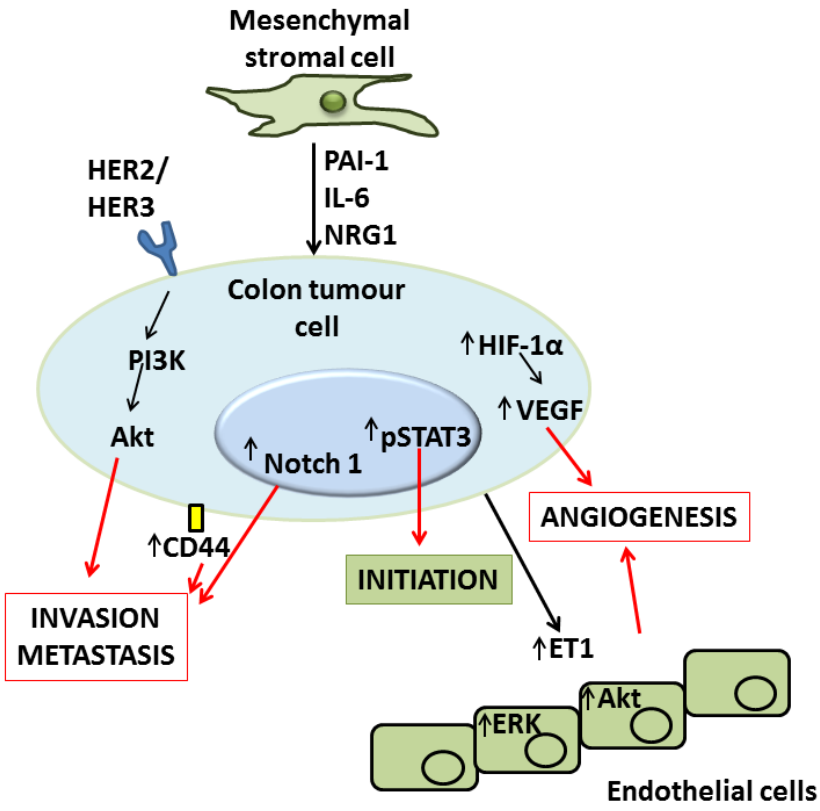


Figure 1.4 Molecular mechanisms of MSC mediated induction of colon tumour cell initiation, angiogenesis, invasion and metastasis *MSCs have been demonstrated to exert direct effects upon tumour cells via secretion of factors like plasminogen activator inhibitor (PAI)-1, interleukin (IL)-6 and neuregulin (NRG)1, or by activation of human epidermal growth factor (HER)2/3 receptor. The result of this signalling is activation of a number of pathways in the tumour, the net effect of which is tumour promotion*

1.2 Mesenchymal stromal cells involvement in modulating the anti-tumour immune response

The theory of cancer immunoediting describes the ability of the immune system to survey the landscape for potential cancerous growths, and to interact with tumours and directly alter their immunogenicity (Dunn et al., 2004a, Dunn et al., 2004b). According to this theory, the immune system can protect the host by recognising immunogenic tumour cells and mounting a

T cell mediated response to actively eliminate these cells (the “elimination” phase) (Shankaran et al., 2001, Matsushita et al., 2012). The corollary of this elimination of immunogenic cells is that the outgrowth of other, less immunogenic tumour cells may be favoured (the “equilibrium” and “escape” phases) (Dunn et al., 2004b, Shankaran et al., 2001, DuPage et al., 2012). There are undoubtedly numerous mechanisms by which tumour cells interact with and avoid clearance by the immune system such as insensitivity to interferon (IFN)-γ, and alteration in tumour cell MHC class I molecules and antigen presentation (Garcia-Lora et al., 2003, Garrido et al., 1997, Restifo et al., 1993). However, evidence now suggests that MSCs may have an important role to play in this process. MSCs possess immune regulatory functions, and in particular have been shown to be immunosuppressive in response to pro-inflammatory cytokines or TLR ligation (Ren et al., 2008, Di Nicola et al., 2002, Bartholomew et al., 2002, Le Blanc et al., 2003). In the colorectal tumour microenvironment, where inflammatory signalling is prevalent, it has been suggested that MSCs interact with immune cells, resulting in dampening of the anti-tumour responses and thereby promoting tumorigenesis (Patel et al., 2010, Ljujic et al., 2013, Ling et al., 2014). Numerous mechanisms have implicated in how MSCs modulate the immune components of the tumour microenvironment. Quite often the focus has been on MSC-T cell interactions, but influences on other immune cell subpopulations such as macrophages and dendritic cells (DCs) have also been identified. In the context of the tumour microenvironment, MSC-mediated influences on the phenotype of these immune cells will dramatically affect tumour progression and a thus a better understanding of the precise mechanisms involved is critical to the development of more efficacious immunotherapies.

1.2.1 The influence of mesenchymal stromal cells on the innate immune response

As noted by Lamagna *et al*., macrophages could be considered the first line of defence against tumours given that they colonise rapidly and secrete cytokines to activate other innate immune components such as dendritic cells (DCs) and natural killer cells (NK cells) (Lamagna et al., 2006). Additionally, macrophages have been shown to be capable of phagocytosing dead tumour cells and cross-presenting tumour antigen to $CD8^+$ T cells (Asano et al., 2011)

1.2.2 Mesenchymal stromal cell-induced polarisation of macrophages towards an M2 phenotype

Evidence shows that MSCs can exert their tumour promoting effects by interacting with macrophages in the tumour microenvironment (Cook and Hagemann, 2013, Mantovani, 2012). Macrophages can be polarised to an M1 phenotype which secrete reactive oxygen and nitrogen species and inflammatory cytokines, or an M2 phenotype which are involved in the suppression of inflammation and tissue remodelling (Mantovani et al., 2013). Tumour-associated macrophages (TAMs) have been found to be of the M2 phenotype which, in the context of the tumour microenvironment, results in dampening of anti-tumour immune responses (Cook and Hagemann, 2013, Mantovani, 2012). It has been shown that MSCs can polarise macrophages towards this "anti-inflammatory" M2 phenotype, characterised by IL-10 production and decreased iNOS and IL-12 expression (Cho et al., 2014, Ryan et al., 2014), and this likely represents a second important mechanism by which MSCs aid tumours in evading immune clearance. Ren *et al*., showed that tumour-educated MSCs greatly enhanced tumour growth, and that this enhancement could be ameliorated by monocyte/macrophage depletion (Ren et al., 2012b). They also showed that these tumour-educated MSCs expressed high levels of CCR2, the major cytokine involved in monocyte chemotaxis (Siebert et al., 2000). Although these studies demonstrate the ability of MSCs to induce and M2 phenotype,

the factors responsible for this switching remain elusive. Factors implicated in this switching include soluble mediators such as CCR2, IL-6 and GM-CSF (Ren et al., 2012b, Zhang et al., 2010). M2-like macrophages could be important in the context of developing novel therapeutics or adjuncts to improve the efficacy of those currently used (Cook and Hagemann, 2013, De Palma and Lewis, 2013, Ryan et al., 2014). However, macrophages perform several essential functions throughout the body and so caution must be taken in therapeutically targeting such a cell. Identification of factors released by MSCs in the tumour microenvironment that are responsible for inducing an M2 macrophage phenotype could be advantageous for the development of cancer immunotherapy, the effect of which is largely dependent on enhancing the macrophage effector functions, including antibody dependent cellular cytotoxicity and phagocytosis.

1.2.3 Mesenchymal stromal cells induce suppression and altered function in dendritic cells, natural killer cells, neutrophils and myeloid derived suppressor cells

Although most studies looking at the importance of the immune cell components to tumour growth focus elsewhere, there is a body of evidence to support the hypothesis that dendritic cells (DCs) and natural killer (NK) cells also have an important role to play in the anti-tumour immune response (Chang et al., 2013, Iwamoto et al., 2003, Vivier et al., 2012). Furthermore, DCs and NK cells are also known important components of the colorectal tumour microenvironment (Peddareddigari et al., 2010). Tumour cell antigens, released either in the form of dying tumour cells or soluble antigen, can be endocytosed by dendritic cells in the tumour microenvironment. Dendritic cells undergo maturation and migrate to secondary lymphoid organs where they can present processed tumor antigens as peptides bound to class I and II MHC molecules to prime effector CD8+ and helper CD4+ T cells, respectively (Guermonprez et al., 2002)

Despite the lack of studies related directly to the effects of MSCs on DCs and NK cell effector functions in the tumour microenvironment, it is likely their effects are similar to those described in other tissues. Aggarwal *et al*., showed using co-cultures that MSCs can suppress both proliferation and cytokine secretion, not only by T cells, but also by DCs, and NK cells, and that these effects can be reversed by PGE_2 synthesis inhibitors, suggesting PGE_2 may be also be important in MSC-induced immunosuppression (Aggarwal and Pittenger, 2005). This is a potentially important finding in the context of CRC. Non-steroidal anti-inflammatory drugs (NSAIDS) have been shown to have a protective effect against the development of CRC (Chan et al., 2005, Flossmann et al., 2007, Friis et al., 2009) owing to their ability to inhibit cyclooxygenase (COX)-2, the enzyme that is responsible for the production of PGE_2 from arachidonic acid (Koehne and Dubois, 2004, Wang and Dubois, 2010). Spaggiari *et al*., showed that MSCs inhibited both the proliferation and effector functions of NK cells, and the maturation and effector functions of DCs, and that these effects were, as in the case of T cells, mediated by IDO and PGE_2, molecules released by MSCs in response to inflammatory signals that are likely prevalent in the colon tumour microenvironment (Spaggiari et al., 2008, Spaggiari et al., 2009).

Myeloid-derived-suppressor cells (MDSCs) are another family of immune cells derived from myeloid progenitors and shown in humans to express varying levels of CD11b, CD14, CD15 and CD33 (Gabrilovich and Nagaraj, 2009, Giallongo et al., 2016). MDSCs are described as having an immature phenotype and the potential to suppress T cell responses via expression of arginase 1, inducible NOS, TGF-β, IL-10, COX2 or the induction of T_{regs} (Gabrilovich and Nagaraj, 2009, Marvel and Gabrilovich, 2015) . Research has shown that these MDSCs can affect tumour growth and progression in a number of ways, including protecting the tumour from immune mediated clearance by the mechanisms mentioned above (Marvel and Gabrilovich, 2015, Giallongo et al., 2016)

Further data suggests that the factors responsible for the accumulation of MDSCs in the tumour microenvironment include GM-CSF, G-CSF, M-CSF, all of which have been shown to be produced by MSCs, giving weight to the notion that MSCs could be responsible, at least in part, for the observed accumulation of MDSCs in the tumour (Marvel and Gabrilovich, 2015, Giallongo et al., 2016)

Additional data has demonstrated that tumour-derived MSCs can have even more pronounced effects on MDSCs, as demonstrated by the induced expansion of MDSCs *in vitro* when in co-culture with tumour derived MSCs. Furthermore, these expanded MDSCs were functionally active and shown to suppress allogeneic T cell proliferation, once again potentially hampering any anti-tumour immune response generated in the host (Giallongo et al., 2016, Yen et al., 2013, Lee et al., 2015a)

Finally, a limited amount of data exists demonstrating a detrimental role for neutrophils in the tumour microenvironment in terms of promoting tumorigenesis and inhibiting apoptosis (Galdiero et al., 2013, Wright et al., 2010). Neutrophils normally play an important role in killing invading microorganisms and although scant, evidence suggests that MSCs have the capacity to interact with this cell type. It has in fact been shown that MSCs can protect neutrophils from apoptosis and that neutrophils activated by tumour-resident MSCs can promote the differentiation of normal MSCs into CAFs, thus contributing to tumour promoting role (Maqbool et al., 2011, Zhang et al., 2014, Fridlender and Albelda, 2012)

This data demonstrates that MSCs in the TME have the capacity to suppress effector functions of numerous innate immune cells which act as a first-line defence in detecting transformed cells. Furthermore, this appears to be orchestrated via release of soluble factors, namely IDO and PGE_2 by the MSCs, meaning that these effects can be functional even in the absence of cell contact with MSCs. Elucidation of these complex interactions is vital in

order to re-activate the host innate immune system to allow the generation of an effective anti-tumour immune response.

1.2.4 The influence of mesenchymal stromal cells on the adaptive immune response

Much of the evidence for MSC-mediated immunomodulation has focused on the effects of MSCs on the tumour- infiltration, proliferation and effector functions of T cells. There are several reasons for such a focus. Firstly, a strong correlation has been identified between a higher frequency of tumour infiltrating T cells and a positive prognosis for a patient in numerous malignancies including colorectal cancer (Hadrup et al., 2013, Azimi et al., 2012, Gooden et al., 2011). This clearly points to an important role for T cells in leading the anti-tumour immune cell charge. In fact, it has now been suggested that the use of an "Immunoscore" is a robust predictor of disease progression and response to therapy in colorectal cancer patients than the traditional TNM (**t**umour burden, presence of tumour cells in lymph **n**odes, evidence of **m**etastasis) tumour staging classification (Galon et al., 2012). Secondly, $CD8^+$ cytotoxic T lymphocytes are capable of recognising, and directly killing tumour cells by release of TNF-α and IFN-γ, inducing apoptosis by engaging death receptors like Fas or by release of the cytotoxic protein perforin and protease granzyme B (Andersen et al., 2006). It has also been shown that $CD4^+$ T cells have an important role to play in the anti-tumour immune response. In particular, the helper lineages of $CD4^+$ T cells Th1 and Th2, have been investigated. Th1 cells produce IFN-γ, TNF-α, monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α), all of which can enhance $CD8^+$ T cell responses and activate macrophages to carry out their effector functions (Kim and Cantor, 2014). A role for Th2 cells in inducing inflammatory responses within a tumour that result in necrosis has been identified, although the mechanism by which this occurs is unclear (Nishimura et al., 1999). It must, however, be noted that

$CD4^+$ T cell responses are not always favourable in the tumour microenvironment. While T helper cells display anti-tumour immune activity, another subset of $CD4^+$ T cells, T regulatory (T_{reg}), which are responsible for maintaining immune homeostasis, have been identified as recruited to the tumour where they act to inhibit anti-tumour immune responses (Kim and Cantor, 2014). These cells may represent a potential target for the development of effective cancer immunotherapy. Finally, immunotherapies targeted towards inhibiting T cell checkpoints, and thereby "rec-activating" T cells and allowing them carry out their effector functions unimpeded, are having unprecedented clinical success, albeit it in a particular group of patients, a caveat which will be discussed in further detail below. The two most commonly targeted T cell receptors are cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death receptor 1 (PD-1) (Buchbinder and Desai, 2016). Treatment with both drugs has been shown to prolong survival in some patients, with positive responses in patients treated with CTLA-4 antibody therapy lasting more than 3 years (Farolfi et al., 2012). Similarly durable responses are beginning to be reported for treatment with PD-1 antibody therapy, once again indicating a critical role for T cells in mediating anti-tumour immunity (Topalian et al., 2014, Wu, 2016).

In light of this pivotal role for T cells as critical mediators of immune-mediated tumour cell clearance, and the fact that the tumour microenvironment is highly stromal in nature with these stromal cells positioned between the vasculature from which the T cells must enter the tumour, and the transformed epithelium, the target of these cells, it is critical that we gain a better understanding of how the stroma can modulate the function of these T cells. To date, a number of important mechanisms by which MSCs interact with T cells have been elucidated, but their relevance to the setting of the tumour microenvironment, not least in the case of colorectal cancer, remains unclear. *The research described in this thesis focuses on developing a better understanding of these cellular interactions in the setting of the tumour microenvironment and provides novel insights*

into the mechanism by which tumour cells dictate the immunomodulatory fate of the stromal compartment.

1.2.5 Mechanisms of MSC mediated T cell suppression

The study of the interactions between MSCs and T cells has gained momentum in recent years. Early experiments by Di Nicola *et al*., found that MSCs could inhibit the proliferation of T cells that had been stimulated by allogeneic peripheral blood lymphocytes (PBLs), dendritic cells (DCs) or phytohemagglutinin (PHA), and that this inhibition was contact-independent and could be reversed using antibodies against TGF-β1 and HGF, suggesting an important role for these cytokines in MSC mediated immunomodulation (Di Nicola et al., 2002). Similar work by Meisel *et al*., identified indoleamine 2,3-dioxygenase (IDO) as important in MSC-mediated T cell suppression, a finding which has been backed up by other separate studies (Meisel et al., 2004, Krampera et al., 2006). These findings are complicated by the fact that they are only applicable to MSCs isolated from humans and non-human primates (Ren et al., 2009). When looking at rodent MSCs, IDO appears dispensable, with nitric oxide (NO) found to be responsible for T cell suppression in this setting (Ren et al., 2008, Ren et al., 2009). More recently, another, IDO-independent mechanism for MSC-mediated immunosuppression has been identified. Chinnadurai *et al*., found that while blocking IDO negated the suppressive effect of MSCs on T cell proliferation, it did not reverse the suppressive effects of MSCs on T cell function as measured by IFN-γ secretion. Instead, the PD-L1 (B7H1) and PD-L2 (B7DC)/PD1 pathways was implicated in the ability of MSCs to suppress T cell function (Chinnadurai et al., 2014b). (This is an important pathway in the context of anti-tumour immune responses and the development of novel cancer immunotherapies and will be discussed in more detail below).

While there are clearly numerous suggested mechanisms responsible for this suppressive effect, there is definitive evidence to show that MSCs inhibit

T cell proliferation, but only following T cell activation, proliferation, and subsequent cytokine production, in particular IFN-γ (Ren et al., 2008, Krampera et al., 2006). T cell stimulation is a common feature of an inflammatory tumour microenvironment (Coussens and Werb, 2002). Furthermore, studies indicate that MSCs are more immunosuppressive when pre-treated with inflammatory cytokines, and as mentioned, can home to sites of inflammation, such as that of the tumour microenvironment (Han et al., 2011, Nakamizo et al., 2005). This implies an important role for MSCs in migrating to the tumour microenvironment (Kolluri et al., 2013) and aiding tumours avoid clearance by interacting with and modulating the host anti-tumour immunity.

1.2.6 MSC mediated immunosuppression allows for tumour promotion

Djouad *et al*., provided some of the earliest evidence to link this MSC-mediated immunosuppression to enhanced tumour growth (Djouad et al., 2003). They found the murine C3 MSC cell line to be immunosuppressive *in vitro,* and when injected with B16 melanoma cells in allogeneic CH3 mice, these MSCs induced greater tumour formation than control groups of MSCs or B16 cells alone. This result was observed regardless of whether the MSCs and B16 cells were co-injected or injected at separate sites, and for numerous ratios of MSC:B16 up to 1:100, which is important in the context of physiological relevance..

More mechanistic insights came from Patel *et al*., who found that MSCs protected breast cancer cells from immune clearance by increasing TGF-β production which caused an upregulation in the induction of regulatory T cells (T_{regs}) (Patel et al., 2010). Ljujic *et al*., found a similar upregulation of T_{regs} along with a decrease in the cytotoxic capacity of $CD8^+$ T cells and NK cells in mice that had been co-administered 4T1 mammary carcinoma cells and hMSCs, and these effects coincided with an increase in tumour growth and metastasis. Mechanistically it was found that the administration of

hMSCs caused significant increases in serum levels of TGF-β, IL-4 and IL-10 and a reduced level of IFN-γ in tumour bearing mice, findings which are consistent with MSCs affecting NK and T cell activity and polarising T cells away from the Th1 phenotype (Patel et al., 2010, Malygin et al., 1993). Han *et al*., showed that co-injection of MSCs with B16 cells increased tumour growth over that of B16 cells alone, and that a further increase in growth occurred when the MSCs were pre-treated with IFN-γ and TNF-α. NO production and T cell suppression was the mechanism cited here as being responsible for the increased growth of tumour cells, owing to the observation that iNOS inhibition reversed the MSC-mediated enhanced tumour growth. As mentioned, however, where MSC NO mediates an immunosuppressive effect in rodents, IDO appears to be the human equivalent (Han et al., 2011, Ren et al., 2009). This discrepancy was overcome by a set of elegant experiments by Ling *et al.,* who "humanised" murine MSCs by transfecting $iNOS^{-/-}$ mouse MSCs with the human IDO gene, leading to constitutive IDO expression in these cells (Ling et al., 2014). In addition, they were able to engineer the cells so that the human IDO expression was inducible and under the control of the promoter of the mouse iNOS gene, thus more accurately resembling the human *in vivo* situation whereby IDO expression is upregulated in response to inflammatory signalling. The conclusion was that IDO produced by MSCs was responsible for the enhanced tumour growth and that suppression of $CD8^{+}$ cells was critical for this effect.

Specifically in the context of colon cancer, murine MSCs treated with IFN-γ and/or TNF-α and co-injected with murine C26 colon cancer cells into a Balb/c mouse model promoter tumourigenesis (Liu et al., 2011). Although this study focused primarily on the pro-angiogenic role of these MSCs, it could be that these cells were also exerting suppressive effects on the immune system. This model, unlike many of the other cited here, is a syngeneic model where all cells used are of Balb/c origin, and so is particularly suitable for studying tumour-immune interactions. The authors

found that cytokine treated MSCs were more potent promoters of angiogenesis, and attributed this effect to increased VEGF expression in MSCs as a result of HIF-1α signalling. While obviously angiogenic, VEGF has more recently been shown to exert immunosuppressive effects on DCs and prevent their maturation (Johnson et al., 2007). The consequences of these effects are two-fold – firstly immature DCs are less effective in presenting tumour antigen to T cells, and secondly can induce anergy in T cells (Kusmartsev et al., 2005). Therefore, it could be that the MSCs administered in this study are having more than one effect on the tumour cells and actually aiding their avoidance of immune clearance as well as promoting angiogenesis (Johnson et al., 2007).

What all of this data demonstrates is that T cells have a clear and central role in immune-mediated clearance of tumour cells, and that their ability to carry out this function is severely hampered by stromal cells in the tumour microenvironment. Identification of the factors expressed or secreted by MSCs that affect T cell proliferation and function will enable the development of more targeted and efficacious immunotherapies, thus reducing reliance on cytotoxic chemotherapeutics and leading to more favourable outcomes for patients. However, to date knowledge in this area is lacking. *The work described in this thesis strives to bridge this gap in the knowledge, and presents and validates data demonstrating a novel method of T cell suppression by stromal cells in the context of the colorectal tumour microenvironment*

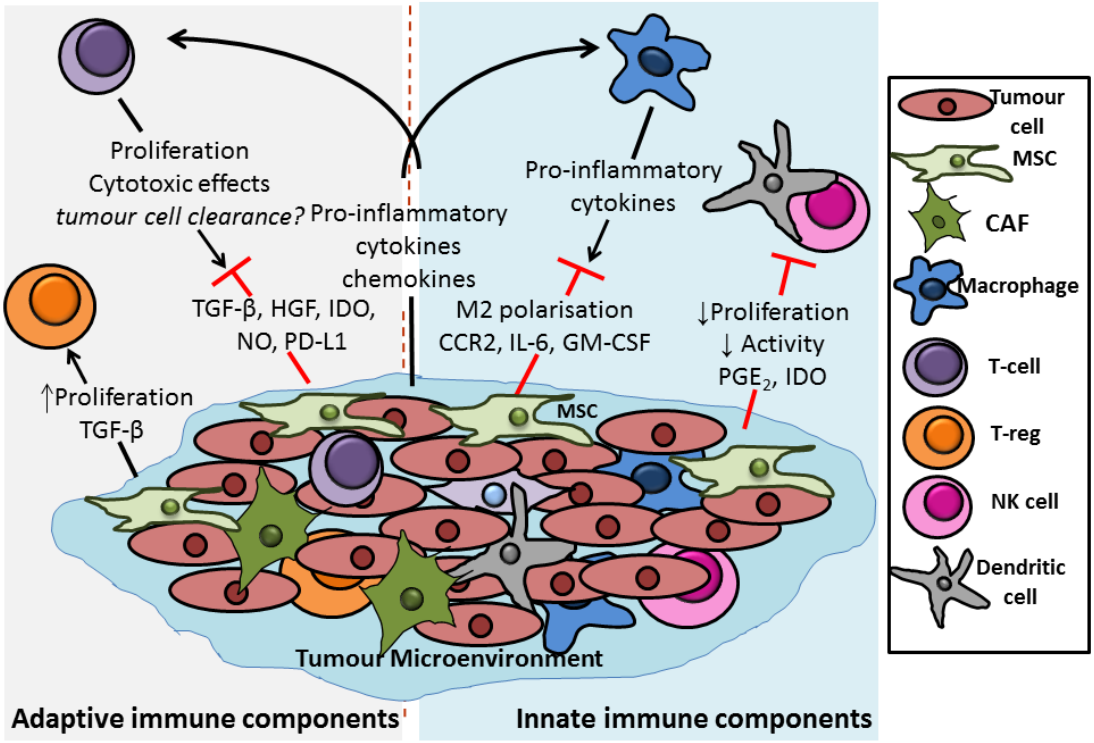


Figure 1.5. Outline of the potential immunomodulatory effects of MSCs in the colon tumour microenvironment. *MSCs have been shown to have potent immunomodulatory effects, acting on components of both the innate and adaptive immune system. In terms of innate immunity it has been shown that MSCs can dampen any early immune response that the host system may mount against a transformed tumour cells due to the ability of the MSCs to decrease the proliferation and activation of dendritic cells (DCs) and natural killer (NK) cells, the potential "first responders" of an anti-tumour immune response, and to skew macrophages to an anti-inflammatory M2 phenotype. With regards to the adaptive immune compartment, evidence from the literature shows that release of factors from and expression of proteins on MSCs directly reduces the proliferation and cytotoxic effects of effector T cells, thus inhibiting their tumour-clearing capacity. In contrast, signalling by MSCs has been reported to increase the proliferation of regulatory T cells (T_{regs}), a population which act to suppress the activity of other effector t cells.*

1.3 Cancer immunotherapy

Immunotherapy is defined as “the approach to treating cancer by generating or augmenting an immune response against it” (Khalil et al., 2016). In recent years the field of cancer immunotherapy has seen a surge in interest. This has resulted in rapid advances in technology and the development of several novel therapeutic agents for use in numerous applications including melanoma, lymphoma, multiple myeloma and non-small cell lung cancer (Achkar and Tarhini, 2017, Zappasodi et al., 2015, Kocoglu and Badros, 2016, Chen, 2017) The forms that these new entities have taken include antibodies (checkpoint inhibiting, immune modulating and tumour targeting), cancer vaccines, cytokines, cell transfer-based therapies and even oncolytic viruses. While each of these therapies involve distinct, and often numerous mechanisms of action for their observed effects, all involve harnessing the power of the patients own immune system and aid it, either actively (checkpoint inhibitors, cytokines, vaccines) or passively (tumour targeted antibodies, cell transfer, viruses) to mount an effective anti-tumour immune response (Morrissey et al., 2016). Current first-line treatment for colorectal cancer generally depends on a number of factors such as the presence of metastases, the likelihood of being able to completely remove these metastatic lesions with surgical intervention and the presence of co-morbidities (Stein and Bokemeyer, 2014). Based upon these criteria a decision will be made to treat with a single agent that will interfere with DNA synthesis like 5-fluorouracil (5-FU) or capecitabin, two drugs in combination, capecitabin with the VEFG-targeting antibody bevacizumab, or a more complex regime such as FOLFOX – Folinic acid, 5-FU and another DNA replication inhibitor oxaliplatin (Stein and Bokemeyer, 2014). As mentioned, bevacizumab is a monoclonal antibody targeted against VEGF and so represents an immunotherapeutic drug indicated for use in colorectal cancer. To date, a limited number of immunotherapeutic agents have been granted FDA approval for use in colorectal cancer, all of which fall into the tumour targeting monoclonal antibody category and are summarised in

table 1.2. However, a number of other immunotherapeutic strategies are being investigated for their potential to treat colorectal cancer, and may well represent the future of treatment for this disease. Checkpoint inhibitors represent the most promising of this therapeutics to date. (Pardoll, 2012)

Agent	**Trade Name**	**Target**	**Indication**
Bevacizumab	Avastin	VEGF	Metastatic CRC Also: cervical cancer, glioblastoma, NSCLC, ovarian, RCC
Cetuximab	Erbitux	EGFR	Metastatic CRC Also: squamous cell carcinoma
Panitumumab	Vectibix	EGFR	Therapy refractory, metastatic CRC
Ramucircumab	Cyramza	VEGFR	Metastatic CRC Also: stomach adenocarcinoma, NSCLC
Nivolumab	Opdivo	PD-1	MSI-High or mismatch repair deficient metastatic CRC that has progressed following other treatment

Table 1.2 Summary of immunotherapeutic agents with FDA approval for use in colorectal cancer CRC: Colorectal cancer, NSCLC: Non-small cell lung cancer, RCC: Renal cell carcinoma

1.3.1 Checkpoint inhibitors

The blockade of immune checkpoints has been cited as one of “the most promising approaches to activating therapeutic anti-tumour immunity”

(Pardoll, 2012, Sharma and Allison, 2015). As mentioned, the immunotherapies currently approved for use in colorectal cancer target one particular protein expressed on the tumour cell **(Table 1.2)**. Although these proteins may be upregulated or dysfunctional on the tumour cell, their expression is not unique to the tumour and so off-target side effects are common and can be severe. Furthermore, resistance to such a single-target therapy develops in many patients thereby reducing the efficacy of the treatment over time (La-Beck et al., 2015). As discussed, there is overwhelming evidence to demonstrate the potential for cytotoxic T lymphocytes to effectively clear transformed and malignant cells, a process that fails only when impeded by the immune escape mechanisms employed by the tumour to evade this response (DuPage et al., 2012, Dunn et al., 2004a). Therefore, the development of a therapy that could overcome this ability of the tumour to render anti-tumour T cells ineffective would allow the generation, in a patient, of an effective anti-tumour immune response, thus clearing the tumour without the off-target effects associated with the administration of non- or partially-specific chemo- or immunotherapeutic agents. Furthermore, an effective and successful anti-tumour immune response would allow for the generation of memory T cells, thus avoiding the complication of resistance and thwarting any future charge by the tumour. The development of immune checkpoint inhibiting drugs represents the most exciting new development in the realm of cancer immunotherapy in this respect.

1.3.2 Immune Checkpoints

In order for a T cell to respond to a stimulus, be it an invading pathogen or malignant tumour cell, antigen from this target cell must be presented to the T cell bound to specialised glycoproteins – the major histocompatibility complex (MHC) molecules (Murphy, 2012). This MHC-antigen complex triggers T cell receptor (TCR) signalling and initiates the process of T cell

activation. However, it is the binding of ligands to the co-stimulatory receptors that determine the fate of the T cell (Chen and Flies, 2013). There is a diverse repertoire of co-stimulatory receptors expressed on the surface of T cells, and when activated by their ligands, can positively (co-stimulatory) or negatively (co-inhibitory) influence T cell behaviour (Chen and Flies, 2013). The most studied of all these co-receptor interactions is that of CD28 expressed on the T cell surface being bound by its ligands CD80 or CD86 on the surface of antigen presenting cells (APCs). This interaction results in T cell proliferation, cytokine production and cell survival (Smith-Garvin et al., 2009). This is only one of a number of co-stimulatory receptors that can be activated on a T cell in order to elicit an effective immune response. (A summary of all of the known T cell co-receptor-ligands pairs is provided in **Figure 1.4**) However, in addition to positively activating T cells, ligation of certain other T cell co-receptors can lead to inhibition of T cell proliferation and cytokine release and can induce T cell exhaustion (Chen and Flies, 2013). These immune checkpoints are of critical importance in maintaining homeostasis in the organism, maintenance of "self" tolerance and prevention of tissue damage that can arises due to excess inflammatory responses (Karachaliou et al., 2015). While these checkpoints are of vital importance in maintaining immune equilibrium, they also represent a convenient vehicle for hijack by tumour cells seeking to avoid immune cell mediated clearance. In fact PD-L1, the cognate ligand for the inhibitory T cell receptor PD-1, has been shown to be overexpressed in several cancers including head and neck, thyroid, lung, colon, pancreas, ovary and skin (Wang et al., 2016, Patel and Kurzrock, 2015). To date, inhibitors targeting two distinct immune checkpoints, namely CTLA-4 and PD-1/PD-L1, have received FDA approval for clinical use (Alexander, 2016)

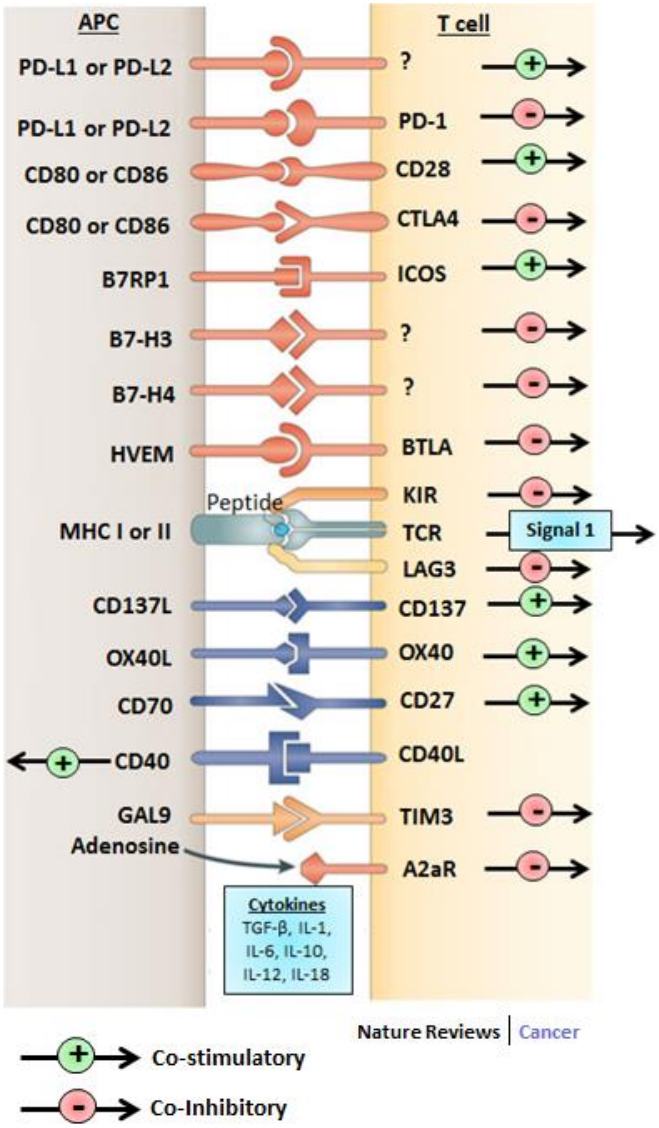


Figure 1.6. Summary of the various positive and negative co-receptors and ligands that have be shown to regulate T cell proliferation and effector functions. *Ligands expressed on the surface of antigen presenting cells (APCs) can bind their corresponding receptor on the T cell surface and induce a positive stimulatory effect (green +) or negative inhibitory effect (red -) on T cell proliferation, cytokine secretion and survival. Some of these ligands, in particular those with an inhibitory effect on T cells such as CTLA-4 and PD-L1, have been shown to be over-expressed on the surface of tumour cells in order for the tumour to dampen the T cell mediated anti-tumour immune response. Adapted from Pardoll 2012*

1.3.3 CTLA-4 based immunotherapy

The first immune checkpoint inhibitor to receive FDA approval for clinical use was ipilimumab (Yervoy, Bristol Myers Squib), a fully human monoclonal antibody targeted against CTLA-4 and tested in 676 patients with unresectable or metastatic melanoma (Hodi et al., 2010). CTLA-4 provided an attractive target as its expression is almost exclusively restricted to T cells, with some limited reports of expression on B cells, and ligation of this receptor by its ligands, CD80 or CD86, decreases T cell activation both by outcompeting the positive co-stimulatory receptor, CD28, for ligand binding and through recruitment of serine or threonine phosphatases (Melero et al., 2007, Pioli et al., 2000). Overall survival in these patients increased from 6.4 months with the control treatment, gp100, to 10.1 months with ipilimumab, as did one and two year survival rates which increased from 24% and 14% respectively with gp100 to 46% and 24% with ipilimumab (Hodi et al., 2010). The more impressive effects on long term survival (< 2 years) compared to median overall survival is indicative of a long term memory T cell response and is very promising in terms of inducing a durable response in patients without the need for additional treatment. While these results showed promise, they did not come without adverse events. Drug related adverse events were reported in 80-90% of patients, with 17-23% of patients experiencing grade 3 or 4 adverse events. 60% of all patients treated with ipilimumab experienced immune related adverse events, with 10-15% of patients experiencing grade 3 or 4 immune related adverse events and 7 patient deaths as a result of these adverse events (Hodi et al., 2010). In spite of these limitations, ipilimumab has since gained additional approval for use in combination with nivolumab (discussed below) and as adjuvant therapy to lower recurrence of stage III melanoma (Alexander, 2016). A second limiting factor in deciding to initiate treatment with anti-CTLA-4 therapy, considering that only a small fraction of patients experience positive responses, is the lack of any particular biomarker that points to increased likelihood of patients to benefit from such treatment. In light of the fact that

CTLA-4 blockade simply prevents competition for the co-stimulatory CD28 receptor and is not specific for the tumour in any way, perhaps genetic profiling of tumours to identify increased mutations or neoantigens likely to induce an immune response, along with comprehensive analysis of the microenvironment supporting the tumour could be useful in determining which patients to treat in this manner (Sharma and Allison, 2015).

1.3.4 PD-1 and PD-L1 based immunotherapy

The second immune checkpoint inhibitor to be developed was pembrolizumab (Keytruda, Merck), an anti-PD-1 blocking antibody. Pembrolizumab was awarded FDA approval for use in advanced or unresectable melanoma in 2014. Since then, additional approvals have been granted for the use of pembrolizumab in certain indications for head and neck squamous cell carcinoma, non-small cell lung cancer and classical Hodgkin lymphoma (FDA). PD-1 is expressed on activated T cells, B cells, NK T cells and myeloid cells. Upon interaction with its ligands, PD-L1 and PD-L2, PD-1 inhibits the proliferation and effector function of T cells and antibody production of B cells (Okazaki et al., 2013). PD-L1 is widely expressed on immune cells including T cells, DCs, macrophages, mast cells and B cells, as well as non-immune cells such as endothelial and epithelial cells. In contrast, PD-L2 expression is more restricted with expression found exclusively on immune cells such as DCs, macrophages and mast cells (Intlekofer and Thompson, 2013). The immunosuppressive function of PD-1/PD-L1/2 is useful in attenuation of an immune response thereby preventing autoimmunity. However, considering the aforementioned overexpression of PD-L1 on several tumour types, the effects upon T cells exerted by PD-1 ligation in this instance can have disastrous consequences for the anti-tumour immune response.

Early clinical trials using pembrolizumab (originally called lambrolizumab) in patients with advanced or unresectable melanoma showed that doses of

either 2mg/kg or 10mg/kg were reasonably well tolerated with 79-82% of patients reporting adverse events of any grade, and 8-17% experiencing grade 3 or 4 adverse events, figures comparable to those observed with CTLA-4 therapy (80-90% any event, 10-15% grade 3 or 4) (Hamid et al., 2013, Robert et al., 2014). Overall response rates varied from 25-56% depending on the dosing regimen used and the method of determining response. However, as was the case for CTLA-4 therapy, the more striking data was with regard to the long term sustainability of the positive response observed, with the Kaplan-Meier estimated overall survival at 1 year found to be 58-63% (Robert et al., 2014). Once again this is indicative of a memory response in patients, the generation of which should, in theory, lead to protracted, durable anti-tumour immune responses in patients, thus lowering greatly the potential for relapse and increasing the likelihood of disease free survival in the long term. Shortly after the original approval of pembrolizumab in 2014, a second PD-1 targeting monoclonal antibody, nivolumab (Opdivo, Bristol Myers Squib) was granted FDA approval (Alexander, 2016). In more recent years three PD-L1 targeting antibodies have joined the FDA list of approved immunotherapies for use in cancer, namely durvalumab (IMFINIZI, AstraZeneca), tezolizumab (Tecentriq, Genetech Inc.) and avelumab (BAVENCIO, EMD Serono Inc.), highlighting the great interest and promise that exit in this burgeoning area of cancer therapeutics (FDA).

1.3.5 Checkpoint inhibition in colorectal cancer

With specific regards to the setting of colorectal cancer, studies are only beginning to emerge on the potential benefit that may be conferred to patients by treatment with single or combined immune checkpoint inhibitors. One of the first large scale clinical trials investigating the use of nivolumab (anti-PD-1 mAb) in combination with a number of other agents, the CheckMate142 trial, is currently recruiting participants

(ClinicalTrials.gov, 2017). From the limited data that has been published to date, it would seem the colorectal cancer does indeed represent a potential indication in which immune checkpoint inhibition, as it currently stands, will be beneficial for patients. In particular, an approach targeting PD-1/PD-L1 signalling appears to be a viable treatment options in this indication, given the observations that colorectal cancer cell express relatively high levels of PD-L1, as do the various immune cells found infiltrating colorectal tumours (Rosenbaum et al., 2016, Masugi et al., 2016). However, further investigations have revealed a potential dependency on microsatellite instability in colorectal tumours in order for immune checkpoint therapy to be efficacious is this disease (Bilgin et al., 2017, Toh et al., 2016, Singh et al., 2015). This could be quite a significant limiting factor in the rolling out of CLTA4, PD-1, PD-L1 or any new checkpoint inhibitor that may be developed for widespread use in colorectal cancer given that only about 15% of all cases of the disease are associated with microsatellite instability (Deschoolmeester et al., 2011). Perhaps novel strategies involving treatment with more than one immune checkpoint inhibitor in combination, the combination of low-dose chemotherapy with immunotherapy, or the use of novel bi-specific or antibody-drug conjugates which can target two proteins or cells simultaneously, or deliver chemotherapeutic agents directly to the tumour, may be potential methods of overcoming this limitation (Chames and Baty, 2009, Thomas et al., 2016).

In spite of these promising data, it is clear that not all patients respond to each, or indeed, any of the currently available immunotherapies, including those not discussed here. This highlights the clear need for a more in-depth investigation of the interactions between the malignant cancer cell and the vast array of infiltrating immune cells we now know to be present in the tumour microenvironment. A better understanding of these interactions will lead to the identification of biomarkers that will better predict disease stage and likelihood to respond to a particular treatment. By extension, this will lead to better stratification of patients for currently available

immunotherapies and therefore more positive outcomes more quickly that is currently achieved. In addition, by fully understanding the key interactions between all the factors that combine to form the tumour microenvironment as we know it will undoubtedly lead to the development of novel therapeutics that can target the key proteins and signalling moieties responsible for the cycle of immunosuppression established in the tumour microenvironment in a more specific manner than is currently achieved. *The use of checkpoint inhibition in colorectal cancer, and the effect of stromal cell immunomodulatory ligand expression on the efficacy of this therapy in colorectal cancer will be explored in this thesis.*

1.4 Limitations of current research

All of the evidence presented here points to an important role for MSCs in the tumour microenvironment in enhancing various aspects of colon tumour cell biology, the net effects of which are tumour growth, survival and progression. While this may indeed be the case, the studies referenced here are not without limitations. As was noted in the review by Hogan *et al*., there is a lack of uniformity in much of this work, particularly with regard to the variation in the numbers of cells and ratios of MSCs to tumour cells being administered in each study, and the physiological relevance of the tumour cell:MSC ratios (Hogan et al., 2012). The physiologically relevant ratio of MSCs present in the colorectal cancer microenvironment is likely to be crucial in fully assessing the specific role MSCs play in the CRC and indeed other cancers. Furthermore, the majority of the preclinical studies in this area rely on the use of xenograft models of cancer, namely human colon cancer cells, often HT29 or HCT116, being co-administered with human MSCs, bone marrow-derived or otherwise, to a mouse lacking a fully competent immune system, commonly athymic nude Balb/c or Swiss nu/nu mice. While this model has its uses and is clearly necessary to facilitate the engraftment and outgrowth of the administered tumour cells that could

otherwise be rejected by the host animal, the autologous nature of the disease is not accurately represented, and aspects of the "anti-tumour-immunity machinery" are lost along with some of the components normally present in the tumour microenvironment. These immune cells components such as T cells, macrophages, dendritic cells and natural killer cells could, in fact, be central players in the story of MSC-mediated tumourigenesis. *The experiments described in this thesis have been designed with these limitations in mind. Our model is entirely syngeneic whereby tumour cells, stromal cells and lymphocytes are of Balb/c origin and so entirely MHC-matched. This will allow us to recapitulate as best we can, in vitro and in vivo, human colorectal cancer which develops within a patient from the patients' own cells*

In addition, in vivo studies were carried out using fully immunocompetent wild type Balb/c mice so that all components of the anti-tumour immune machinery were present.

1.5 Knowledge Gaps

1. The role of stromal cell immunomodulation on the anti-tumour immune response

2. The mechanism by which the malignant tumour interacts with and dictates the immunomodulatory fate of the stromal compartment of the tumour microenvironment

3. The influence of inflammation and tumour cell NF-κB signalling on tumour-stromal interactions

4. The role of stromal cell PD-L1 expression in dictating response to PD-l immunotherapy in colorectal cancer

1.6 Hypothesis and Aims

Hypothesis

Tumour cell NF-κB mediated inflammation enhances the immunosuppressive potential of stromal cells, thus aiding tumours evade the anti-tumour immune response

Aims

1. To elucidate the effect of tumour conditioning on the immunosuppressive capacity of mesenchymal stromal cells *in vitro*
2. To determine the mechanism of enhanced immunosuppressive stromal cell phenotype
3. To establish a role for tumour-mediated inflammation and NF-κB signalling on the observed changes in stromal cell immunomodulatory potential
4. To test an validate *in vitro* findings in a fully immunocompetent, syngeneic murine model
5. To identify novel stromal cell markers as targets for immunotherapy and to better stratify patients for such treatment regime

CHAPTER TWO

Materials and Methods

2.1 Animal strains and ethical approval

8 to 14-week-old female Balb/c mice were purchased from Harlan. Experimental animals were housed in a specific pathogen-free facility and fed a standard chow diet. All experimental procedures were carried out under licence from the Health Products Regulatory authority and approved by the NUI Galway Animal Care Research Ethics Committee

2.2 MSC isolation

Mice were euthanised by CO_2 inhalation and the femur and tibia were removed, cleaned of connective tissue and placed in MSC culture medium, MEM-α (Biosciences-Gibco) supplemented with 10% FBS (Fisher – Hyclone), 10% Equine serum (Fisher – Hyclone) and 1% penicillin/streptomycin (Sigma). The ends of the bones were cut to expose the marrow and cells were flushed out with culture medium using a 35-gauge needle. Clumps were removed by filtering through a 70µm mesh filter. Cells were then centrifuged at 400 x g for 5 minutes, re-suspended in 25ml culture medium and plated at a density of $9x10^5$ per cm^2 in a T175. Cells were incubated at 37°C, 5% CO_2, and non-adherent cells were removed 24 hours later. This process was repeated 3 times per week until cells reached confluency. At this point trypsin was added and incubated at 37°C for 5 minutes. Culture medium was added to neutralise trypsin. Cells no longer adhering to the flask were removed, centrifuged and re-plated. Characterisation was carried out at passage 4

2.3 Cell culture conditions

All experiments were carried out with Balb/c MSCs between passage 5 and 11. MSCs were grown to 70% confluency in MEM-α (Biosciences-Gibco)

supplemented with 10% FBS (Fisher – Hyclone), 10% Equine serum (Fisher – Hyclone) and 1% penicillin/streptomycin (Sigma)

The Balb/c colon carcinoma cell line (CT26) was obtained from ATCC. Cell were cultured in DMEM (Biosciences-Gibco) supplemented with 10% FBS (Sigma) and 1% penicillin/streptomycin (Sigma).

HCT116 and HT29 were kindly donated by Ms. Coralie Mureau and cultured in McCoys 5A medium (Sigma) supplemented with 10% FBS (Fisher – Hyclone), L-glutamine and 1% penicillin/streptomycin (Sigma). Human MSCs were kindly donated by Dr. Joana Cabral and cultured in MEM-α (Biosciences-Gibco) supplemented with10% FBS (Sigma), 1% penicillin/streptomycin (Sigma) and 1ng/ml FGF_2. Cells were characterised as being plastic adherent, capable of tri-lineage differentiation (data not shown) and cell surface marker expression

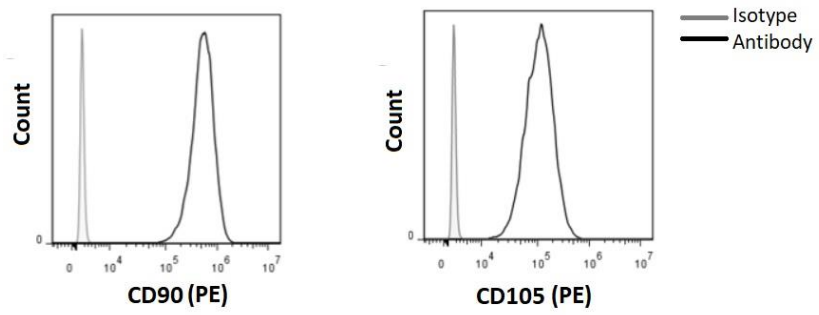


Figure 2.1 Cell surface marker expression on human MSCs

Transfection of CT26 with IκB-α superrepressor plasmid

The NF-κB Luciferase reporters driven by 5 × wild type (5 × NF-κB-Luc) (pNF-κB-Luc plasmid, Stratagene, Santa Clara, CA, USA) or negative control (no NF-κB binding sites-Luc) (pCIS-CK, Stratagene) were used in this study. Protocol details can be found in Ryan et al., 2014. Briefly CT26/Parental, CT26/EV and CT26/IκB-α SR cells were plated in 96-well plates and were co-transfected with 500 ng of pNF-κB-Luc, 500 ng of an expression vector and 50 ng of RSV-pRL reporter (Promega Corp., Madison, WI, USA). Twenty-four hours after transfection, cells were treated with TNF-α (10–100 ng/ml) for 24 h.

Luciferase activity was determined with the Dual-Glo Luciferase Reporter Assay System according to the manufacturer's instructions

2.4 MSC characterization

Adipogenesis

MSCs were plated at a density of $2x10^5$ cells per well in a 6 well plate with 2ml of standard MSC culture medium. Cells were incubated at 37°C, 5% CO_2 until confluency was reached. Once confluent, MSC medium was removed and replaced with 2ml per well of adipogenic induction medium (see below). Control wells received standard MSC medium. After 3 days the medium was removed from each well and replaced with 2ml per well of adipogenic maintenance medium (see below). Control wells received standard MSC medium. This cycle of media changes was repeated until 3 cycles of induction and maintenance medium were completed. For the final stint in maintenance medium cells were left in this medium for 5 days.

After 5 days, the medium was removed and cells were washed twice in PBS. Cells were then fixed in 10% neutral buffered formalin for 30 minutes at room temperature. Formalin was removed and wells rinsed with distilled water. A thin layer of Oil Red O working stock (Sigma) (6 parts Oil Red O stock solution mixed with 4 parts distilled water) was added to each well. The plate was slowly rotated to ensure even coverage of each well, and let stand for 5 minutes at room temperature. The stain was then discarded and excess stain removed by adding 2ml of 60% isopropanol (Sigma) to each well, swirling the plate and removing the isopropanol. Wells were next rinsed with tap water. Hematoxylin (Sigma) solution was diluted 1:5 with distilled water and a thin layer of this 1:5 solution was pipetted on to each well and left to stand for 1 minute. Wells were then washed in warm tap water and covered with

a thin layer of water in order for images to be taken on an inverted light microscope.

After photography was complete the water was removed. 500µl of 99% isopropanol (Sigma) was added to each well, rinsed several times over the surface of the well, and transferred to an eppenforf tube. This process was repeated with a second 500µl of 99% isopropanol. Debris was pelleted by centrifuging samples at 500 x g for 2 minutes. 200µl of stain extracted from each sample was added to wells of a 96 well flat bottom plate in triplicate. Absorbance was measured at 520nm on a Wallac 1410 plate reader (Perkin Elmer). Results were plotted as mean absorbance.

Table 2.1 Adipogenic Induction Medium

Reagent	Volume (for 100ml)	Final Concentration
DMEM (High glucose)	87.6ml	
Dexamethasone 1mM	100µl	1µM
Insulin 1mg/ml	1ml	10µg/ml
Indomethacin 100mM	200µl	200µM
500mM MIX	100µl	500µM
Penicillin/streptomycin	1ml	100U/mL penicillin 100µg/mL streptomycin
FBS	10ml	10%

Table 2.2 Adipogenic Maintenance Medium

Reagent	Volume (for 100ml)	Final Concentration
DMEM (High glucose)	88ml	
Insulin 1mg/ml	1ml	10µg/ml
Penicillin/streptomycin	1ml	100U/mL penicillin 100µg/mL streptomycin
FBS	10ml	10%

Chondrogenesis

MSCs were counted and $5x10^5$ cells were added to 1.5ml screw cap tube per condition 500µl of incomplete chondrogenic medium (ICM)(see below) and centrifuged at 100 x g for 5 minutes. Cells were resuspended in 500µl of complete chondrogenic medium (CCM) (see below) and centrifuged at 100 x g for 5 minutes. The cap on each tube was loosened slightly and incubated upright at 37°C, 5% CO_2. Medium was changed 3 times per week by aspirating off as much medium as possible without disturbing the pellet and replaced with 500µl of fresh CCM. After 21 days in culture the pellet was harvested by aspirating off all medium and washing twice in PBS. Pellets were air dried and glycosaminoglycan (GAG) content measured by DMMB assay

Table 2.3 Incomplete Chondrogenic Medium (ICM)

Reagent	**Volume (for 100ml)**	**Final Concentration**
DMEM (High glucose)	94ml	
Dexamethasone 1mM	10µl	100nM
Ascorbic acid 2-P: 5mg/ml	1ml	50µg/ml
L-Proline: 4mg/ml	1ml	40µg/ml
ITS+ supplement	1ml	6.25 µg/mL bovine insulin 6.25 µg/mL transferrin 6.25 µg/mL selenous acid 5.33 µg/mL linoleic acid 1.25 mg/mL BSA
Sodium pyruvate	1ml	1mM
Penicillin/Streptomycin	1ml	100U/mL penicillin 100µg/mL streptomycin 0.25µg/ml amphotericin B

Complete Chondrogenic medium (CCM)
To 1ml of ICM add 0.5µl of TGFβ-3 to give a final concentration of 10ng/ml and 2µl BMP-2 to give a final concentration of 100ng/ml

DMMB Assay

16mg of 1,9 Dimethylmethylene blue (DMMB) was dissolved overnight in 5ml of reagent grade 100% ethanol. 2.73g of sodium chloride and 3.04g of glycine were added to 975ml of deionised water. 0.69ml of concentrated hydrochloric acid (HCl) (11.6M) was added to this solution and mixed. The dissolved DMMB solution was then added and mixed again. The pH was adjusted to 3.0 with 1M HCl. The volume was brought to 1 litre with deionised water, and the container was stored protected from light.

1mg of papain was dissolved in 9.75ml of DMMB assay dilution buffer. 250µl of this solution was added to a further 10ml of dilution buffer. 200µl of this solution was added to each pellet and digested overnight at 60^{o}C. Samples were then vortexed to ensure the pellet is dispersed. Assay standards were prepared by adding 4mg of Chondroitin-6-sulphate to 10ml of assay dilution duffer. This solution was diluted 1:10 to give a 40µg/ml working stock. Standards were made as follows:

Table 2.4 DMMB Assay Standards

Chondroitin sulphate solution	**Dilution buffer**	**Concentration of GAG /well (50µl)**
200µl	0µl	2µg
180µl	20µl	1.8µg
160µl	40µl	1.6µg
120µl	80µl	1.2µg
80µl	120µl	0.8µg
40µl	160µl	0.4µg
0µl	200µl	0µg

50µl of each standard or sample was added to the wells of a 96 well flat bottom plate in triplicate. 200µl of DMMB stock solution was added to each well and incubated at room temperature for 5 minutes. Absorbance was measured at 595nm on a Wallac 1420 plate reader (Perkin Elmer)

Osteogenesis

MSCs were plated at a density of $2x10^5$ cells per well in a 6 well plate with 2ml of standard MSC culture medium. Cells were incubated at 37°C, 5% CO_2 until confluency was reached. Once confluent, MSC medium was removed and replaced with osteogenic medium (see below). Control wells received standard MSC medium. Medium was changed every 2 days. Cells were harvested between days 10 and 17 depending on the condition of the monolayer (cells will begin to peel away and need to be harvested before this point is reached). Osteogenesis was determined by Alizarin Red Staining

Table 2.5 Osteogenic Medium

Reagent	Volume (for 100ml)	Final Concentration
Iscoves MEM	77.5ml	
Dexamethasone 1mM	10µl*	100nM
Ascorbic acid 2-P 10mM	0.5ml	50µM
β glycerophosphate 1M	2ml	20mM
L-thyroxine	50µl	50ng/ml
FBS**	9ml	9%
Equine serum	9ml	9%
L-glutamine	1ml	2mM
Penicillin/Streptomycin 100x solution	1ml	100U/mL penicillin 100µg/mL streptomycin

Alizarin Red Staining

A 2% Alizarin Red solution was prepared by dissolving 2g of Alizarin Red S in 100ml dH_2O. This was mixed well and the pH adjusted to 4.1 with 1% ammonium hydroxide. Medium was removed from cells and wells were washed twice with PBS for 5 minutes. Cells were fixed in 95% ice cold methanol for 10 minutes. Wells were then washed with dH_2O and incubated for 5 minutes in 2% Alizarin Red solution. Wells were rinsed once more with dH_2O and left to dry on the bench before imaging by microscopy. A small amount of dH_2O was added to each well before taking images.

2.5 MSC Conditioning

CT26/EV and CT26/IκB-αSR tumour cells were seeded in a T175 flask (Nunc – Fisher) at a density of 1 x 10^6 cells per flask in 25ml of cell culture medium. Cells were left to grow at 37^{o}C at 5% CO_2 (normoxia) for a total of 72 hours at which point conditioned medium was collected, spun at 1000g to pellet any cellular debris and stored at -80^{o}C. For TNF-α conditioning, 100ng/ml TNF-α was added to tumour cell cultures 24 hours prior to removing medium.

For conditioning MSCs were seeded at a density of 0.035 x 10^6 cells per well of a 6 well plate in 2ml culture medium. 24 hours after seeding the culture medium was removed and replaced with 40% fresh MSC medium and 60% tumour conditioned medium. Fresh DMEM and DMEM with 100ng/ml TNF-α were added as controls. MSCs were analysed at 24, 48 and 72 hours after addition of conditioned medium. *In vitro* immunosuppression assays and *in vivo* experiments were all carried out using MSCs that had been conditioned for 72 hours.

To determine cell viability following conditioning cells were trypsanised, counted and added to FACS tubes at 100,000 cells/tube. Cells were washed twice in PBS and resuspended in Annexin V binding buffer (BD Biosciences) with 5µl Annexin V per sample. 10µl Propidium iodide (PI 1:10,000 dilution) was then added to each sample and after a 5 minute incubation samples

were vortexed and run in the Accuri® C6 flow cytometer (BD Biosciences). Analysis of viability was performed on CFlow Sampler® software.

2.6 T cell immunosuppression assay

Lymph nodes and spleen were harvested from Balb/c mice and single cell suspensions were obtained by mechanical disruption of the tissue in culture medium, RPMI 1640 (Fisher-Lonza) supplemented with 10% FBS (Sigma), 1% sodium pyruvate, 1% non-essential amino acids, L-glutamine, 1% penicillin/streptomycin (all Sigma) and 0.01% β-mercaptoethanol.

Cells were washed, re-suspended in PBS and erythrocytes lysed using ammonium-chloride-potassium lysing buffer for 5 minutes on ice. Cells were re-suspended in culture medium and a combination of 90% lymphocyte/10% splenocyte was used for immunosuppression assays.

To assess proliferation cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) using CellTrace cell proliferation kit® (Invitrogen). Cells were counted and re-suspended in pre-warmed 0.1% BSA/PBS at a concentration of $1x10^7$cells/ml. CFSE was reconstituted in dimethyl sulfoxide and 2µl of reconstituted dye were added for each 1ml BSA/PBS. Cells were protected from light and incubated at 37^{o}C for 6 minutes. Ice cold culture medium was added to neutralise the CFSE and cells were washed twice more. Cells were then activated using CD3/CD28 Mouse T-Activator Dynabeads® (Life Technologies).

0.1 x 10^6 CFSE labelled and activated cells (90% lymphocytes/10% splenocytes) were co-cultured with naïve or tumour-conditioned Balb/c MSCs at a ratio of 1 MSC:50 lymphocytes in a 96 well round bottom cell culture plate (Sarstedt).

2.6.1 Analysis of proliferation

After 72 hours, medium was removed from co-cultures and stored at -80^{o}C for further analysis. Cells were re-suspended in FACS buffer (PBS supplemented with 2% FBS and 0.05% sodium azide). Cells were incubated

with CD8a-APC or CD4-PerCP Biolegend – Medical Supply Company) for 10 minutes at 4°C. Cells were washed twice in FACS buffer and proliferation was measured using a FACSCanto® cytometer (Becton Dickinson). Data was analysed using FlowJo® software (TreeStar Inc.). T cell suppression was calculated according to the formula: 100-(%PosCtrlDivided/%SampleDivided)*100

2.7 Enzyme-linked immunosorbent assay (ELISA)

Supernatants from co-cultures were analysed using Ready-SET-Go!® ELISA kits (Affymetrix – eBioscience) for IFN-γ and TNF-α, Granzyme B and TGF-β. Flat bottom 96 well ELISA plates were coated overnight at 4°C with capture antibody. The following morning plates were washed with wash buffer (1 x PBS with 0.05% Tween-20). Plates were blocked using the supplied assay diluent for 1 hour and then washed. Standards and samples were added to the plates and incubated for 24 hours at 4°C. Following 3 washes detection antibody was added and incubated at room temperature for 1 hour. Plates were washed, Avidin-Horse radish peroxidase (HRP) added for 30 minutes prior to 3 more washes. Tetramethylbenzidine substrate solution was then added and left at room temperature for 15 minutes before stop solution (2N sulfuric acid) was added and the plates were read at 450 and 570nm on a Wallac 1420 plate reader (Perkin Elmer).

2.8 Cytokine quantification by Bioplex® analysis

Supernatants were analysed using the Bio-Rad Bioplex 200® (Bio-Rad – Fannin Healthcare). Assay was run according to manufacturer's instructions. Briefly, standards and beads were reconstituted in dilution buffer and standards were incubated on ice for 30 minutes, before performing serial dilution. The wells of the Bioplex plate were pre-wet with 150µl of assay dilution buffer and this was removed prior to adding standards and samples. Next, 50µl of beads were added to appropriate wells, and these wells were

then washed twice with 100µl of wash buffer. Following washes, standards and samples were added to the plate at 50µl per well, the plate was sealed and shook a 1,100 rpm for 30 seconds, then 300rpm for 30 minutes protected from light.

Detection antibody was diluted in assay buffer, vortexed, and added to the plate at 25µl per well following 3 more washes. The plate was then sealed, shook at 1,000 rpm for 30 seconds and 300rpm for 30 minutes protected from light.

Streptavidin-PE was diluted, vortexed, and added to the plate at 50µl per well. The plate was then sealed, shook at 1,000 rpm for 30 seconds and 300rpm for 10 minutes protected from light. Following incubation wells were washed 3 times with 100µl of wash buffer, beads were resuspended in 125µl per well of assay buffer and the plate was sealed and read following one final shake at 1,100 rpm for 30 seconds. Cytokine concentrations were calculated and plotted as pg/ml based on the standard curve generated by the Boplex software (Bio-Rad – Fannin Healthcare).

2.9 In vivo tumour model

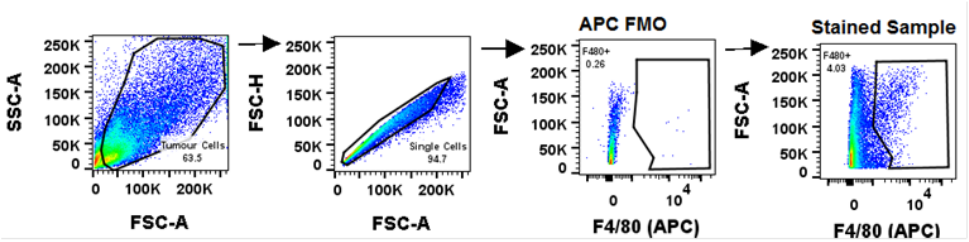
Tumours were induced in 8-14 week old female Balb/c mice by subcutaneous injection into the left flank of $2x10^5$ CT26 cells +/- $0.5x10^5$ MSCs (+/- *in vitro* tumour pre-conditioning) in a total volume of 100µl PBS. Animals receiving PD-1 antibody therapy received an intraperitoneal injection of 200µg of anti-PD-1 mAb (Clone RMP1-14; Bio X Cell – 2B Scientific) in 100µl PBS at days 7 and 14 post tumour induction. Tumour growth was monitored daily until sacrifice on day 21. At day 21 animals were euthanized and tumours harvested from left flank and, where invasion had occurred, the peritoneum. Measurements of tumour length and width were taken using a digital callipers and tumour volume calculated according to the rational ellipse formula: $(M^{1\wedge2}xM^2 \times \pi/6)$. Tumours were then transported to the lab in PBS, where they were placed on filter paper to remove excess fluid.

Tumour specimens were then weighed on an electric balance and mass recorded in grams

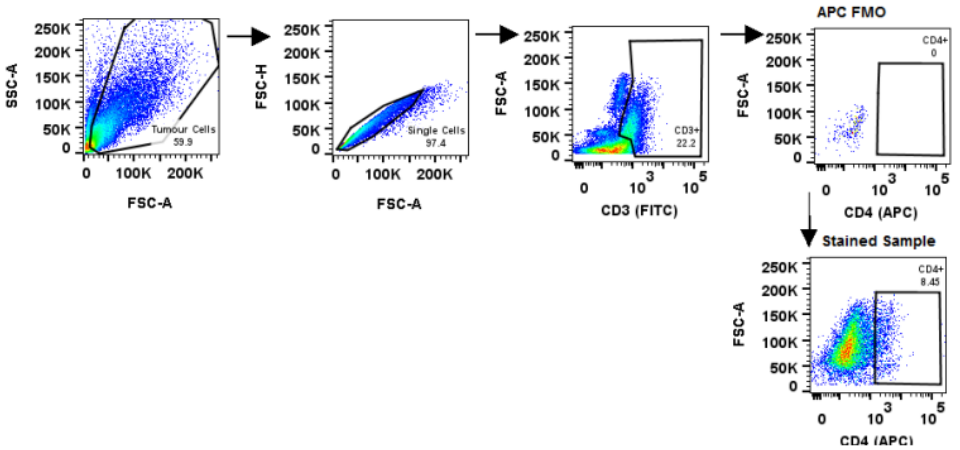
2.9.1 Flow cytometric analysis of tumour immune cell infiltrate

Tumours were digested in 1ml HBSS (Gibco-Biosciences) containing 150U/ml collagenase IV (Biosciences) and 200U/ml DNase (Sigma). Samples were placed on the heat block at 37^{o}C for 2 hours with gentle agitation and then filtered through 40µm cell strainers and washed with PBS. Single cell suspensions were counted and stained with markers of interest: CD3 (FITC), CD8 (APC), CD4 (APC), Granzyme B (PE). Cell surface staining was carried out as described previously. To stain for intracellular Granzyme B cells were washed twice after surface staining had been completed and fixed for intercellular staining. Fixing was carried out by incubating cells in 2% paraformaldehyde for 10 minutes at 4^{o}C. Cells were then re-suspended in permeabilisation buffer (1% BSA/PBS with 0.5% saponin (Sigma)) containing the intracellular antibody of interest. Gating strategies for tumour and spleen immune cell analysis are described in **Figure 2.1** and **2.2**.

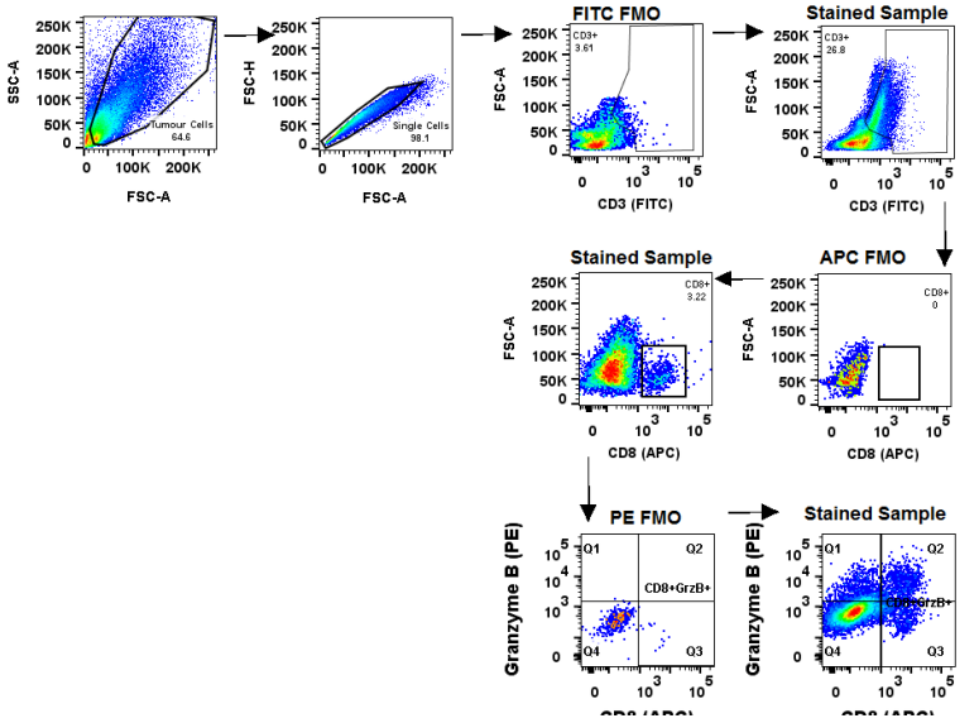
Tumour infiltrating macrophage staining panel



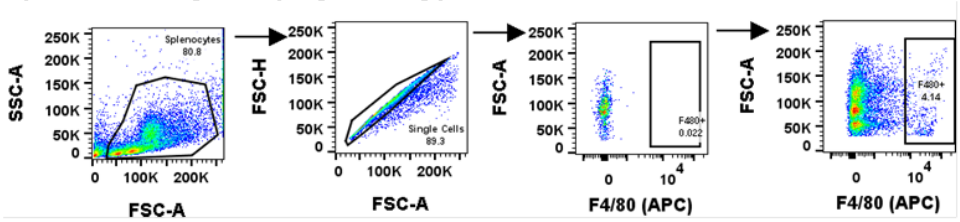
Tumour infiltrating CD4^{+} T cell staining panel



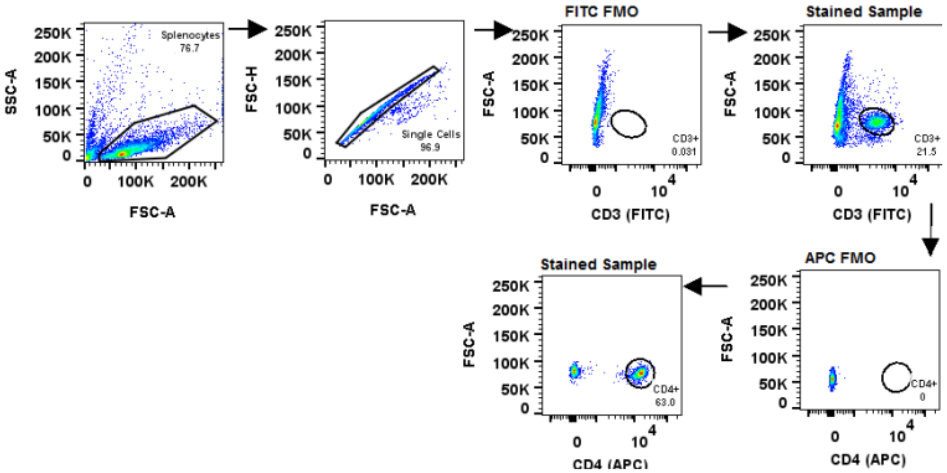
Tumour infiltrating CD8^{+} T cell staining panel



Spleen infiltrating macrophage staining panel



Spleen infiltrating CD4^{+} T cell staining panel



Spleen infiltrating CD8^{+} T cell staining panel

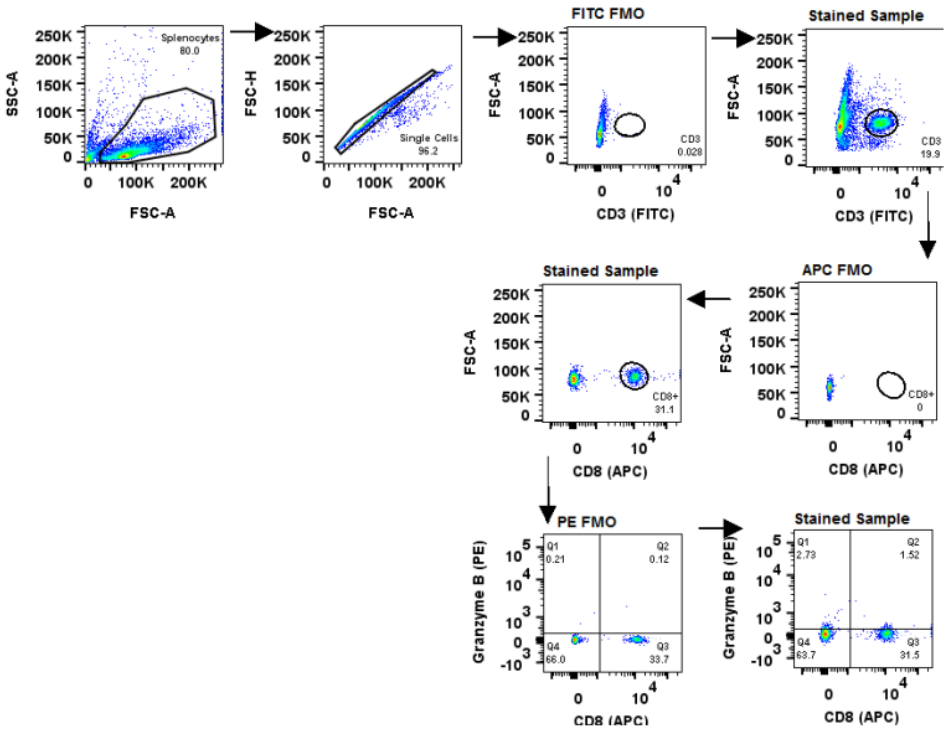


Figure 2.2 Gating strategies used to assess tumour immune cell infiltrate and frequency of immune cell populations in the spleen of tumour burdened animals

2.10 Western blotting

2.10.1 Cell lysis

Cells were trypsinised, counted and resuspended in a volume of 1X SDS PAGE Lysis buffer to yield a concentration of $5x10^6$ cells per ml.

Table 2.6 Lysis Buffer

Reagent	**Volume (100ml)**
10% SDS solution	20ml
100mM Tris HCl pH6.8	5ml
0.1% Bromophenol Blue	0.05g
20% glycerol	10ml
Water	15ml
***1X Buffer**:* Dilute 2X 1:2 with 95% H_2O and 5% β-mercaptoethanol	

Following resuspension in 1X Lysis buffer samples were left on ice for 5 minutes and then boiled at 100^oC for 5 minutes. Samples were placed on ice for a further 2 minutes and stored at -20^oC.

2.10.2 Western blotting of cell lysates

Gels were assembled and poured on the evening before the blot was to be run. The electrophoresis rig was assembled, filled with 1L of running buffer (see below) and the gel loaded with 15µl of protein. The power pack was set to 55V until protein had run through the stacking gel, and this was increased

to 90V until samples had reached the bottom of the gel. While running, transfer buffer was made and stored at 4°C.

When the samples reached the bottom of the gel the rig was disassembled, and the gel incubated in transfer buffer for 5 minutes. Nitrocellulose membrane was cut to size and also incubated in transfer buffer along with 2 pieces of filter paper and 2 sponges. Sandwich transfer apparatus was assembled, and the transfer was run at 110V for 90 minutes in the cold room. The apparatus was then disassembled, and the membrane blocked in 5% non-fat milk in 0.01% PBS/Tween for 1 hour at room temperature with gentle rocking.

Following blocking the membrane was incubated in 10ml of primary antibody overnight at 4°C with gentle rocking. Standard proteins were made up in 5% w/v non-fat milk in PBS/0.01% Tween and phosphor-proteins made in 5% w/v BSA in PBS/0.01% Tween.

Table 2.7 10% Resolving gel

Reagent	**Volume (1 gel)**
H_2O	2ml
30% acrylamide mix	1.7ml
1.5 M Tris pH 8.8	1.3
10% SDS	50µl
10% APS	50µl
TEMED	2µl

Table 2.8 Stacking gel

Reagent	**Volume (1 gel)**
H_2O	1.4ml
30% acrylamide mix	330µl
1 M Tris pH 6.8	250µl
10% SDS	20µl
10% APS	20µl
TEMED	2µl

Table 2.9 Primary and secondary antibody concentrations for western blotting

Target	Isotype	**Supplier**	**Cat. No.**	Dilution
STAT 1	Rabbit	Cell Signaling	9172	1/1000
p-STAT 1	Rabbit	Cell Signaling	7649	1/1000
STAT 3	Mouse	Cell Signaling	9139	1/1000

p-STAT 3	Mouse	Cell Signaling	4113	1/500
α-Rabbit HRP		Cell Signaling	7074	1/2000
α-Mouse HRP		Cell Signaling	7076	1/2000

2.10.3 Development

The membrane was washed three times 3 times for 5 minutes PBS/0.01% Tween and incubated with 10 mL of the appropriate horseradish peroxidase conjugated-secondary antibody diluted in 5% w/v non-fat skimmed milk powder in PBS/0.01% Tween for 1 hour at room temperature. Following this, membranes were washed with PBS/0.01% Tween and then with PBS alone. Following washes, 500µl of ELC (reagent 1 and 2 in a 1:1 ratio) was pipetted over the membrane. After 5 minutes a sheet of X-ray film was placed over the membrane and exposed for various lengths of time before being placed into developing machine.

Pharmacological inhibition of TGF-β, NF-κB STAT1 and STAT3 signalling pathways in MSCs

MSCs were seeded in at $0.05x10^6$ cells in a 12 well flat bottom plate in 1ml of medium and left to adhere for 24 hours. For NF-κB inhibition, cells were then treated with the previously optimised dose of 5µM BMS345541 for 2, 12 and 24 hours of pre-treatment. Medium was then removed and replaced with 40% and 60% conditioned medium, also containing BMS345541. PD-L1 expression was measured 24 hours later.

For TGF-β neutralisation 0.25 (low dose) and 1.25 (high dose) µg/ml of TGF-β neutralising antibody was added to a sufficient quantity of conditioned medium needed to treat MSCs. This was left to incubate at room temperature for 30 minutes, and 60% conditioned medium was then added to MSCs as previously described. D-L1 expression was measured 24 hours later.

STAT1 inhibition was attempted using fludarabine phosphate at concentrations of 2.5, 5 and 10µM. STAT3 inhibition was attempted using p3971 at concentrations of 400 and 800nM. Cells were treated with each inhibitor alone, or in combination, for 24 hours. Medium was then removed and replaced with 40% and 60% conditioned medium, also containing either or both inhibitors. PD-L1 expression was measured 24 hours later.

2.11 Statistical Analysis

Statistical analysis was carried out using GraphPad® Version 6 (GraphPad Software, CA, USA). Data were assessed for normal distribution using D'Agostino-Pearson omnibus normality test. Data sets with 2 groups were analysed using an unpaired t test. Data sets with more than 2 groups were analysed by ordinary one-way ANOVA followed by Tukey's multiple comparisons test. For analysis of correlation Pearson correlation coefficient was calculated. Results were considered statistically significant at $p<0.05$.

Table 2.10: Antibodies used for flow cytometry

FITC Antibodies				
Marker	**Clone**	**Supplier**	**Cat. No.**	**Volume for $1x10^6$ cells (µl)**
CD3	17A2	Biolegend	100203	
Streptavidin		eBiosciences	11-4317-87	1ul neat
Ly-6A/E (Sca-1)	D7	Biolegend	108105	2ul
CD326 (Ep-CAM)	G8.8	Biolegend	118207	0.5
Annexin V		Biolegend	640905	
APC Antibodies				
Marker	**Clone**	**Supplier**	**Cat. No.**	**Volume for $1x10^6$ cells (µl)**
F4/80	BM8	Biolegend	123115	0.625
CD4	Gk1.5	Biolegend	100412	0.16
CD8	53-6.7	Biolegend	100712	0.16
CD73	TY/11.8	Biolegend	127209	0.5
CD105	MJ7/18	Biolegend	120413	0.5
CD274 (PD-L1)	10F.9G2	Biolegend	124311	0.3
MHC-I ($H2K^d$)	SF1-1.1	Biolegend	116619	0.625

CD11b	M1/70	Biolegend	101212	1.25
PE Antibodies				
Marker	**Clone**	**Supplier**	**Cat. No.**	**Volume for $1x10^6$ cells (µl)**
CD273 (PD-L2)	TY25	Biolegend	107205	0.5
Streptavidin		eBioscience	12-4317-87	1ul of 1/8 dilution
CD80	16-10A1	Biolegend	104707	0.625
CD86	PO3	Biolegend	105105	0.625
Granzyme B	REA226	Miltenyi	130-101-351	5
Biotin Antibodies				
Marker	**Clone**	**Supplier**	**Cat. No.**	**Volume for $1x10^6$ cells (µl)**
I-Ad (MHC-II)	39-10-8	Biolegend	115003	0.125
CD178 (FasL)	MFL4	BD Biosciences	556998	1
PerCP Antibodies				
Marker	**Clone**	**Supplier**	**Cat. No.**	**Volume for $1x10^6$ cells (µl)**
CD4	GK1.5	Biolegend	100431	0.16
PE-Cy7 Antibodies				
Marker	**Clone**	**Supplier**	**Cat. No.**	**Volume for $1x10^6$ cells (µl)**
Brilliant Violet 421 Antibodies				
Marker	**Clone**	**Supplier**	**Cat. No.**	**Volume for $1x10^6$ cells (µl)**
CD25	PC61	Biolegend	102033	0.625
Brilliant Violet 510 Antibodies				
Marker	**Clone**	**Supplier**	**Cat. No.**	**Volume for $1x10^6$ cells (µl)**
CD44	IM7	Biolegend	103043	0.625

CHAPTER THREE:

Exposure to the inflammatory tumour secretome induced an enhanced immunosuppressive phenotype in stromal cells which is dependent on stromal cell PD-L1 expression

3.1 Introduction

As discussed in Chapter 1, the stromal cell compartment of the tumour microenvironment has been shown to be an important prognostic factor for cancer patients. In fact, in a study of 263 lung adenocarcinoma patients, higher expression of α-SMA, not only on the tumour cells but also in the stroma, was found to correlate to a lower rate of overall survival and metastasis-free survival for patients (Lee et al., 2013). Further studies specifically in the setting of colorectal cancer have identified that a stromal cell signature from colon tumours is associated with tumour progression and a poorer outcomes in colon cancer patients (Calon et al., 2015, Isella et al., 2015). While this observation of an association between higher stromal cells density and poor outcomes for patients is clear, the mechanisms underlying this association are unclear. It remains to be determined if the reason for the observed correlation of stromal density and genetic signatures with poor outcomes are due to an inherent tumour promoting property of these stromal cells, or their capacity to interact with and modulate components of the anti-tumour immune response. A vast array of data now exists to demonstrate the immunomodulatory potential of naïve or cytokine activated stromal cells and so it is entirely plausible that these cells will have a dramatic effect on anti-tumour immunity, in particular when present at a high density (Krampera, 2011). Considering the location of these stromal cells, positioned between the epithelial cells and the underlying vasculature, they are in a prime location to passively or actively impair immune cell activation and trafficking to neoplastic epithelial cells. It is poorly understood, however, how inflammation in the tumour microenvironment and the inflammatory tumour secretome affect these stromal-immune cell interactions.

In light of the fact that T cells are key mediators of the anti-tumour immune response due to the correlation between tumour infiltrating T cells and positive patient outcomes, the numerous immunotherapies in development

that aim to target the T cell response and knowing that T cells can directly kill tumour cells, we sought to determine the effect of tumour conditioning on the ability of stromal cells to modulate the proliferation and effector function of T cells. *To this end, the experiments described in this chapter were carried out with the aim of investigating the role of factors present in the tumour microenvironment in altering the proliferation and effector function of T cells. In addition, we examined the role of tumour cell inflammatory signalling on this process.*

There is a well-established and strong link between inflammatory bowel disease and the development of colorectal cancer (Terzic et al., 2010). The transcription factor NF-κB, which is a key regulator of both inflammation and cancer has been cited as a critical mediator in this process (Karin et al., 2002, Egan and Toruner, 2006). NF-κB regulates the expression of many pro-inflammatory chemokines and cytokines, including CCL-2, CXCL-2, IL-6, TNF-α and IL-1β in the tumour microenvironment and the activation of these molecules in cancer cells has been associated with colon cancer progression and metastasis (Greten et al., 2004, Luo et al., 2004, Gujral et al., 2014). Additionally, TNF-α can itself activate NF-κB signalling and has been shown, in the context of cellular therapy, to activate stromal cells to inhibit adaptive and innate immune effector mechanisms (Ren et al., 2012a). Furthermore, ligands known to be potent inhibitors of T cell proliferation and effector functions, PD-L1, PD-L2 and FasL, have all been linked to NF-κB signalling, and in the case of PD-L1, by TNF-α mediated activation of NF-κB in particular (Kucharczak et al., 2003, Liang et al., 2003, Bi et al., 2016, Lim et al., 2016) . However, although TNF-α and NF-κB are inextricably linked, the role of TNF-α in colon cancer is controversial and poorly understood. In addition, the cues and stimuli in the inflammatory colon microenvironment that influence mesenchymal cell phenotype and function in this setting remain unknown. *The data presented in this chapter provides an insight into the influence of tumour cell NF-κB signalling in response to TNF stimulation on the capacity of the tumour to influence the immunomodulatory capacity of stromal cells.*

Finally, as mentioned in Chapter 1, several immunomodulatory ligands have been shown to be expressed on tumour cells and responsible for enabling tumour cells to avoid clearance by the anti-tumour immune response (Wang et al., 2016, Patel and Kurzrock, 2015). PD-L1, in particular, has shown to be of significant importance in a number of malignancies, and represents an attractive therapeutic target (Patel and Kurzrock, 2015). However, this strategy is not always effective and needs refinement and better patient stratification, particularly when it is noted that some patients for whom the treatment is ineffective will in fact be negatively affected following PD-1 immunotherapy (Dunne et al., 2016). Furthermore, it has, in been observed that some patients whose tumour cells are deemed PD-L1 negative do in fact respond positively to PD-1 immunotheraoy (Ma et al., 2016). This highlights a need for better understanding of tumour microenvironmental PD-L1 expression, particularly in the immunomodulatory stromal cell comparment. *In this chapter we investigate the role of immunomodulatory ligand expression, in particular PD-L1, on the ability of stromal cells to modulate lymphocyte phenotype and function*.

Chapter 3 specific aims:

1. To determine the effects of the tumour secretome on the ability of stromal cells to modulate T cell proliferation and effector function
2. To investigate the role of tumour inflammatory signalling and NF-κB activation on this process
3. To identify the alterations in stromal cells in response to inflammatory tumour conditioning and block or reverse these changes with pharmacological intervention

3.2 Results

3.2.1 Inflammatory tumour conditioning of stromal cells results in a significantly enhanced capacity to inhibit T cell proliferation To test the functional consequences of tumour conditioning on the ability of stromal cells to inhibit lymphocyte proliferation and effector function we set up an *ex vivo* syngeneic culture system involving primary Balb/c stromal cells and lymphocytes and CT26 tumour cells, also of Balb/c origin. Primary murine stromal cells were isolated from Balb/c bone marrow from mice aged between 8 and 14 weeks. Primary bone marrow stromal cells represent a robust model cell type for examination of the effects of tumour conditioning on stromal cell components of the tumour microenvironment considering they share numerous characteristics with intestinal stromal cells (Mishra et al., 2008, Spaeth et al., 2009, Spaeth et al., 2013) . These cells can be successfully isolated with relative ease and are present in high enough density in the bone marrow to make numerous experiments feasible. These cells were characterised according to cell surface marker expression and the capacity for tri-lineage cells in terms of surface marker expression, gene signature s and differentiation **(Figure 3.1)**

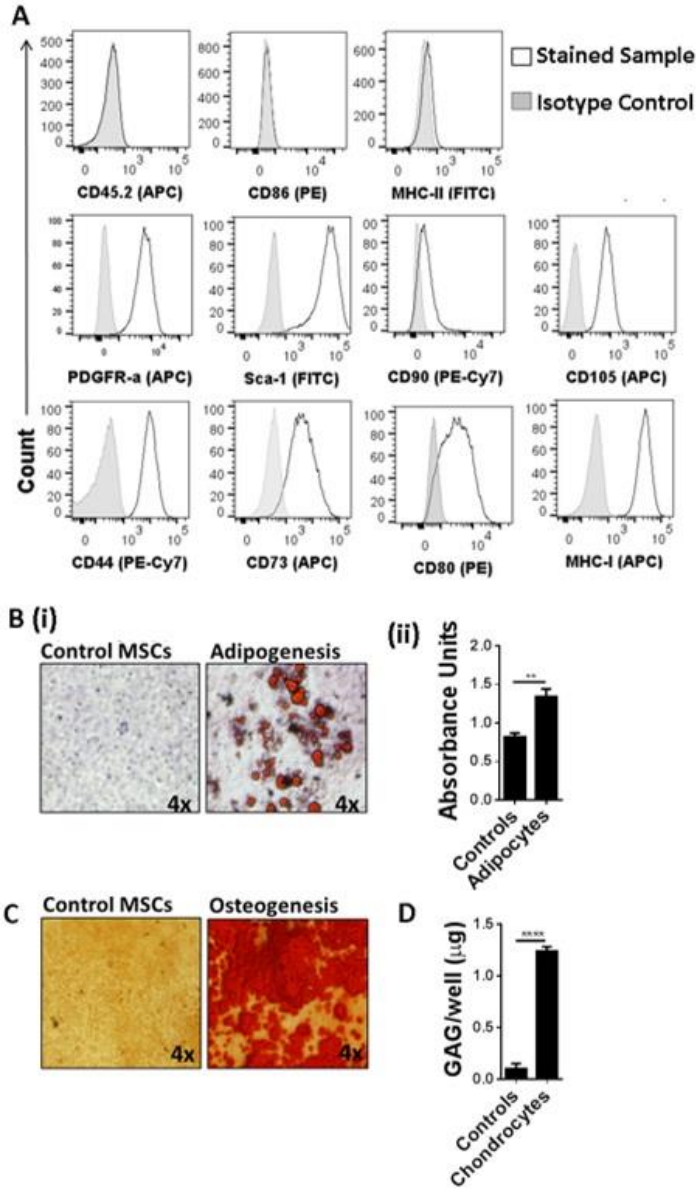
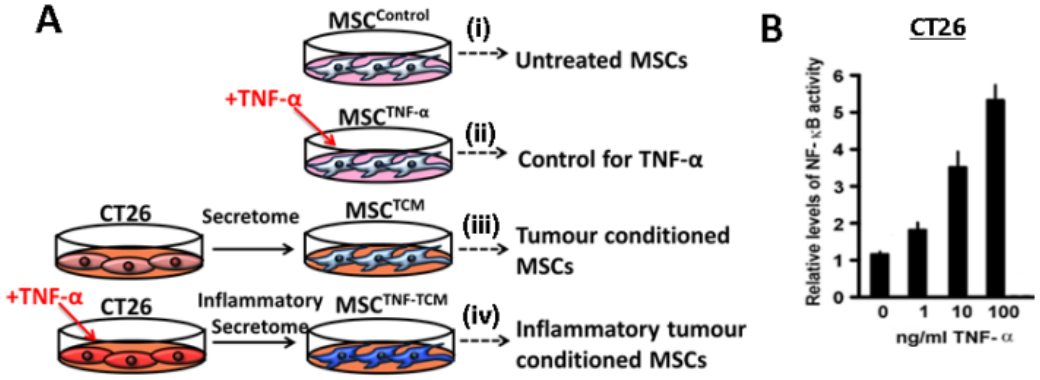


Figure 3.1 Characterisation of primary Balb/c MSCs isolated from bone marrow
(A) Bone marrow cultures were analysed by flow cytometry for their expression of cell surface markers necessary to confirm mesenchymal stromal cell phenotype. Black lines indicate antibody-specific staining, filled grey peaks represent isotype control staining. Tri-lineage differentiation capacity of MSCs was confirmed by the ability of MSCs to form **(B)(i)** adipocytes, (**C)** osteoblasts and **(D)** chondrocytes
Error bars: mean +/- SEM **p<0.01 ***p<0.001 one-way ANOVA, Tukey's Post Hoc Test n=3

To test the consequences of inflammatory tumour signalling upon these stromal cells, conditioned medium was harvested from CT26 cells and used to treat stromal cells *in vitro* (MSC^{TCM}). In order to better mimic the

inflammatory tumour microenvironment and to activate NF-κB signalling in tumour cells, CT26 were pre-activated with TNF-α and the treatment of stromal cells was repeated to yield "inflammatory tumour conditioned stromal cells" ($MSC^{TNF\text{-}TCM}$). A luciferase reporter gene assay confirmed robust, dose-dependent activation of NF-κB in CT26 following treatment with TNF-α. Naïve and TNF-α treated stromal cells served as controls. This treatment strategy is summarised in **Figure 3.2**.



3.2 Experimental set up
(A) MSC were seeded at 0.035 x 10^6 cells per well of a 6 well plate in 2ml culture medium. 24 hours after seeding the culture medium was removed and replaced with 40% fresh MSC medium and 60% tumour conditioned medium. Fresh DMEM and DMEM with 100ng/ml TNF-α were added as controls. MSCs were analysed at 24, 48 and 72 hours after addition of conditioned medium in 6 well plates, left to adhere for 24 hours and subsequently treated with 60% tumour conditioned medium for 72 hours. Treatment groups were as follows: (i) Untreated ($MSC^{Control}$) (ii) TNF-α treated ($MSC^{TNF\text{-}\alpha}$) (iii) CT26 conditioned medium (MSC^{TCM}) (iv) Conditioned medium from CT26 pre-activated with TNF-α ($MSC^{TNF\text{-}TCM}$) **(B)** NF-κB activation in CT26 in response to TNF-α treatment was measured by luciferase reporter gene assay. N=3

Our *ex*-vivo co-culture system consisted of CFSE stained primary Balb/c lymphocytes co-cultured with these syngeneic, pre-conditioned stromal cells. Lymphocytes were activated and stimulated to proliferate by the addition of CD3/CD28 so-stimulatory beads. Following 72 hours in co-culture, proliferation of $CD4^+$ and $CD8^+$ T cells, as defined by antibody staining, was analysed as described in **Figure 3.3**. Exposure to the

inflammatory tumour secretome did not alter the viability of stromal cells **(Figure 3.4)**.

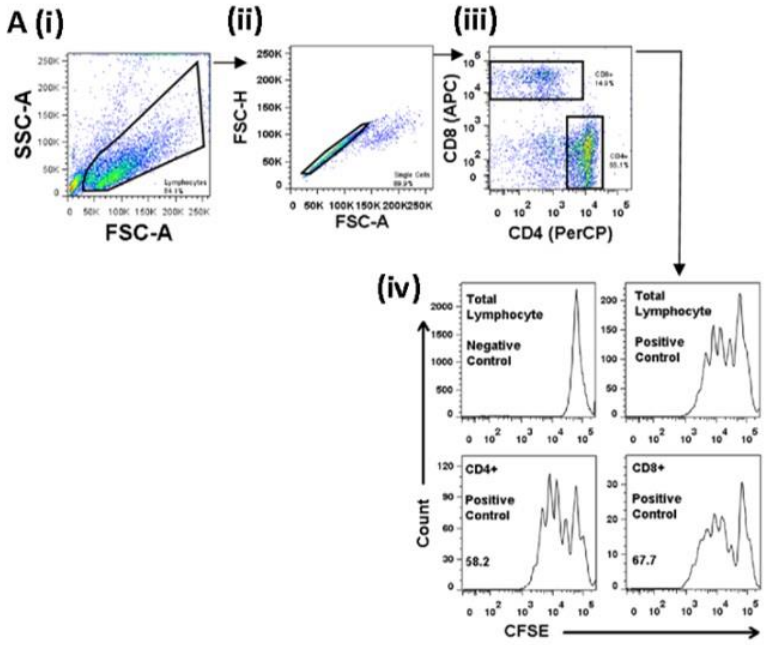


Figure 3.3 Gating strategy used to identify $CD4^+$ and $CD8^+$ T cell subset proliferation following co-culture with stromal cells
(A) T cells were identified by **(i)** size and granularity, **(ii)** doublets excluded and **(iii)** populations stained for CD4 (PerCP) and CD8 (APC)**(iv)** Representative histograms of positive (bead-stimulated) and negative (unstimulated, no beads) controls based on CFSE expression

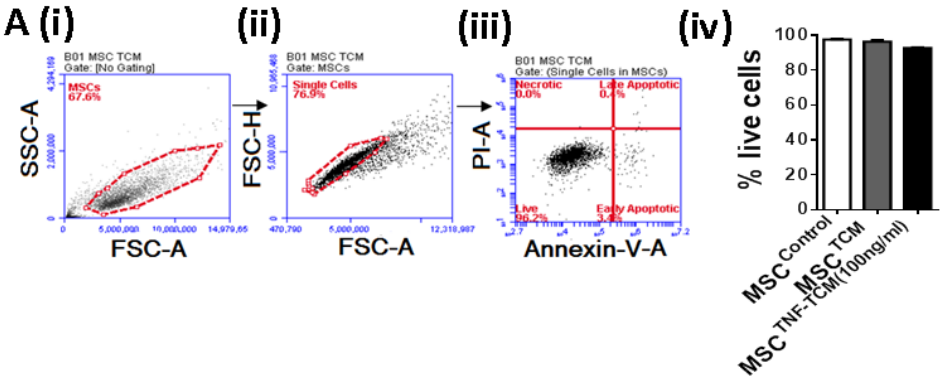


Figure 3.4 Tumour conditioning did not alter stromal cell viability
(A) Gating strategy used to analyse cell death in MSCs following tumour conditioning for 72 hours. MSCs were identified based on **(i)** size and granularity **(ii)** Doublets were excluded by plotting FSC-A vs FSC-H **(iii)** Death was measured by plotting Annxin V (FITC) vs PI (PE). Cells were identified as: Early Apoptotic AnnexinV^{+}PI^{-}, Late Apoptotic AnnexinV^{+}PI^{+} or necrotic AnnexinV^{-}PI^{+} **(v)** Bar chart of stromal cell viability following treatment with tumour conditioned medium.
Error bars: mean +/- SEM N=3

As previously documented, control MSCs were mildly suppressive in terms of their ability to reduce the proliferation of CD4^{+} and CD8^{+} T cells relative to positive control (stimulated T cells, no stromal cells), with a slight, but non-significant increase in this suppressive capacity with high-dose TNF-α treatment (Lee et al., 2015b) **(Figure 3.5)**. There was no significant increase in T cell suppressive capacity for stromal cells exposed to the control, non-inflamed tumour secretome compared to TNF-α treated stromal cells. However, this suppressive capacity was significantly increased by stromal cell exposure to the inflammatory tumour secretome. Furthermore, there was a dose dependent response to increasing concentrations of TNF-α pre-stimulation of CT26 tumour cells, with more potent suppression of CD4+ and CD8+ T cells observed with 100ng/ml TNF-α treatment of CT26 **(Figure 3.5 (iv))**. This highlights a central role for tumour mediated inflammation in dictating the immunomodulatory role of the stromal compartment. For this reason, 100ng/ml TNF-α pre-activation of CT26 was chosen for all subsequent experiments.

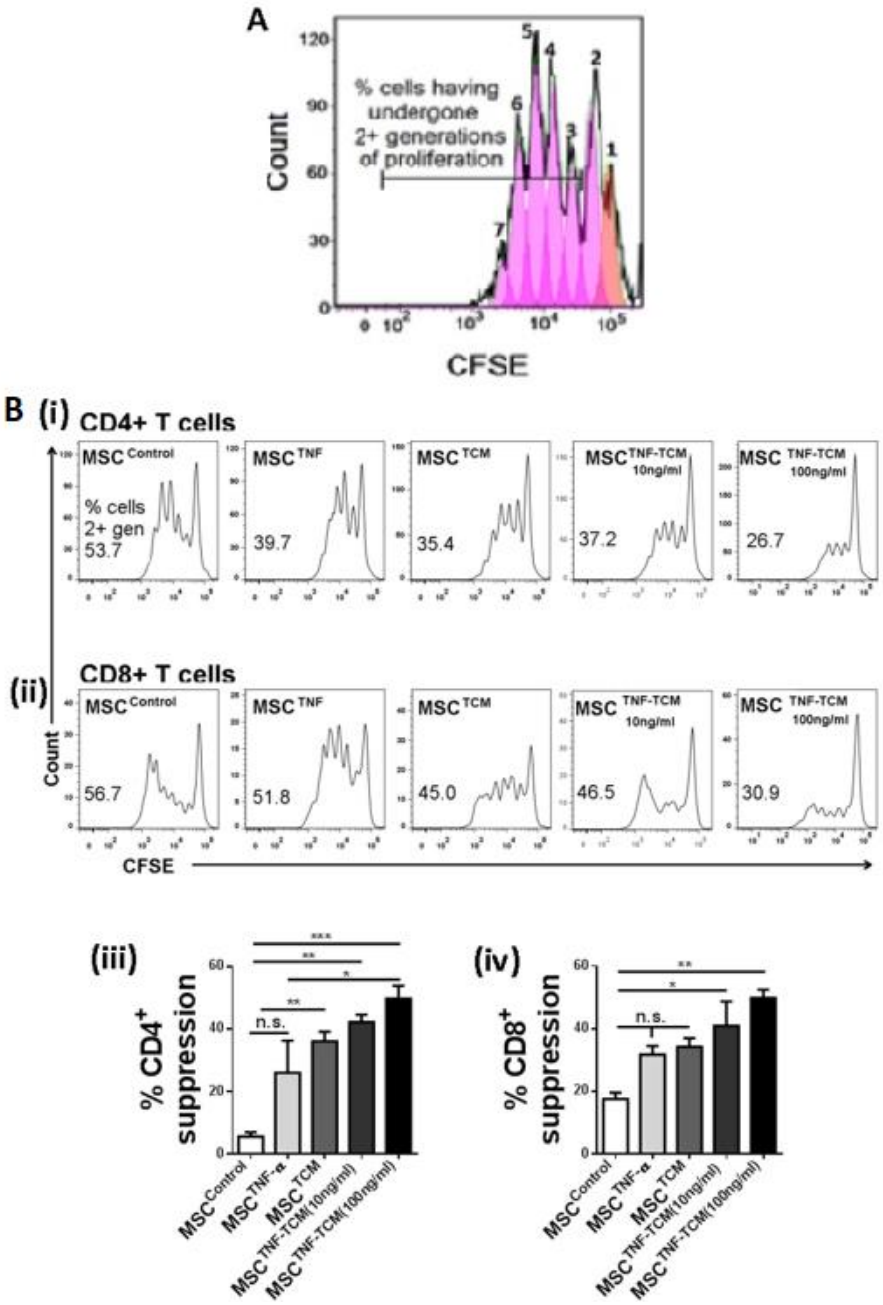


Figure 3.5 Inflammatory tumour conditioning of stromal cells results in a significantly enhanced capacity to inhibit T cell proliferation

(A) To accurately determine MSC mediated effects on T cell proliferation FlowJo®V7 proliferation software was used to account for the percentage of lymphocytes in each generation of proliferation, and analysis was carried out and plotted for cells having undergone 2 or more generations of proliferation. Percentage suppression was calculated by: 100-(%PositiveCtrlDivided/%SampleDivided)*100

(B) Representative histograms displaying **(i)** $CD4^+$ and **(ii)** $CD8^+$ T cell suppression following 72 hour co-culture with tumour conditioned MSCs. Bar charts for **(iii)** $CD4^+$ and **(iv)** $CD8^+$ suppression greater than 2 generations.

Error bars: mean +/- SEM *p<0.05, **p<0.01 ***p<0.001 one-way ANOVA, Tukey's Post Hoc Test n=3

Cytotoxic T lymphocytes (CTLs) are critical effectors of the anti-tumour immune response, and can carry out their cell killing function in a number of different ways. Two important mechanism by which CTLs clear transformed or malignant cells is via indirect killing by release of cytokines such as IFN-γ or TNF-α, or direct killing by release of cytotoxic proteases such as granzyme B (Andersen et al., 2006, Hodge et al., 2014). In addition to enhanced T cell suppression, inflammatory tumour conditioned stromal cells, compared to control stromal cells, also significantly reduced lymphocyte activation as measured by cytokine and effector molecule release **(Figure 3.6)**. ELISA analysis of cell culture supernatants showed that co-culture of lymphocytes with inflammatory tumour conditioned stromal cells resulted in a significant reduction in TNF-α and IFN-γ release two important cytokines employed by T cells in mounting a response against transformed cells and in stimulating other components of the anti-tumour immune machinery. There was also a significant reduction in the cytolytic capacity of lymphocytes when cultured with inflammatory tumour conditioned stromal cells, as measured by a reduction in granzyme B, a cytotoxic protease released by T cells for the purpose of direct killing of target cells, **(Figure 3.6A)**. The overall inhibitory effect exerted by tumour conditioned stromal cells on T cell proliferation and effector phenotype was most potent following stromal cell exposure to the secretome from TNF-α pre-treated CT26, indicating a requirement for inflammatory signalling in the tumour microenvironment in dictating stromal cell immunomodulatory function. These data demonstrate an enhanced ability of stromal cells to prevent the proliferation, activation and effector function of T cells following exposure to the inflammatory tumour secretome. Considering the close contact between the tumour cells and stromal niche, an immunosuppressive stromal compartment could impede anti-tumour immune effector subsets, such as $CD8^+$ cytotoxic T lymphocytes to eliminate transformed or malignant cells.

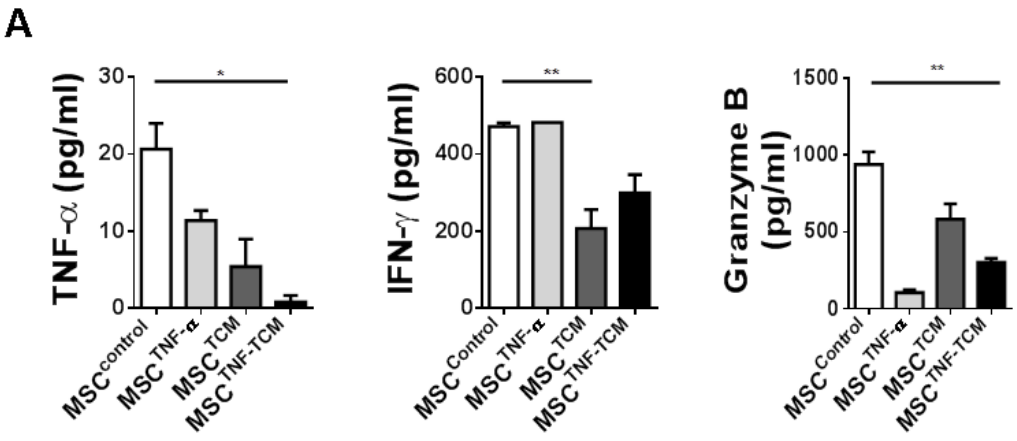


Figure 3.6 Inflammatory tumour conditioning of stromal cells results in a significantly enhanced capacity to inhibit T cell activation and cytolytic capacity
(A) Cell culture supernatants were analysed for the presence of cytokines and T cell effector molecule release following 72 hour co-culture. Error bars: mean +/- SEM *p<0.05, **p<0.01 one-way ANOVA, Tukey's Post Hoc Test n=3

3.2.2 Exposure to the inflammatory tumour secretome induces markers of an enhanced immunosuppressive ability on the stromal cell surface

Stromal cells have been shown to inhibit T cell responses by both cell contact-dependent mechanisms via cell surface protein expression, and cell contact independent mechanisms via release of soluble mediators (Chinnadurai et al., 2014a, Di Nicola et al., 2002). Early preliminary experiments in our lab involving the use of transwells to separate MSCs and T cells indicated that the mechanism for our observations here were contact-dependent. Furthermore, our findings of an enhanced immunosuppressive stromal cell potential following exposure to the inflammatory tumour secretome was observed following contact-dependent interactions between the stromal cells and lymphocytes, so this prompted a comprehensive analysis of an array of ligands on the stromal cell surface known to have immunomodulatory capacity. This analysis was carried out at for an early time point – 24 hours **(Figure 3.7A, B, C)** and the time at which stromal cells were added to the lymphocyte co-culture system – 72 hours **(Figure 3.7D, E, F)**. These time points were selected as we believed such analysis would allow

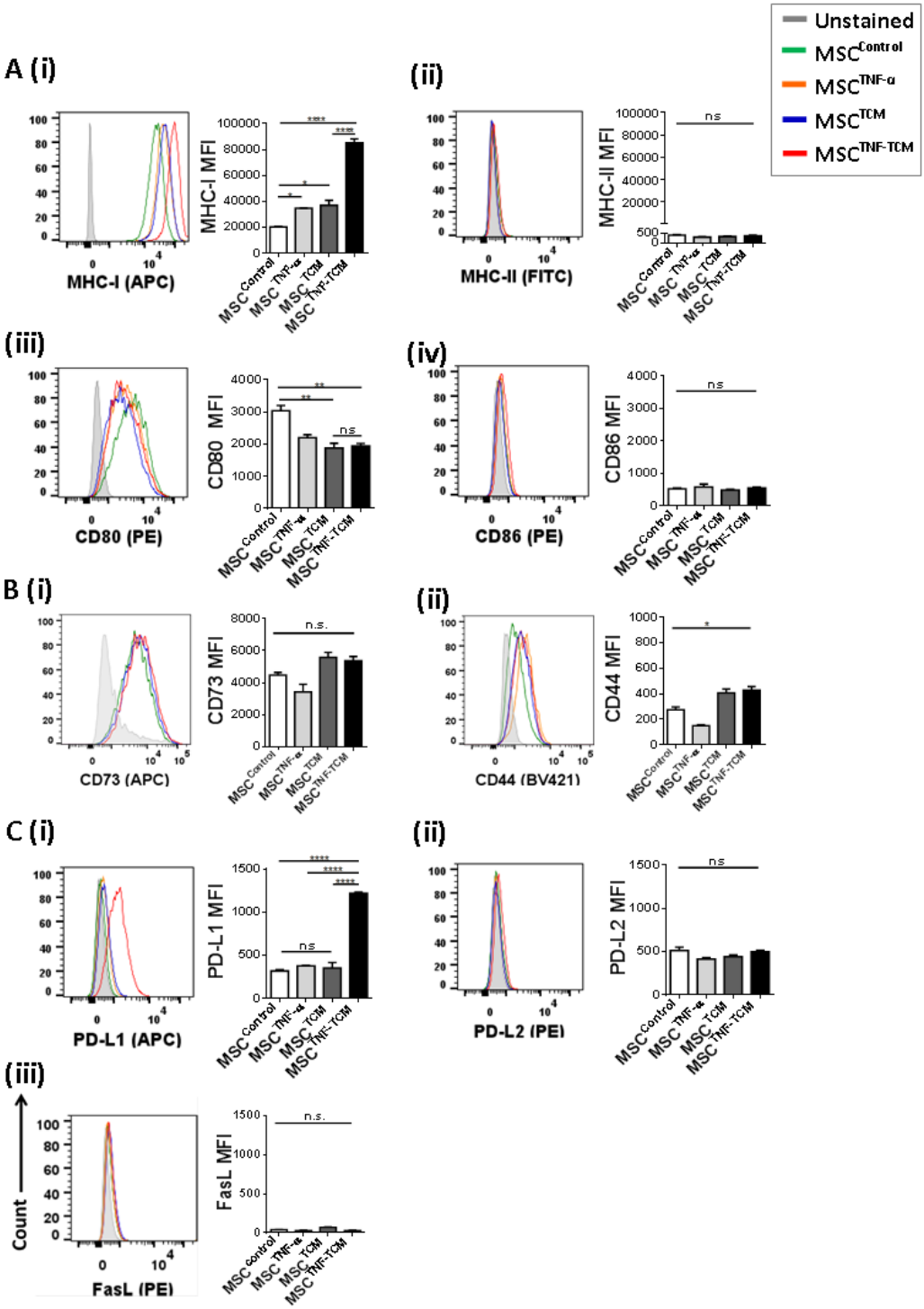
us to identify the cell surface proteins responsible for the enhanced immunosuppressive phenotype we had observed in previous experiments, but also provide insight into the dynamics of protein expression on the stromal cell surface following initial interaction with the factors present in the inflammatory tumour microenvironment and thus, potentially provide insight as to the primary signalling pathways activated in stromal cells by this inflammatory tumour signalling (discussed in detail in Chapter 5).

We began by measuring the expression levels of the ligands for the T cell receptor – MHC-I and MHC-II, and co-receptor – CD80 and CD86 **(Figure 3.7A (24hr), D (72hr)).** Compared to control cells, tumour conditioning, in the presence or absence of inflammation, had no effect on the surface expression of MHC-II or CD86, with expression of these two markers remaining very low or negative for both time point analysed **(Figure 3.7A(ii),(iv), D(i),(iv))**. CD80 expression was decreased by tumour conditioning, irrespective of inflammation, but with expression levels remaining high at both 24 and 72 hours **(Figure 3.7A(iii), D(iii))**. Interestingly, there was a significant increase in MHC-I expression following stromal cell exposure to the tumour secretome, and this effect was significantly enhanced by inflammatory tumour activation and was sustained over 72 hours **(Figure 3.7A(i), D(i))**. This observation may have important consequences, considering tumour antigens can be presented on MHC-I complexes. This increased MHC-I expression can potentially result in dysfunctional activation of antigen-specific $CD8^+$ T cells whereby tumour antigen is presented as "self", thus inducing tolerance to the tumour, or alternatively, the increased MHC-I will present tumour antigen to T cells, but the lack of a concomitant increase in co—stimulatory molecule expression will lead to T cell anergy or death (Hirosue and Dubrot, 2015).

The next markers we investigated were CD73 and CD44 **(Figure 3.7B and E)**. CD73 has been shown to inhibit activation, clonal expansion and homing of tumour-specific T cells and its expression in the tumour stromal compartment is associated with poor patient survival (Antonioli et al., 2016,

Huang et al., 2014). CD44 is a multifunctional glycoprotein shown to be important in stromal cells acquiring a tumour-promoting, activated fibroblast phenotype (Spaeth et al., 2013). At the earlier time point CD73 expression remained unchanged following tumour conditioning **(Figure 3.7B(i))** with a decrease in expression observed at 72 hours **(Figure 3.7E(i))**. However, this increase was independent of inflammatory tumour conditioning, as a similar decrease was observed for stromal cell treatment with TNF-α, the control tumour secretome, or inflammatory tumour secretome. There was a very moderate increase in CD44 expression following exposure to the inflammatory tumour secretome at 24 hours **(Figure 3.7B(ii))**, but this increase was no longer evident at 72 hours **(Figure 3.7E(ii))**.

Finally, we assessed the expression levels of PD-L1, PD-L2 and Fas Ligand, three known inhibitors of T cell proliferation and effector function **(Figure 3.7C and F)** (Igney and Krammer, 2005, Latchman et al., 2001, Nguyen and Ohashi, 2015). Expression of PD-L2 and Fas Ligand, were found to be low or almost absent respectively, and remained unchanged following exposure to the tumour secretome for both time points assessed **(Figure 3.7C(ii)(iii) and 3.7F(ii)(iii))**. However, PD-L1, a T cell inhibitory ligand known to contribute to tumour immune escape, was found to be dramatically increased following inflammatory tumour conditioning at the earlier time point **(Figure 3.7C(i))** and this increase was sustained over 72 hours **(Figure 3.7F(i))** (He et al., 2015, Juneja et al., 2017). Taken together these results point to induced PD-L1 expression on stromal cells as a potentially important mechanism underpinning the observation of an enhanced immunosuppressive capacity in these cells, and thus we felt, warranted further investigation.


Unstained
MSC^Control
MSC^TNF-α
MSC^TCM
MSC^TNF-TCM
A (i)
MHC-I (APC)
MHC-I MFI
(ii)
MHC-II (FITC)
MHC-II MFI
n.s
(iii)
CD80 (PE)
CD80 MFI
(iv)
CD86 (PE)
CD86 MFI
B (i)
CD73 (APC)
CD73 MFI
n.s.
(ii)
CD44 (BV421)
CD44 MFI
C (i)
PD-L1 (APC)
PD-L1 MFI
(ii)
PD-L2 (PE)
PD-L2 MFI
(iii)
Count
FasL (PE)
FasL MFI

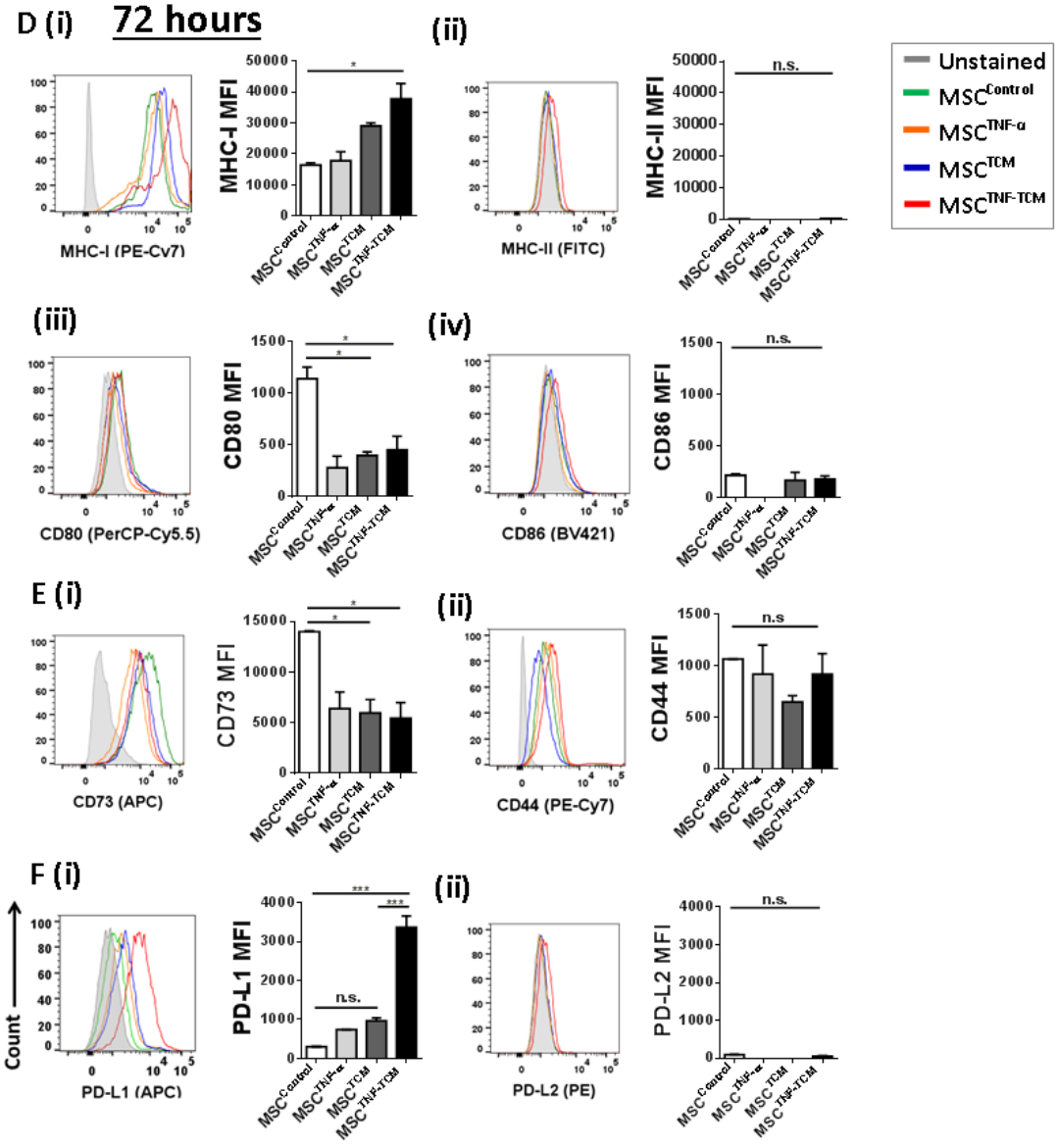


Figure 3.7 Exposure to the inflammatory tumour secretome induces markers of an enhanced immunosuppressive ability on the stromal cell surface Representative histograms and bar charts displaying median fluorescent intensity (MFI) for flow cytometric analysis of the stromal cell surface following **(A-C)** 24 hour and **(D-F)** 72 hour exposure to the inflammatory tumour secretome Error bars: mean +/- SEM *p<0.05, **p<0.01 ***p<0.001 one-way ANOVA, Tukey's Post Hoc Test n=3

3.2.3 The tumour secretome mediated induction of PD-L1 on the stromal cell surface is stable under conditions of normoxia and hypoxia

In response to the published finding that PD-L1 expression on MDSCs and other immune cells in tumour bearing mice was dependent on hypoxia-inducible factor (HIF)-1α, and in light of the fact that hypoxia is a common feature of many solid tumours, we next sought to test the effects of hypoxic culture conditions on the induction of PD-L1 expression on stromal cells **(Figure 3.8A)** (Noman et al., 2014, Semenza, 2011). To this end, stromal cells were cultured, passaged and grown to the same confluency in hypoxia as for previous experiments in normoxia described thus far. We compared the level of PD-L1 induced by control and inflammatory tumour conditioned medium under normoxic (21% O_2) and hypoxic (5% O_2) conditions in terms of fold induction so that we could normalise to control MSCs on different days of analysis. The induction of PD-L1 on the stromal cell surface following culture and treatment under hypoxic conditions was similar to that observed under normoxic culture conditions, with no differences detected in the level of PD-L1 induced on the stromal cell surface **(Figure 3.8A(iii))**. Since we did not observe any difference in PD-L1 induction between normoxic and hypoxic culture conditions, and the fact that our earlier immunosuppression results were obtained following culture under normoxic culture conditions, we carried out all further analysis using stromal cells grown under conditions of normal oxygen concentration.

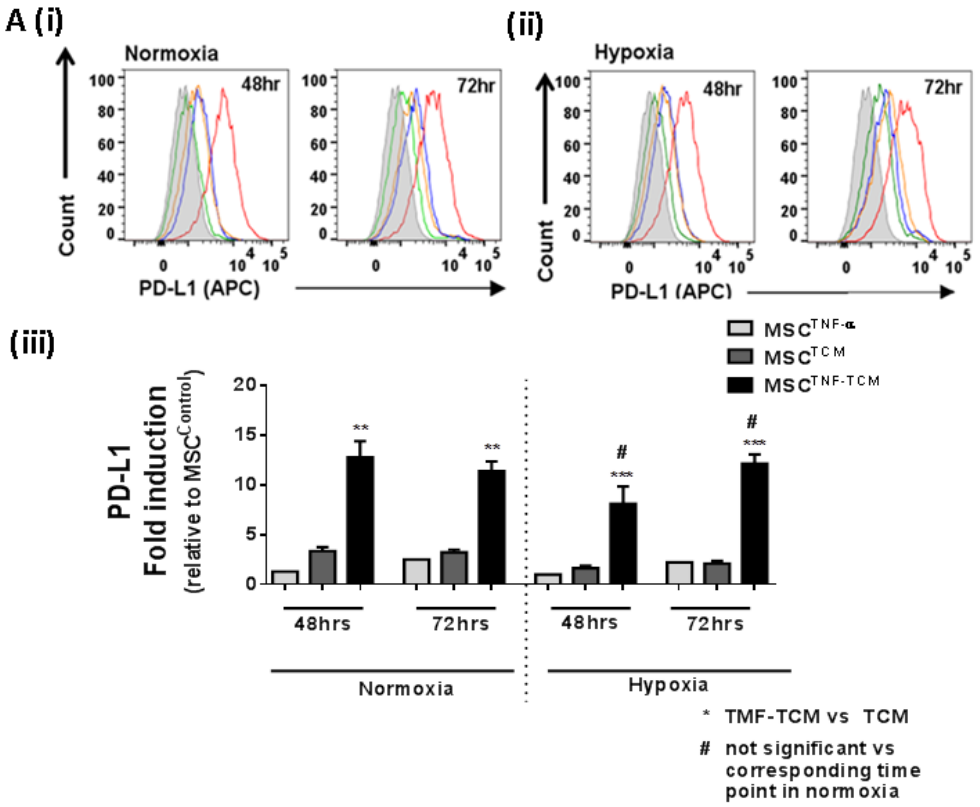


Figure 3.8 The tumour secretome mediated induction of PD-L1 on the stromal cell surface is stable under conditions of normoxia and hypoxia **(A)** Representative histograms displaying median fluorescent intensity (MFI) for flow cytometric analysis of the stromal cell surface PD-L1 expression under **(i)** normoxic and **(ii)** hypoxic culture conditions **(iii)** The magnitude of PD-L1 induction expressed relative to the level expressed on control stromal cells for each time point and culture condition **(B)**
Error bars: mean +/- SEM **p<0.01 ***p<0.001 one-way ANOVA, Tukey's Post Hoc Test N=3

3.2.4 The level of PD-L1 induced on stromal cells following exposure to the inflammatory tumour secretome is comparable to that expressed on CT26 tumour cells, and correlates directly with their *in vitro* immunosuppressive capacity

CT26 tumour cells are described as immunogenic and have been shown to express robust levels of PD-L1 (Lau et al., 2017). We next sought to determine how the level of PD-L1 expression induced on the stromal cell surface compared to the level expressed on CT26 tumour cells, since it is

now well documented that it is not only PD-L1 expression on tumour cells, but accessory cells in the tumour microenvironment, that can predict patient outcomes and responses to immune checkpoint therapy (Kim et al., 2016, Santarpia and Karachaliou, 2015). We found that the level of PD-L1 induced on the stromal cell surface following exposure to the inflammatory tumour secretome was comparable to the level of basal expression of PD-L1 on CT26 **(Figure 3.9A)**. This finding again points to a potential important role for the stromal niche in dictating the fate of tumour infiltrating PD-1 receptor expressing immune cells. In order to test the dependency of this observed enhanced immunosuppressive capacity, we next aimed to block PD-L1/PD-1 signalling interactions in our co-culture system. Before doing this, and to validate the targeting of this signalling pathway, we carried out a correlative analysis to verify a link between PD-L1 expression and immunosuppressive ability. When the level of stromal cell PD-L1 expression was plotted against the ability to suppress $CD8^+$ T cell suppression a Pearson correlation coefficient of 0.9088 was obtained with a p value of 0.0003, indicating an almost linear, very strong positive correlation between the level of PD-L1 induced on the stromal cell surface and the ability to inhibit $CD8^+$ T cell proliferation **(Figure 3.9B (ii))**. Although weaker, there was also a high degree of correlation between PD-L1 expression level and the ability of stromal cells to suppress $CD4^+$ proliferation, as demonstrated by a Pearson correlation coefficient of 0.7728 **(Figure 3.9B (i))**. We felt that these data provided compelling rationale for blocking PD-L1/PD-1 signalling interactions in our co-culture system in order to confirm a role for stromal cell immunosuppression in response to signals released from the inflammatory tumour microenvironment.

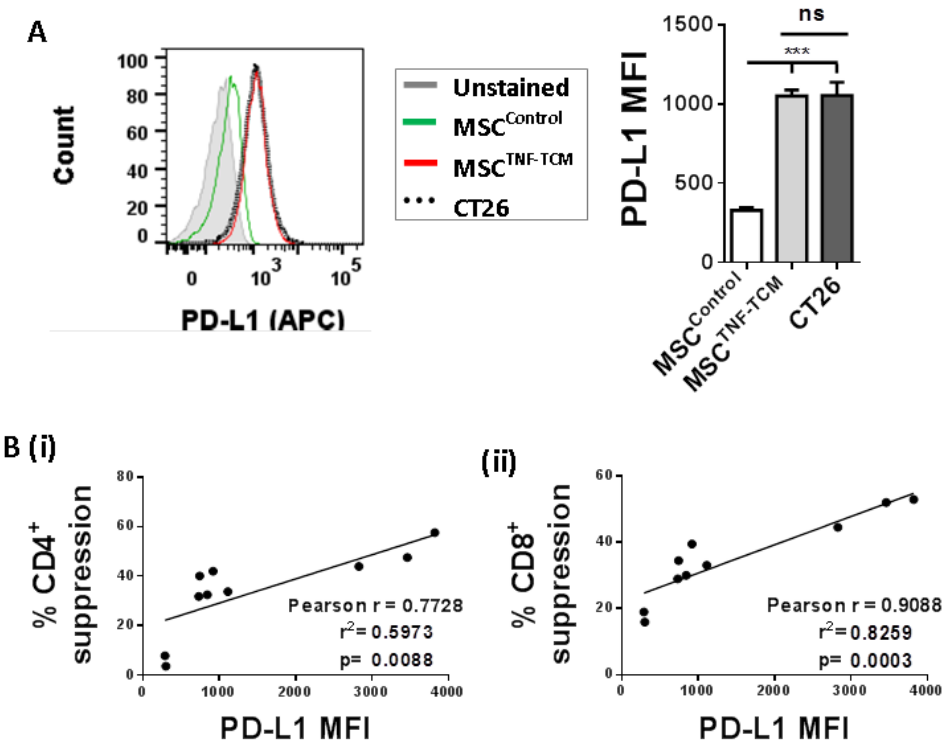
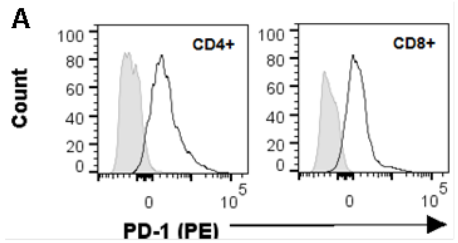


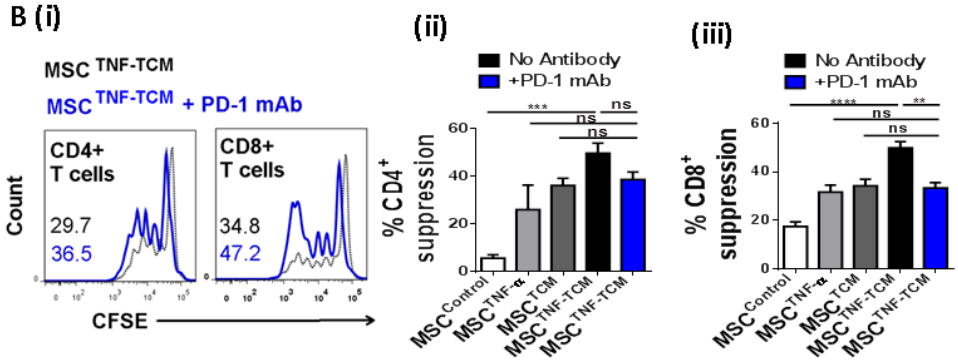
Figure 3.9 The level of PD-L1 induced on stromal cells following exposure to the inflammatory tumour secretome is comparable to that expressed on CT26 tumour cells, and correlates directly with their *in vitro* immunosuppressive capacity (A) Representative histogram and bar chart for PD-L1 expression on stromal and CT26 tumour cells **(B)** Pearson correlation coefficients of 0.7728 and 0.9088 were calculated for PD-L1 MFI on stromal cells after 72 hour exposure to the inflammatory tumour secretome (from **Figure 3.7F)** plotted against percentage $CD4^+$ T cell suppression and CD8+ T cell suppression (calculated in **Figure 3.5 (iii)** and **(iv)**) respectively
Error bars: mean +/- SEM ***p<0.001 one-way ANOVA, Tukey's Post Hoc Test N=3

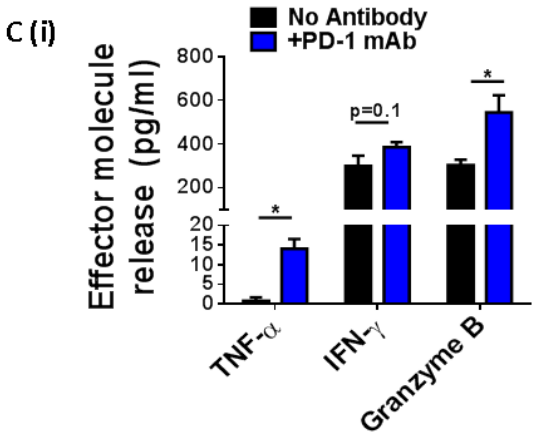
3.2.5 Blocking PD-1 signalling reverses the increased CD8+ T cell suppression induced by inflammatory tumour conditioned stromal cells

Since we had observed strong correlations between the level of PD-L1 expressed on the stromal cell surface, we next aimed to confirm a definitive role for role for stromal cell PD-L1 in the suppression of T cell proliferation and activation. In order to assess this we targeted the PD-1/PD-L1 signalling axis using a monoclonal blocking antibody to PD-1 receptor. For this reason, we began our analysis by measuring the expression of the PD-L1 receptor, PD-1, on primary murine lymphocytes. We observed high PD-1 expression on both $CD4^+$ and $CD8^+$ T cells **(Figure 3.10A)** in our culture system.

Treatment with anti-PD-1 antibody significantly reduced the ability of inflammatory tumour conditioned stromal cells to suppress the proliferation of $CD8^+$ T cells **(Figure 3.10B(iii))** In fact, following α-PD-1 antibody treatment, $CD8^+$ T cell suppression elicited by stromal cells exposed to the inflammatory tumour secretome was comparable to that induced by stromal cells exposed to the tumour secretome alone or TNF-α treated MSCs. These findings indicate that the enhanced $CD8^+$ T cell suppressive capacity of these cells is dependent on stromal PD-L1 expression induced by inflammatory signalling in the tumour microenvironment. A similar trend was seen with a decrease in the capacity of inflammatory tumour conditioned stromal cells to inhibit $CD4^+$ T cell suppression upon treatment with anti-PD-1, but this change was not statistically significant indicating a separate, as yet unknown mechanism by which stromal cells in the tumour microenvironment modulated $CD4^+$ T cells **(Figure 3.10B(ii))**. Additionally, PD-1 blockade was sufficient to restore lymphocyte activation and cytolytic potential, as determined by ELISA based measurements of IFN-γ, TNF-α and granzyme B secretion **(Figure 3.10C)**. This demonstrates the critical role of the PD-1/PD-L1 signalling axis in the ability of tumour-conditioned stromal cells to inhibit $CD8^+$ T cell mediated anti-tumour immune effector functions. Secretion of a panel of sever other pro- and anti-inflammatory cytokines measured by Bioplex analysis showed that secretion of one other cytokine, IL-5, a cytokine important in aiding B cell maturation and antibody production, was enhanced by the blockade of the PD-1/PD-L1 signalling axis. (Takatsu, 2011). This is potentially an important finding worthy of further investigation given the ability of IL-5 to activate and aid the migration of eosinophils, another population of immune cells with known anti-tumour capabilities (Carretero et al., 2015)


A
Count
CD4+
CD8+
PD-1 (PE)


B (i)
MSC TNF-TCM
MSC TNF-TCM + PD-1 mAb
CD4+ T cells
29.7
36.5
CD8+ T cells
34.8
47.2
Count
CFSE
(ii)
No Antibody
+PD-1 mAb
% CD4+ suppression
(iii)
% CD8+ suppression


C (i)
No Antibody
+PD-1 mAb
Effector molecule release (pg/ml)
p=0.1
TNF-α
IFN-γ
Granzyme B

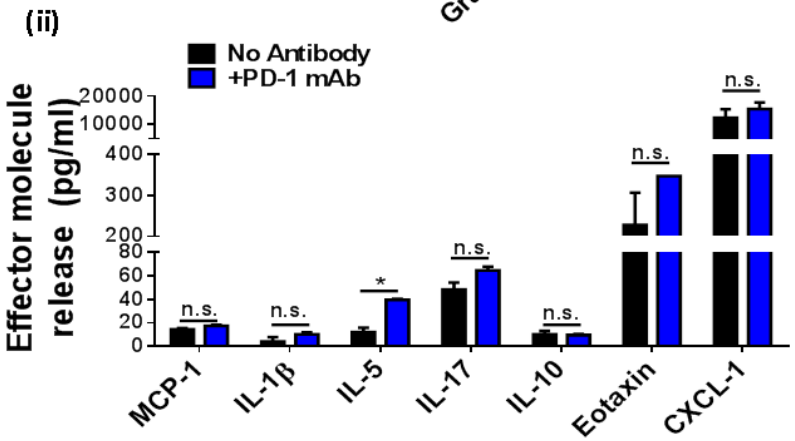
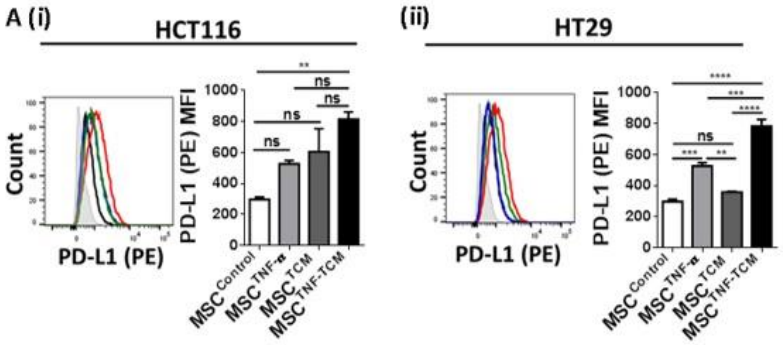

(ii)
No Antibody
+PD-1 mAb
Effector molecule release (pg/ml)
n.s.
MCP-1
IL-1β
IL-5
IL-17
IL-10
Eotaxin
CXCL-1

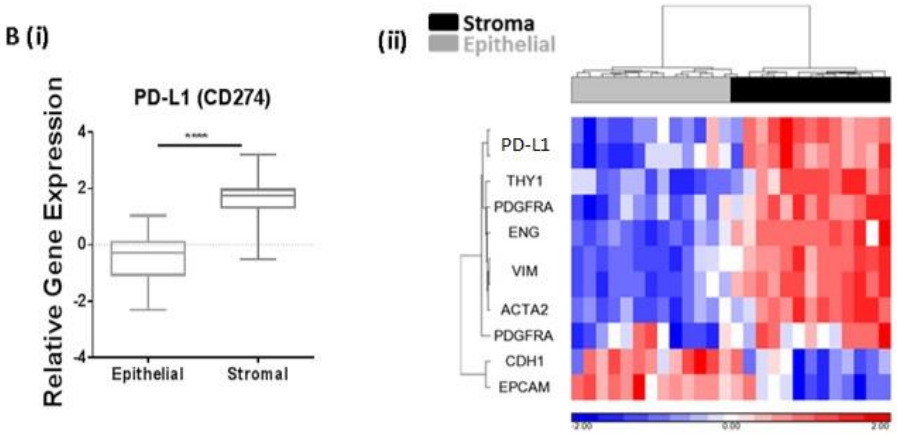
Figure 3.10 Blocking PD-1 signalling reverses the increased CD8+ T cell suppression induced by inflammatory tumour conditioned stromal cells (A) Representative histograms of PD-1 expression on murine CD^{4+} and $CD8^{+}$ T cells **(B)(i)** Representative histograms of $CD4^{+}$ and $CD8^{+}$ T cells with (blue histogram/bars) and without (black dotted histogram/black bars) PD-1 blocking antibody **(ii)** and **(iii)** Bar charts showing % $CD4^{+}$ and $CD8^{+}$ T cell suppression following treatment with PD-1 blocking antibody **(C)** Measurement of cytokines and T cell effector molecules secrete into culture supernatants following treatment with anti-PD-1 mAb (blue bars) as measured by **(i)** ELISA and **(ii)** Bioplex analysis
Error bars: mean +/- SEM *p<0.05, **p<0.01, one-way ANOVA, Tukey's Post Hoc Test N=3

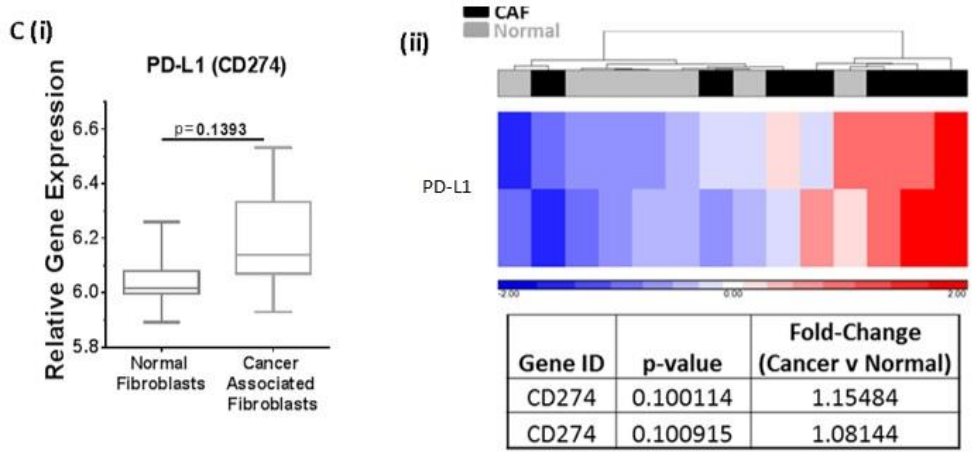
3.2.6 PD-L1 is also induced on human stromal cells exposed to the inflammatory tumour secretome, irrespective of microsatellite instability, and is differentially expressed on cancerous stroma in clinical samples

Since our experiments to date had been carried out using murine tissue, and before proceeding to *in vivo* studies, we next wanted to confirm the phenomenon of stromal cell PD-L1 induction in response to the inflammatory tumour secretome in human cells. Using primary human bone marrow stromal cells, we observed a significant increase in PD-L1 expression upon exposure to the inflammatory tumour secretome irrespective of microsatellite (HCT116) or chromosomal (HT29) instability **(Figure 3.11A (i) and (ii))** (Dunican et al., 2002). In addition to this, we collaborated with Dr. Philip Dunne from the Centre for Cancer Research and Cell Biology in Queen's University Belfast, who provided us with transcriptional profiles of CRC resection samples, which had been laser capture microdissected (LCM) to isolate the stromal and epithelial fractions prior to microarray profiling. Assessment of PD-L1 gene expression (CD274) indicated a significantly higher level of PD-L1 in the stromal compartment of CRC compared to the epithelial cells **(Figure 3.11 B(i))**. Importantly, assessment of EpCam, E-caderhin (CDH1) as markers of epithelial lineage, and, PDGFRA, THY1 (CD90), ENG (CD105), α-SMA (ACTA2) and Vimentin (VIM) as markers of mesenchymal lineages further confirmed the purity of the samples following LCM **(Figure 3.11 B(ii))**.

Our data in both mouse and human models has indicated that PD-L1 expression is elevated in MSCs following exposure to conditioned media from inflammatory stimulated colon cancer cells. Cancer associated fibroblasts (CAFs) represent a major component of CRC stroma, and as such provides a suitable model to test our findings in CRC clinical samples. Therefore we utilised gene expression profiles derived from patient-matched primary fibroblasts (n=14 samples from 7 patients) isolated from within CRC tissue (CAFs), to represent MSCs following cancer-cell exposure, or from adjacent, albeit distant, normal mucosal tissue (Normal) to represent unconditioned MSCs. Analysis of overall gene expression profiles indicated a non-significant trend (p-value 0.10; range 1.15 – 1.08 fold-change) towards increased PD-L1 gene expression in CAFs compared to normal **(Figure 3.11C (i) and (ii))**. Importantly, a pairwise analysis of patient matched samples further confirmed this finding, with 6 of the 7 matched CAFs displaying an increase in PD-L1 gene expression compared to their patient-matched normal fibroblast **(Figure 3.11D (i) and (ii))**.







Gene ID	p-value	Fold-Change (Cancer v Normal)
CD274	0.100114	1.15484
CD274	0.100915	1.08144

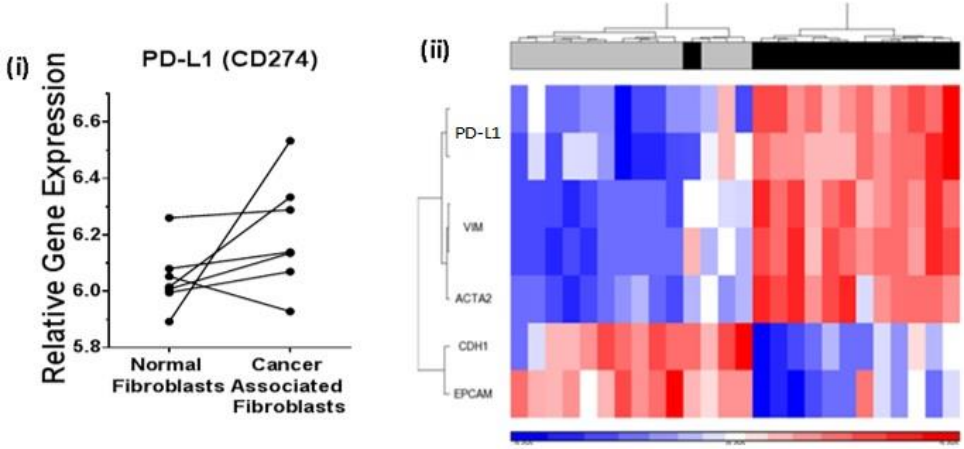


Figure 3.11 PD-L1 is also induced on human stromal cells exposed to the inflammatory tumour secretome, irrespective of microsatellite instability, and is differentially expressed on cancerous stroma in clinical samples **(A)** Representative histograms and bar charts of PD-L1 expression on human MSCs following exposure to the inflammatory tumour secretome from **(i)** HCT116 and **(ii)** HT26 tumour cells

Error bars: mean +/- SEM *p<0.05, **p<0.01, one-way ANOVA, Tukey's Post Hoc Test N=3

(B)(i) Relative PD-L1 gene expression profile on epithelial and stromal cells from CRC patients **(ii)** Clustering for gene expression profiles of PDGFRA, CD274 (PD-L1), THY1 (CD90), ENG (CD105), Vimentin, ACTA2 (α-SMA), CDH1 (E-cadherin) and EPCAM **(C)(i)** Relative PD-L1 gene expression profile on normal and cancer associated fibroblasts CRC patients **(ii)** Clustering for gene expression profile of CD274 (PD-L1) between normal and cancer-associated fibroblasts (CAFs). **(D)** Pairwise analysis of relative PD-L1 gene expression between normal and cancer-associated fibroblasts from patients with CRC **(ii)** Clustering for gene expression profile of CD274 (PD-L1), Vimentin and ACTA2 (α-SMA) (mesenchymal lineage) and CDH1 (E-cadherin) and EPCAM (epithelial lineage).

Error bars: Min-Max ****p<0.0001, unpaired T test

These findings are particularly interesting in the context of immunotherapy where biologics targeting PD-L1 and PD-1 are showing great promise. So much so that nivolumab, an antibody targeting PD-1, received FDA approval for metastatic colon cancer in August of this year (FDA, 2017). However, while success in treating patients is evident, this is only true for a subset of patients. A number of clinical trials have relied on measurements of PD-L1 expression on tumour cells, or tumour infiltrating immune cells in an effort to predict those patients that will respond best, but this method of stratification has had only limited success, with evidence now showing that some patients deemed PD-L1 negative will in fact respond to this kind of therapy (Ma et al., 2016, Ribas and Hu-Lieskovan, 2016). Our findings may highlight an important role for stromal cell PD-L1 expression in modulating anti-tumour T cell responses and in selecting optimal immunotherapeutic strategies for patients.

3.3 Discussion

In this chapter we show for the first time that exposure to the inflammatory tumour secretome induces an enhanced immunosuppressive phenotype in stromal cells. Furthermore, we present the novel finding that this enhanced immunosuppressive phenotype observed in inflammatory tumour conditioned stromal cells is mediated by PD-L1 induction on the stromal cell surface.

Tumour conditioning enhanced MSC capacity to suppress $CD4^+$ and $CD8^+$ T cells

In agreement with previously published data, we show that mesenchymal stromal cells are mildly suppressive in terms of their capacity to inhibit T cell proliferation, and this capability is not dramatically affected by TNF-α treatment (Keating, 2008, Ren et al., 2008). However, we show in this chapter that this immunosuppressive capacity is significantly enhanced by exposure to the tumour secretome **(Figure 3.5)**. Furthermore, we show for the first time that this enhancement of stromal cell immunomodulatory capacity is dependent on signalling taking place in the tumour in response to inflammatory activation. Although the actual physiological levels of TNF-α in the colon during inflammation or cancer, (we based our selection of TNF-α dose on that in the literature), monocytes from colorectal cancer patients have been shown to produce significantly more TNF-α than those from healthy patients. Furthermore, the level of TNF-α produced by these cells increased to as high as 2298pg/ml when taken from those patients with later stage disease (Stanilov et al., 2014)Given that this is only one of the several TNF-α producing cells in the inflamed colon we estimate the actual levels present to be very high. We hypothesise that tum our cell NF-κB activation is the mechanism responsible for our observed results, for two main reasons. Firstly, a vast body of evidence exists to show a strong link between constitutive NF-κB activation in the colon, as occurs in many patients with inflammatory bowel disease, with the development of

colorectal cancer (Ben-Neriah and Karin, 2011, Greten et al., 2004, Karin, 2009). Furthermore, data from our own lab shows that targeting colon tumour cell NF-κB reduces tumour growth, an effect which is partially dependent on an induced M1 macrophage phenotype (Ryan et al., 2015). This reduction in tumour burden is, however, not entirely reversed by depletion of macrophages, indicating the involvement of at least one more immunomodulatory cell type, whose immunomodulatory function is also under the influence of NF-κB. Secondly, the inflammatory stimulus used to activate the tumour cells in our experiments was TNF-α.This cytokine is a known potent, and commonly used activator of NF-κB signalling (Schutze et al., 1995, Zhou et al., 2003). Additionally, we show by luciferase reporter gene assay, a dose-dependent induction of NF-κB signalling in CT26 tumour cells in response to treatment with TNF-α **(Figure 3.2B)**. In order to confirm the pivotal role for NF-κB signalling in tumour cells in modulating the immunomodulatory capacity of the tumour stromal compartment, it will be necessary to target NF-κB before exposing stromal cells to the tumour secretome. This will be further discussed in Chapter 5.

Tumour conditioning stromal cells reduce activation and cytolytic capacity of T cells

In addition to showing an increased ability of inflammatory tumour conditioned stromal cells to inhibit the proliferation of $CD4^+$ and $CD8^+$ T cells, we also show that these stromal cells reduce the activation and cytolytic capacity of T cells, as measured by the release of T cell effector molecules **(Figure 3.6)**. As mentioned earlier, TNF-α and IFN-γ have important roles to play in the anti-tumour immune response, and in particular, in stimulating other anti-tumour immune effector cells. Granzyme B is a cytotoxic protease release by cytotoxic T lymphocytes for the purpose of killing tumour cells (Rousalova and Krepela, 2010, Hodge et al., 2014). A diminished secretion of these important anti-tumour immune effector molecules indicates a T cell

phenotype less capable of clearing transformed or malignant cells. As was the case for inhibition of T cell proliferation, this inhibition of effector molecule release was, in the case of TNF-α and granzyme B, dependant on tumour cell inflammatory activation, where the observed reduction in IFN-γ was dependent only on tumour conditioning, and independent of inflammatory tumour signalling. Taken together these data suggest that in an inflammatory tumour microenvironment, not only could T cell proliferation be dramatically inhibited, but also that those T cells are likely to have diminished cytolytic capacity and determined by release of proteolytic compounds.

The increased immunosuppressive phenotype observed for inflammatory tumour conditioned stromal cells is dependent on stromal cell PD-L1 expression

The finding that the immunomodulatory potential of stromal cells in the tumour microenvironment is, in the case of $CD8^+$ T cell suppression and effector molecule release, dependent on PD-L1 has particular relevance in light of recent developments in the field of cancer immunotherapy. The uses of PD-1 or PD-L1 immune checkpoint inhibitor are beginning to enter widespread use in the clinic. Our finding of a lack of a significant restoration of $CD4^+$ T cell proliferation could point to a second mechanism of T cell suppression employed by tumour conditioned stromal cells. It is known that TGF-β can induce a regulatory $CD4^+$ T cell phenotype (T_{reg}), and a more comprehensive analysis of these signalling pathways may shed further light on this observation (Moo-Young et al., 2009).

From the initial approval for pembrolizumab in unresectable melanoma only three years ago, four more agents targeting this signalling pathway have already joined the list of approved tumour immunotherapeutics (discussed in Chapter 1), and the indications in which these drugs have been approved is increasing rapidly, with FDA approval for use in colon cancer having been granted very recently (Alexander, 2016, FDA, 2017). This highlights the

importance of the PD-1/PD-L1 signalling axis in tumour progression. However, not all patients respond to this therapy and much remains to be elucidated in terms of patient stratification for therapy and the mechanism of action and cellular targets of PD-1/PD-L1 inhibition. To date very few studies exist examining these effects *in vitro or in vivo*. The experiments described by Wang *et al*., go some way as to investigating the mechanism of action of PD-1 inhibition using an *ex-vivo* culture system similar to that used in this chapter with DCs used in place of stromal cells (Wang et al., 2014a). The authors in this case show that their PD-1 antibody bound PD-1 with high affinity and specificity and prevented the interaction with ligands of this receptor. They did not detect any antibody-mediated cell cytotoxicity but did observe an increase in IFN-γ release in their DC/T cell co-culture. (Wang et al., 2014a). This increase in IFN-γ release from T cells following PD-1 blockade agrees with the data presented in this chapter. Data is lacking to demonstrate granzyme B release *in vitro* following PD-1 blockade, but this protease has been shown to be enhanced in $CD8^+$ T cells in those patents in which a positive response to PD-1 immunotherapy was observed (Tumeh et al., 2014). With regards TNF-α release, this remains largely unexplored in the setting of cancer immunotherapy, but in a set of experiments whereby T cells were transfected with PD-1 RNA, it was observed that increasing expression of PD-1 correlated with increased suppression of TNF-α expression (Wei et al., 2013). Our data suggests that simply ligation of PD-1 by its receptor PD-L1, rather than expression of PD-1 on the T cell, is sufficient to diminish TNF-α production **(Figure 3.6A, 3.10C)**. While the roles of IFN-γ, TNF-α and granzyme B in anti-tumour immune responses are well documented, data for the role of IL-5 is lacking, but some reports suggest that this cytokine can play a role in tumour suppression and augmented tumour eosinophil infiltration, suggesting an as yet unknown mechanism of anti-tumour immune stimulation by PD-1 blockade (Ikutani et al., 2012).

A more recent study by Grenga *et al*., shows that addition of a PD-L1 blocking antibody enhanced antigen specific T cell responses (Grenga et al., 2016).

Although targeting the ligand, PD-L1, rather than the receptor, PD-1 as was done in our case, this observation raises some interesting questions that, although outside the scope of this thesis, warrant further investigations in the future. In examining CFSE proliferation peaks following PD-1 blockade in our *in vitro* system, it appears that the restoration of T cell proliferation is specific to one particular population of $CD8^+$ T cell, as indicated by a particularly large final peak **(Figure 3.10 B(i) Blue lines on histogram)**. This large fourth/fifth peak is indicative of a particular subset of the $CD8^+$ T cell population that, when the PD-1 checkpoint is lifted, proliferates to a much greater extent that the rest of the $CD8^+$ T cells. It is possible that these proliferative T cells are indeed antigen specific and responding to a particular antigen expressed on the stromal cell surface, but this will need to be further explored before any conclusions can be drawn. This leads us to question the antigen presentation capacity of mesenchymal stromal cells. Some limited data does exist to show that MSCs are capable of antigen uptake and presentation, though little has been done to further these initial findings (Francois et al., 2009, Chan et al., 2006). Furthermore, since our stromal cells are only exposed to the tumour secretome and not in direct contact with tumour cells, this may suggest that the tumour sheds soluble antigen, possibly in response to inflammatory stimulus. Interestingly, we observed a dramatic and significant increase in MHC-I expression on the stromal cells following inflammatory tumour conditioning, which as mentioned, could allow for presentation of tumour antigens and dysfunctional $CD8^+$ T cell activation (Hirosue and Dubrot, 2015).

As regards T_H1/T_H2 $CD4^+$ T cell polarisation following PD-1 blockade and restoration of T cell proliferation, our data is not definitive. We observe a significant restoration of TNF-α secretion and a trend towards restored IFN-γ production from the lymphocytes in our cultures, two cytokines indicative of a T_H1 type response (Cavalcanti et al., 2012). However, at the same time as seeing restored TNF-α and IFN-γ production, we also observed a significant increase in IL-5 release. This cytokine, along with IL-4 and IL-13

are characteristic of a T_H2 type response (Linch et al., 2016). This is further complicated by the observation that T_H2 type inflammatory responses have implicated in facilitating tumour growth, with the opposite effect attributed to a T_H1 type responses (Nishimura et al., 1999, Nishimura et al., 2000). Studies using peripheral blood from patients with melanoma and prostate cancer show that, in agreement with our findings, PD-1 blockade induces IFN-γ release or induction of a T_H1 type response in T cells, but also that PD-1 blockade results in diminished production of T_H2 type cytokines (Dong et al., 2017, Dulos et al., 2012). The differences between the results we obtained in our studies and those referenced here could be attributed to species, cell type or disease-specific differences given that our cells are isolated from murine lymph nodes and in contact with colon cancer conditioned stromal cells, compared to those referenced whereby T cells were isolated from human peripheral blood from patients with melanoma or pancreatic cancer. However, the effects of PD-1 blockade on T_H1/ T_H2 polarisation in the setting of colorectal cancer certainly warrants further investigation.

PD-L1 is induced on human stromal cells by exposure to the secretome from inflammatory activated human colon cancer cells, irrespective of microsatellite instability

We were able to show this phenomenon of inflammatory tumour induced PD-L1 expression on human stromal cells in response to the secretome from microsatellite (HCT116) or chromosomal HT29 instable human colon tumour cells. This is particularly important in light of data showing a better response to PD-1 immunotherapy in MSI-high colon cancer patients. We suggest that this is, as was discussed by Huang and Wu, a result of a lower mutational load attracting much fewer tumour infiltrating $CD8^+$ T cells (Huang, 2017). In this setting, PD-1 inhibition is of limited use since there is a paucity of T cells whose effector function can be disinhibited by such therapy. However, as the authors suggest, treatment with a second, immunostimulatory agent such as MEKi could lead to $CD8^+$ T cell infiltration, and thus synergistic and very

favourable responses in these patients (Huang, 2017, Ebert et al., 2016). Similarly, several other agents have been suggested for use in PD-1 targeting combinational strategies, the immunomodulatory functions of which may serve to convert a "cold", T cell deficient tumour microenvironment, to one in which T cell infiltrate is present and ready for activation (Bu, 2017).

Summary: Interpretation, Limitations and Further Studies

We have shown in this chapter that naïve stromal cells can be polarised to a more immunosuppressive phenotype with enhanced ability to suppress T cell proliferation and effector function by exposure to the inflammatory tumour secretome. We identify PD-L1 induction on the stromal cell surface as a mechanism, at least in part, for this enhanced immunosuppressive phenotype, as confirmed by blockade of the PD-1 receptor resulting in restored $CD8^+$ T cell proliferation and effector molecule release.

We did not, however, see a significant restoration in the proliferation of $CD4^+$ T cells following PD-1 blockade, despite a similar level of $CD4^+$ T cell suppression observed upon co-culture with inflammatory tumour conditioned stromal cells. This point to a probable second mechanism of enhanced immunosuppression by inflammatory tumour conditioned stromal cells. This mechanism may be better elucidated in further studies probing more deeply the stromal cell surface, the factors present in the tumour secretome, and the signalling pathways activated in and soluble mediators released from inflammatory tumour conditioned stromal cells.

We hypothesise that this induction of PD-L1 is dependent on NF-κB signalling in the tumour cell, but further studies targeting tumour cell NF-κB are necessary to confirm this. This will be discussed further in Chapter 5. Furthermore, the molecular mechanism of the induced PD-L1 expression on stromal cells is not clear at this point and will also be further discussed in Chapter 5.

We show a decrease in the cytolytic capacity of $CD8^+$ T cells following co-culture with inflammatory tumour conditioned stromal cells as defined by a reduction in effector cytokine release. A second way in which this could be measured may be to sort back T cells after this 72 hour co-culture for inclusion in a direct killing assay with CT26 tumour cells.

Finally, we chose to use murine Balb/c stromal cells and murine CT26 tumour cells, also of Balb/c origin, so that when investigating our hypotheses *in vivo* we can use a fully immunocompetent and fully MHC-matched murine model without the need for ablation of certain populations of immune cells or suppression of graft-rejection by other means. We believed that that this would allow us recapitulate the host anti-tumour immune response in a model *in vivo* system as best we could in order to accurately determine the immunomodulatory role of tumour conditioned stromal cells. However, future studies will look at the ability of human colorectal cancer cells to induce an immunomodulatory phenotype in primary human bone marrow cells, and furthermore, stromal cells isolated from fresh tumour specimens. This will expand our understanding of the complex interactions taking place in the tumour microenvironment and lead to better insights into patient stratification and therapeutic targeting of tumour microenvironmental components leading to better clinical prognosis.

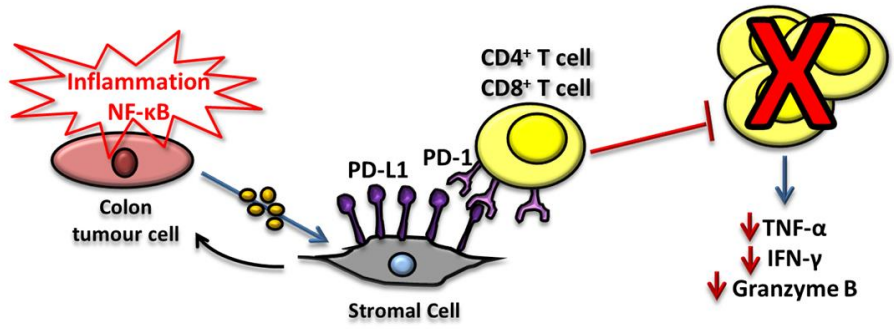


Figure 3.12 Summary

In the inflammatory tumour microenvironment, stromal cells are under the influence of factors secreted by the malignant tumour cell. In response to these factors, PD-L1 expression is induced on the stromal cell surface, resulting in an inhibition of T cell proliferation and effector function. This inhibition is dependent, at least in part, by the induced stromal cells PD-L1 expression as it can be partially reversed by the addition of a PD-1 blocking antibody.

CHAPTER FOUR

Inflammatory tumour conditioned stromal cells promote tumour growth and invasiveness *in vivo*, an effect which is partially dependent on PD-L1/PD-1 signalling

4.1 Introduction

In Chapter 3 we observed an enhanced ability for inflammatory tumour conditioned stromal cells to inhibit T cell proliferation and effector molecule release. We next aimed to test this finding *in vivo*. The effect of stromal cell on the progression of colon cancer has been previously tested *in vivo*, but much of this work has relied on the use of immunocompromised mice, limiting our ability to fully assess the anti-tumour immune response (Huang et al., 2013, Lin et al., 2013). *The work described in this chapter overcomes this limitation and allowed us to assess the anti-tumour immune response by using a fully immunocompetent and syngeneic murine model.*

In order to induce tumour growth in mice we optimised a subcutaneous injection of the immunogenic CT26 tumour cell line into the right flank of Balb/c. These cells are of Balb/c origin, and so are MHC-matched to the host animal, and have been described as being similar to "aggressive, undifferentiated, refractory " human colorectal cancer (Castle et al., 2014). Furthermore, these cells have a kRAS mutation, which is similar to 35-45% of all colorectal cancer patients (Tan and Du, 2012).

To test the functional consequences of induced stromal cell PD-L1 expression on anti-tumour effector T cell responses *in vivo*, CT26 tumour cells were co-administered with or without inflammatory tumour conditioned stromal cells ($MSC^{TNF\text{-}TCM}$). Untreated stromal cells were used as controls since the PD-L1 expression on these cells was not different to $MSC^{TNF\text{-}\alpha}$ or MSC^{TCM}. Additionally, the use of control stromal cells allowed us to fully control the factors to which stromal cells were expressed, and to determine a definitive role for the inflammatory tumour secretome in altering the effector function of these stromal cells.

In order to confirm a role for stromal cell PD-L1 on any tumour-promoting effects observed upon co-administration of stromal cells with CT26 tumour cells, a second group of animals were treated with a PD-1 monoclonal antibody (mAb) at days 7 and 14 post tumour/stromal cell injection. Each mouse, whose average weight was 20g, was administered a 200µg intraperitoneal injection of anti-PD-1 mAb as this dose is reflective of the upper dose of 10mg/kg used clinically in patients, and is a does at which therapeutic efficacy has been observed in melanoma, non-small cell lung cancer and renal cell carcinoma (Jazirehi et al., 2016, Guo et al., 2017).

Chapter 4 specific aims:

1. To investigate the ability of stromal cells to promote tumour growth
2. To examine the effect of tumour conditioning on the ability of stromal cells to promote tumour growth
3. To determine the cellular mechanism of tumour promotion by inflammatory tumour conditioned stromal cells

4.2 Results

4.2.1 Inflammatory tumour conditioned stromal cell decrease the cytolytic capacity of tumour infiltrating $CD8^+$ t cells *in vivo*

To investigate the tumour promoting effects of stromal cells we optimised a subcutaneous tumour model as described in **Figure 4.1**. Since our *in vitro* analysis showed that dramatically increased PD-L1 expression was unique to stromal cells exposed to the inflammatory tumour secretome, we assessed this group compared to control MSCs *in vivo* Untreated MSCs were used as controls since the PD-L1 expression on these cells was not different to $MSC^{TNF\text{-}\alpha}$ or MSC^{TCM} , thus allowing us to minimise the number of variables in our experiments **(Figure 4.1A)**.

Animal health was monitored daily, with body weights recorded every 5 days. Injection of CT26 alone or in combination with control or inflammatory tumour conditioned stromal cells did not cause animals significant distress or weight loss over the 21-day period of the study **(Figure 4.1C)**.

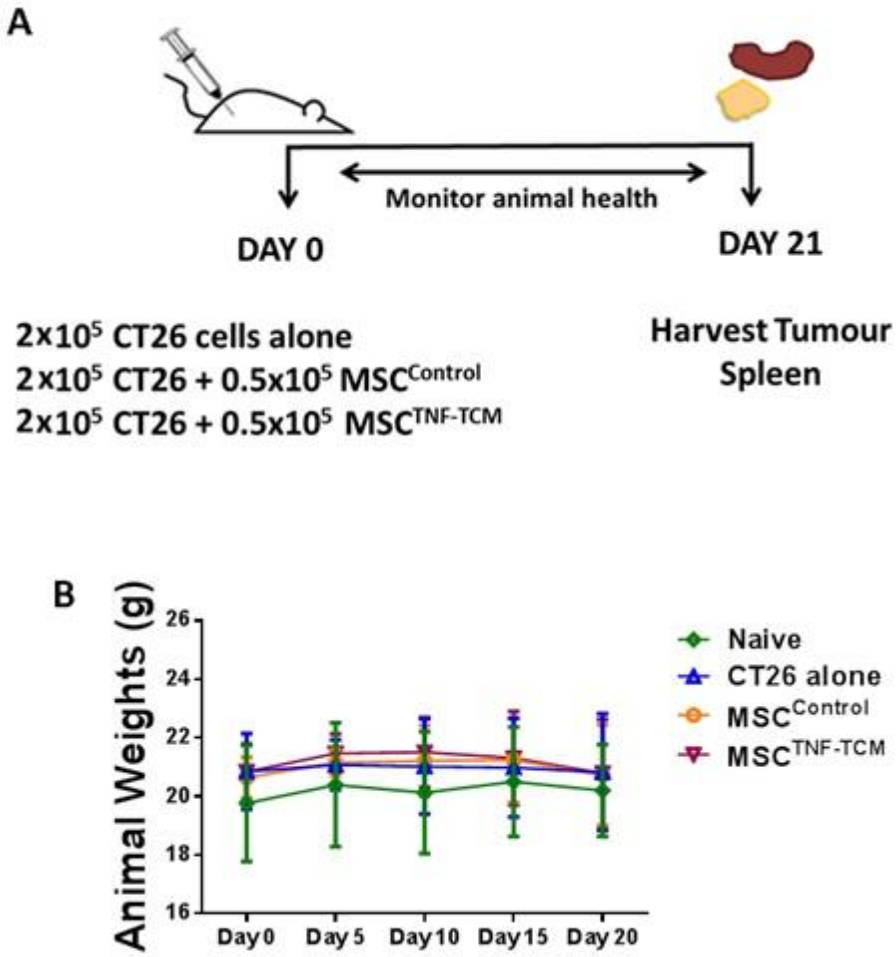


Figure 4.1 Schematic of subcutaneous tumour model and its impact on animal health

(A) Balb/c mice were injected subcutaneously into the flank with $2x10^5$CT26 cells alone, or in combination with $0.5x10^5$ control or inflammatory tumour conditioned stromal cells. Animals were monitored daily. Tumours and spleens were harvested at 21 when tumours size was measured and both tumours and spleens were analysed by flow cytometry for immune cell infiltrate **(B)** There was no significant weight loss in animals irrespective of tumour or stromal cell injection
Error bars: +/- StDev, n=3-9

A vast body of evidence exits to suggest that tumour infiltrating lymphocytes contribute significantly to the prognosis, responsiveness to treatment and likelihood of disease relapse in cancer patients (Galon et al., 2006). For this reason, and in light of our earlier finding of significant suppression of T cell proliferation and effector function by inflammatory tumour conditioned

stromal cells, we measured the frequency of tumour infiltrating $CD3^+$ T cells, as well as the frequency of $CD4^+$ and $CD8^+$ lymphocytes within this population. We observed no significant changes in the frequency of $CD3^+$, $CD3^+CD4^+$, or $CD3^+CD8^+$ T cells between animals injected with CT26 tumour cells alone, or in combination with control or conditioned stromal cells **(Figure 4.2 (i)-(iii))**. There was also no change in the level of CD69, an early T cell activation marker (de la Fuente et al., 2014), expressed on tumour infiltrating $CD8^+$ T cells. **(Figure 4.2 (iv))**. We observed a downward trend in the frequency of $CD8^+GranzymeB^+$ cells **(Figure 4.2 (v))**. This did not reach statistical significance, but considering the previous observation of increased levels of granzyme B expression in colon tumours correlating with favourable outcome for patients (Prizment et al., 2016), we measured the intensity of granzyme B expression on $CD8^+$ T cells by median fluorescent intensity (MFI) and found a significant reduction in the level of granzyme B expressed on CD8+ T cells infiltrating tumours formed from the co-administration of CT26 with inflammatory tumour conditioned stromal cells ($MSC^{TNF\text{-}TCM}$) compared to CT26 alone **(Figure 4.2 (iv))**. This result was dependent on previous exposure of the stromal population to the inflammatory tumour secretome and so suggests a role for PD-L1 in this effect. Cytotoxic $CD8^+$ T lymphocytes, defined by granzyme B expression, are of particular importance in the anti-tumour immune response, and so a reduction in granzyme B expression could result in an inhibition of their tumour cell clearing capacity (Giordano et al., 2015).

Macrophages represent another important cell type in mediating a host anti-tumour immune response (Ryan et al., 2015). In fact it has recently been demonstrated that tumour-associated macrophages (TAMs) express PD-1, and that the upregulation of PD-L1 on tumour cells can impede the ability of TAMs to phagocytose tumour cells (Gordon et al., 2017). In light of these studies we also assessed the frequency and phenotype of tumour infiltrating macrophages. We found no difference in the frequency of $F4/80^+$ cells in mouse tumours (Lin et al., 2010a), regardless of the cell type administered

Figure 4.2 (vii). Furthermore, we did not observe any significant difference in the level of MHC-II, a putative marker of tumour-supressing macrophage phenotype (Wang et al., 2011), on tumour infiltrating F4/80^{+} cells **(Figure 4.2 (viii))**.

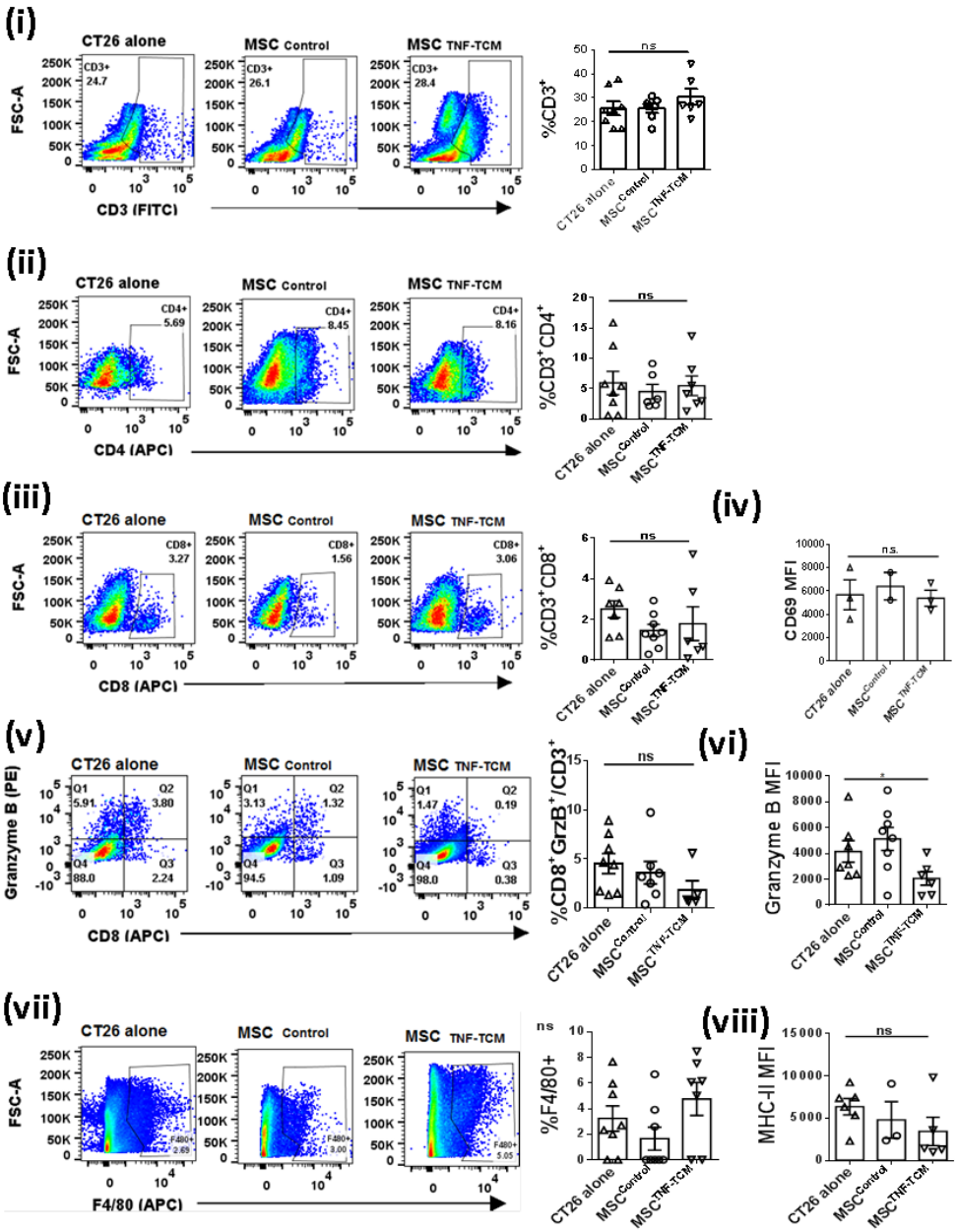


Figure 4.2 Inflammatory tumour conditioned stromal cells inhibit tumour infiltrating $CD8^+$ t cell cytolytic capacity *in vivo*
Tumours were processed to single cell suspension and frequency and phenotype of infiltrating immune cell populations were assessed by flow cytometry. Populations measured were **(i)** $CD3^+$ T cells **(ii)** $CD3^+CD4^+$ T cells **(iii)-(vi)** $CD3^+CD8^+$ T cells and **(vii)** and **(viii)** $F4/80^+$ mononuclear cells Error bars: mean +/- SEM *p<0.05, **p<0.01 ***p<0.001 one-way ANOVA, Tukey's Post Hoc Test n=3-8

4.2.2 Inflammatory tumour conditioned stromal cells significantly promote tumour growth and invasiveness *in vivo*

In addition to altering tumour immune cell infiltrate, stromal cells have been shown to promote the growth and invasion of tumours (Shinagawa et al., 2010, De Boeck et al., 2013). In our model, injection of CT26 alone induced subcutaneous tumour formation with no evidence of tumour cell invasion and metastasis. Interestingly, in 50% of animals receiving control stromal cells tumours were observed to have invaded tissue surrounding the primary tumour site and to have metastasised from the flank to the peritoneal cavity **(Figure 4.3A)**. This effect was significant potentiated by the co-administration of stromal cells previously exposed to the inflammatory tumour secretome **(Figure 4.3A)**. In the clinical setting, an invasive or metastatic tumour is associated with a much less favourable outcome for patient survival (Luchini et al., 2016, Lin et al., 2015). This holds true in the setting of colorectal cancer, where the emergence of metastasis is particularly grave (Riihimaki et al., 2016). Our finding of an invasive tumour phenotype in animals co-administered inflammatory tumour conditioned stromal cells points to a potential role for PD-L1 expression in the promotion of this effect.

In addition to promoting an invasive tumour phenotype, co-administration of inflammatory tumour conditioned stromal cells induced an increase in tumour burden, as measured by tumour weight **(Figure 4.3B)**. There was an increase in tumour burden with control stromal cell administration, but this did not reach statistical significance **(Figure 4.3B(i))**. When tumour volumes were measured, a significant increase in tumour size was observed upon the

co-injection of stromal cells, with inflammatory tumour conditioned stromal cells inducing greater increases in volume than their control counterparts **(Figure 4.3B(ii))**. These results highlight a clear role for inflammatory tumour signalling in altering the functional characteristics of the tumour stroma.

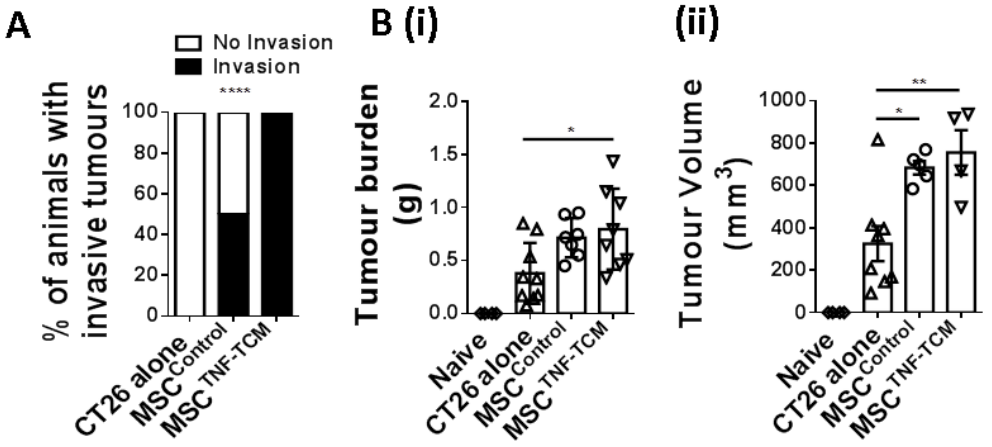


Figure 4.3 Inflammatory tumour conditioned stromal promote tumour invasiveness and growth *in vivo*

(A) Graph displaying the percentage of animals in which an invasive tumour phenotype was observed. An invasive tumour was defined as any tumour which had penetrated skin tissue or grown across the peritoneal membrane with tumour deposits found attached to the liver and colon

****P<0.0001 Chi Squared test for trend n=8

(B)(i) Graph of tumour burden, as measured by weight in grams after brief blotting on filter paper to remove excess fluid. **(ii)** Graph of tumour volume, as measured by the rational ellipse formula ($M^{1\wedge2}xM^2 \times \pi/6$)

Error bars: mean +/- SEM *P<0.05, **P<0.01 one-way ANOVA, Tukey's Post Hoc Test n=3 for naïve animals 8/9 for experimental groups

To confirm the immunomodulatory role of inflammatory tumour conditioned stromal cell and test the effect of increasing stromal cell number, a second group of animals were co-administered CT26 with double the number of stromal cells used previously ($0.5x10^5$ vs $1x10^5$). This was investigated using both control stromal cells and those exposed to the inflammatory tumour secretome. No differences were found in tumour invasiveness, tumour weighs or tumour volumes between animals that had received the lower or higher stromal cell number **(Figure 4.4).** Hence all further experiments and analysis were carried out using the lower MSC dose.

These data confirm that the presence of an immunosuppressive stromal niche in a tumour is sufficient to have significant effects upon the ability of immune cells to access and eliminate that tumour. We have shown that the presence of this stromal population cause biologically relevant and potent effects on tumour promotion even when present in low numbers.

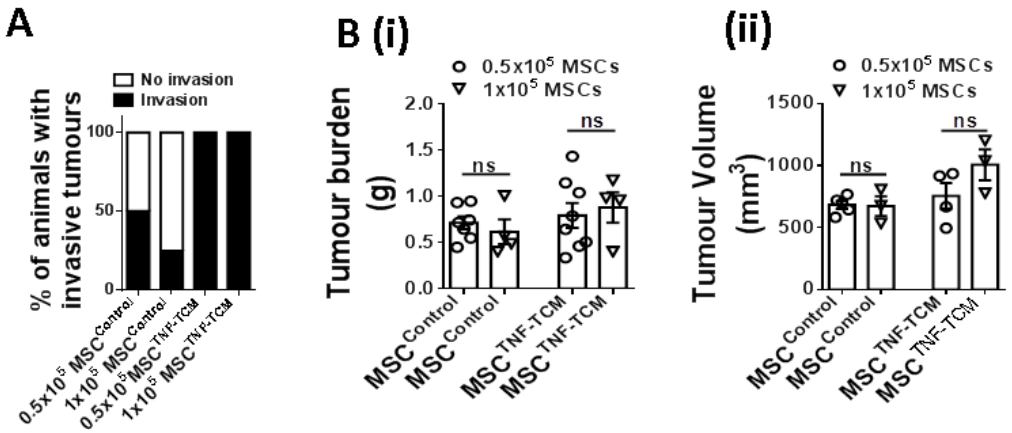


Figure 4.4 Co-injection of $0.5x10^5$ MSCs (20% of total cell number) was sufficient to induce observed tumour promotion
(A) The percentage of animals in which invasive tumours were observed
Chi Squared test for trend n=8
(B)(i) Tumour burden, as measured by weight in grams and tumour volume **(B)(ii)**, as measured by the rational ellipse formula ($M^{1\wedge 2}xM^2$ x $\pi/6$) were compared between animals administered $0.5x10^5$ and $1x10^5$ MSCs.
Error bars: mean +/- SEM one-way ANOVA, Tukey's Post Hoc Test n=3 for naïve animals 8/9 for experimental groups

4.2.3 Treatment with anti-PD-1 therapy reversed stromal mediated inhibition of $CD8^+$ T cell cytolytic capacity and was associated with decreased tumour volume and invasive potential

To confirm a role for PD-L1 in the enhanced immunosuppressive and tumour promoting ability of co-administered stromal cells observed in vivo, three additional groups of animals were treated an anti-PD-1 monoclonal antibody

at 7 and 14 days post tumour induction **(Figure 4.5)**. Once again, there was no significant impact on animal health over the 21 day course of the study.

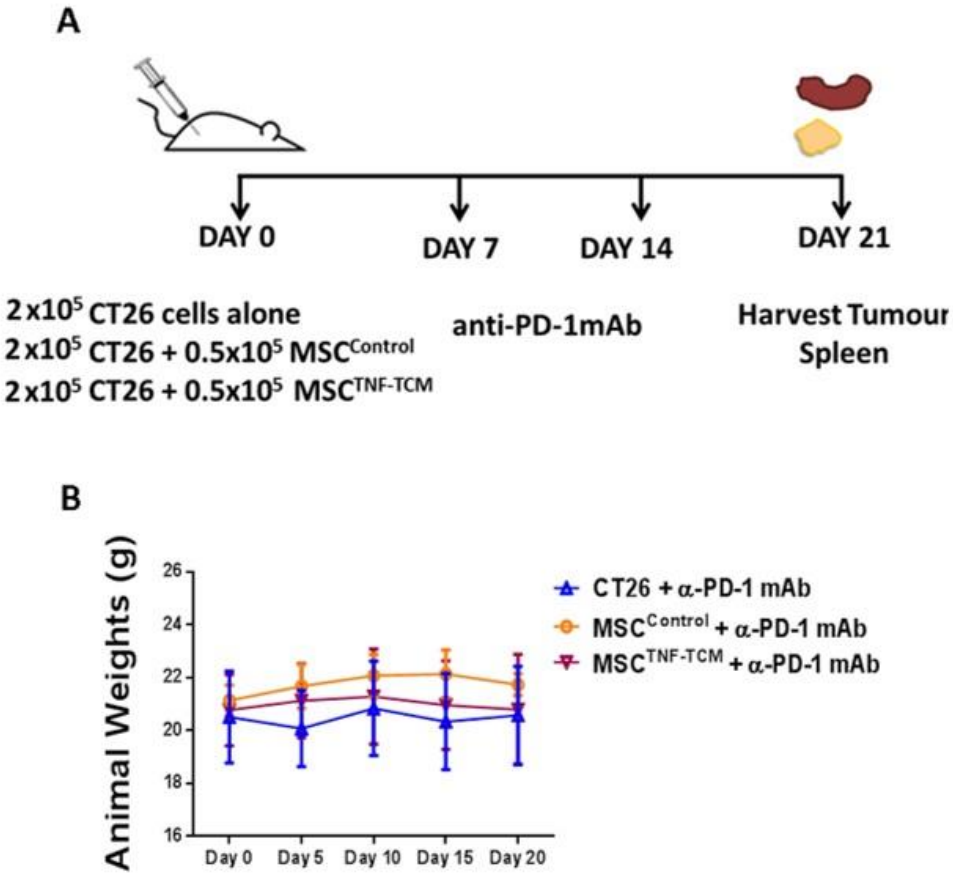


Figure 4.5 Schematic of subcutaneous tumour model and its impact on animal health

(A) Balb/c mice were injected as in **Figure 4.1**, with the addition of an anti-PD-1 monoclonal antibody at days 7 and 14 post-tumour cell injection **(B)** There was no significant weight loss in animals irrespective of treatment with PD-1 monotherapy

Error bars: +/- StDev, n=3 for naïve animals 8/9 for experimental groups

In line with previous data demonstrating only mild responses to PD-1 monotherapy in CT26 derived tumours, PD-1 antibody treatment had no significant effect on the immune cell infiltrate of tumours formed from CT26 alone **(Figure 4.6)**. Treatment with anti-PD-1 mAb did not alter the frequency of $CD3^+$, $CD3^+CD4^+$ or $CD3^+CD8^+$ infiltrating tumours **(Figure 4.6 (i) – (iii))**, nor was there any change in the early activation marker CD69 on $CD8^+$ T cells **(Figure 4.6 (iv))**. There was, however, a clear trend towards a restoration of the cytolytic capacity of intratumoural $CD8^+$ T cells, as measured by the frequency of CD8+GranzymeB+ cells infiltrating tumours **(Figure 4.6 (v))** and the level of granzyme B expressed on these cells, as measured by MFI **(Figure 4.6 (vi))** following treatment with PD-1 mAb therapy. The frequency of tumour infiltrating macrophages remained unchanged following treatment with PD-1 mAb therapy, as did the level of MHC-II expression on these cells **(Figure 4.6 (vi)(vii))**.

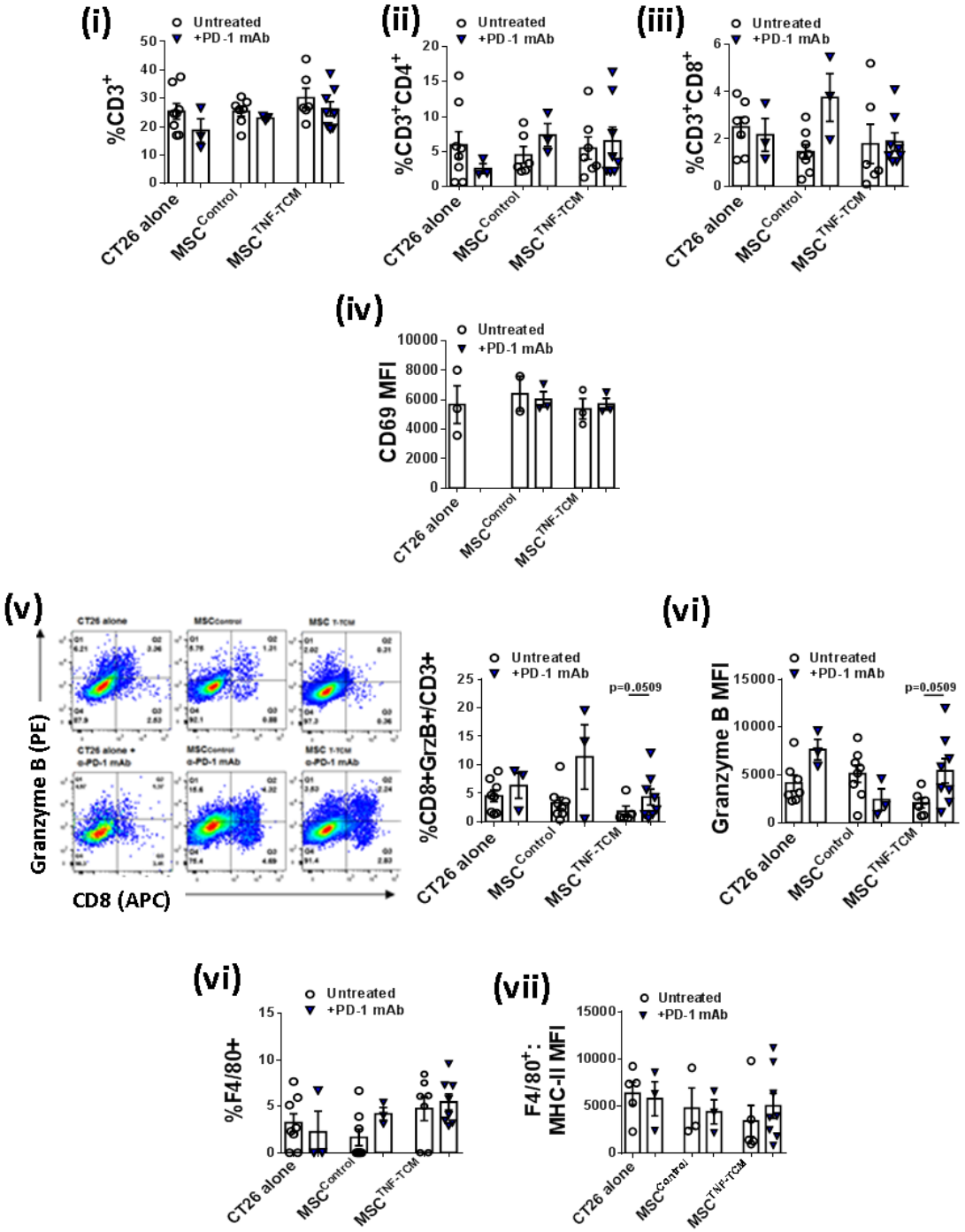


Figure 4.6 Treatment with PD-1 mAb restored CD8$^+$ T cell cytolytic capacity but did not alter the frequency of immune cell populations infiltrating tumours

Tumours were processed to single cell suspension and frequency and phenotype of infiltrating immune cell populations were assessed by flow cytometry. Populations measured were **(i)** CD3$^+$ T cells **(ii)** CD3$^+$CD4$^+$ T cells **(iii)-(vi)** CD3$^+$CD8$^+$ T cells and **(vii)** and **(viii)** F4/80$^+$ mononuclear cells Error bars: mean +/- SEM Multiple T tests of Untreated vs +PD-1 mAb groups n=3 for naïve animals 8/9 for experimental groups

Perhaps most importantly, treatment of animals bearing tumours formed from co-injection of CT26 with inflammatory tumour conditioned stromal cells with PD-1 monotherapy was sufficient to reverse the increased tumour invasiveness that had been observed upon co-administration of inflammatory tumour conditioned stromal cells **(Figure 4.7A)**. This suggests that stromal cell PD-L1 expression may have a central role in enabling the tumour to inhibit CD8+ T cell mediated inhibition of metastatic tumour spread. A similarly significant restoration of tumour volume was observed upon treatment with PD-1 monotherapy **(Figure 4.7B)**. In fact, tumour volume in animals administered inflammatory tumour conditioned stromal cells treated with PD-1 monoclonal antibody was no different to that of animals administered CT26 alone. This data points to a central role for stromal cell PD-L1 expression in obstructing the activity of anti-tumour $CD8^+$ immune responder cells and thereby enabling tumours to grow by immune evasion.

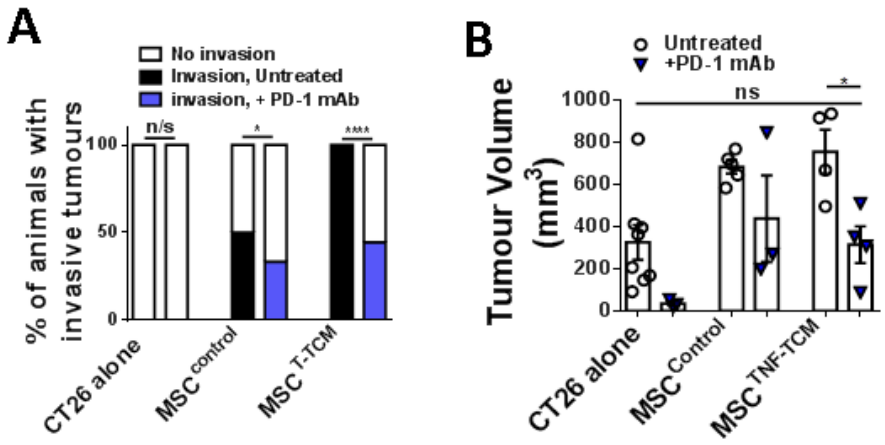


Figure 4.7 Treatment with PD-1 mAb resulted in a decrease in invasive phenotype and tumour volume

(A) Graph displaying the percentage of animals in which an invasive tumour phenotype was observed.

* p<0.01 ****P<0.0001 Fisher's Exact Test

(B) Graph of tumour volume, as measured by the rational ellipse formula ($M^{1\wedge2}xM^2 \times \pi/6$)

Error bars: mean +/- SEM *P<0.05 multiple T tests n=3 for naïve animals 8/9 for experimental groups

4.2.4 Effects of stromal cell administration and PD-1 monoclonal antibody treatment upon CD8$^+$ T cell phenotype were restricted to the tumour and not observed in splenic T cells

In order to test for any systemic effect of tumour or stromal cell administration, spleens were harvested from animals at the time of tumour recovery and analysed for a similar panel of infiltrating immune cells. Administration of CT26 alone or in combination with control or inflammatory tumour conditioned stromal cells resulted in a decreased frequency of CD3$^+$ T cells present in the spleen of mice compared to naïve animals **(Figure 4.8 (i))**. The proportion of these cells which were CD4$^+$ and CD8$^+$ remained unchanged, as did the granzyme B expression on CD8$^+$ splenic T cells **(Figure 4.8 (ii)-(iv))**.

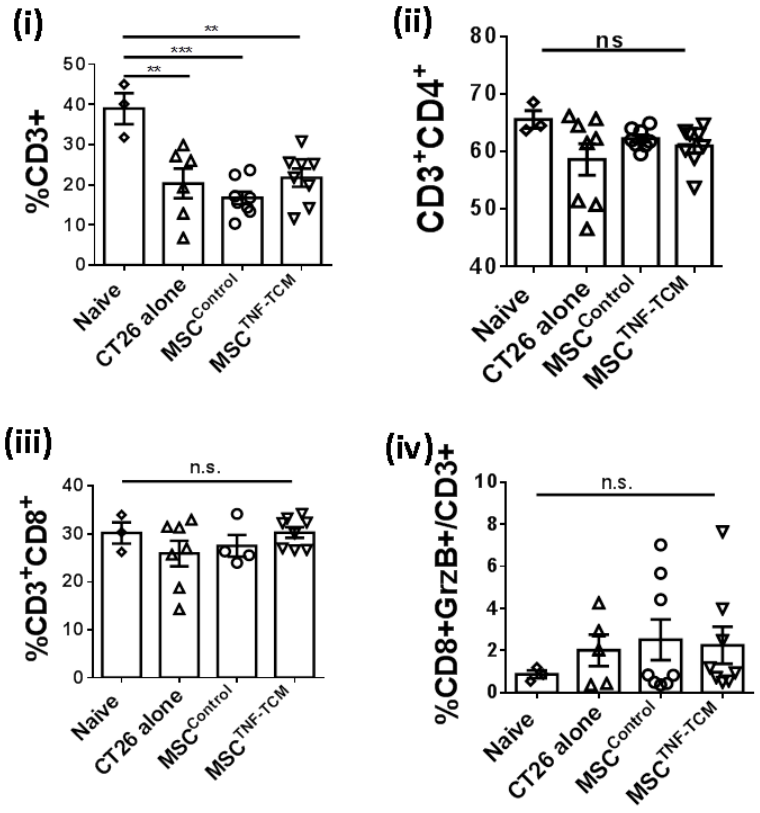


Figure 4.8 The administration of tumour or stromal cell reduced the frequency of splenic CD3$^+$ T cells

Spleens were processed to single cell suspension and frequency and phenotype of infiltrating immune cell populations were assessed by flow cytometry. Populations measured were **(i)** CD3$^+$ T cells **(ii)** CD3$^+$CD4$^+$ T cells **(iii),(iv)** CD3$^+$CD8$^+$ T cells Error bars: mean +/- SEM one way ANOVA, Tukey's Post Hoc Test n=3-8

The administration of anti-PD-1 immunotherapy did not alter the frequency of any of the immune cell populations present in the spleen, indicating that any effects of PD-1 immunotherapy on restoring $CD8^+$ T cell cytolytic capacity was specific to tumour infiltrating T cells **(Figure 4.9)**.

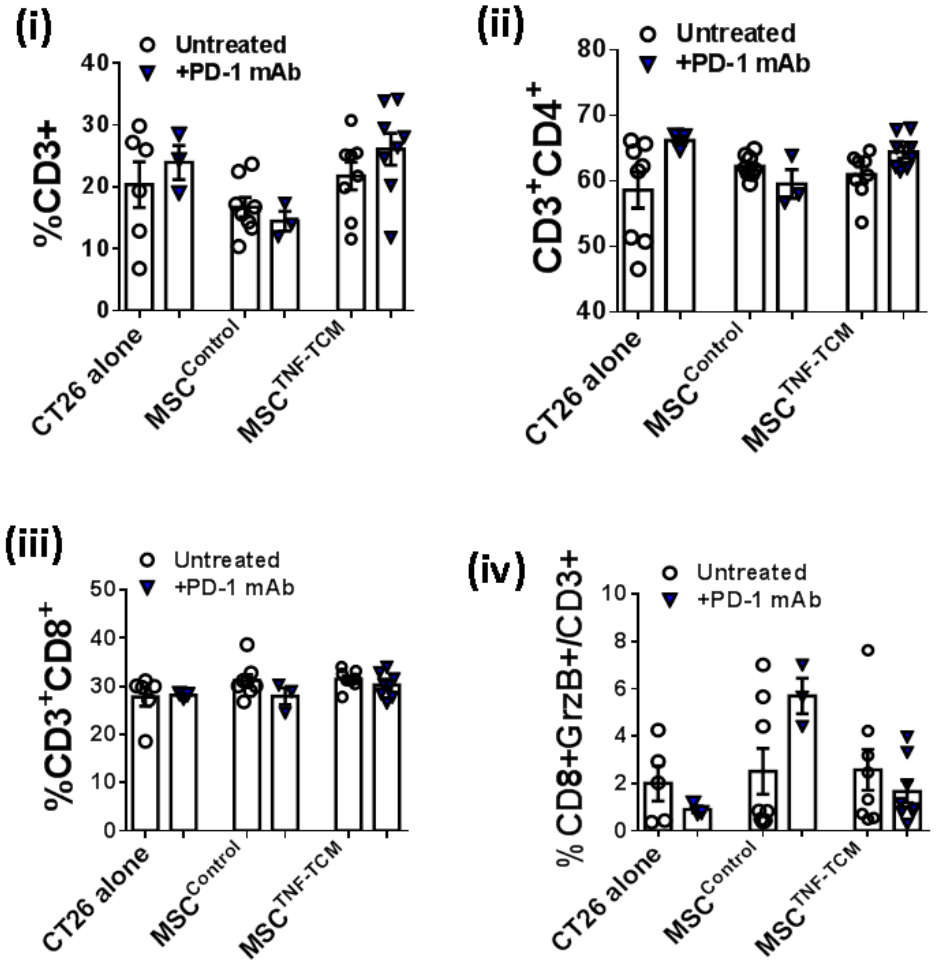


Figure 4.9 PD-1 immunotherapy did not affect the splenic immune cell frequency in treated animals

Spleens were processed to single cell suspension and frequency and phenotype of infiltrating immune cell populations were assessed by flow cytometry. Populations measured were **(i)** $CD3^+$ T cells **(ii)** $CD3^+CD4^+$ T cells **(iii),(iv)** $CD3^+CD8^+$ T cells Error bars: mean +/- SEM multiple T tests, Tukey's Post Hoc Test n=3-8

4.3 Discussion

In this chapter we show for the first time, a definitive role for the stromal compartment in the dampening of the $CD8^+$ T cell mediated anti-tumour immune response and in promoting tumour growth and invasiveness. This finding is of particular importance when it is considered that the stroma is located adjacent to both the cancerous epithelial cells and the colonic vasculature and lymphatic network, and so represents a physical barrier to entry for immune cells in response to inflammation or epithelial transformation (Turley et al., 2015).

Inflammatory tumour conditioned stromal cells reduce the cytolytic capacity of tumour infiltrating T cells and promotes tumour growth and invasiveness

By using an immunocompetent, syngeneic murine model and immunogenic tumour cells, we could overcome the limitations in assessing immune cell infiltrate associated with the use of xenografts in immunocompromised mice. This model allowed us to identify a clear role for the tumour stromal compartment in dampening the anti-tumour immune response as was observed by a decrease in tumour infiltrating $CD8^+$ T cell cytolytic capacity as defined by a reduction in granzyme B expression on these cells. With specific regard to colon cancer, higher intra-tumoural expression of granzyme B has been shown to correlate with improved survival in patients (Prizment et al., 2016). This effect on T cell effector molecule expression was only observed in animals administered inflammatory tumour conditioned stromal cells and not control stromal cells, suggesting a dependency on stromal cell PD-L1 expression for this effect **(Figure 4.2 (vi))**. Indeed, a central role for PD-L1 in this granzyme B inhibition was confirmed by the restoration of $CD8^+$ T cell granzyme B expression following treatment with PD-1 monoclonal antibody therapy **(Figure 4.6 (vi))**.

In addition to the effect on $CD8^+$ T cell cytolytic capacity, the co-injection of stromal cells was associated with a 50% increase in the number of animals in which an invasive tumour phenotype was observed **(Figure 4.3A)**. There

was a further dramatic increase in tumour invasiveness with the administration of inflammatory tumour conditioned stromal cells, and a concomitant increase in tumour volume **(Figure 4.3A, B)** with tumour deposits recovered from the peritoneum and extrahepatic sites, a finding consistent with that in patients (Riihimaki et al., 2016). This is of particular importance in the setting of colorectal cancer, where the presence of tumour metastasis is associated with a particular poor outcome for patients. In fact, as many as 20% of colorectal cancer patients present with metastasis at diagnosis and metastatic disease was previously viewed as incurable (Riihimaki et al., 2016). A role for tumour-stromal interactions in dictating this metastatic spread has been suggested and studied, though the precise factors responsible remain largely unknown (Langley and Fidler, 2011). We now show a clear and important role for stromal cell PD-L1 expression the invasion of colorectal tumours to distant sites in a murine model of subcutaneous tumour development given that the highest number of animals in which invasive tumours were observed were in the group administered PD-L1 expressing inflammatory tumour conditioned stromal cells. Furthermore, at least part of this invasiveness could be reversed by administration of a PD-1 blocking antibody.

The observation of an invasive phenotype in animals administered control, untreated stromal cells could be suggestive of *in vivo* induction of PD-L1 as the tumour forms in the animal over the duration of the study. Similarly, tumours formed by co-administration of these control stromal cells were indeed larger than those formed by CT26 administration alone, once again suggesting potential *in situ* stromal cell PD-L1 induction. Furthermore, though not statistically significant, there was a trending decrease in tumour volume and a small but significant decrease in invasiveness in these animals co-administered control stromal cells when treated with PD-1 immunotherapy. However, the increases in invasiveness and tumour size were not as dramatic or as statistically significant when control stromal cells were administered when compared to their inflammatory tumour

conditioned counterparts. This may be indicative of the importance of timing of PD-L1 expression and induction of immunomodulation by the tumour stromal compartment. Our data may suggest that stromal cell expression at the early stages of tumour formation is critical to hampering anti-tumour immunity and allowing tumours grow unimpeded by the immune system, as would be the case for our animals administered high-PD-L1 expressing inflammatory tumour conditioned stromal cells. Any induction of PD-L1 that may occur later on in the process of tumour formation appears to also have a role to play in promoting tumour growth an invasiveness given the increases in these parameters that we observed, but that the earlier existence of an immunosuppressive stromal compartment, i.e. at the time of injection, may be the critical factor in determining the effectiveness of the anti-tumour immune response. This, however, will need further investigation. Immunohistochemical analysis of stromal cell PD-L1 expression on embedded tumours may begin to address this point, but further experiments involving different timings of stromal cell administration with and without prior tumour exposure, along with experiments where exogenous stromal cell are not administered, and a slower growing tumour model used, before any conclusions can be drawn.

<u>Assessment of stromal PD-L1 expression may lead to better stratification of patients for immunotherapeutic regiments</u>

The use of checkpoint inhibitors as a therapeutic strategy for the treatment of various cancers has gained momentum, with PD-1 providing an attractive target. Antibodies specifically targeting PD-1 signalling are currently under clinical investigation for melanoma, renal cell carcinoma, non-small-cell lung cancer, head and neck squamous cell cancer and haematological malignancies (Philips and Atkins, 2015). The most recent indication for which PD-1 immunotherapy has been granted FDA approval is colorectal cancer (FDA, 2017). Indeed, evidence shows that a subset of these patients will respond positively to PD-1 therapy, but this is not without caveat – Dunne *et* al., have identified a second cohort of patients who will in fact be harmed by

PD-1 blockade (Dunne et al., 2016). Interestingly this group noted that it is not only epithelial tumour cell expression of PD-L1 that is important in dictating a positive response to this therapy. Our data demonstrate a central role for stromal PD-L1 expression in inhibiting anti-tumour immune responses and enabling tumours to grow and metastasis. In light of studies showing positive responses to PD-1 immunotherapy in patients whose tumours have been deemed PD-L1 negative, we provide a clear rationale for the assessment of stromal cell PD-L1 expression in order to better stratify patients for immunotherapy.

Stromal cell PD-L1 mediated immunosuppression is likely not limited to $CD8^+$ T cells

Our data clearly points to a role for inhibition of $CD8^+$ T cells and their effector molecule granzyme B in the observed effects of stromal cell mediated tumour promotion. However, it is highly unlikely that these cells are the only anti-tumour immune effector cells affected by stromal cell PD-L1 expression. In fact PD-1 expression has been shown to also inhibit B cells natural killer cells and dendritic cells, the study of which were beyond the scope of these experiments, but certainly warrant further investigation in the future(Thibult et al., 2013, Benson et al., 2010). More recently it was demonstrated that tumour resident macrophages express PD-1 in tumours formed from administration of CT26 tumour cells, and that these macrophages are of a more "M2" pro-tumourigenic phenotype with decreased MHC-II and increased CD206 expression (Gordon et al., 2017). Although we did not observe a significant decrease in MHC-II expression on F4/80+ tumour infiltrating macrophages, we did see a trend towards lower MHC-II expression **(Figure 4.8 (viii))**. The reason for this discrepancy could be related to timing and tumour cell number. The authors show that early on it tumour formation tumour-associated macrophages (TAMs) don't express PD-1. It begins to be expressed about two weeks after tumour engraftment, and increases over time. While the tumours in our study were assessed at day 21, a time point at which the authors of this study saw about

80% PD-1 TAM positivity, it could be that the kinetics of expression and tumour injection site were slightly different in our study and we may have noticed more significant changes in macrophage populations at a slightly later time point. In addition, in this study animals received in injection of $1x10^6$ tumour cells, whereas in our study mice were administered a lower cell number of $2x10^5$. Thus effects on certain immune populations may have occurred more quickly with the increased cell number injected. For these reasons the effect of stromal cell PD-L1 expression on macrophage frequency, phenotype and phagocytic capacity is an avenue worth pursuing in further studies.

Summary: Interpretation, Limitations and Further Studies

In summary, the data presented in this chapter demonstrate a clear and important role for stromal cell PD-L1 in altering CD8+ T cell tumour infiltrate, and in promoting tumour invasiveness and growth in a syngeneic murine tumour model, given that these two parameters were significantly increased by co-administration of inflammatory tumour conditioned stromal cells and at least partly reversed by PD-1 monotherapy. The model we chose has particular relevance to therapy refractory, metastasis prone human colorectal cancer as the CT26 cell line used has been characterised as such and has be shown to bear a Kras mutation, also similar to the human condition (Castle et al., 2014)

In addition to the aforementioned investigation of other immune cell populations likely affected by stromal cell PD-L1 expression including B cells and macrophages, the *in vivo* model used to study these effects could be altered to give more insights. Although a fully immunocompetent and MHC-matched model, there are some limitations to the use of such a tumour injection strategy. Firstly, exogenous administration of a bolus of fast growing and invasive tumour cells does not accurately represent the many genetic and epigenetic changes that take place over several weeks, month

and years in the inflamed colon to give rise to clinical symptoms of colorectal cancer. The administration of a carcinogen such as azoxymethane or dextran sulfate sodium could overcome some of these limitations, as would the use of a genetic knockout mouse such as the Msh2 knockout or mice homozygous for the M1h1 gene which are predisposed to the development of tumour of the gastrointestinal tract (De Robertis et al., 2011, Karim and Huso, 2013). Secondly, mouse models of cancer, particularly those in which a homogenous fast growing cell line such as CT26 is administered does not fully recapitulate the numerous changes that have taken place in an environment as complex and heterogeneous as the human colon tumour microenvironment. In this instance an immunocompromised mouse model can be advantageous in that human clinical samples can be implanted and actual responses to therapy measured, however, immune responses will be lacking. Finally, orthotropic implantation can be useful in that the site of tumour development and anatomical location of metastatic spread more accurately represents the clinical disease (Tseng et al., 2007).

Another aspect of this model that could be further refined is to isolate stromal cells from the intestine of mice and carry out the conditioning protocol on these cells to gain further insights into the colon-specific effects of tumour conditioning on stromal cells. A number of protocols have now been published detailing stromal cell isolation from murine and human colonic tissue (Khalil et al., 2013). Although a large proportion of these stromal fibroblasts are of mesenchymal origin and similar to those used throughout our studies, stromal fibroblasts of colonic origin may offer additional and novel insights into the effects of the colon-resident stromal population at the time of tumour formation. Furthermore, these cells could be advantageous in studying the effects of long term inflammation, the likes of which arises in Crohn's disease or ulcerative colitis, on the immunomodulatory capacity of intestinal stromal cells, and their subsequent contribution to the tumour microenvironment.

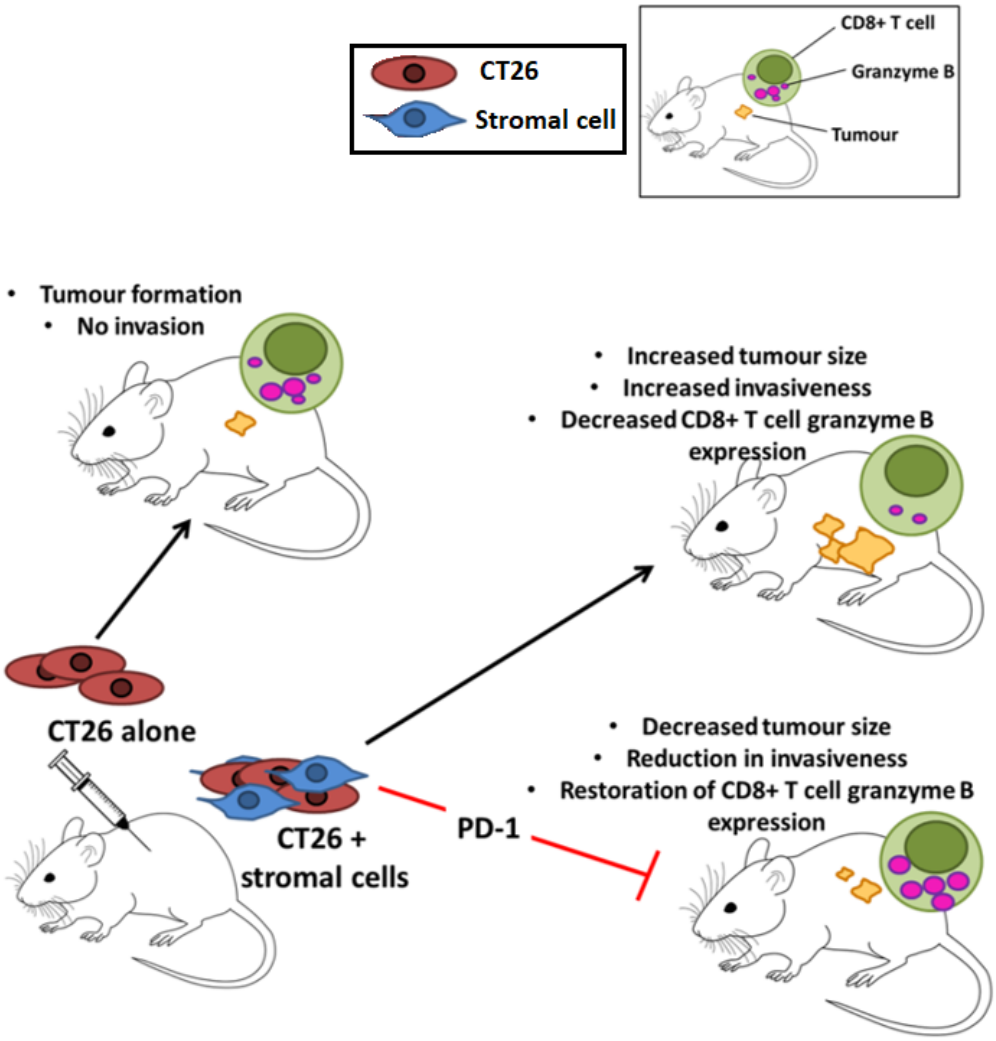


Figure 4.10 Summary
Co-administration of inflammatory tumour conditioned stromal cells resulted in an inhibition of intratumoural CD8+ T cell granzyme B expression and was associated with increased tumour size and invasiveness. These effects were partially reversed by treatment with a PD-1 monoclonal antibody

CHAPTER FIVE

Investigating the molecular mechanisms responsible for tumour-induced stromal cell PD-L1 expression

Part 1: The induction of stromal cell PD-L1 is partially dependent on tumour cell NF-κB signalling

Part 2: PD-L1 induction on stromal cells is likely a two-signal process involving stromal cell NF-κB and STAT signalling, and independent of TGF-β signalling

5.1 Introduction

The data we have presented thus far demonstrates a clear role for the inflammatory tumour microenvironment in dictating the immunomodulatory potential of the tumour stromal compartment via robust induction of stromal cell PD-L1 expression. We have shown that this induced PD-L1 expression is responsible for an increased capacity for inflammatory tumour conditioned stromal cells to inhibit $CD8^+$ T cell proliferation and effector molecule release *in vitro*, diminished granzyme B expression *in vivo*, and increased tumour growth and invasiveness in immunocompetent mice.

Following on from these observations we next aimed to determine the molecular regulation of the induced PD-L1 expression when stromal cells are exposed to the inflammatory tumour secretome.

In the setting of colorectal cancer, NF-κB is a transcription factor that has garnered a lot of attention for a number of reasons. Constitutive NF-κB has been shown to be present in 40% of colorectal cancer tissues and 67% of cell lines (Sakamoto et al., 2009). Furthermore, NF-κB has been identified as a critical link between the presence of inflammation and the development of cancer (Karin, 2009, Karin et al., 2002). This finding is likely to be of particular importance in the setting of colorectal cancer where it has been observed that patients with a history of inflammatory bowel disease are at a higher risk of developing this disease (Kim and Chang, 2014). Along with these direct links between NF-κB activation and colorectal cancer development it also notable that, in addition NF-κB itself, the various molecular signals identified as important in recruiting stromal cells to the tumour microenvironment are often regulated by or themselves activate NF-κB signalling (Wong et al., 2005, Uchibori et al., 2013). Finally, data from our own lab has shown that targeting tumour cell NF-κB prolongs survival in animals and induces an anti-tumour M1 phenotype in macrophages (Ryan

et al., 2015). The effects of tumour cell NF-κB signalling on the immunomodulatory capacity of tumour resident stromal cells remains to be elucidated. *The experiments described in the first half of this chapter will explore the influence of tumour cell NF-κB signalling on the ability of the tumour to modulate the anti-inflammatory potential of stromal cells.*

Although the role of stromal cells in promoting tumour growth and metastasis is beginning to be explored, and significant data exists identifying mesenchymal stromal cells as precursors for cancer-associated fibroblasts (CAFs), little is known of the molecular changes that take place in the stromal cells in response to signals in the tumour microenvironment. To date TGF-β is the cytokine most strongly associated with both colorectal cancer and an alteration in stromal cell and CAF function (Calon et al., 2012, Calon et al., 2014). While the research described in published studies identifies an important role for TGF- β in modulating the potential of CAFs to promote tumour cell proliferation, survival, invasion and epithelial to mesenchymal transition, data is lacking on the role of TGF-β on altering the immunomodulatory capacity of CAFs or stromal cells. Furthermore, it has been shown that TGF-β and TNF-α counter-regulate PD-L1 expression on monocytes (Ou et al., 2012). *The work described in the second half of this chapter assess the effect of TGF- β on stromal cell PD-L1 expression.*

Finally, signal transducer and activator of transcription (STAT) proteins are now known to be potent regulators of the anti-tumour immune response, in particular STAT1 and STAT3 (Yu et al., 2009, Shou et al., 2016). Furthermore, these STAT proteins have been implicated in regulating the immunomodulatory potential of mesenchymal stromal cells, and have been also been identified as central regulators of PD-L1 expression (Shou et al., 2016, Noh et al., 2015, Vigo et al., 2017). However, it remains to be elucidated if inflammatory tumour conditioning of stromal cells induces STAT protein signalling, and if so, whether or not this signalling has any bearing on stromal cell PD-L1 expression. *The experiments presented in the*

second half of this chapter were designed to identify STAT protein induction in stromal cells following inflammatory tumour activation.

Specific Aims

1. To investigate the effect of tumour cell NF-κB signalling on stromal cell PD-L1 induction
2. To determine the molecular mechanisms of PD-L1 induction in stromal cells

5.2 PART 1

The experiments presented in the first part of this chapter were designed to explore the role of tumour cell NF-κB signalling on the induction of stromal cell PD-L1 expression and enhanced immunosuppressive capacity

5.2.1 Stromal cell PD-L1 induction is partially dependent on tumour cell NF-κB signalling

As mentioned, NF-κB signalling is of particular importance in the setting of colorectal cancer, and has been described as important in altering macrophage anti-tumour immune responses (Ryan et al., 2015). However, the effect, if any, of tumour cell NF-κB signalling upon the tumour stromal compartment is not well understood. To this end we repeated our earlier stromal cell conditioning experiments using medium generated from CT26 stably transfected with an IκB-α super-repressor plasmid (CT26/IκB-αSR). An empty vector was transfected as a control (CT26/EV) **(Figure 5.1)**. Following the same treatment protocol outlined in Chapter 3, stromal cells were conditioned by the secretome from both CD26/EV and CT26/IκB-αSR cells, with and without TNF-α pre-treatment of these cells. **(Figure 5.1C)**.

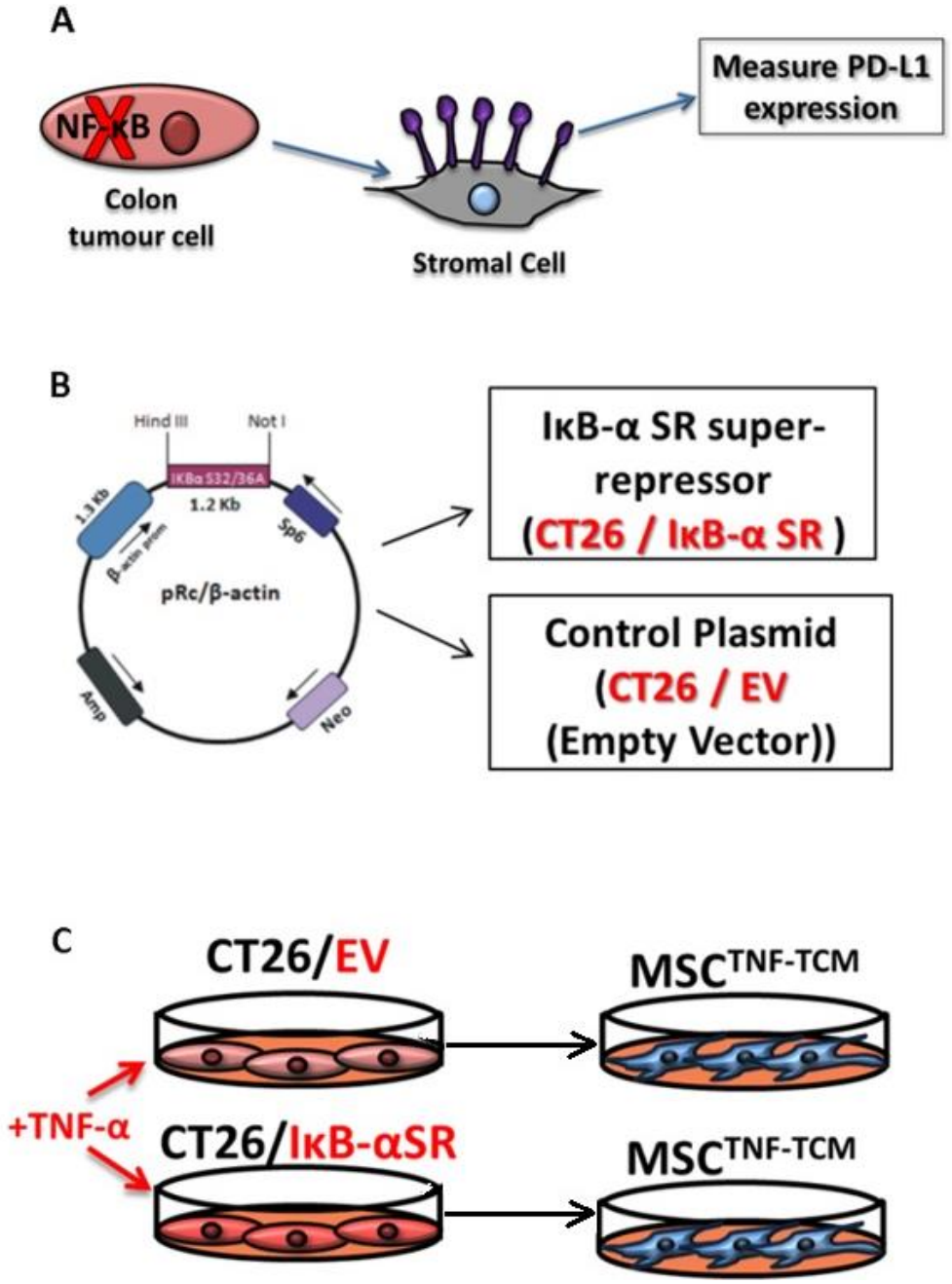


Figure 5.1 Experimental design to test the effects of tumour cell NF-κB signalling on stromal cell PD-L1 expression and immunomodulatory potential
(A) Schematic of experimental design whereby tumour cell NF-κB was targeted using the **(B)** IκB-α Super Repressor plasmid. **(C)** CT26/EV or CT26/IκB-αSR cells were seeded as in previous experiments, pre-treated with TNF-α, and the secretome collected and used to treat stromal cells.

Flow cytometric analysis of stromal cells at 24, 42 and 72 hours revealed no change in PD-L1 expression for stromal cells treated with the control medium from either CT26/EV or CT26/IκB-αSR cells as was expected and in line with our previous findings in Chapter 3. However, following inflammatory tumour cell activation by TNF-α, only the secretome from control tumour cells in which NF-κB signalling was still active was capable of inducing a robust increase in stromal cell PD-L1 expression **(Figure 5.2)**. There was a trend towards an increase with the secretome from CT26/IκB-αSR cells, but this never reached statistical significance, and was significantly lower than the level of PD-L1 induced by the CT26/EV secretome for all time points analysed **(Figure 5.2 (ii))**. These results suggest a clear dependence on tumour cell NF-κB activation and signalling for the enhanced stromal cell expression we have observed thus far.

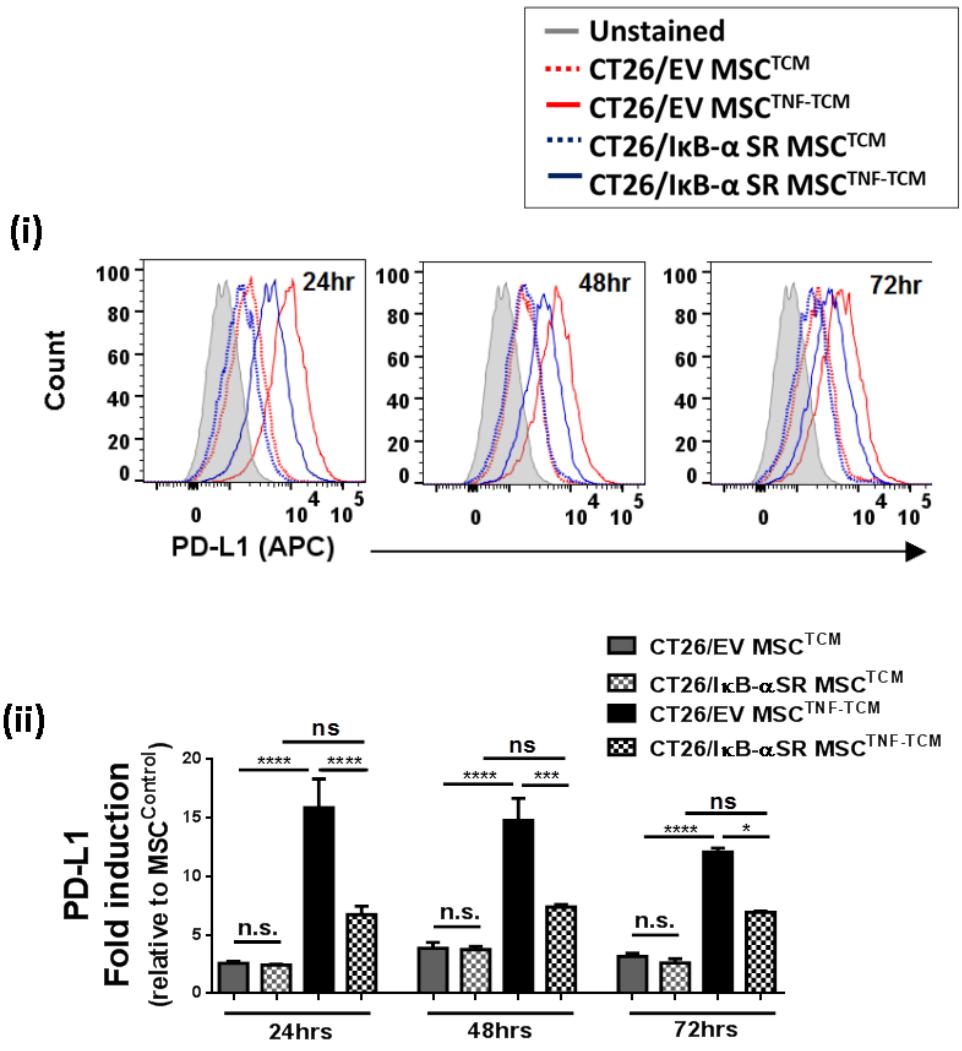


Figure 5.2 Stromal cell PD-L1 induction is dependent on tumour cell NF-κB signalling (i) Representative histograms and **(ii)** bar charts showing PD-L1 induction on the stromal cell surface following 24, 48 and 72 hour exposure to the inflammatory tumour secretome. Error bars: mean +/- SEM *p<0.05***p<0.001****p<0.0001 one-way ANOVA, Tukey's Post Hoc Test n=3

5.2.2 The enhanced $CD4^+$, but not $CD8^+$ T cell suppression by inflammatory tumour conditioned stromal cells can be ameliorated by targeting tumour cell NF-κB

Following on from the observation that stromal cell PD-L1 induction is significantly diminished when tumour cell NF-κB signalling is blocked; we next aimed to test the effects of tumour cell NF-κB inhibition on the capacity of the tumour secretome to enhance the T cell immunosuppressive capacity of stromal cells. Using a similar protocol to that outlined in Chapter 3, we co-cultured stromal cells pre-conditioned with the medium from control and TNF-α pre-treated CT26/EV and CT26IκB-αSR tumour cells with CFSE stained murine lymphocytes. In terms of $CD4^+$ T cell suppression, targeting tumour cell NF-κB resulted in a trend towards a reduction in the ability of stromal cells to suppress $CD4^+$ T cell proliferation **(Figure 5.3B (i) black bar vs checked bar)**. In fact, although also not significantly different, this trending decrease is $CD4^+$ T cell suppression by stromal cells when tumour cell NF-κB was inhibited was greater than that which resulted from PD-1 blockade **(Figure 5.3B (i) blue bar vs checked bar)**. However, the same did not told true for $CD8^+$ T cell suppression, where stromal cells exposed to the secretome from TNF-α pre-treated NF-κB proficient or deficient cell lines induced a similar level of $CD8^+$ T cell suppression **(Figure 5.3B (ii) black bar vs checked bar)**, and only treatment with our PD-1 blocking antibody was effective in reversing this $CD8^+$ T cell suppression **(Figure 5.3B (ii) blue bar)**. Since CT26/IκB-αSR conditioned stromal cells were equally proficient in suppressing $CD8^+$ T cell proliferation as their CT26/EV counterparts despite a significantly lower level of PD-L1 surface expression, we hypothesised that

this observation pointed to a compensatory mechanism of stromal cell modulation by tumour cells in which NF-κB signalling has been inhibited. A more compressive analysis of the surface of CT26/IκB-αSR conditioned stromal cells and their immunomodulatory ligand expression will be necessary to yield more insights into the potential mechanisms responsible. There may well be induction of cell surface proteins not measured here, as well as secreted factors which may synergise with cell surface proteins to inhibit T cell proliferation. Finally, although much smaller, there is a slight induction of PD-L1 expression on stromal cells following conditioning with medium from CT26/IκB-α/SR. There is a chance that this level is all that is needed for physiological activity, and that any increases over this are surplus. In order to test some of these hypotheses, the PD-1 blocking antibody could be introduced to the CT26/IκB-α/SR conditioned stromal cell/T cell co-culture. Furthermore, only the canonical NF-κB pathway is inhibited by the IκB-α/SR. TNF-α can also exert its effects via the non-canonical signalling pathway and so this will need to be blocked too in order to fully validate the effect of NF-κB on out observed effects. Finally, the pathways other than NF-κB which are activated by TNF-α, including JNK, MAPK etc., will also need to be investigated (Moo-Young et al., 2009).

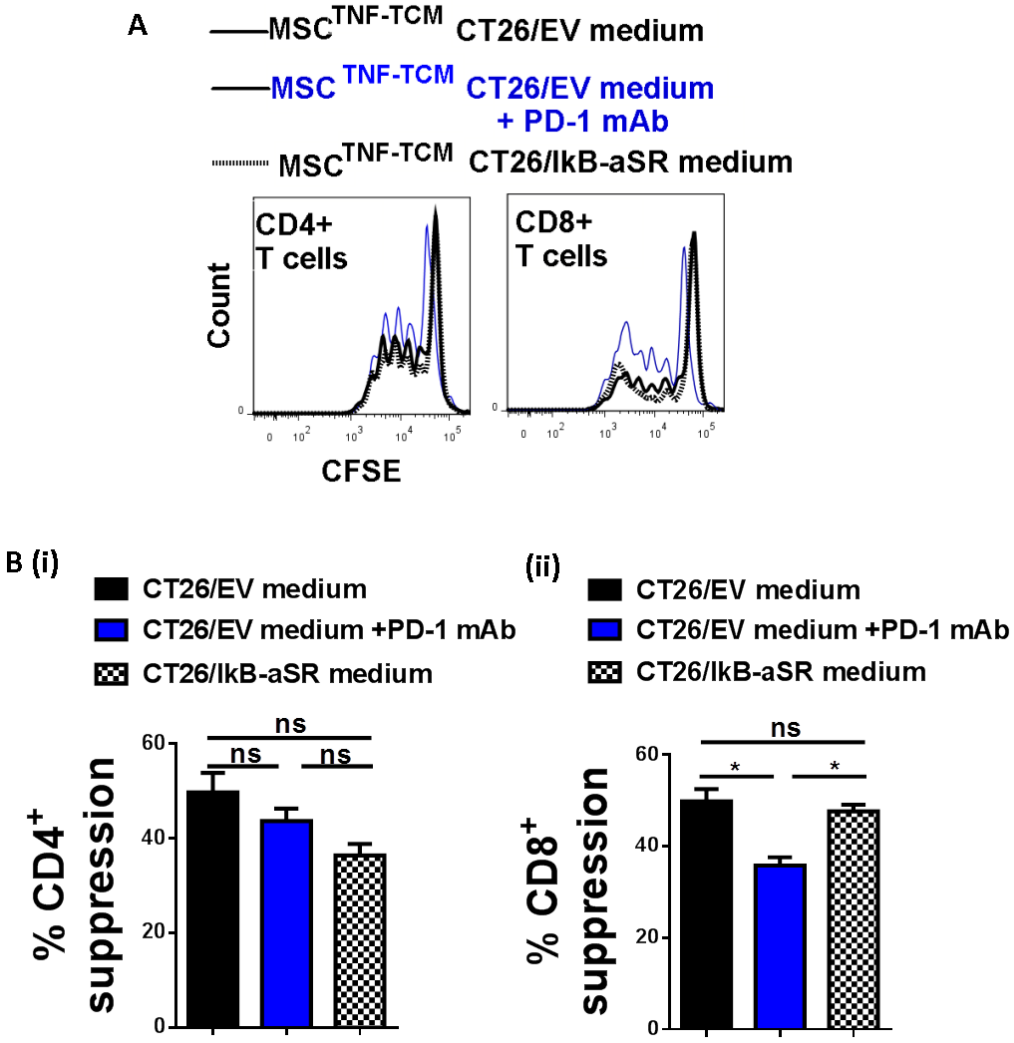


Figure 5.3 The enhanced CD4$^+$, but not CD8$^+$ T cell suppression by inflammatory tumour conditioned stromal cells can be ameliorated by targeting tumour cell NF-κB (i) Representative histograms and bar charts showing the extent of **(i)** CD4+ and **(ii)** CD8+ T cell suppression following 72 hour co-culture with stromal cells conditioned by the secretome from NF-κB proficient (sold bars) or deficient (chequered bars) tumour cells.
Error bars: mean +/- SEM one-way AVOVA, Tukey's Post Hoc Test *p<0.05**p<0.01***p<0.001 multiple t tests, n=3

5.3 PART 2

The work described in second part of this chapter was carried out in order to investigate the molecular signalling pathways activated in tumour conditioned stromal cells and identify those responsible for the induction of PD-L1 expression

5.3.1 Stromal cell PD-L1 induction is likely a two signal process partly dependent on TNF-α but independent of TGF-β signalling

As outlined in the introduction, TGF-β signalling has an important role to play in the colon tumour microenvironment and in regulating the immunomodulatory potential of CAFs in this niche (Calon et al., 2012, Calon et al., 2014) . Importantly, TGF-β has been shown to be an key driver of stromal cells in their pro-metastatic activities in colon cancer, a finding which we believed may have particular relevance to our observations of increased invasiveness in tumours formed from the co-administration of inflammatory tumour conditioned stromal cells **(Chapter 4, Figure 4.3A)** (Calon et al., 2012). Furthermore, it was found that PD-L1 expression on monocytes was regulated by the opposing actions of TNF-α and TGF-β (Ou et al., 2012). With these observations in mind, we next aimed to determine the effect of TGF-β signalling on stromal cell PD-L1 induction.

We began our investigations by measuring the levels of TNF-α and TGF-β secreted by CT26 tumour cells and present in the medium from control (TCM) and TNF-α treated CT26 (TNF-TCM) used to condition stromal cells. We observed TNF-α production from CT26 tumour cells, and a slight but not significant increase in this level following TNF-α treatment of the tumour cells **(Figure 5.4A (i))**. Interestingly we found almost 10 times more TGF-β than TNF-α present in TCM, and this was decreased following TNF-α treatment of the CT26 tumour cells **(Figure 5.4A (ii))**. Although the increase in TNF-α and decrease in TGF-β did not reach statistical significance, the

trends were opposite to each other and in line with the observations by Ou *et al*, who observed an increased expression of PD-L1 on macrophages when TFG-β was inhibited and TNF-α was present (Ou et al., 2012).

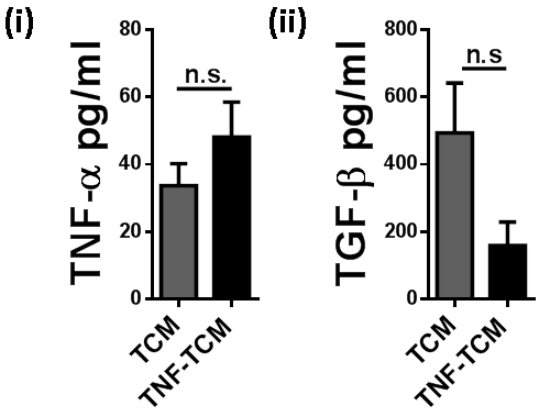


Figure 5.4 TNF-α and TGF-β are present in the secretome of control and TNF-α treated CT26 tumour cells, as measured by ELISA following 72 hour treatment with TNF-α Error bars: mean +/- SEM t test n=3

In all experiments to this point we had included TNF-α treated stromal cells whereby stromal cells were treated for 24 and 72 hours with the highest concentration of TNF-α used to condition stromal CT26 (100ng/ml) and observed no increase in PD-L1 expression at any time **(Chapter 3 Figure 1.7C(i))** and (**Figure 5.5A second, light grey bar)**. Similarly, we did not observe any significant increase in PD-L1 expression upon treatment of stromal cells with the secretome from control CT26 **(Figure 5.5A third, dark grey bar)**. However, when these two treatments were combined – control TCM and TNF-α, we observed a significant induction of stromal cell PD-L1 expression as early as 24 hours **(Figure 5.5A green bar)** and to a level similar to that observed upon stromal cell exposure to the inflammatory tumour secretome **(Figure 5.5A black bar)**. This suggested to us that there was a two signal process of stromal cell PD-L1 induction, one of which is TNF-α or TNF-α-like in the signalling cascades activated. We hypothesised that one of two things is true about the second signal – (1) The second, as yet unknown signal

is likely present in TCM but not capable of PD-L1 induction in the absence of the second signal only present in TNF-TCM or (2) There exists an inhibitory signal for stromal cell PD-L1 induction in the TCM that is not present in TNF-TCM. Since TGF-β is decreased in TNF-TCM when compared to TCM, we next aimed to block TGF-β signalling in the secretome from control CT26 i.e. TCM. We began by ruling out a positive role for TGF-β in promoting stromal cell PD-L1 expression **(Figure 5.5B)**. We then added a TGF-β neutralising antibody to the TCM to test whether blocking our hypothesised "negative regulator" would be sufficient to allow for the induction of PD-L1 on the stromal cell surface. We found no increase in the level of stromal cell PD-L1 with TGF-β in TCM **(Figure 5.5C)**. Next, we aimed to test if the inhibition of TGF-β in TCM would lead to an additive increase in the observed induction of stromal cell PD-L1 with the addition of TNF-α to this medium. As before we did not observe any effect on stromal cell PD-L1 expression with TGF-β neutralisation **(Figure 5.5D)**. To test if TGF-β did indeed exert an inhibitory effect on stromal cell PD-L1 induction we added TGF-β to the TNF-TCM and used this to condition stromal cells. Once again, we did not observe any inhibition of stromal cell PD-L1 induction by TNF-TCM **(Figure 5.5E)**. Finally, since TNF-α addition to control TCM was sufficient to induce stromal cell PD-L1 induction, we investigated whether inhibition of NF-κB signalling in stromal cells would be sufficient to prevent the induction of PD-L1 expression as had been the case for tumour cell NF-κB inhibition. In this case, pharmacological inhibition of NF-κB by BM-S345541, a selective IκB kinase inhibitor, resulted on only a slight, but not statistically significantly inhibition of PD-L1 induction by the inflammatory tumour secretome **(Figure 5.5F**) (Burke et al., 2003). Taken together these data suggest an important role for TNF-α independent of NF-κB signalling, but not TGF-β in inducing stromal cell PD-L1 expression. However, we have not validated, in our own hands, the efficacy of BMS345541, in inhibiting NF-κB signalling in our stromal cells, and this will need to be done before we draw any definite conclusions.

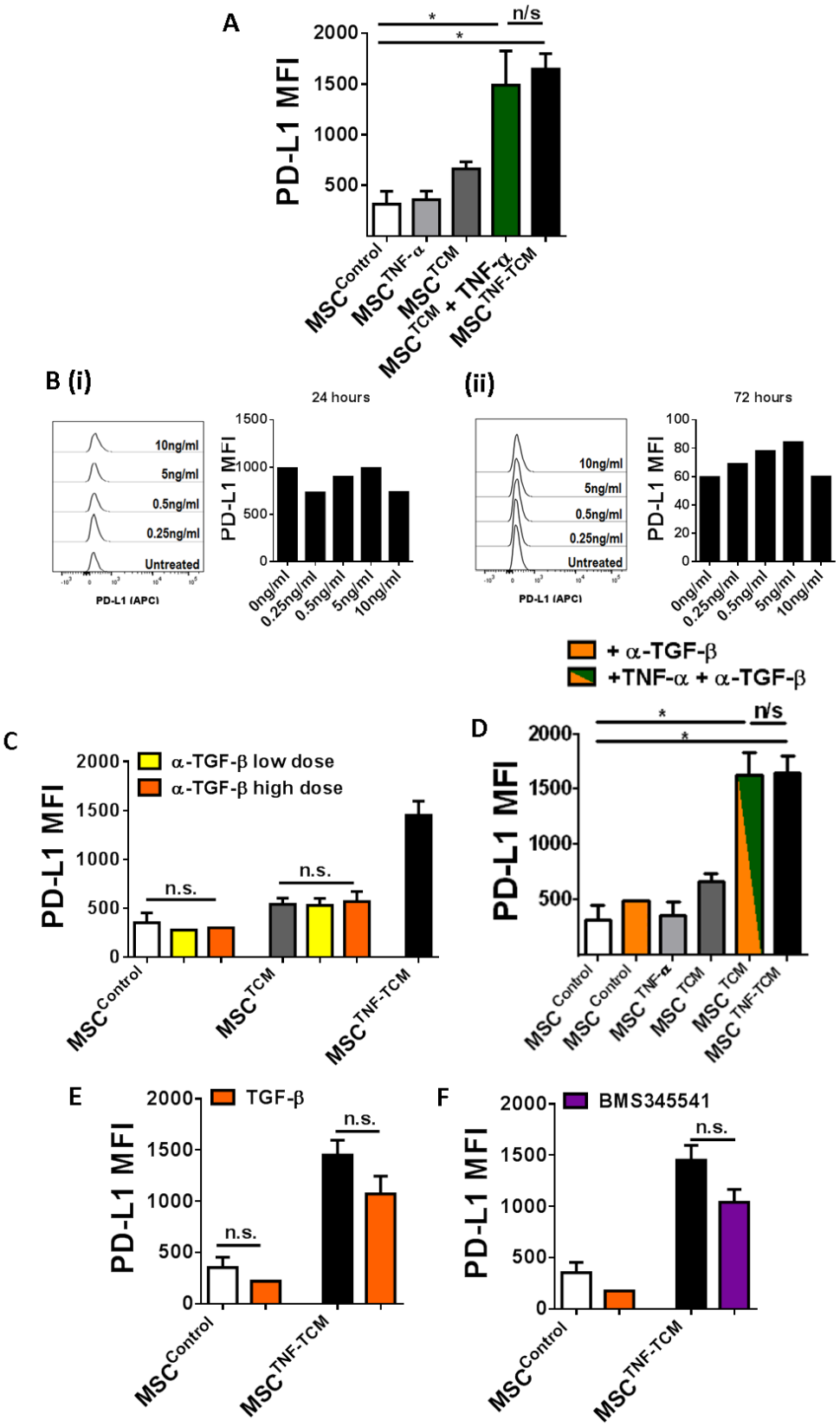

A
PD-L1 MFI
2000
1500
1000
500
*
*
n/s
MSC Control
MSC TNF-α
MSC TCM
MSC TCM + TNF-α
MSC TNF-TCM
B (i)
24 hours
10ng/ml
5ng/ml
0.5ng/ml
0.25ng/ml
Untreated
PD-L1 (APC)
PD-L1 MFI
1500
1000
500
0
0ng/ml
0.25ng/ml
0.5ng/ml
5ng/ml
10ng/ml
(ii)
72 hours
10ng/ml
5ng/ml
0.5ng/ml
0.25ng/ml
Untreated
PD-L1 (APC)
PD-L1 MFI
100
80
60
40
20
0
0ng/ml
0.25ng/ml
0.5ng/ml
5ng/ml
10ng/ml
+ α-TGF-β
+TNF-α + α-TGF-β
C
α-TGF-β low dose
α-TGF-β high dose
PD-L1 MFI
2000
1500
1000
500
0
n.s.
n.s.
MSC Control
MSC TCM
MSC TNF-TCM
D
PD-L1 MFI
2000
1500
1000
500
*
*
n/s
MSC Control
MSC Control
MSC TNF-α
MSC TCM
MSC TCM
MSC TNF-TCM
E
TGF-β
PD-L1 MFI
2000
1500
1000
500
0
n.s.
n.s.
MSC Control
MSC TNF-TCM
F
BMS345541
PD-L1 MFI
2000
1500
1000
500
0
n.s.
MSC Control
MSC TNF-TCM

Figure 5.5 Neither TGF-β treatment nor neutralisation had any effect on stromal cell PD-L1 expression (A) Stromal cell PD-L1 expression following the addition of TNF-α to control TCM **(B)** The effect of TGF-β on basal stromal cell PD-L1 expression **(C)** PD-L1 expression on the stromal cell surface following TGF-β neutralisation in control TCM (low dose: 0.25 μg/ml, high dose 1.25 μg/ml) **(D)** Stromal cell PD-L1 expression following both TNF-α addition to and TGF-β neutralisation in control TCM **(E)** Stromal cell PD-L1 expression following addition of 5ng/ml TGF-β to TNF-TCM **(F)** Stromal cell PD-L1 expression following pharmacological inhibition (2 hour incubation with 5μM BMS345541 prior to adding conditioned medium) of NF-κB prior to exposure to the inflammatory tumour secretome (TNF-TCM).
Error bars: +/- SEM *p<0.05 one-way ANOVA, Tukey's Post Hoc test n=3

5.3.2 STAT1 and STAT3 proteins are phosphorylated by inflammatory tumour conditioning of stromal cells

Around the time at which we were investigating the molecular regulation of stromal cell PD-L1 induction a paper was published showing that IFN-γ controlled the suppressive ability of murine MSCs via early phosphorylation of STAT1 and STAT3 (Vigo et al., 2017). Furthermore, this group noticed a decrease in PD-L1 (CD274) mRNA in their MSCs following STAT1 knockdown. As mentioned in this introduction to this chapter, STAT proteins are known regulators of the anti-tumour immune response (Yu et al., 2009, Shou et al., 2016). In response to these findings, we measured the amount of IFN-γ secreted by CT26 tumour cells without and without TNF-α treatment. Interestingly we found the level of IFN-γ production to be almost negligible at less than 3pg/ml in our cultures, with no statistical difference between that produced by control or TNF-α treated CT26 **(Figure 5.6A)**. Since IFN-γ is not the only cytokine known to activate STAT signalling, we next measured early STAT1 and STAT3 phosphorylation in our tumour conditioned stromal cells, and found induction of both STAT1 and STAT3 as early as 30 minutes **(Figure 5.6B)**. Perhaps most interestingly, the pattern of STAT1 induction correlated with stromal cell PD-L1 induction, whereby there was a very slight

induction with control TCM but a dramatic induction upon stromal cell exposure to the inflammatory tumour secretome **(Figure 5.6B)**.

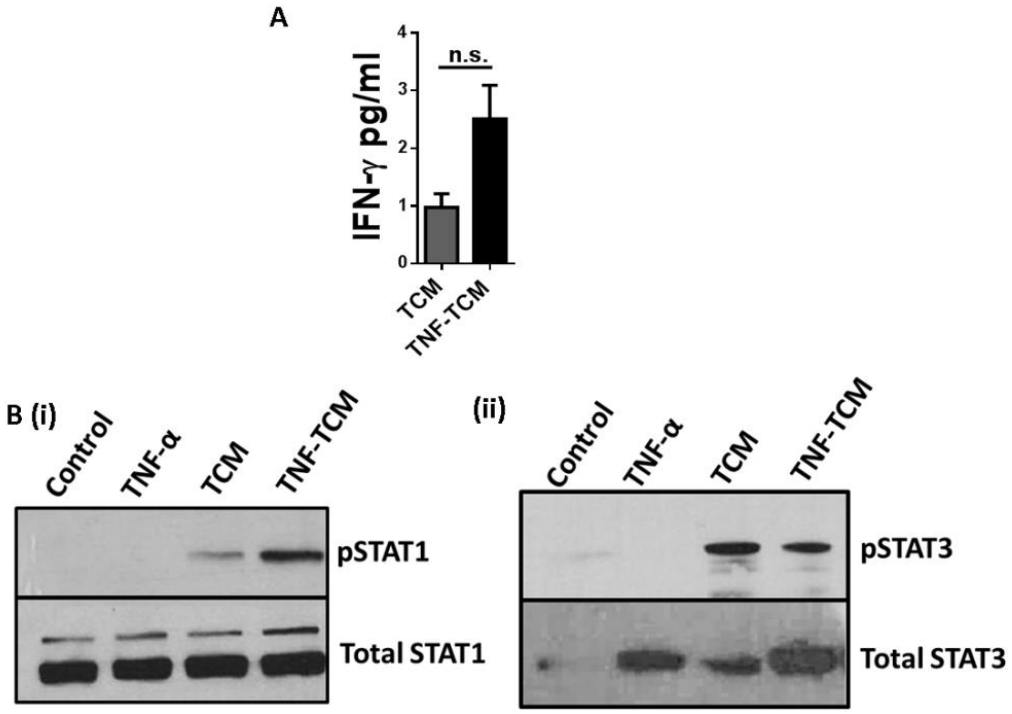


Figure 5.6 STAT1 and STAT3 phosphorylation occurs in stromal cells following exposure to the inflammatory tumour secretome, likely independent of tumour cell IFN-γ production (A) Measurement of IFN-γ concentration produced by CT26 tumour cells with and without TNF-α treatment **(B)** Representative western blots of **(i)** STAT1 and **(ii)** STAT3 phosphorylation in stromal cells following 30 minute exposure to the inflammatory tumour secretome
Error bars: +/- SEM t test n=3

This finding concurs with that of Vigo *et* al, and certainly warrants further investigation via pharmacological inhibition or genetic knockdown of STAT1 and 3 signalling proteins and subsequent investigation of MSC immunomodulatory potential. Additional support for the importance of STAT1 and STAT3 in colon cancer can be seen in images presented in the Human Protein Atlas, where significant STAT1 and STAT3 staining is observed on the stromal compartment in samples from patients diagnosed with colorectal adenocarcinoma **(Figure 5.7)** (Uhlen et al., 2015)

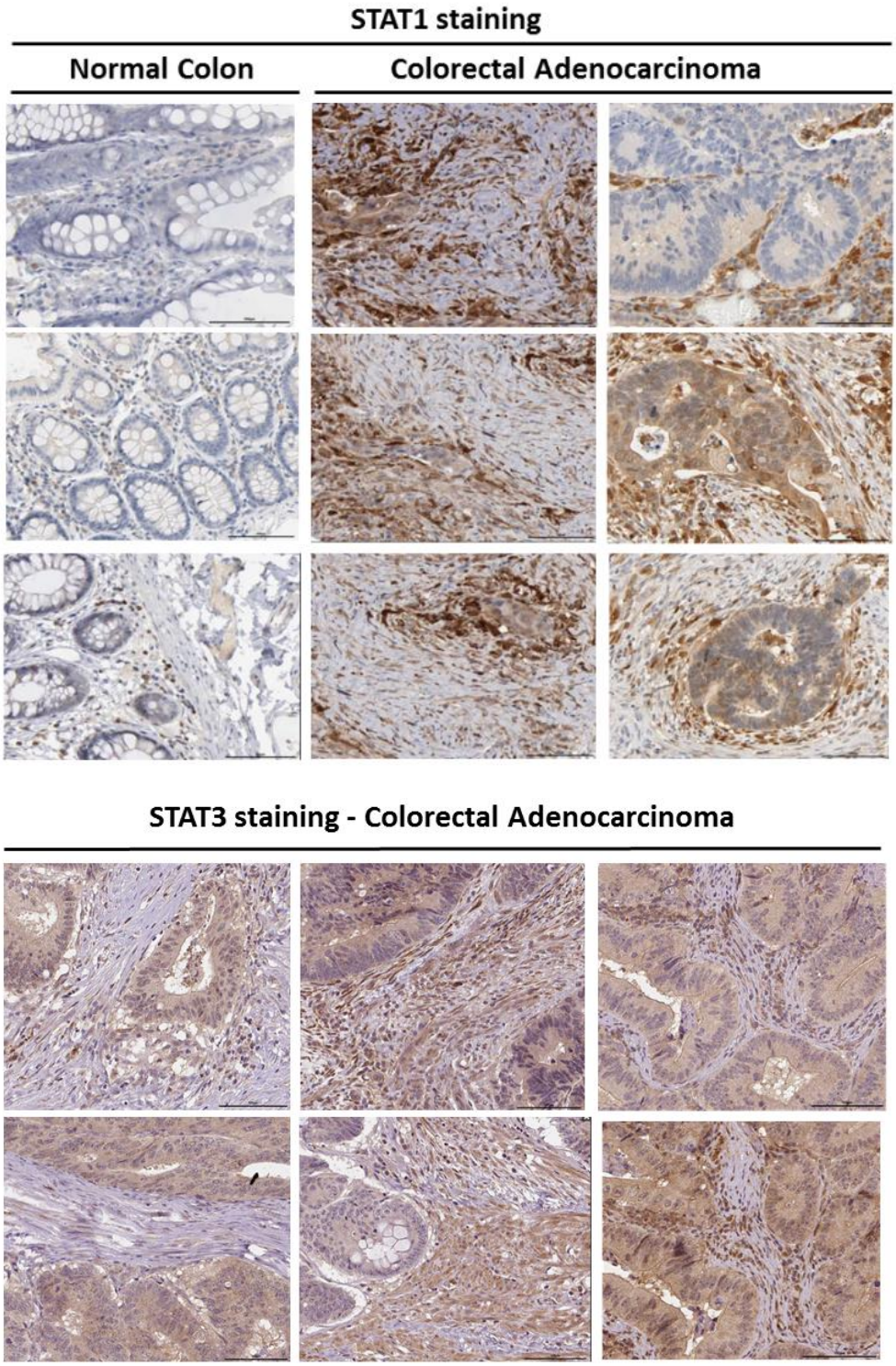


Figure 5.7 STAT1 and STAT3 expression in colon cancer samples
Brown staining indicates **(A)** STAT1 or **(B)** STAT3 staining in colon adenocarcinoma samples. Each panel represents a separate patient (Uhlen et al., 2015)

The preliminary findings presented here suggest that stromal cell STAT signalling, and STAT1 in particular may represent an important target in the quest to modulate the anti-tumour immune response. Initial early experiments carried out to address this question relied on the use of pharmacological inhibitors to target STAT1 (Fuldarabine) and STAT3 (p3971) at varying doses 24 hours prior to treatment with tumour conditioned medium, also containing the inhibitor, as per previous experiments in our lab (unpublished). However, we did not observe a decrease in PD-L1 expression following treatment with either drug **(Figure 5.8 A,B)**, or the two in combination **(Figure 5.8C)**.

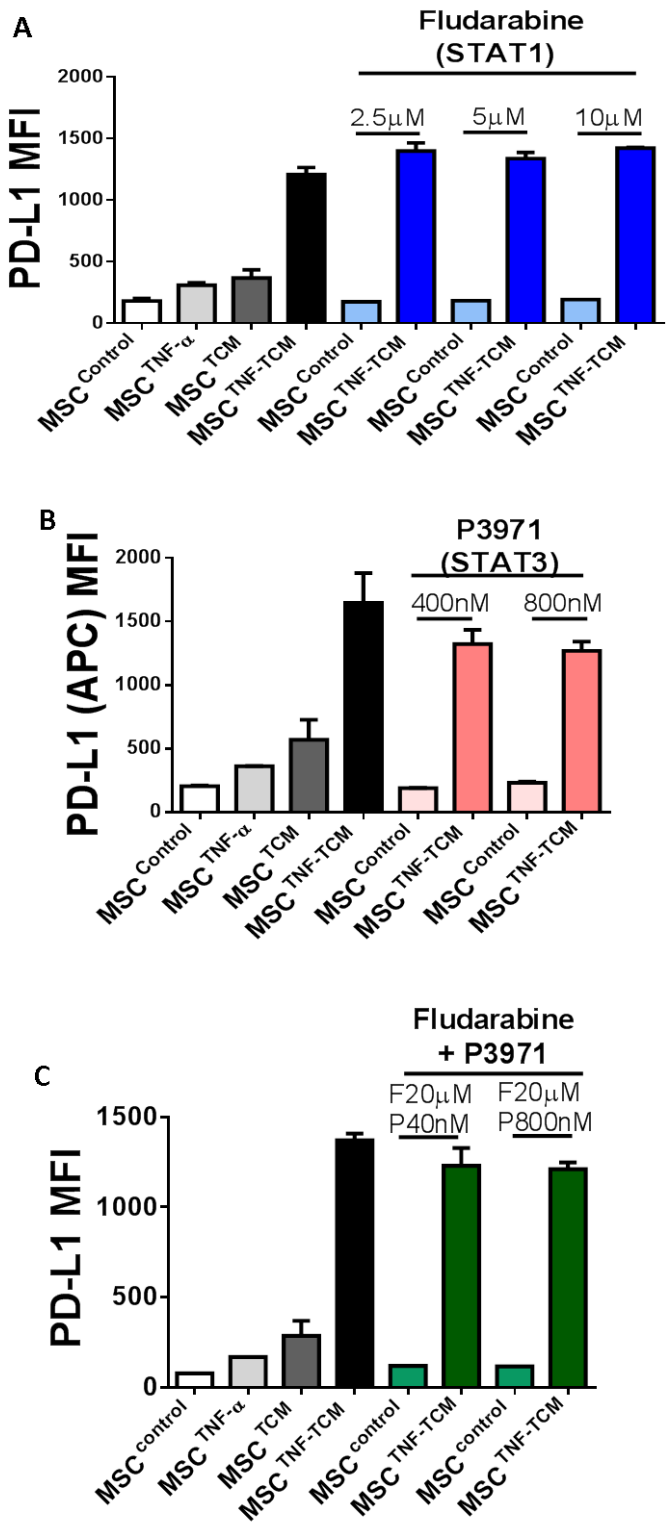


Figure 5.8 Blockade of STAT1 with fludarabine or STAT 3 with p3971, alone or in combination, had no effect on stromal cell PD-L1 induction by inflammatory tumour conditioned medium (A) Measurement of stromal cell PD-L1 expression following STAT1 inhibition by fludarabine **(B)** Measurement of stromal cell PD-L1 expression following STAT3 inhibition by p3971 **(C)** Measurement of stromal cell PD-L1 expression following a combination of STAT1 and STAT3 inhibition Error bars: +/- SEM t test n=3

L1 induction. For this reason, we decided to confirm the inhibition of STAT1 and STAT3 phosphorylation in our cells before proceeding to try different dosing and timing strategies for these drugs. In order to do this, we treated stromal repeated the treatment protocol as before, with 24hour STAT inhibition followed by treatment with inflammatory tumour conditioned medium. Interestingly, and indeed unexpectedly, we observed an induction of STAT1 phosphorylation after fludarabine treatment followed by conditioned medium **(Figure 5.9A)**, and a similar induction of STAT3 phosphorylation when cells were treated with conditioned medium after p3971 treatment **(Figure 5.9B)**. While outside the scope and time constraints of this thesis, this finding certainly warrants further investigation. Perhaps, this question may be best addressed by more specific targeting of STAT1 or 3 by shRNA or RNAi, rather than using less specific pharmacological inhibitors. Fludarabine has been shown to lead to inhibition of STAT1 activation and a loss of STAT1 protein and mRNA in human blood lymphocytes (Frank et al., 1999). We did observe an apparent inhibition of total STAT1 with the highest concentration of fludarabine (20μM) **(Figure 5.9A)**. However, there was still significant STAT1 phosphorylation in our cells after this treatment. It is possible that this is a species specific effect since the data showing STAT1 protein and mRNA loss were taken from human blood samples (Frank et al., 1999). Fludarabine has been used therapeutically in mice in several studies, in particular in the setting of leukemia, and appears to have the same clinical effect that is observed in human patients. However, the effects of this drug on STAT1 phosphorylation specifically are not examined in these studies (Johnson et al., 2006, Weiss et al., 2003). P3971 is cited as capable of HIF-1α and STAT3 inhibition and so is not specific to STAT3 (Godse et al., 2013). Furthermore, experiments carried out by this group to test STAT3 inhibition were carried out in HeLa cells using a luciferase reporter gene assay, and so once again may not represent the STAT3 expected in murine stromal cells. Also, it will be worthwhile measuring total STAT3 in these cells. While we believe it is the phosphorylated protein that is indicative of activation and potentially

responsible for PD-L1 induction, this may be interesting in terms of better elucidating the STAT3 inhibitory potential of p3971.In addition to specifies specificity, the timing and concentration of these drugs may not be optimal and perhaps could be further assessed. However, STAT1 and 3 targeting by shRNA currently represents an avenue of interest in our lab and these experiments will be carried out in the coming months.

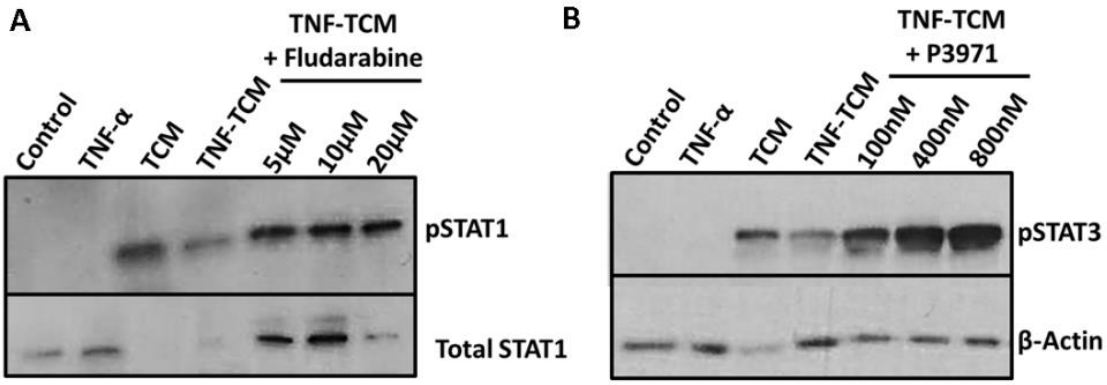


Figure 5.9 2 hour pre-treatment with fludarabine and p3971 prior to treatment with inflammatory tumour conditioned medium result in induction of phosho-STAT1 and phosphor-STAT3 respectively
(A) Western blot of phospho-STAT1 following pre-treatment with fludarabine **(B)** Western blot of phospho-STAT3 following pre-treatment with p3971

Finally, while IFN-γ appears to be absent in the secretome from control or TNF-α activated CT26 tumour cells, further analysis of this medium revealed the presence of a number of proteins, many of which have been differentially regulated by TNF-α activation of CT26 tumour cells **(Figure 5.10A)**. All of these proteins have been linked to STAT1 activation, often in T cells and other immune cells, but their role in modulating PD-L1 expression and stromal cells immunosuppressive capacity remains to be elucidated (Miyamoto et al., 2012, Ylikoski et al., 2005, van der Bruggen et al., 1995, Wang et al., 2004, Wong and Fish, 1998, Vegran et al., 2014).In addition, a number of other proteins, although not significantly different between the control and TNF-α pre-treated CT26 tumour secretome, were also produced,

and again, this group of proteins include activators of STAT1 phosphorylation such as G-CSF and IL-6 **(Figure 5.10B)** (Duarte and Frank, 2000, Zhang et al., 2003).

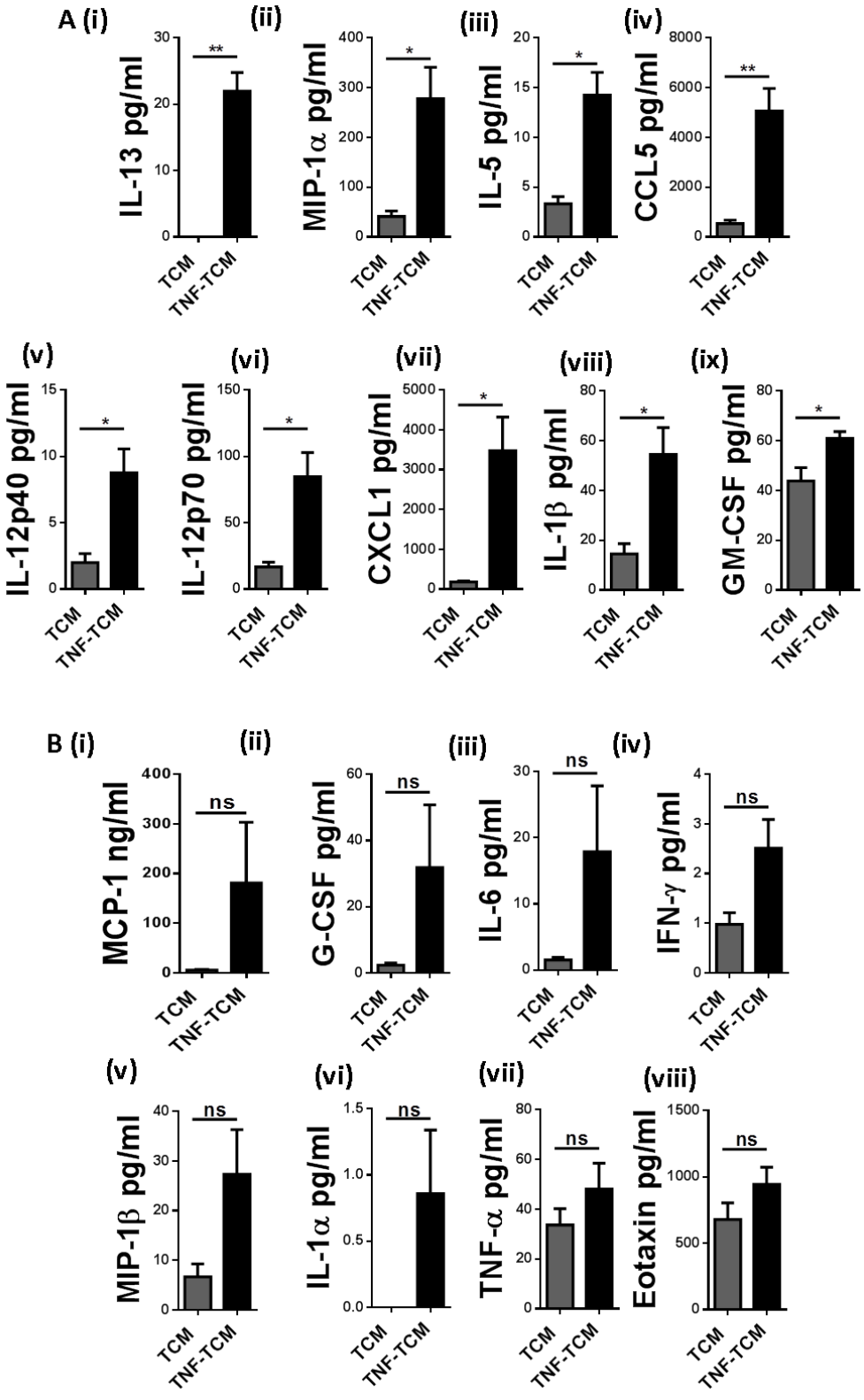


Figure 5.10 Bioplex analysis of the secretome from control (TCM) and TNF-α treated (TNF-TCM) CT26 tumour cells (A) Proteins differentially regulated by TNF-α pre-treatment of CT26 **(B)** Proteins for which no difference was observed with or without TNF-α pre-treatment of CT26
Error bars: +/- SEM t test n=3

5.4 Discussion

Prior to carrying out the experiments described in this chapter we had shown that inflammatory tumour conditioning of stromal cells induced robust PD-L1 expression on the stromal cell surface, and that expression of this protein endowed stromal cells with and enhanced T cell suppressive capability *in vitro*, and correlated with increased tumour volume and invasiveness *in vivo*. However, the mechanism by which tumour cells exerted this effect on stromal cells was not clear and thus we designed the experiments presented here in order to gain a better insight into the molecular regulation of this process. We addressed this question from two separate standpoints – (1) signalling pathways activated in tumour cells that may be responsible for the release of critical factors into the secretome responsible for stromal cell PD-L1 induction and (2) Signalling pathways activated in stromal cells prior to PD-L1 induction.

5.4.1 Part 1

Stromal cell PD-L1 expression is dependent on tumour cell NF-κB signalling

As mentioned at the outset, NF-κB is a transcription factor of critical importance in the development of a number of cancers, not least colorectal, and is upregulated in inflammatory bowel disorders, the presence of which may predispose patients to the development of colon cancer (Lawrence, 2009, Kim and Chang, 2014, Karin, 2009). Furthermore, data from our own lab has shown that targeting tumour cell NF-κB leads to decreased tumour volume *in vivo*, owing in part to the switch from an M2 "pro-tumourigenic" macrophage phenotype to that of an M1 "anti-tumour" macrophage phenotype (Ryan et al., 2015). In this model, macrophage depletion from NF-κB proficient tumours resulted in a significant reduction in tumour growth. However, selective depletion of macrophages from NF-κB deficient tumours did not cause growth to the same extent as untreated NF-κB proficient

tumours, highlighting the existence of at least one other cell type influenced by tumour cell NF-κB signalling and responsible for aiding tumour growth under the influence of such signals (Ryan et al., 2015). It was here that we hypothesised that tumour cell NF-κB also affect the immunomodulatory capacity of tumour resident stromal cells. Our findings show that inhibition of tumour cell NF-κB signalling did indeed prevent the secretome-induced stromal cell PD-L1 induction we had observed previously **(Figure 5.2)**. This result concurs somewhat with published data showing a dependency on NF-κB activation for PD-L1 expression, however, these studies only investigate the effect of tumour cell NF-κB signalling on tumour cell PD-L1 expression (Gowrishankar et al., 2015, Lim et al., 2016). Our data shows for the first time that activation of this signalling pathway also has an important role to play in inducing stromal cell PD-L1 expression. This novel finding may support the notion of pharmacological targeting of tumour cell NF-κB leading to a number of "complementary actions that inhibit the metastatic spread of colorectal cancer" (Ryan et al., 2015). However, this is not clear from our preliminary *in vitro* data, which could in fact suggest that targeting tumour cell NF-κB may be futile in so far as augmenting stromal cell $CD8^+$ T cell suppressive capacity is concerned. Although we found a trend towards a diminished ability of inflammatory tumour conditioned stromal cells to inhibit $CD4^+$ T cell suppression when exposed to the secretome from NF-κB deficient tumour cells, the same did not hold true for $CD8^+$ T cell suppression, where both control (CT26/EV) and NF-κB deficient (CT26/IκB-αSR) conditioned stromal cells were equally suppressive **(Figure 5.3)**. This may suggest an alternative, compensatory mechanism of stromal cell modulation employed by tumour cells in which NF-κB has been inhibited. In fact, this is highly likely, given the fact that our CT26 tumour cells do not rely on NF-κB for survival, as is the case for several other cell lines. Furthermore, our NF-κB deficient CT26 grow and proliferate well, at a rate comparable to their control counterparts, further confirming the existence of compensatory mechanisms in these cells. As yet, this mechanism remains unknown and will require more in-depth analysis of stromal cells exposed to

the NF-κB deficient tumour cell secretome. Alternatively, this sustained immunosuppressive capacity of stromal cells even when exposed to the secretome from NF-κB deficient tumour cells could be due to a very low threshold of PD-L1 expression being required for $CD8^+$ T cell suppression. In **Figure 5.2 (ii)** we observed a slight induction of PD-L1 on the stromal cell surface following conditioning with TNF-α pre-treated NF-κB deficient CT26. Although significantly less than the level induced with NF-κB proficient CT26, it could possibly be that this small induction is sufficient to reach the threshold of that necessary to suppress $CD8^+$ T cell proliferation. In order to confirm this, co-cultures can be repeated with the addition of out PD-1 blocking antibody to all samples, including those conditioned with the secretome from NF-κB deficient cells. Additionally, cytokine levels can be measured to determine if it is only proliferation that is affected, or effector molecule release too.

Limitations and future studies (Part 1)

In this chapter we were able to show quite convincingly, a dependency on tumour cell NF-κB signalling for stromal cell PD-L1 induction. However, the inhibition in stromal cell PD-L1 induction following tumour cell NF-κB inhibition was not sufficient to inhibit the $CD8^+$ T cell suppressive capacity of inflammatory tumour conditioned stromal cells. MSCs in solid tumours have been shown to regulate the immune response via CD73 expression, IDO (human) or NO (rodent) release, or secretion of proteins including TGF-β, IL-10 and PGE_2 (Poggi et al., 2014). Each of these factors, in addition to countless others not mentioned here could represent potential mediators of $CD8^+$ T cell suppression by CT26/IκB-αSR conditioned stromal cells and will only be identified by a wider analysis of the NF-κB dependent differential regulation of protein release from CT26/EV and CT26/IκB-αSR tumour cells following treatment with TNF-α. Secondly, it may be necessary to test the *in vivo* tumour promoting effect of CT26/IκB-αSR conditioned stromal cells to

confirm a similarly immunomodulatory, tumour and invasiveness promoting phenotype as that observed for stromal cells conditioned by the NF-κB inflammatory tumour secretome. In addition to effects on $CD8^+$ T cells, which were perhaps predictable for stromal cells found to express high levels of PD-L1, should NF-κB deficient tumour conditioning of stromal cells induce one or more of the factors mentioned above, or indeed others, it is likely that these cells will also exert differential effects on distinct immune cell populations to their NF-κB proficient tumour conditioned counterparts. Finally, the emergence of this similar, but as yet poorly understood immunosuppressive stromal cell phenotype upon exposure to the secretome from NF-κB deficient tumour cells may mean that, while targeting tumour cell NF-κB for the purpose of modulating macrophage anti-tumour immune responses may be effective, may be impractical and even deleterious from the point of view of altering the immunomodulatory capacity of the tumour stromal compartment. We propose that a comprehensive analysis of the signalling pathways activated in stromal cells in response to inflammatory tumour conditioning and identification of those responsible for the induction of PD-L1 expression and enhanced immunosuppressive capacity may reveal one or more viable targets for therapeutic intervention. The second part of this chapter describes the preliminary experimental work we have carried out with the aim of identification of these factors.

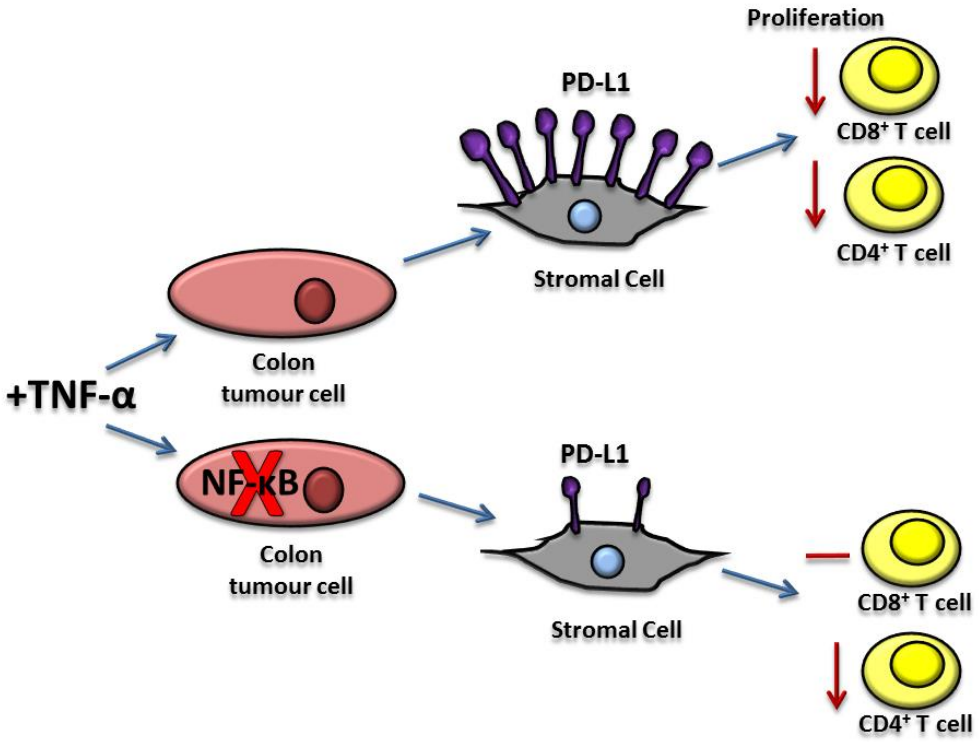


Figure 5.11 Summary (Part 1) Targeting tumour cells NF-κB prevents stromal cell PD-L1 induction by the inflammatory tumour secretome and partly reduces the ability of inflammatory tumour conditioned stromal cells to inhibit $CD8^+$ T cell proliferation

5.4.2 Part 2

Tumour produced TNF-α, but not TGF-β may have an important role in stromal cell PD-L1 induction

Our data showed that, although TNF-α alone is insufficient to induce stromal cell PD-L1 by itself, when added into the medium from control CT26 (TCM) it was sufficient to induce robust PD-L1 expression on stromal cells. The level of PD-L1 expression following addition of TNF-α to control TCM was in fact comparable to that induced by the medium from TNF-α pre-treated CT26 (TNF-TCM). This finding is in line with that recently published showing increased macrophage PD-L1 expression in response to tumour conditioned medium (Hartley et al., 2017). Unlike our observations, in this study the

authors found that TNF-α alone was sufficient to induce macrophage PD-L1 expression. However, in a separate experiment whereby a TNF-α neutralising antibody was added to the tumour conditioned medium used to treat macrophages they found a reduction in the level of PD-L1 expressed, but the expression was not completely abrogated and remained much higher than control macrophages, indicating the presence of at least one more signal in the tumour conditioned medium responsible for macrophage PD-L1 induction (Hartley et al., 2017). Although the authors here did not investigate further any additional tumour secreted signals that could be responsible for macrophage PD-L1 induction, they identified macrophage produced TNF-α as the source of TNF-α responsible for PD-L1 induction on macrophages resident in tumour samples *in vivo*. They found this TNF-α to act in self-promoting feed-forward loop on macrophages. This is an interesting observation and one which suggests that a comprehensive analysis of the secretome of tumour conditioned stromal cell secretome is warranted and may reveal further insights into the molecular regulation of stromal cell PD-L1 expression, though data is lacking to show any capability of mesenchymal stromal cells to produce TNF-α. This is not to say, however, that one or more other factors are not present and playing an active role in the induction of stromal cell PD-L1 expression.

Additionally, the concentration of TNF-α used to treat TNF-α control stromal cells ($MSC^{TNF-\alpha}$) was always the same as the highest concentration used to condition CT26 tumour cells, i.e. 100ng/ml. Given that the concentration of free TNF-α remaining in the conditioned medium from CT26 (TNF-TCM) is several orders lower than this (40-60pg/ml), this increases the likelihood that more than one signal is necessary for stromal cell PD-L1 induction, one of which is TNF-α, or indeed some other cytokine that will activate the same signalling pathways as TNF-α in stromal cells.

With regard to TGF-β, this cytokine has been shown to be important in modulating the ability of CAFs to promote tumour growth, but not from an immunomodulatory point of view (Calon et al., 2012, Calon et al., 2014). We

found TGF-β to have no effect on basal stromal cell PD-L1 expression. While the recently published study by Ou *et* al., identified a role for TGF-β in negatively regulating TNF-α mediated increases in monocyte PD-L1 expression, we did not find TGF-β inhibition to be effective in decreasing the enhanced stromal cell PD-L1 expression we observed in response to TNF-α treatment **(Figure 5.6)** (Ou et al., 2012). In looking at renal tubular epithelial cells, a tissue known to express PD-L1, Starke *et al*., found that TGF-β was only effective in modulating PD-L1 expression in when PD-L1 expression was induced by IFN-γ treatment (Starke et al., 2007, Ding et al., 2005). Although we did not observe an appreciable level of IFN-γ in the secretome from CT26 tumour cells, even when pre-treated with TNF-α, it could be that there exists a second factor present in the tumour secretome which is in part responsible for PD-L1 induction and may be antagonised by TGF-β. Furthermore, in light of the study by Hartley *et al*. there may be factors produced by the stromal cells acting in a feed forward manner to promote PD-L1 induction, although, as was the case for TNF-α, data is lacking to show evidence of IFN-γ production by MSCs (Madrigal et al., 2014).

There is a well published association between STAT signalling and PD-L1 expression on various cell types, and in particular STAT1 and STAT3 (Meissl et al., 2017, Horlad et al., 2016). Thus, the finding of STAT1 and STAT3 phosphorylation in stromal cells in following exposure to the tumour secretome was unsurprising **(Figure 5.7)**. STAT1 looks to be of particular interest with regards the robust stromal cell PD-L1 induction we observed in response to the inflammatory tumour conditioning as the pattern of STAT1 phosphorylation correlates with that of PD-L1 induction, with greatest STAT1 phosphorylation induction observed where the greatest PD-L1 protein induction was seen.

With regard to the factors we identified as secreted by inflammatory activated tumour cells, as well as links to STAT signalling, several of the proteins we identified have been shown to be important mediators of colorectal cancer metastasis. Most notable of these is CCL5, neutralisation

of which has been shown to restrict tumour growth in colorectal cancer (Cambien et al., 2011). Furthermore, a recent study showed that CCR5 blockade can polarise macrophages to an anti-tumour phenotype (Bronte and Bria, 2016). Data are lacking to show if the same is true for MSC polarisation, but given our findings of significantly elevated CCL5 in TNF-TCM, and a significanty more immunosuppressive stromal cell phenotype following exposure to this medium, CCL5 represents a potentially important signalling protein worthy of further investigation. In addition to CCL5, CXCL1 secretion is a marker of poor prognosis in colorectal cancer patients (le Rolle et al., 2015). Perhaps even more interestingly in the context of the studies presented here, elevated CXCL1 levels were found to promote growth, not only of tumour cells, but also murine fibroblasts, adding weight to the idea that it may be another important factor in the tumour promoting stromal cell effects we saw *in vivo* (le Rolle et al., 2015). IL-5 and GM-CSF, another two of the cytokines we found to be secreted by tumour cells in response to inflammation stimulus, have been shown to enhance metastasis in breast cancer (Quail et al., 2017). This, as is the case for the other proteins mentioned here, could represent an important mechanism by which tumour cells altered the biology of stromal cells and culminated in the enhanced metastatic disease we observed *in vivo*. One caveat to this data is that it only examines the factors secreted by inflammatory conditioned CT26 tumour cells. This is important from the point of view of it being the secretome we used to condition our stromal cells prior to experiments. In the future it will be necessary to measure the factors subsequently released from these inflammatory tumour conditioned stromal cells, as this may give greater insight into the soluble factors employed by the tumour stromal compartment to aid tumour growth or supress anti-tumour immune responses. As mentioned, carcinogenesis is a complex, multi-factorial process, and it is likely that several, or indeed all of the proteins mentioned here, along with others not studied have an important role to play in this disease.

Limitations and future studies (Part 2)

Taken together, the data in the second part of this chapter serves to highlight the existence of a complex process of stromal cell PD-L1 induction in response to factors present in the inflammatory tumour microenvironment. Data from part 1 of this chapter may suggest that targeting tumour cell NF-κB could be of benefit in colorectal cancer. This, however, is not without limitations, irrespective of any detrimental effects this may have on stromal cell immunomodulatory potential. Widespread NF-κB inhibition would result in potent immunosuppression in patients, thereby increasing greatly their risk of opportunistic infection (Baud and Karin, 2009). Also, NF-κB signalling itself is very complex and differs from cell to cell and cancer to cancer, not to mention the excess inflammasome activation and inflammatory cytokine production shown to result from pharmacological NF-κB inhibition (Lin et al., 2010b, Greten et al., 2007). Thus, targeting NF-κB in colorectal cancer with the hope of overcoming the immunosuppressive milieu set up by the stromal compartment is not straightforward. We propose that more specific targeting of downstream signalling molecules or released proteins may represent a more viable therapeutic option in this regard. To this end we investigated signalling pathways activated in stromal cells in order to identify potentially targetable candidates. The experiments described in the second part of this chapter address this question to a point, but considerably more work is necessary in order to identify optimum targets. While the Bioplex analysis of the tumour secretome shown here **(Figure 5.8)** identifies some potentially important factors responsible for the observed changes in stromal cell immunomodulatory potential, a more comprehensive analysis of this secretome by a proteomics or mass spectrometry based approach will yield a better overview of the factors involved (Kratchmarova et al., 2002, Finoulst et al., 2011, Sarkar et al., 2012)

While the western blot analysis of STAT phosphorylation is suggestive of a correlation between STAT1 phosphorylation, PD-L1 induction and enhanced stromal cell immunomodulatory potential, it is likely that one or more separate signalling pathways are involved in this process, especially in light of our data showing a likely 2 signal process of PD-L1 induction. Thus, as we suggested for the secretome, a more well-rounded analysis of all the signalling pathways altered in stromal cells in response to the inflammatory tumour secretome will yield more comprehensive data and identify those pathways most suitable for targeting.

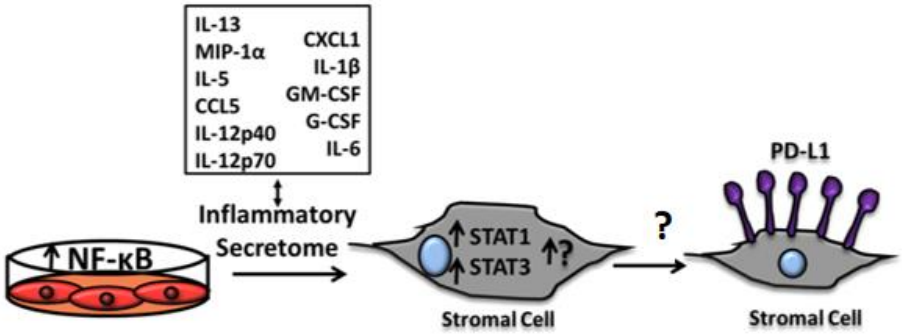


Figure 5.12 Summary (Part 2) STAT1 and STAT3 phosphorylation take place in stromal cells following exposure to the inflammatory tumour secretome. The pattern of STAT1 phosphorylation in particular, correlates with that of STAT1 expression, and may represent an important mechanism by which stromal cell PD-L1 in induced in the tumour microenvironment

CHAPTER 6

Discussion and Further Perspectives

As mentioned at the outset, T cells are critical mediators of the anti-tumour immune response (Hadrup et al., 2013, Galon et al., 2012, Galon et al., 2006). In an immunocompetent host, CD8+ cytotoxic T cells are capable of recognising and killing tumour cells by release of cytokines or proteases, or induction of apoptosis (Andersen et al., 2006) A. CD4+ T cells can also have a role to play in enhancing CD8+ T cell effector function (Kim and Cantor, 2014). Clearly, these anti-tumour immune effector responses are not always efficient, and tumours succeed in avoiding immune mediated clearance. Numerous mechanisms have been implicated in this immune escape including secretion of factors by the tumour which act to suppress lymphocytes, APCs and other immune cells, downregulation of surface expression of MHC-I or insensitivity to anti-tumour effector cytokines such as IFN-γ (Garcia-Lora et al., 2003, Stewart and Abrams, 2008). However, the role of stromal cells in mediating the process of colon tumour immune evasion remains unclear and studies investigating the tumour promoting effects of stromal cells to date have been largely observational or focused on mechanisms such as MSC-mediated enhancement of tumour angiogenesis (Huang et al., 2013, Liu et al., 2011). Little progress has been made on elucidating the role of the immunomodulatory potential of these stromal cells in the tumour microenvironment. Given the location of these stromal cells which are positioned between the epithelial cells and underlying vasculature, and the fact that they have a known immunomodulatory capacity, we chose to investigate the ability of stromal cells to affect the proliferation and effector function of T cells. Additionally, since there is a strong link between inflammatory diseases and the development of colorectal cancer, and with the knowledge that NF-κB is an important transcription factor implicated in both, we investigated the role of the inflammatory tumour microenvironment in modulating this process.

6.1 Exposure to the inflammatory tumour secretome results in an enhanced immunomodulatory phenotype in stromal cells

The aim of this thesis at the outset was to investigate the immunomodulatory changes that take place in stromal cells when they encounter the various factors secreted by tumour cells in the inflammatory tumour microenvironment. By measuring T cell proliferation, we found that stromal cells conditioned by the inflammatory tumour secretome had an enhanced ability to suppress both $CD4^+$ and $CD8^+$ T cell proliferation compared to control MSCs, or those treated with TNF-α or the control, non TNF-α activated tumour secretome. In addition to suppressing $CD4^+$ and $CD8^+$ T cell proliferation, inflammatory tumour conditioned stromal cells also significantly inhibited the release of effector molecules by T cells. This finding has a number of important ramifications in terms of the anti-tumour immune response. Firstly, T cell infiltrate into tumours has been shown to correlate with a better prognosis for patients, so a reduction in T cell proliferation as a result of encountering the suppressive stromal cell compartment could have detrimental outcomes on patient survival (Nosho et al., 2010). Secondly, in order to carry out their anti-tumour effector functions, it is necessary that T cells secrete effector molecules, some of which will induce direct cell killing (e.g. proteases such as granzyme B), others which serve to recruit and activate other important anti-tumour immune cell components (e.g. IFN-γ, TNF-α) (Hadrup et al., 2013, Andersen et al., 2006, Hung et al., 1998, Prevost-Blondel et al., 2000). Inhibition of the release of these effector molecules could have dramatic consequences, not only on the ability of T cells to kill transformed or malignant cells, but also on the capacity of these cells to recruit and activate other important anti-tumour immune effector cells such as macrophages (Mosser and Edwards, 2008). Finally, our data using secretome-mediated conditioning of stromal cells highlights a lack of need for contact between the tumour and stromal cells in order for the development of this potently immunosuppressive stromal cell phenotype. This finding is unsurprising in light of published

studies showing an alteration in stromal cell behaviour following treatment with tumour conditioned medium, and indeed data from our own lab whereby an increased immunosuppressive capacity can be induced in stromal cells simply by treating with pro- and anti-inflammatory cytokines (Gao et al., 2009, Sun et al., 2014) (Lynch *et al*., Murphy *et al.,* unpublished). Although not unexpected, this finding could mean that stromal cells at the fringes of a solid tumour and not necessarily in direct contact with malignant tumour cells could be induced to be equally as immunosuppressive as those closer to the core of the tumour. In particular in a large tumour this could mean the existence of quite a substantial immunosuppressive milieu (Kornprat et al., 2011). The presence of an immunomodulatory stromal compartment such as we have described here represents a significant barrier to entry for immune cells and so will undoubtedly have negative implications for anti-cancer therapies, and particularly the various types of immunotherapies which rely on entry to a tumour of activated anti-tumour effector immune cells.

Indeed, in validation of this claim, we did observe an altered immune cell infiltrate in the tumours harvested in our *in vivo* studies when tumour cells were co-administered with inflammatory tumour conditioned stromal cells. Specifically, in a result that correlated with our earlier *in vitro* findings, we observed diminished $CD8^+$ T cell granzyme B expression with the co-administration of inflammatory tumour conditioned stromal cells. As described, granzyme B is an important effector molecule of the cytotoxic T cell anti-tumour response (Andersen et al., 2006). Perhaps most importantly, the co-administration of inflammatory tumour conditioned stromal cells with CT26 tumour cells resulted in a significant increase in tumour invasiveness and growth. Given that metastasis in colorectal is often fatal, this finding may be of critical importance when deciding on the optimal treatment, and which cells to target most specifically, in a patient with metastatic colon cancer. Interestingly, we did not see a diminished infiltrate of $CD4^+$ or $CD8^+$ T cells in tumour *in vivo*, despite observing a significant

inhibition of the proliferation of these cells *in vitro*. This likely represents the complex, multicellular environment that is the tumour microenvironment. Our *in vitro* experiments, while indispensable for investigating mechanism and studying precise interactions between specific cell populations, are more limited in their scope in terms of the numerous populations present *in vivo* in an environment as complex as the tumour microenvironment. For example, bone marrow derived antigen presenting cells (APCs) have been shown to be important in the priming of de novo T cell responses and amplification of the effector response by processing and presenting tumour antigen to T cells, and tumour associated macrophages can augment T cell behaviour in the tumour microenvironment (Hung et al., 1998, Noy and Pollard, 2014). Each of these cellular populations is largely absent from our *in vitro* cultures, apart from a small number of APCs in the lymph node and spleen, and so their stromal cell modulating effects have not been assessed in our system. However, these populations no doubt have an active role to play in the findings we obtained *in vivo*, the study of which will form the basis of future studies.

6.2 Stromal cell PD-L1 may represent a viable target for immunotherapeutic targeting and may be useful in better stratifying patients for immunotherapeutic regiments

We show here that inflammatory tumour conditioning induces robust PD-L1 expression on stromal cells. Furthermore, PD-1 blockade was sufficient to reverse the enhanced $CD8^+$ T cell suppression and inhibition of effector molecule release observed *in vitro*, indicating a dependency on induced PD-L1 expression for these T cell inhibitory effects in response to inflammatory tumour conditioning. Furthermore, by utilising an immunocompetent, syngeneic murine model, we could overcome the limitations in assessing immune cell infiltrate associated with the use of xenografts in immunocompromised mice, and successfully identified an important role for stromal cell PD-L1 in the observed increases in tumour invasiveness and growth given that we saw a significant reduction in both upon administration

of PD-1 monotherapy. In our immunogenic model, these results were associated with increased CD8+ T cell granzyme B expression, suggesting a role for these anti-tumour immune effector cells in modulating the observed response to PD-1 antibody therapy.

The use of checkpoint inhibitors as a therapeutic strategy for the treatment of various cancers has gained momentum, with PD-1 providing an attractive target. Antibodies specifically targeting PD-1 signalling are currently under clinical investigation for colorectal cancer, melanoma, renal cell carcinoma, non-small-cell lung cancer, head and neck squamous cell cancer and haematological malignancies (Philips and Atkins, 2015). With regards specifically to colorectal cancer, evidence now shows that there is a particular patient cohort that will respond favourably to PD-1 therapy, and may in fact be harmed by standard chemotherapy (Dunne et al., 2016). Moreover it was observed in this work that this PD-L1 expression is not restricted to the epithelial tumour cell, and in fact was located in the stromal compartment. It is these stromal PD-L1-expressing patients that were found to respond best to PD-1 antibody therapy (Dunne et al., 2016). In fact, PD-1 immunotherapy has very recently been granted FDA approval for use in metastatic colon cancer that has been unresponsive to standard chemotherapy (FDA, 2017)

Our data demonstrate a central role for stromal PD-L1 expression in inhibiting anti-tumour immune responses and enabling tumours to grow and metastasise. In light of studies such as the one just mentioned showing positive responses to PD-1 immunotherapy in patients whose tumours have been deemed PD-L1 negative or for whom PD-L1 is expressed on cells other than the cancerous epithelium, we provide a clear rationale for the assessment of stromal cell PD-L1 expression in order to better stratify patients for immunotherapy.

Furthermore, we observed high CD80 expression on stromal cells, even in following inflammatory tumour conditioning. CD80 is a ligand for the T cell

co-receptor CD28, the blockade of which by CTLA-4 immunotherapy has yielded positive results in malignancies such as melanoma (Hodi et al., 2010). As mentioned in the introduction, ipilimumab, a CTLA-4 antibody, has recently been shown to have beneficial effects for another cohort of patients when combined with PD-1 inhibition (Alexander, 2016). Given the robust CD80 expression we observed on these stromal cells, it is plausible that CTLA-4 blockade may have additional benefits in our model.

Furthermore, it is well known that PD-L1 or PD-1 expression is not limited to stromal and T cells respectively (Gordon et al., 2017). In a setting as complex and heterogeneous as the colon tumour microenvironment, targeting one cell is isolation may be insufficient in generating a tangible clinical benefit. However, the use of stromal cell PD-L1 expression to, as we have suggested, better stratify patients and identify optimum therapeutic strategies, whether monotherapy or various combinations of immune/chemotherapy, will lead to faster and more durable patient responses and avoid the harm observed by Dunne *et al*., caused by the administration of inappropriate therapies to these very sick patients.

The precise molecular mechanisms underpinning this enhanced PD-L1 expression on stromal cells remains to be elucidated and represents an important avenue of pursuit for the future in our laboratory. The preliminary data presented in Chapter 5 of this thesis begins to address this large unknown gap in our knowledge, however, a more broad and systematic approach to tackling this question will likely yield more robust and clinically useful data.

6.3 MSCs as APCs?

One interesting finding that came to light over the course of these experiments, but the study of which was outside the scope and time constraints of this project, was the robust increase in MHC-I expression, along with PD-L1, upon stromal cell exposure to the inflammatory tumour conditioning. This provoked the comparison of MSCs to antigen presenting

cells, given that the role of MHC-I is indeed to present antigen to T cells (Janeway CA, 2001). It has in fact been shown that MSCs can behave as 'non-professional antigen presenting cells' as demonstrated by their ability to sense bacteria via TLRs/NLRs, their expression of MHC class II and their aforementioned ability to alter T cell function (Owens, 2015, Pinchuk et al., 2008) . Evidence in support of this includes, IFN-γ induction of MHC-I and MHC-II expression and the ability to present soluble exogenous antigen leading to the activation or anergy of $CD8^+$ and $CD4^+$ T cells. (Francois et al., 2009, Chan et al., 2006, Stagg et al., 2006). Furthermore, $CD90^+$ intestinal stromal cells have been shown to be capable of bacteria uptake and phagocytosis (Owens et al., 2013). This has provoked the question of whether MSCs are immune cells. A recent review by Hoogduijn addresses this question in detail (Hoogduijn, 2015), with the conclusion that MSCs, though immunomodulatory, are not in fact immune cells. In spite of this, the data presented does highlight some interesting idiosyncrasies related to MSC behaviour that does suggest they have "immune-like" functions, although to a different extent than professional immune cells. Hoogduijn points out that an immune cell is classified as a cell that protects the host against pathogens and removes debris or diseased cells. To date, data demonstrating these functions in MSC is limited or even absent. At present, in the tumour microenvironment, MSCs act as the "sensors and switchers of inflammation" described by Bernardo and Fibbe in the sense that they detect altered immune activity, respond to it, and as demonstrated above, interact with and influence the behaviour of components of the innate and adaptive immune system (Bernardo and Fibbe, 2013), This is an attempt, presumably, to protect the host, albeit from excessive inflammation rather than a pathogenic threat. However, little is known about the other immune cell-like functions of MSCs and even less so the influence of tumour conditioning on these processes. Can MSCs phagocytose transformed or apoptotic tumour cells? Can they induce tolerance by presenting tumour antigen as "self"? Is it possible even that MSCs could be polarised by tumour conditioning to a pro- or anti-tumorigenic phenotype, as is the case for

M1/M2 type macrophages. These questions require much investigation before we can fully understand the immunomodulatory capacity of MSCs in maintaining tissue homeostasis and more importantly, their role in dictating the anti-tumour immune response in the colon cancer microenvironment.

6.4 Conclusion

We show that stromal cells will be induced to express PD-L1 in response to factors released in the inflammatory tumour microenvironment. This conditioning results in a heightened ability for T cell suppression and tumour promotion by these stromal cells, an effect which is at least partly dependent on tumour cell NF-κB signalling and reversed by blockade of PD-1. Identification of the factors released from tumour cells under conditions of inflammation, and the signalling pathways activated in MSCs may open more doors for the development of novel adjuvants for immunotherapy to enhance the clinical therapeutic effects. In summary, we believe that targeting stromal cell PD-L1 may be a novel and important aspect to consider in efforts to break the cycle of immune evasion and immunosuppression established by the stromal compartment of the tumour microenvironment. This may represent an exciting target for combinatorial approaches to cancer therapy, leading to more favourable and durable outcomes for patients.

6.5 Further Perspectives

6.5.1 MSCs in the tumour microenvironment – a double-edged sword?

MSCs are being investigated for their potential to therapeutically modulate inflammatory diseases of the intestine, including Crohn's disease (Dalal et al., 2012). This clinical application, along with a small number of recent publications add a further level of complexity to the colorectal cancer-MSCs narrative. Studies by Chen *et al*. and Nasumo *et al*., looked at the impact of MSCs on the development of colon cancer following treatment with azoxymethane (AOM) alone, or in combination with dextran sulphate

sodium (DSS) (Nasuno et al., 2014, Chen et al., 2014). The DSS-AOM model is a well-accepted model of inflammation-associated colorectal cancer whereby DSS provides the inflammatory insult and is followed by the carcinogen AOM to induce colon tumour formation (Tanaka et al., 2003). In each of these studies, administration of MSCs at different stages of the DSS-AOM protocol resulted in an inhibition of tumourigenesis. Chen *et al*. attributed this reduction to decreased IL-6 and pSTAT3 signalling in colon cells, whereas Nasuno *et al*., found MSCs prevented initiating cells from sustaining DNA insults and induced G1 arrest in initiated cells via TGF-β signalling (Nasuno et al., 2014, Chen et al., 2014). Undoubtedly these two studies bring to light some interesting aspects of MSC-colorectal cancer interactions, and raise the question of how MSCs can be anti-tumourigenic in the setting of healthy tissue exposed to a carcinogenic insult, but be pro-tumourigenic once the early tumour initiation phase has passed. Could it be that the MSCs, being immunosuppressive, dampen any inflammatory response that so often leads to tumour development in the colon (Grivennikov, 2013, Terzic et al., 2010), but that excess anti-inflammatory signalling hinders the tumour-immune response? These models could represent the "elimination" phase of the tumour-immune response mentioned earlier. In the clinical setting colorectal tumours don't usually develop quickly or in response to an acute chemical insult as provided by the DSS-AOM models, and perhaps MSCs are not recruited to the site until later on in the process in contrast to these studies where MSCs were administered in high numbers very early on in the process of tumour development. Further studies refining the precise contributions of stromal cells at each stage of colon cancer development, from adenomatous polyp to overt clinical disease, are necessary in order to better understand the precise contributions of this population of cells at each stage of the process, and indeed to understand more fully the implications of therapeutically targeting these cells.

6.5.2 MSCs as therapeutic vectors for cancer therapy

Interestingly, MSCs are also being investigated for their potential use as delivery vehicles for anti-tumour therapy. Some early evidence for the anti-tumour potential for MSCs came from studies from Khakoo *et al*., demonstrating that human MSCs injected into a mouse model of Kaposi's sarcoma caused a dose-dependent inhibition of tumour growth (Khakoo et al., 2006). This reduction was attributed to the ability of MSCs to inactivate Akt signalling in tumour cells. A limited number of other studies noted similar effects with separate malignancies (Kidd et al., 2010, Qiao et al., 2008). A broader knowledge of the particular tumour microenvironment may be an essential consideration in interpreting these data, as the presence or absence of high levels of inflammatory signalling could be critical in dictating the capacity of the stromal compartment in these tumours to modulate infiltrating immune cells. More recent research has aimed to harness the tumour-homing property of MSCs to deliver cancer-specific drug or gene therapy to patients, with some positive outcomes being observed (Chen et al., 2008, Kosaka et al., 2012, Xin et al., 2007, Studeny et al., 2004, Mueller et al., 2011). This concept arose from MSCs remarkable ability to 'home' to tumours. In fact the first clinical trial using MSCs as an anti-cancer therapy is currently underway in Germany (eudract_number:2012-003741-15). Although no results have been published to date, this is yet another interesting facet of the MSC-tumour story where the immunological consequences of MSCs may have more important effects. The rationale behind this study is to engineer a patient's own MSCs to express therapeutic gene of interest and make use of the tumour-homing capacity of MSCs so as to allow for IV administration of the engineered MSCs (Niess et al., 2015). These MSCs will, however, also be transfected with a replication-incompetent and self-inactivating vector system so as to avoid any potential adverse effects of a bolus of MSCs being recruited to the TME, one of which effects would presumably include immunosuppression and potential tumour-immune evasion (Niess et al., 2015). This trial is based upon a number of pre-clinical studies showing potential efficacy in inhibiting tumour growth or metastasis of MSCs engineered to express anti-cancer

therapies such as CX3CL-1, TRAIL and IFN-β (Xin et al., 2009, Loebinger et al., 2009, Studeny et al., 2004, Studeny et al., 2002). However, as with all pre-clinical studies, these results were obtained over a short period of time in animals with exogenously administered tumours displaying a much accelerated growth rate compared to that in patients. For these reasons, and the fact that the follow up periods in these studies were short and the so long term immunological consequences were not fully investigated, these results must be interpreted with caution. The data we have presented in this thesis suggests that exogenous MSC administration to cancer patients may in fact be a very high risk approach to therapy, at least in certain instances such as that of our model whereby the microenvironment is highly inflammatory and capable of dramatically altering MSC biology and immunomodulatory potential. However, data that will emerge from studies such as this where the tumour-homing capacity of MSCs is investigated and better understood will undoubtedly have clinical benefit, be it via exploitation to deliver therapeutic agents, or for targeting in itself in order to halt the process of stromal cell recruitment by tumours.

6.5.3 PD-1 therapy may be a useful adjuvant for T cell therapy

In addition to this role in predicting response to immunotherapy, stromal cell PD-L1 expression in the colon tumour microenvironment may also have important implications for the use of adoptive T cell therapy which involves harvesting T cells from patients, expanding them and infusing these T cells back into the patient (Lynch and Murphy, 2016). This process relies on T cells leaving the blood and lymphatic system and infiltrating the tumour to exert their effect. A dense stromal compartment in which cells express high levels of PD-L1 may represent a significant hindrance to the effectiveness of this therapy. We propose that co-administration of an immune checkpoint inhibitor such as anti-PD-1 may have a synergistic effect in this setting.

In summary of the data cited in this section, and indeed that presented throughout the course of this thesis, we suggest that the role of MSCs in the

tumour microenvironment may be tumour and context specific, highlighting the urgent need for standardised models. A better understanding of the process of stromal cell recruitment, phenotype induction and effector function, some of which we have shed light on in this thesis and presented novel findings upon, will undoubtedly lead the development of more targeted and efficacious cancer immunotherapies and ultimately to better clinical outcomes for patients.

Appendices

Appendix One: Publications, Presentations and Achievements

Publications

- **O'Malley G**, Naicker SD, Lynch K, Lohan P, Shaw G, Rigalou A, Ritter T, Egan LJ, Ryan AE
  Stromal cell immunomodulatory potential in the tumour microenvironment is regulated by inflammatory signalling and stromal PD-L1 expression **Under consideration – Cancer Immunology Research**, October 2017

- **O'Malley G**, Heijltjes M, Houston AM, Rani S, Ritter T, Egan LJ, Ryan AE.
  Mesenchymal Stromal Cells and colorectal cancer – a troublesome twosome for the anti-tumour immune response? **Oncotarget,** June 2016 PMID 27542276

- Ryan AE, Colleran A, O'Gorman A, O'Flynn L, Pindjacova J, Lohan P, **O'Malley G**, Nosov M, Mureau C, Egan LJ.
  Targeting colon cancer cell NF-κB promotes an anti-tumour M1-like macrophage phenotype and inhibits peritoneal metastasis. **Oncogene**. 2014 PMID: 24704833

- Barron V, Neary M, Mohammed KM, Ansboro S, Shaw G, **O'Malley G**, Rooney N, Barry F, Murphy M.
  Evaluation of the Early In Vivo Respinse of a Functionally Graded Macroporous Scaffold in an Osteochondral Defect in a Rabbit Model. **Ann Biomed Eng,** 2015 PMID 26438451

- Barron V, Merghani K, Shaw G, Coleman CM, Hayes JS, Ansboro S, Manian A, **O'Malley G**, et al.,
  Evaluation of Cartilage repair by mesenchymal stem cells seeded on a PEOT scaffold in an Osteochondral Defect. **Ann Biomed Eng,** 2015 PMID 25589372

Oral presentations

- **O'Malley G**, Naicker SD, Lynch K, Lohan P, Shaw G, Rigalou A, Ritter T, Egan LJ, Ryan AE
  Stromal cell immunomodulatory potential in the tumour microenvironment is regulated by inflammatory signalling
  EAS Special Conference – "From cancer biology to the clinic" – hosted by the European Association for Cancer Research (EACR), American Association for Cancer Research (AACR) and Italian Cancer Society (SIC), Florence, Italy, June 2017

- **O'Malley G**, Naicker SD, Lynch K, Lohan P, Shaw G, Rigalou A, Ritter T, Egan LJ, Ryan AE

Stromal cell PD-L1 inhibits CD8+ T cell anti-tumour immune response and promotes colon cancer
College of Medicine, Nursing and Health Science research day, Galway, May 2017

- **O'Malley G**, Naicker SD, Lynch K, Lohan P, Shaw G, Rigalou A, Ritter T, Egan LJ, Ryan AE
  Stromal cell PD-L1 inhibits CD8+ T cell anti-tumour immune response and promotes colon cancer
  Irish Epithelial Physiology Group Annual Conference, Kilkenny, October 2016

- **O'Malley G**, Naicker SD, Lynch K, Lohan P, Shaw G, Rigalou A, Ritter T, Egan LJ, Ryan AE
  Stromal cell PD-L1 inhibits CD8+ T cell anti-tumour immune response and promotes colon cancer
  Irish Association for Cancer Research (IACR) National Conference, Kilkenny, February 2017

- **O'Malley G**, Naicker SD, Lynch K, Lohan P, Shaw G, Ritter T, Egan LJ, Ryan AE
  Tumour conditioning induces PD-L1 expression on stromal cells thereby inhibiting anti-tumour immunity
  College of Medicine, Nursing and Health Science research day, Galway, June 2016

- **O'Malley G**, Naicker SD, Lynch K, Lohan P, Shaw G, Ritter T, Egan LJ, Ryan AE
  Tumour conditioning induces PD-L1 expression on stromal cells thereby inhibiting anti-tumour immunity
  Irish Association for Cancer Research (IACR) National Conference, Cork, February 2016

- **O'Malley G**, Lohan P, Rani S, Lynch K, Shaw G, Ritter T, Egan LJ, Ryan AE
  The effect of NF-κB on tumour-stromal interactions in colorectal cancer
  European Congress of Immunology (ECI), Vienna, September 2015

- **O'Malley G**, Lohan P, Rani S, Lynch K, Naicker S, Shaw G, Ritter T, Egan LJ, Ryan AE
  The effect of NF-κB on tumour-stromal interactions in colorectal cancer
  College of Medicine, Nursing and Health Science research day, Galway, May 2015

Poster presentations

- **O'Malley G**, Naicker SD, Lynch K, Lohan P, Shaw G, Rigalou A, Ritter T, Egan LJ, Ryan AE
  Inflammatory signalling in the colon tumour microenvironment enhances stromal cell mediated suppression of anti-tumour immune responses
  American Association for Cancer Research (AACR) annual conference, Washington, April 2017

- **O'Malley G**, Naicker SD, Lynch K, Lohan P, Shaw G, Rigalou A, Ritter T, Egan LJ, Ryan AE

Tumour conditioning induces PD-L1 expression on stromal cells and inhibits CD8+ T cell mediated anti-tumour immunity
Irish Cytometry Society Annual Conference, TBSI, Dublin, November 2016

- **O'Malley G**, Naicker SD, Lynch K, Lohan P, Shaw G, Ritter T, Egan LJ, Ryan AE
  NF-κB activity in colon cancer cells induces an immunosuppressive and tumour promoting phenotype in stromal cells mediated by PD-L1
  Keystone Symposium: Cancer Pathophysiology, Colorado, USA, March 2016

- **O'Malley G**, Rani S, Lohan P, Lynch K, Shaw G, Ritter T, Egan LJ, Ryan AE
  The effect of NF-κB on tumour-stromal interactions in colorectal cancer
  Irish Association for Cancer Research (IACR) National Conference, Limerick, February 2015
  Awarded prize for best poster

- **O'Malley G**, Mc Stravick G, Lohan P, Lynch K, Shaw G, Ritter T, Egan LJ, Ryan AE
  The effect of tumour-cell NF-kB on MSC immunomodulatory phenotype
  NUI Galway College of Medicine Research Day, Galway 2014

Awards and Prizes

- **Winner Best Oral presentation**
  College of Medicine, Nursing and Health Science research day, May 2017
- **Winner of Local Heats and 3rd place in Regional Finals,**
  Participant at National Finals of "Thesis in 3", November 2016
- **Winner Best Poster Presentation**,
  Cytometry Society of Ireland National Conference, November 2016
- **Winner Best Oral presentation**
  College of Medicine, Nursing and Health Science research day, June 2016
- **Winner Best Oral presentation**
  College of Medicine, Nursing and Health Science research day, June 2015
- **Winner Best Poster Presentation**
  Irish Association for Cancer Research national conference, February 2015

Successfully Funded Grants

Date	Funding Agency	Funding Type	Role
May 2017	Health Research Board (HRB)	Summer Scholarship	Co-supervisor
May 2017	Wellcome Trust	Summer Scholarship	Co-supervisor
September 2015	European Federation of Immunological Societies (EFIS)	Travel Grant (European Congress of Immunology)	Primary Applicant
October 2013	Irish Research Council	PhD Scholarship	Primary Applicant
May 2012	Wellcome Trust	Summer Scholarship	Primary Applicant

Appendix Two: Reagents and plastics

Cell Culture Medium and Additives		
Item	**Supplier**	**Cat. No.**
alpha-MEM	Gibco-Biosciences	32561029
Ascorbic acid 2-Phosphate	Sigma	A8960
Bone Morphogenetic Protein		
Bovine Serum Albumin	Sigma	A2153
Dexamethasone	Sigma	D4902
DMEM (high glucose)	Gibco-Biosciences	31966-021
Dumethyl Sulfoxide (DMSO)	Sigma	2650
Equine Serum	Fisher	10407223
Fetal Bovine Serum (FBS)	Sigma	F7524
ITS+ Supplement	Sigma	I3143
L-Proline	Sigma	P5607
Penicillin/Streptomycin	Sigma	P4333
Sodium Pyruvate	Gibco	15240062
TGF-β3		
TNF-α	Peprotech	400-14
Trypsin 0.25% EDTA	Biosciences	25200056
Hematoxylin	Sigma	H3136
L-Glutamine	Biosciences	25030024
Mouse activatror CD3/CD28 Dynabeads	Life Technogies	11456D
Plastics		
Item	**Supplier**	**Cat. No.**
15ml tubes	Sarstedt	62.554.502
25ml blow out pipette	Sarstedt	86.1685.001
5ml blow out pipette	Sarstedt	86.1253.001
5ml Facscan	Sarstedt	55.1578
6 well plates, flat bottom	Sarstedt	83.392
T175 Culture Flask	Fisher	10246131
24 well plates, flat bottom	Sarstedt	83.3922
96 well plates, round bottom	Sarstedt	83.3926
50ml tubes	Sarstedt	62.547.254
ELISAs and Bioplex		
Item	**Supplier**	**Cat. No.**
human/mouse TGF-beta ELISA	eBioscience	88-8350-22
IFN-g ELISA	eBioscience	88-7314
Granzyme B ELISA	eBioscience	88-8022
PGE_2 ELISA	Abcam	ab133021
Bioplex Plate and Reagents	Fannin Heaalthcare	M60-009RDPD
Western Blotting		

Item	Supplier	Cat. No.
30% acrylamide mix	Sigma	A3699 5x100ml
Amersham Protran 0.2um NC	Biosciences	15249794
ammonium persulfate	Sigma	A3678 - 100g
Anti-mouse HRP	Cell Signalling	7076s
Anti-rabbit HRP	Cell Signalling	7074s
a-SMA antibody	MSC	PA5-19465
CL-Xposure Film	Sigma	34088
ECL Western Blotting Substrate	Sigma	32106
Glycerol	Sigma	G2025 500ml
Hydrochloric Acid		H1758-100ml
Lamin B1 antibody	MSC	PA5-19468
Methanol	Sigma	34860 - 2.5L
N,*N*',*N*'-Tetramethylethylenediamine	Sigma	T9281 - 50ml
Sodium dodecyl sulfate	Sigma	L3771-100G
X2 PageRuler prestained protein ladder	Fisher	11832124

Appendix Three: Media and Buffer Formulations

CT26 Tumour cell medium	
DMEM	
Fetal bovine serum (FBS)	10%
Penicillin-streptomycin	1%

HCT116/HT29 Tumour cell medium	
McCoy's 5A	
Fetal bovine serum (FBS)	10%
Penicillin-streptomycin	1%
L-Glutamine	1%

T cell meduym	
RPMI-1640	
Fetal bovine serum (FBS)	10%
Penicillin-streptomycin	1%
L-Glutamine	1%
Non-essential amino acids	1%
β-Mercaptoethanol	0.10%

Freezing medium	
FBS	
DMSO	10%

Murine MSC medium	
MEM-α	
Fetal bovine serum (FBS)	10%
Equine serum	10%
Penicillin-streptomycin	1%

Human MSC Medium	
MEM-α	
Fetal bovine serum (FBS)	10%
Penicillin-streptomycin	1%
FGF_2	1ng/ml

FACS Buffer	
PBS	
FBS	2%
Sodium Azide	0.05%

Western Blot Lysis Buffer 2X	
10% SDS solution	20ml
100mM Tris HCl pH6.8	5ml
0.1% Bromophenol Blue	0.05g
20% glycerol	10ml
Water	15ml

1X Buffer: Dilute 1:2 with 95% H_2O and 5% β-mercaptoethanol

Resolving Gel	
Reagent	**Volume (1 gel)**
H_2O	2ml
30% acrylamide mix	1.7ml
1.5 M Tris pH 8.8	1.3
10% SDS	50µl
10% APS	50µl
TEMED	2µl

Stacking Gel	
Reagent	**Volume (1 gel)**
H_2O	1.4ml
30% acrylamide mix	330µl
1 M Tris pH 6.8	250µl
10% SDS	20µl
10% APS	20µl
TEMED	2µl
TEMED	2µl

NATURE PUBLISHING GROUP LICENSE TERMS AND CONDITIONS

Nov 08, 2017

This Agreement between Grace O'Malley ("You") and Nature Publishing Group ("Nature Publishing Group") consists of your license details and the terms and conditions provided by Nature Publishing Group and Copyright Clearance Center.

License Number	4224261256537
License date	Nov 08, 2017
Licensed Content Publisher	Nature Publishing Group
Licensed Content Publication	Nature Reviews Drug Discovery
Licensed Content Title	TNF receptor 2 pathway: drug target for autoimmune diseases
Licensed Content Author	Denise Faustman, Miriam Davis
Licensed Content Date	May 21, 2010
Licensed Content Volume	9
Licensed Content Issue	6
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
High-res required	no
Figures	Figure 1
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	Cellular interactions in the tumour microenvironment during inflammation associated carcinogenesis
Expected completion date	Sep 2017

References

ACHKAR, T. & TARHINI, A. A. 2017. The use of immunotherapy in the treatment of melanoma. *J Hematol Oncol,* 10**,** 88.

AGGARWAL, B. B., GUPTA, S. C. & KIM, J. H. 2012. Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey. *Blood,* 119**,** 651-65.

AGGARWAL, S. & PITTENGER, M. F. 2005. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood,* 105**,** 1815-22.

AL OBEED, O. A., ALKHAYAL, K. A., AL SHEIKH, A., ZUBAIDI, A. M., VAALI-MOHAMMED, M. A., BOUSHEY, R., MCKERROW, J. H. & ABDULLA, M. H. 2014. Increased expression of tumor necrosis factor-alpha is associated with advanced colorectal cancer stages. *World J Gastroenterol,* 20**,** 18390-6.

ALEXANDER, W. 2016. The Checkpoint Immunotherapy Revolution: What Started as a Trickle Has Become a Flood, Despite Some Daunting Adverse Effects; New Drugs, Indications, and Combinations Continue to Emerge. *P T,* 41**,** 185-91.

ALGE, D. L., ZHOU, D., ADAMS, L. L., WYSS, B. K., SHADDAY, M. D., WOODS, E. J., GABRIEL CHU, T. M. & GOEBEL, W. S. 2010. Donor-matched comparison of dental pulp stem cells and bone marrow-derived mesenchymal stem cells in a rat model. *J Tissue Eng Regen Med,* 4**,** 73-81.

ANDERSEN, M. H., SCHRAMA, D., THOR STRATEN, P. & BECKER, J. C. 2006. Cytotoxic T cells. *J Invest Dermatol,* 126**,** 32-41.

ANTONIOLI, L., YEGUTKIN, G. G., PACHER, P., BLANDIZZI, C. & HASKO, G. 2016. Anti-CD73 in cancer immunotherapy: awakening new opportunities. *Trends Cancer,* 2**,** 95-109.

ASANO, K., NABEYAMA, A., MIYAKE, Y., QIU, C. H., KURITA, A., TOMURA, M., KANAGAWA, O., FUJII, S. & TANAKA, M. 2011. CD169-positive macrophages dominate antitumor immunity by crosspresenting dead cell-associated antigens. *Immunity,* 34**,** 85-95.

AZIMI, F., SCOLYER, R. A., RUMCHEVA, P., MONCRIEFF, M., MURALI, R., MCCARTHY, S. W., SAW, R. P. & THOMPSON, J. F. 2012. Tumor-infiltrating lymphocyte grade is an independent predictor of sentinel lymph node status and survival in patients with cutaneous melanoma. *J Clin Oncol,* 30**,** 2678-83.

BARTHOLOMEW, A., STURGEON, C., SIATSKAS, M., FERRER, K., MCINTOSH, K., PATIL, S., HARDY, W., DEVINE, S., UCKER, D., DEANS, R., MOSELEY, A. & HOFFMAN, R. 2002. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol,* 30**,** 42-8.

BAUD, V. & KARIN, M. 2009. Is NF-kappaB a good target for cancer therapy? Hopes and pitfalls. *Nat Rev Drug Discov,* 8**,** 33-40.

BEN-NERIAH, Y. & KARIN, M. 2011. Inflammation meets cancer, with NF-kappaB as the matchmaker. *Nat Immunol,* 12**,** 715-23.

BENSON, D. M., JR., BAKAN, C. E., MISHRA, A., HOFMEISTER, C. C., EFEBERA, Y., BECKNELL, B., BAIOCCHI, R. A., ZHANG, J., YU, J., SMITH, M. K., GREENFIELD, C. N., PORCU, P., DEVINE, S. M., ROTEM-YEHUDAR, R., LOZANSKI, G., BYRD, J. C. & CALIGIURI, M. A. 2010. The PD-1/PD-L1 axis modulates the natural killer cell versus multiple myeloma effect: a

therapeutic target for CT-011, a novel monoclonal anti-PD-1 antibody. *Blood,* 116**,** 2286-94.
BERNARDO, M. E. & FIBBE, W. E. 2013. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell,* 13**,** 392-402.
BI, X. W., WANG, H., ZHANG, W. W., WANG, J. H., LIU, W. J., XIA, Z. J., HUANG, H. Q., JIANG, W. Q., ZHANG, Y. J. & WANG, L. 2016. PD-L1 is upregulated by EBV-driven LMP1 through NF-kappaB pathway and correlates with poor prognosis in natural killer/T-cell lymphoma. *J Hematol Oncol,* 9**,** 109.
BILGIN, B., SENDUR, M. A., BULENT AKINCI, M., SENER DEDE, D. & YALCIN, B. 2017. Targeting the PD-1 pathway: a new hope for gastrointestinal cancers. *Curr Med Res Opin,* 33**,** 749-759.
BOLAND, C. R. & GOEL, A. 2010. Microsatellite instability in colorectal cancer. *Gastroenterology,* 138**,** 2073-2087 e3.
BRENNER, H., KLOOR, M. & POX, C. P. 2014. Colorectal cancer. *Lancet,* 383**,** 1490-502.
BRONTE, V. & BRIA, E. 2016. Interfering with CCL5/CCR5 at the Tumor-Stroma Interface. *Cancer Cell,* 29**,** 437-439.
BU, X., MAHONEY, KM, FREEMAN, GJ 2017. Learning from PD-1 Resistance: New Combination Strategies. *Trends in Molecular Medicine,* 22**,** 448-451.
BUCHBINDER, E. I. & DESAI, A. 2016. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *Am J Clin Oncol,* 39**,** 98-106.
BURKE, J. R., PATTOLI, M. A., GREGOR, K. R., BRASSIL, P. J., MACMASTER, J. F., MCINTYRE, K. W., YANG, X., IOTZOVA, V. S., CLARKE, W., STRNAD, J., QIU, Y. & ZUSI, F. C. 2003. BMS-345541 is a highly selective inhibitor of I kappa B kinase that binds at an allosteric site of the enzyme and blocks NF-kappa B-dependent transcription in mice. *J Biol Chem,* 278**,** 1450-6.
CALON, A., ESPINET, E., PALOMO-PONCE, S., TAURIELLO, D. V., IGLESIAS, M., CESPEDES, M. V., SEVILLANO, M., NADAL, C., JUNG, P., ZHANG, X. H., BYROM, D., RIERA, A., ROSSELL, D., MANGUES, R., MASSAGUE, J., SANCHO, E. & BATLLE, E. 2012. Dependency of colorectal cancer on a TGF-beta-driven program in stromal cells for metastasis initiation. *Cancer Cell,* 22**,** 571-84.
CALON, A., LONARDO, E., BERENGUER-LLERGO, A., ESPINET, E., HERNANDO-MOMBLONA, X., IGLESIAS, M., SEVILLANO, M., PALOMO-PONCE, S., TAURIELLO, D. V., BYROM, D., CORTINA, C., MORRAL, C., BARCELO, C., TOSI, S., RIERA, A., ATTOLINI, C. S., ROSSELL, D., SANCHO, E. & BATLLE, E. 2015. Stromal gene expression defines poor-prognosis subtypes in colorectal cancer. *Nat Genet,* 47**,** 320-9.
CALON, A., TAURIELLO, D. V. & BATLLE, E. 2014. TGF-beta in CAF-mediated tumor growth and metastasis. *Semin Cancer Biol,* 25**,** 15-22.
CAMBIEN, B., RICHARD-FIARDO, P., KARIMDJEE, B. F., MARTINI, V., FERRUA, B., PITARD, B., SCHMID-ANTOMARCHI, H. & SCHMID-ALLIANA, A. 2011. CCL5 neutralization restricts cancer growth and potentiates the targeting of PDGFRbeta in colorectal carcinoma. *PLoS One,* 6**,** e28842.
CARRETERO, R., SEKTIOGLU, I. M., GARBI, N., SALGADO, O. C., BECKHOVE, P. & HAMMERLING, G. J. 2015. Eosinophils orchestrate cancer rejection by normalizing tumor vessels and enhancing infiltration of CD8(+) T cells. *Nat Immunol,* 16**,** 609-17.
CASTLE, J. C., LOEWER, M., BOEGEL, S., DE GRAAF, J., BENDER, C., TADMOR, A. D., BOISGUERIN, V., BUKUR, T., SORN, P., PARET, C., DIKEN, M., KREITER, S.,

TURECI, O. & SAHIN, U. 2014. Immunomic, genomic and transcriptomic characterization of CT26 colorectal carcinoma. *BMC Genomics,* 15**,** 190.
CAVALCANTI, Y. V., BRELAZ, M. C., NEVES, J. K., FERRAZ, J. C. & PEREIRA, V. R. 2012. Role of TNF-Alpha, IFN-Gamma, and IL-10 in the Development of Pulmonary Tuberculosis. *Pulm Med,* 2012**,** 745483.
CHAMES, P. & BATY, D. 2009. Bispecific antibodies for cancer therapy: the light at the end of the tunnel? *MAbs,* 1**,** 539-47.
CHAN, A. T., GIOVANNUCCI, E. L., MEYERHARDT, J. A., SCHERNHAMMER, E. S., CURHAN, G. C. & FUCHS, C. S. 2005. Long-term use of aspirin and nonsteroidal anti-inflammatory drugs and risk of colorectal cancer. *JAMA,* 294**,** 914-23.
CHAN, J. L., TANG, K. C., PATEL, A. P., BONILLA, L. M., PIEROBON, N., PONZIO, N. M. & RAMESHWAR, P. 2006. Antigen-presenting property of mesenchymal stem cells occurs during a narrow window at low levels of interferon-gamma. *Blood,* 107**,** 4817-24.
CHANG, A. Y., BHATTACHARYA, N., MU, J., SETIADI, A. F., CARCAMO-CAVAZOS, V., LEE, G. H., SIMONS, D. L., YADEGARYNIA, S., HEMATI, K., KAPELNER, A., MING, Z., KRAG, D. N., SCHWARTZ, E. J., CHEN, D. Z. & LEE, P. P. 2013. Spatial organization of dendritic cells within tumor draining lymph nodes impacts clinical outcome in breast cancer patients. *J Transl Med,* 11**,** 242.
CHEN, L. & FLIES, D. B. 2013. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nat Rev Immunol,* 13**,** 227-42.
CHEN, X., LIN, X., ZHAO, J., SHI, W., ZHANG, H., WANG, Y., KAN, B., DU, L., WANG, B., WEI, Y., LIU, Y. & ZHAO, X. 2008. A tumor-selective biotherapy with prolonged impact on established metastases based on cytokine gene-engineered MSCs. *Mol Ther,* 16**,** 749-56.
CHEN, Y., ZHANG, L., LIU, W. & LIU, X. 2015. Prognostic Significance of the Tumor-Stroma Ratio in Epithelial Ovarian Cancer. *Biomed Res Int,* 2015**,** 589301.
CHEN, Y. M. 2017. Immune checkpoint inhibitors for nonsmall cell lung cancer treatment. *J Chin Med Assoc,* 80**,** 7-14.
CHEN, Z., HE, X., CHEN, X., LIN, X., ZOU, Y., WU, X. & LAN, P. 2014. Bone marrow mesenchymal stem cells ameliorate colitis-associated tumorigenesis in mice. *Biochem Biophys Res Commun*.
CHINNADURAI, R., COPLAND, I. B., PATEL, S. R. & GALIPEAU, J. 2014a. IDO-independent suppression of T cell effector function by IFN-gamma-licensed human mesenchymal stromal cells. *J Immunol,* 192**,** 1491-501.
CHINNADURAI, R., COPLAND, I. B., PATEL, S. R. & GALIPEAU, J. 2014b. IDO-Independent Suppression of T Cell Effector Function by IFN-γ-Licensed Human Mesenchymal Stromal Cells. *J Immunol*.
CHO, D. I., KIM, M. R., JEONG, H. Y., JEONG, H. C., JEONG, M. H., YOON, S. H., KIM, Y. S. & AHN, Y. 2014. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. *Exp Mol Med,* 46**,** e70.
CIRRI, P. & CHIARUGI, P. 2011. Cancer associated fibroblasts: the dark side of the coin. *Am J Cancer Res,* 1**,** 482-97.
CLINICALTRIALS.GOV. 2017. *An Investigational Immuno-therapy Study of Nivolumab, and Nivolumab in Combination With Other Anti-cancer Drugs, in Colon Cancer That Has Come Back or Has Spread (CheckMate142)* [Online]. Available: https://clinicaltrials.gov/ct2/show/NCT02060188 [Accessed 05/05/2017 2017].

COLLERAN, A., RYAN, A., O'GORMAN, A., MUREAU, C., LIPTROT, C., DOCKERY, P., FEARNHEAD, H. & EGAN, L. J. 2011. Autophagosomal IkappaB alpha degradation plays a role in the long term control of tumor necrosis factor-alpha-induced nuclear factor-kappaB (NF-kappaB) activity. *J Biol Chem,* 286**,** 22886-93.

COMPTON, C. C. & GREENE, F. L. 2004. The staging of colorectal cancer: 2004 and beyond. *CA Cancer J Clin,* 54**,** 295-308.

CONDEELIS, J. & POLLARD, J. W. 2006. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell,* 124**,** 263-6.

COOK, J. & HAGEMANN, T. 2013. Tumour-associated macrophages and cancer. *Curr Opin Pharmacol,* 13**,** 595-601.

COUSSENS, L. M. & WERB, Z. 2002. Inflammation and cancer. *Nature,* 420**,** 860-7.

DALAL, J., GANDY, K. & DOMEN, J. 2012. Role of mesenchymal stem cell therapy in Crohn's disease. *Pediatr Res,* 71**,** 445-51.

DE BOECK, A., PAUWELS, P., HENSEN, K., RUMMENS, J. L., WESTBROEK, W., HENDRIX, A., MAYNARD, D., DENYS, H., LAMBEIN, K., BRAEMS, G., GESPACH, C., BRACKE, M. & DE WEVER, O. 2013. Bone marrow-derived mesenchymal stem cells promote colorectal cancer progression through paracrine neuregulin 1/HER3 signalling. *Gut,* 62**,** 550-60.

DE LA FUENTE, H., CRUZ-ADALIA, A., MARTINEZ DEL HOYO, G., CIBRIAN-VERA, D., BONAY, P., PEREZ-HERNANDEZ, D., VAZQUEZ, J., NAVARRO, P., GUTIERREZ-GALLEGO, R., RAMIREZ-HUESCA, M., MARTIN, P. & SANCHEZ-MADRID, F. 2014. The leukocyte activation receptor CD69 controls T cell differentiation through its interaction with galectin-1. *Mol Cell Biol,* 34**,** 2479-87.

DE PALMA, M. & LEWIS, C. E. 2013. Macrophage regulation of tumor responses to anticancer therapies. *Cancer Cell,* 23**,** 277-86.

DE ROBERTIS, M., MASSI, E., POETA, M. L., CAROTTI, S., MORINI, S., CECCHETELLI, L., SIGNORI, E. & FAZIO, V. M. 2011. The AOM/DSS murine model for the study of colon carcinogenesis: From pathways to diagnosis and therapy studies. *J Carcinog,* 10**,** 9.

DE WEVER, O., DEMETTER, P., MAREEL, M. & BRACKE, M. 2008. Stromal myofibroblasts are drivers of invasive cancer growth. *Int J Cancer,* 123**,** 2229-38.

DESCHOOLMEESTER, V., BAAY, M., LARDON, F., PAUWELS, P. & PEETERS, M. 2011. Immune Cells in Colorectal Cancer: Prognostic Relevance and Role of MSI. *Cancer Microenviron,* 4**,** 377-92.

DI NICOLA, M., CARLO-STELLA, C., MAGNI, M., MILANESI, M., LONGONI, P. D., MATTEUCCI, P., GRISANTI, S. & GIANNI, A. M. 2002. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood,* 99**,** 3838-43.

DING, H., WU, X. & GAO, W. 2005. PD-L1 is expressed by human renal tubular epithelial cells and suppresses T cell cytokine synthesis. *Clin Immunol,* 115**,** 184-91.

DIREKZE, N. C. & ALISON, M. R. 2006. Bone marrow and tumour stroma: an intimate relationship. *Hematol Oncol,* 24**,** 189-95.

DIREKZE, N. C., HODIVALA-DILKE, K., JEFFERY, R., HUNT, T., POULSOM, R., OUKRIF, D., ALISON, M. R. & WRIGHT, N. A. 2004. Bone marrow contribution to tumor-associated myofibroblasts and fibroblasts. *Cancer Res,* 64**,** 8492-5.

DJOUAD, F., PLENCE, P., BONY, C., TROPEL, P., APPARAILLY, F., SANY, J., NOEL, D. & JORGENSEN, C. 2003. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood,* 102**,** 3837-44.

DOMINICI, M., LE BLANC, K., MUELLER, I., SLAPER-CORTENBACH, I., MARINI, F., KRAUSE, D., DEANS, R., KEATING, A., PROCKOP, D. & HORWITZ, E. 2006. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy,* 8**,** 315-7.

DONG, Y., SUN, Q. & ZHANG, X. 2017. PD-1 and its ligands are important immune checkpoints in cancer. *Oncotarget,* 8**,** 2171-2186.

DUARTE, R. F. & FRANK, D. A. 2000. SCF and G-CSF lead to the synergistic induction of proliferation and gene expression through complementary signaling pathways. *Blood,* 96**,** 3422-30.

DULOS, J., CARVEN, G. J., VAN BOXTEL, S. J., EVERS, S., DRIESSEN-ENGELS, L. J., HOBO, W., GORECKA, M. A., DE HAAN, A. F., MULDERS, P., PUNT, C. J., JACOBS, J. F., SCHALKEN, J. A., OOSTERWIJK, E., VAN EENENNAAM, H. & BOOTS, A. M. 2012. PD-1 blockade augments Th1 and Th17 and suppresses Th2 responses in peripheral blood from patients with prostate and advanced melanoma cancer. *J Immunother,* 35**,** 169-78.

DUNICAN, D. S., MCWILLIAM, P., TIGHE, O., PARLE-MCDERMOTT, A. & CROKE, D. T. 2002. Gene expression differences between the microsatellite instability (MIN) and chromosomal instability (CIN) phenotypes in colorectal cancer revealed by high-density cDNA array hybridization. *Oncogene,* 21**,** 3253-7.

DUNN, G. P., OLD, L. J. & SCHREIBER, R. D. 2004a. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity,* 21**,** 137-48.

DUNN, G. P., OLD, L. J. & SCHREIBER, R. D. 2004b. The three Es of cancer immunoediting. *Annu Rev Immunol,* 22**,** 329-60.

DUNNE, P. D., MCART, D. G., O'REILLY, P. G., COLEMAN, H. G., ALLEN, W. L., LOUGHREY, M., VAN SCHAEYBROECK, S., MCDADE, S., SALTO-TELLEZ, M., LONGLEY, D. B., LAWLER, M. & JOHNSTON, P. G. 2016. Immune-Derived PD-L1 Gene Expression Defines a Subgroup of Stage II/III Colorectal Cancer Patients with Favorable Prognosis Who May Be Harmed by Adjuvant Chemotherapy. *Cancer Immunol Res,* 4**,** 582-91.

DUPAGE, M., MAZUMDAR, C., SCHMIDT, L. M., CHEUNG, A. F. & JACKS, T. 2012. Expression of tumour-specific antigens underlies cancer immunoediting. *Nature,* 482**,** 405-9.

DVORAK, H. F. 1986. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med,* 315**,** 1650-9.

DWYER, R. M., POTTER-BEIRNE, S. M., HARRINGTON, K. A., LOWERY, A. J., HENNESSY, E., MURPHY, J. M., BARRY, F. P., O'BRIEN, T. & KERIN, M. J. 2007. Monocyte chemotactic protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells. *Clin Cancer Res,* 13**,** 5020-7.

EBERT, P. J., CHEUNG, J., YANG, Y., MCNAMARA, E., HONG, R., MOSKALENKO, M., GOULD, S. E., MAECKER, H., IRVING, B. A., KIM, J. M., BELVIN, M. & MELLMAN, I. 2016. MAP Kinase Inhibition Promotes T Cell and Anti-tumor Activity in Combination with PD-L1 Checkpoint Blockade. *Immunity,* 44**,** 609-21.

EGAN, L. J. & TORUNER, M. 2006. NF-kappaB signaling: pros and cons of altering NF-kappaB as a therapeutic approach. *Ann N Y Acad Sci,* 1072**,** 114-22.

ENGLISH, K., BARRY, F. P., FIELD-CORBETT, C. P. & MAHON, B. P. 2007. IFN-gamma and TNF-alpha differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett,* 110**,** 91-100.

EREZ, N., TRUITT, M., OLSON, P., ARRON, S. T. & HANAHAN, D. 2010. Cancer-Associated Fibroblasts Are Activated in Incipient Neoplasia to Orchestrate Tumor-Promoting Inflammation in an NF-kappaB-Dependent Manner. *Cancer Cell,* 17**,** 135-47.

ERICES, A., CONGET, P. & MINGUELL, J. J. 2000. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol,* 109**,** 235-42.

FAROLFI, A., RIDOLFI, L., GUIDOBONI, M., NICOLETTI, S. V., PICIUCCHI, S., VALMORRI, L., COSTANTINI, M., SCARPI, E., AMADORI, D. & RIDOLFI, R. 2012. Ipilimumab in advanced melanoma: reports of long-lasting responses. *Melanoma Res,* 22**,** 263-70.

FAUSTMAN, D. & DAVIS, M. 2010. TNF receptor 2 pathway: drug target for autoimmune diseases. *Nat Rev Drug Discov,* 9**,** 482-93.

FDA. *Hematology/Oncology (Cancer) Approvals & Safety Notifications* [Online]. Available: https://www.fda.gov/drugs/informationondrugs/approveddrugs/ucm279174.htm [Accessed 05/05/2017 2017].

FDA. 2017. *FDA grants nivolumab accelerated approval for MSI-H or dMMR colorectal cancer* [Online]. Available: https://www.fda.gov/Drugs/InformationOnDrugs/ApprovedDrugs/ucm569366.htm [Accessed 09/08/2017 2017].

FEIG, C., JONES, J. O., KRAMAN, M., WELLS, R. J., DEONARINE, A., CHAN, D. S., CONNELL, C. M., ROBERTS, E. W., ZHAO, Q., CABALLERO, O. L., TEICHMANN, S. A., JANOWITZ, T., JODRELL, D. I., TUVESON, D. A. & FEARON, D. T. 2013. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. *Proc Natl Acad Sci U S A,* 110**,** 20212-7.

FERLAY J, S. I., ERVIK M, DIKSHIT R, ESER S, MATHERS C, REBELO M, PARKIN DM, FORMAN D, BRAY, F. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet].

Lyon, France: International Agency for Research on Cancer; 2013. Available from: http://globocan.iarc.fr, accessed on 12/08/2015.

FINOULST, I., VINK, P., ROVERS, E., PIETERSE, M., PINKSE, M., BOS, E. & VERHAERT, P. 2011. Identification of low abundant secreted proteins and peptides from primary culture supernatants of human T-cells. *J Proteomics,* 75**,** 23-33.

FLOSSMANN, E., ROTHWELL, P. M., BRITISH DOCTORS ASPIRIN, T. & THE, U. K. T. I. A. A. T. 2007. Effect of aspirin on long-term risk of colorectal cancer: consistent evidence from randomised and observational studies. *Lancet,* 369**,** 1603-13.

FORAN, E., GARRITY-PARK, M. M., MUREAU, C., NEWELL, J., SMYRK, T. C., LIMBURG, P. J. & EGAN, L. J. 2010. Upregulation of DNA methyltransferase-mediated gene silencing, anchorage-independent growth, and migration of colon cancer cells by interleukin-6. *Mol Cancer Res,* 8**,** 471-81.

FRANCOIS, M., ROMIEU-MOUREZ, R., STOCK-MARTINEAU, S., BOIVIN, M. N., BRAMSON, J. L. & GALIPEAU, J. 2009. Mesenchymal stromal cells cross-present soluble exogenous antigens as part of their antigen-presenting cell properties. *Blood,* 114**,** 2632-8.

FRANK, D. A., MAHAJAN, S. & RITZ, J. 1999. Fludarabine-induced immunosuppression is associated with inhibition of STAT1 signaling. *Nat Med,* 5**,** 444-7.

FRIDLENDER, Z. G. & ALBELDA, S. M. 2012. Tumor-associated neutrophils: friend or foe? *Carcinogenesis,* 33**,** 949-55.

FRIIS, S., POULSEN, A. H., SORENSEN, H. T., TJONNELAND, A., OVERVAD, K., VOGEL, U., MCLAUGHLIN, J. K., BLOT, W. J. & OLSEN, J. H. 2009. Aspirin and other non-steroidal anti-inflammatory drugs and risk of colorectal cancer: a Danish cohort study. *Cancer Causes Control,* 20**,** 731-40.

GABRILOVICH, D. I. & NAGARAJ, S. 2009. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol,* 9**,** 162-74.

GALDIERO, M. R., BONAVITA, E., BARAJON, I., GARLANDA, C., MANTOVANI, A. & JAILLON, S. 2013. Tumor associated macrophages and neutrophils in cancer. *Immunobiology,* 218**,** 1402-10.

GALON, J., COSTES, A., SANCHEZ-CABO, F., KIRILOVSKY, A., MLECNIK, B., LAGORCE-PAGES, C., TOSOLINI, M., CAMUS, M., BERGER, A., WIND, P., ZINZINDOHOUE, F., BRUNEVAL, P., CUGNENC, P. H., TRAJANOSKI, Z., FRIDMAN, W. H. & PAGES, F. 2006. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science,* 313**,** 1960-4.

GALON, J., PAGES, F., MARINCOLA, F. M., ANGELL, H. K., THURIN, M., LUGLI, A., ZLOBEC, I., BERGER, A., BIFULCO, C., BOTTI, G., TATANGELO, F., BRITTEN, C. M., KREITER, S., CHOUCHANE, L., DELRIO, P., ARNDT, H., ASSLABER, M., MAIO, M., MASUCCI, G. V., MIHM, M., VIDAL-VANACLOCHA, F., ALLISON, J. P., GNJATIC, S., HAKANSSON, L., HUBER, C., SINGH-JASUJA, H., OTTENSMEIER, C., ZWIERZINA, H., LAGHI, L., GRIZZI, F., OHASHI, P. S., SHAW, P. A., CLARKE, B. A., WOUTERS, B. G., KAWAKAMI, Y., HAZAMA, S., OKUNO, K., WANG, E., O'DONNELL-TORMEY, J., LAGORCE, C., PAWELEC, G., NISHIMURA, M. I., HAWKINS, R., LAPOINTE, R., LUNDQVIST, A., KHLEIF, S. N., OGINO, S., GIBBS, P., WARING, P., SATO, N., TORIGOE, T., ITOH, K., PATEL, P. S., SHUKLA, S. N., PALMQVIST, R., NAGTEGAAL, I. D., WANG, Y., D'ARRIGO, C., KOPETZ, S., SINICROPE, F. A., TRINCHIERI, G., GAJEWSKI, T. F., ASCIERTO, P. A. & FOX, B. A. 2012. Cancer classification using the Immunoscore: a worldwide task force. *J Transl Med,* 10**,** 205.

GAO, H., PRIEBE, W., GLOD, J. & BANERJEE, D. 2009. Activation of signal transducers and activators of transcription 3 and focal adhesion kinase by stromal cell-derived factor 1 is required for migration of human mesenchymal stem cells in response to tumor cell-conditioned medium. *Stem Cells,* 27**,** 857-65.

GARCIA-LORA, A., ALGARRA, I. & GARRIDO, F. 2003. MHC class I antigens, immune surveillance, and tumor immune escape. *J Cell Physiol,* 195**,** 346-55.

GARRIDO, F., RUIZ-CABELLO, F., CABRERA, T., PÉREZ-VILLAR, J. J., LÓPEZ-BOTET, M., DUGGAN-KEEN, M. & STERN, P. L. 1997. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today,* 18**,** 89-95.

GIALLONGO, C., TIBULLO, D., PARRINELLO, N. L., LA CAVA, P., DI ROSA, M., BRAMANTI, V., DI RAIMONDO, C., CONTICELLO, C., CHIARENZA, A., PALUMBO, G. A., AVOLA, R., ROMANO, A. & DI RAIMONDO, F. 2016. Granulocyte-like myeloid derived suppressor cells (G-MDSC) are increased in multiple myeloma and are driven by dysfunctional mesenchymal stem cells (MSC). *Oncotarget*.

GILBERT, S., ZHANG, R., DENSON, L., MORIGGL, R., STEINBRECHER, K., SHROYER, N., LIN, J. & HAN, X. 2012. Enterocyte STAT5 promotes mucosal wound healing via suppression of myosin light chain kinase-mediated loss of barrier function and inflammation. *EMBO Mol Med,* 4**,** 109-24.

GIORDANO, M., HENIN, C., MAURIZIO, J., IMBRATTA, C., BOURDELY, P., BUFERNE, M., BAITSCH, L., VANHILLE, L., SIEWEKE, M. H., SPEISER, D. E., AUPHAN-ANEZIN, N., SCHMITT-VERHULST, A. M. & VERDEIL, G. 2015. Molecular profiling of CD8 T cells in autochthonous melanoma identifies Maf as driver of exhaustion. *EMBO J,* 34**,** 2042-58.

GODSE, P., KUMAR, P., YEWALKAR, N., DEORE, V., LOHAR, M., MUNDADA, R., PADGAONKAR, A., MANOHAR, S., JOSHI, A., BHATIA, D., DESAI, N., DAMRE, A., BHONDE, M., JOSHI, K., SHARMA, R. & KUMAR, S. 2013. Discovery of P3971 an orally efficacious novel anticancer agent targeting HIF-1alpha and STAT3 pathways. *Anticancer Agents Med Chem,* 13**,** 1460-6.

GOODEN, M. J., DE BOCK, G. H., LEFFERS, N., DAEMEN, T. & NIJMAN, H. W. 2011. The prognostic influence of tumour-infiltrating lymphocytes in cancer: a systematic review with meta-analysis. *Br J Cancer,* 105**,** 93-103.

GORDON, S. R., MAUTE, R. L., DULKEN, B. W., HUTTER, G., GEORGE, B. M., MCCRACKEN, M. N., GUPTA, R., TSAI, J. M., SINHA, R., COREY, D., RING, A. M., CONNOLLY, A. J. & WEISSMAN, I. L. 2017. PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature,* 545**,** 495-499.

GOUT, S. & HUOT, J. 2008. Role of cancer microenvironment in metastasis: focus on colon cancer. *Cancer Microenviron,* 1**,** 69-83.

GOWRISHANKAR, K., GUNATILAKE, D., GALLAGHER, S. J., TIFFEN, J., RIZOS, H. & HERSEY, P. 2015. Inducible but not constitutive expression of PD-L1 in human melanoma cells is dependent on activation of NF-kappaB. *PLoS One,* 10**,** e0123410.

GRENGA, I., DONAHUE, R. N., LEPONE, L. M., RICHARDS, J. & SCHLOM, J. 2016. A fully human IgG1 anti-PD-L1 MAb in an in vitro assay enhances antigen-specific T-cell responses. *Clin Transl Immunology,* 5**,** e83.

GRETEN, F. R., ARKAN, M. C., BOLLRATH, J., HSU, L. C., GOODE, J., MIETHING, C., GOKTUNA, S. I., NEUENHAHN, M., FIERER, J., PAXIAN, S., VAN ROOIJEN, N., XU, Y., O'CAIN, T., JAFFEE, B. B., BUSCH, D. H., DUYSTER, J., SCHMID, R. M., ECKMANN, L. & KARIN, M. 2007. NF-kappaB is a negative regulator of IL-1beta secretion as revealed by genetic and pharmacological inhibition of IKKbeta. *Cell,* 130**,** 918-31.

GRETEN, F. R., ECKMANN, L., GRETEN, T. F., PARK, J. M., LI, Z. W., EGAN, L. J., KAGNOFF, M. F. & KARIN, M. 2004. IKKbeta links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. *Cell,* 118**,** 285-96.

GRIVENNIKOV, S. I. 2013. Inflammation and colorectal cancer: colitis-associated neoplasia. *Semin Immunopathol,* 35**,** 229-44.

GUERMONPREZ, P., VALLADEAU, J., ZITVOGEL, L., THERY, C. & AMIGORENA, S. 2002. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol,* 20**,** 621-67.

GUJRAL, T. S., CHAN, M., PESHKIN, L., SORGER, P. K., KIRSCHNER, M. W. & MACBEATH, G. 2014. A noncanonical Frizzled2 pathway regulates epithelial-mesenchymal transition and metastasis. *Cell,* 159**,** 844-56.

GUO, L., ZHANG, H. & CHEN, B. 2017. Nivolumab as Programmed Death-1 (PD-1) Inhibitor for Targeted Immunotherapy in Tumor. *J Cancer,* 8**,** 410-416.

HADRUP, S., DONIA, M. & THOR STRATEN, P. 2013. Effector CD4 and CD8 T cells and their role in the tumor microenvironment. *Cancer Microenviron,* 6**,** 123-33.

HAMID, O., ROBERT, C., DAUD, A., HODI, F. S., HWU, W. J., KEFFORD, R., WOLCHOK, J. D., HERSEY, P., JOSEPH, R. W., WEBER, J. S., DRONCA, R., GANGADHAR, T. C., PATNAIK, A., ZAROUR, H., JOSHUA, A. M., GERGICH, K., ELASSAISS-SCHAAP, J., ALGAZI, A., MATEUS, C., BOASBERG, P., TUMEH, P. C., CHMIELOWSKI, B., EBBINGHAUS, S. W., LI, X. N., KANG, S. P. & RIBAS, A. 2013. Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. *N Engl J Med,* 369**,** 134-44.

HAN, Z., TIAN, Z., LV, G., ZHANG, L., JIANG, G., SUN, K., WANG, C., BU, X., LI, R., SHI, Y., WU, M. & WEI, L. 2011. Immunosuppressive effect of bone marrow-derived mesenchymal stem cells in inflammatory microenvironment favours the growth of B16 melanoma cells. *J Cell Mol Med,* 15**,** 2343-52.

HANAHAN, D. & COUSSENS, L. M. 2012. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer Cell,* 21**,** 309-22.

HARTLEY, G., REGAN, D., GUTH, A. & DOW, S. 2017. Regulation of PD-L1 expression on murine tumor-associated monocytes and macrophages by locally produced TNF-alpha. *Cancer Immunol Immunother,* 66**,** 523-535.

HARTNETT, L. & EGAN, L. J. 2012. Inflammation, DNA methylation and colitis-associated cancer. *Carcinogenesis,* 33**,** 723-31.

HE, J., HU, Y., HU, M. & LI, B. 2015. Development of PD-1/PD-L1 Pathway in Tumor Immune Microenvironment and Treatment for Non-Small Cell Lung Cancer. *Sci Rep,* 5**,** 13110.

HELBIG, G., CHRISTOPHERSON, K. W., 2ND, BHAT-NAKSHATRI, P., KUMAR, S., KISHIMOTO, H., MILLER, K. D., BROXMEYER, H. E. & NAKSHATRI, H. 2003. NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *J Biol Chem,* 278**,** 21631-8.

HIROSUE, S. & DUBROT, J. 2015. Modes of Antigen Presentation by Lymph Node Stromal Cells and Their Immunological Implications. *Front Immunol,* 6**,** 446.

HODGE, G., BARNAWI, J., JURISEVIC, C., MOFFAT, D., HOLMES, M., REYNOLDS, P. N., JERSMANN, H. & HODGE, S. 2014. Lung cancer is associated with decreased expression of perforin, granzyme B and interferon (IFN)-gamma by infiltrating lung tissue T cells, natural killer (NK) T-like and NK cells. *Clin Exp Immunol,* 178**,** 79-85.

HODI, F. S., O'DAY, S. J., MCDERMOTT, D. F., WEBER, R. W., SOSMAN, J. A., HAANEN, J. B., GONZALEZ, R., ROBERT, C., SCHADENDORF, D., HASSEL, J. C., AKERLEY, W., VAN DEN EERTWEGH, A. J., LUTZKY, J., LORIGAN, P., VAUBEL, J. M., LINETTE, G. P., HOGG, D., OTTENSMEIER, C. H., LEBBE, C., PESCHEL, C., QUIRT, I., CLARK, J. I., WOLCHOK, J. D., WEBER, J. S., TIAN, J., YELLIN, M. J., NICHOL, G. M., HOOS, A. & URBA, W. J. 2010. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med,* 363**,** 711-23.

HOFSTETTER, C. P., SCHWARZ, E. J., HESS, D., WIDENFALK, J., EL MANIRA, A., PROCKOP, D. J. & OLSON, L. 2002. Marrow stromal cells form guiding

strands in the injured spinal cord and promote recovery. *Proc Natl Acad Sci U S A,* 99**,** 2199-204.
HOGAN, N. M., DWYER, R. M., JOYCE, M. R. & KERIN, M. J. 2012. Mesenchymal stem cells in the colorectal tumor microenvironment: recent progress and implications. *Int J Cancer,* 131**,** 1-7.
HOGAN, N. M., JOYCE, M. R., MURPHY, J. M., BARRY, F. P., O'BRIEN, T., KERIN, M. J. & DWYER, R. M. 2013. Impact of mesenchymal stem cell secreted PAI-1 on colon cancer cell migration and proliferation. *Biochem Biophys Res Commun,* 435**,** 574-9.
HOOGDUIJN, M. J. 2015. Are mesenchymal stromal cells immune cells? *Arthritis Res Ther,* 17**,** 88.
HORLAD, H., MA, C., YANO, H., PAN, C., OHNISHI, K., FUJIWARA, Y., ENDO, S., KIKUKAWA, Y., OKUNO, Y., MATSUOKA, M., TAKEYA, M. & KOMOHARA, Y. 2016. An IL-27/Stat3 axis induces expression of programmed cell death 1 ligands (PD-L1/2) on infiltrating macrophages in lymphoma. *Cancer Sci,* 107**,** 1696-1704.
HORWITZ, E. M., PROCKOP, D. J., FITZPATRICK, L. A., KOO, W. W., GORDON, P. L., NEEL, M., SUSSMAN, M., ORCHARD, P., MARX, J. C., PYERITZ, R. E. & BRENNER, M. K. 1999. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med,* 5**,** 309-13.
HOULIHAN, D. D., MABUCHI, Y., MORIKAWA, S., NIIBE, K., ARAKI, D., SUZUKI, S., OKANO, H. & MATSUZAKI, Y. 2012. Isolation of mouse mesenchymal stem cells on the basis of expression of Sca-1 and PDGFR-alpha. *Nat Protoc,* 7**,** 2103-11.
HU, Y., CHENG, P., XUE, Y. X. & LIU, Y. H. 2012. Glioma cells promote the expression of vascular cell adhesion molecule-1 on bone marrow-derived mesenchymal stem cells: a possible mechanism for their tropism toward gliomas. *J Mol Neurosci,* 48**,** 127-35.
HUANG, K., WU, J 2017. Targeting the PD-1 Pathway in MSI-Stable Metastatic Colorectal Cancer. *Chemo Open Access,* 6.
HUANG, R., WU, D., YUAN, Y., LI, X., HOLM, R., TROPE, C. G., NESLAND, J. M. & SUO, Z. 2014. CD117 expression in fibroblasts-like stromal cells indicates unfavorable clinical outcomes in ovarian carcinoma patients. *PLoS One,* 9**,** e112209.
HUANG, W. H., CHANG, M. C., TSAI, K. S., HUNG, M. C., CHEN, H. L. & HUNG, S. C. 2013. Mesenchymal stem cells promote growth and angiogenesis of tumors in mice. *Oncogene,* 32**,** 4343-54.
HUNG, K., HAYASHI, R., LAFOND-WALKER, A., LOWENSTEIN, C., PARDOLL, D. & LEVITSKY, H. 1998. The central role of CD4(+) T cells in the antitumor immune response. *J Exp Med,* 188**,** 2357-68.
IGNEY, F. H. & KRAMMER, P. H. 2005. Tumor counterattack: fact or fiction? *Cancer Immunol Immunother,* 54**,** 1127-36.
IKUTANI, M., YANAGIBASHI, T., OGASAWARA, M., TSUNEYAMA, K., YAMAMOTO, S., HATTORI, Y., KOURO, T., ITAKURA, A., NAGAI, Y., TAKAKI, S. & TAKATSU, K. 2012. Identification of innate IL-5-producing cells and their role in lung eosinophil regulation and antitumor immunity. *J Immunol,* 188**,** 703-13.
INTLEKOFER, A. M. & THOMPSON, C. B. 2013. At the bench: preclinical rationale for CTLA-4 and PD-1 blockade as cancer immunotherapy. *J Leukoc Biol,* 94**,** 25-39.

IRISHCANCERSOCIETY. 2017. *Bowel (colon and rectum) cancer* [Online]. Available: https://www.cancer.ie/cancer-information/bowel-colon-rectum-cancer#sthash.NDxYuGcH.dpbs.

ISELLA, C., TERRASI, A., BELLOMO, S. E., PETTI, C., GALATOLA, G., MURATORE, A., MELLANO, A., SENETTA, R., CASSENTI, A., SONETTO, C., INGHIRAMI, G., TRUSOLINO, L., FEKETE, Z., DE RIDDER, M., CASSONI, P., STORME, G., BERTOTTI, A. & MEDICO, E. 2015. Stromal contribution to the colorectal cancer transcriptome. *Nat Genet,* 47**,** 312-9.

IWAMOTO, M., SHINOHARA, H., MIYAMOTO, A., OKUZAWA, M., MABUCHI, H., NOHARA, T., GON, G., TOYODA, M. & TANIGAWA, N. 2003. Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. *Int J Cancer,* 104**,** 92-7.

JANEWAY CA, T. P., WALPORT M ET AL. 2001. *Immunobiology: The Immune System in Health and Disease*, Garland Science.

JAZIREHI, A. R., LIM, A. & DINH, T. 2016. PD-1 inhibition and treatment of advanced melanoma-role of pembrolizumab. *Am J Cancer Res,* 6**,** 2117-2128.

JOHNSON, A. J., LUCAS, D. M., MUTHUSAMY, N., SMITH, L. L., EDWARDS, R. B., DE LAY, M. D., CROCE, C. M., GREVER, M. R. & BYRD, J. C. 2006. Characterization of the TCL-1 transgenic mouse as a preclinical drug development tool for human chronic lymphocytic leukemia. *Blood,* 108**,** 1334-8.

JOHNSON, B. F., CLAY, T. M., HOBEIKA, A. C., LYERLY, H. K. & MORSE, M. A. 2007. Vascular endothelial growth factor and immunosuppression in cancer: current knowledge and potential for new therapy. *Expert Opin Biol Ther,* 7**,** 449-60.

JUNEJA, V. R., MCGUIRE, K. A., MANGUSO, R. T., LAFLEUR, M. W., COLLINS, N., HAINING, W. N., FREEMAN, G. J. & SHARPE, A. H. 2017. PD-L1 on tumor cells is sufficient for immune evasion in immunogenic tumors and inhibits CD8 T cell cytotoxicity. *J Exp Med*.

KALLURI, R. & ZEISBERG, M. 2006. Fibroblasts in cancer. *Nat Rev Cancer,* 6**,** 392-401.

KARACHALIOU, N., CAO, M. G., TEIXIDO, C., VITERI, S., MORALES-ESPINOSA, D., SANTARPIA, M. & ROSELL, R. 2015. Understanding the function and dysfunction of the immune system in lung cancer: the role of immune checkpoints. *Cancer Biol Med,* 12**,** 79-86.

KARIM, B. O. & HUSO, D. L. 2013. Mouse models for colorectal cancer. *Am J Cancer Res,* 3**,** 240-50.

KARIN, M. 2009. NF-kappaB as a critical link between inflammation and cancer. *Cold Spring Harb Perspect Biol,* 1**,** a000141.

KARIN, M., CAO, Y., GRETEN, F. R. & LI, Z. W. 2002. NF-kappaB in cancer: from innocent bystander to major culprit. *Nat Rev Cancer,* 2**,** 301-10.

KEATING, A. 2008. How do mesenchymal stromal cells suppress T cells? *Cell Stem Cell,* 2**,** 106-8.

KHAKOO, A. Y., PATI, S., ANDERSON, S. A., REID, W., ELSHAL, M. F., ROVIRA, I. I., NGUYEN, A. T., MALIDE, D., COMBS, C. A., HALL, G., ZHANG, J., RAFFELD, M., ROGERS, T. B., STETLER-STEVENSON, W., FRANK, J. A., REITZ, M. & FINKEL, T. 2006. Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *J Exp Med,* 203**,** 1235-47.

KHALIL, D. N., SMITH, E. L., BRENTJENS, R. J. & WOLCHOK, J. D. 2016. The future of cancer treatment: immunomodulation, CARs and combination immunotherapy. *Nat Rev Clin Oncol,* 13**,** 273-90.

KHALIL, H., NIE, W., EDWARDS, R. A. & YOO, J. 2013. Isolation of primary myofibroblasts from mouse and human colon tissue. *J Vis Exp*.

KIDD, S., CALDWELL, L., DIETRICH, M., SAMUDIO, I., SPAETH, E. L., WATSON, K., SHI, Y., ABBRUZZESE, J., KONOPLEVA, M., ANDREEFF, M. & MARINI, F. C. 2010. Mesenchymal stromal cells alone or expressing interferon-beta suppress pancreatic tumors in vivo, an effect countered by anti-inflammatory treatment. *Cytotherapy,* 12**,** 615-25.

KIM, E. R. & CHANG, D. K. 2014. Colorectal cancer in inflammatory bowel disease: the risk, pathogenesis, prevention and diagnosis. *World J Gastroenterol,* 20**,** 9872-81.

KIM, H. J. & CANTOR, H. 2014. CD4 T-cell subsets and tumor immunity: the helpful and the not-so-helpful. *Cancer Immunol Res,* 2**,** 91-8.

KIM, H. R., HA, S. J., HONG, M. H., HEO, S. J., KOH, Y. W., CHOI, E. C., KIM, E. K., PYO, K. H., JUNG, I., SEO, D., CHOI, J., CHO, B. C. & YOON, S. O. 2016. PD-L1 expression on immune cells, but not on tumor cells, is a favorable prognostic factor for head and neck cancer patients. *Sci Rep,* 6**,** 36956.

KOCOGLU, M. & BADROS, A. 2016. The Role of Immunotherapy in Multiple Myeloma. *Pharmaceuticals (Basel),* 9.

KOEHNE, C. H. & DUBOIS, R. N. 2004. COX-2 inhibition and colorectal cancer. *Semin Oncol,* 31**,** 12-21.

KOLIARAKI, V., PASPARAKIS, M. & KOLLIAS, G. 2015. IKKbeta in intestinal mesenchymal cells promotes initiation of colitis-associated cancer. *J Exp Med,* 212**,** 2235-51.

KOLLURI, K. K., LAURENT, G. J. & JANES, S. M. 2013. Mesenchymal stem cells as vectors for lung cancer therapy. *Respiration,* 85**,** 443-51.

KORNPRAT, P., POLLHEIMER, M. J., LINDTNER, R. A., SCHLEMMER, A., REHAK, P. & LANGNER, C. 2011. Value of tumor size as a prognostic variable in colorectal cancer: a critical reappraisal. *Am J Clin Oncol,* 34**,** 43-9.

KOSAKA, H., ICHIKAWA, T., KUROZUMI, K., KAMBARA, H., INOUE, S., MARUO, T., NAKAMURA, K., HAMADA, H. & DATE, I. 2012. Therapeutic effect of suicide gene-transferred mesenchymal stem cells in a rat model of glioma. *Cancer Gene Ther,* 19**,** 572-8.

KRAMAN, M., BAMBROUGH, P. J., ARNOLD, J. N., ROBERTS, E. W., MAGIERA, L., JONES, J. O., GOPINATHAN, A., TUVESON, D. A. & FEARON, D. T. 2010. Suppression of antitumor immunity by stromal cells expressing fibroblast activation protein-alpha. *Science,* 330**,** 827-30.

KRAMPERA, M. 2011. Mesenchymal stromal cell 'licensing': a multistep process. *Leukemia,* 25**,** 1408-14.

KRAMPERA, M., COSMI, L., ANGELI, R., PASINI, A., LIOTTA, F., ANDREINI, A., SANTARLASCI, V., MAZZINGHI, B., PIZZOLO, G., VINANTE, F., ROMAGNANI, P., MAGGI, E., ROMAGNANI, S. & ANNUNZIATO, F. 2006. Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells,* 24**,** 386-98.

KRATCHMAROVA, I., KALUME, D. E., BLAGOEV, B., SCHERER, P. E., PODTELEJNIKOV, A. V., MOLINA, H., BICKEL, P. E., ANDERSEN, J. S., FERNANDEZ, M. M., BUNKENBORG, J., ROEPSTORFF, P., KRISTIANSEN, K., LODISH, H. F., MANN, M. & PANDEY, A. 2002. A proteomic approach for

identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Mol Cell Proteomics,* 1**,** 213-22.
KUCHARCZAK, J., SIMMONS, M. J., FAN, Y. & GELINAS, C. 2003. To be, or not to be: NF-kappaB is the answer--role of Rel/NF-kappaB in the regulation of apoptosis. *Oncogene,* 22**,** 8961-82.
KUSMARTSEV, S., NAGARAJ, S. & GABRILOVICH, D. I. 2005. Tumor-associated CD8+ T cell tolerance induced by bone marrow-derived immature myeloid cells. *J Immunol,* 175**,** 4583-92.
LA-BECK, N. M., JEAN, G. W., HUYNH, C., ALZGHARI, S. K. & LOWE, D. B. 2015. Immune Checkpoint Inhibitors: New Insights and Current Place in Cancer Therapy. *Pharmacotherapy,* 35**,** 963-76.
LAMAGNA, C., AURRAND-LIONS, M. & IMHOF, B. A. 2006. Dual role of macrophages in tumor growth and angiogenesis. *J Leukoc Biol,* 80**,** 705-13.
LANGLEY, R. R. & FIDLER, I. J. 2011. The seed and soil hypothesis revisited--the role of tumor-stroma interactions in metastasis to different organs. *Int J Cancer,* 128**,** 2527-35.
LATCHMAN, Y., WOOD, C. R., CHERNOVA, T., CHAUDHARY, D., BORDE, M., CHERNOVA, I., IWAI, Y., LONG, A. J., BROWN, J. A., NUNES, R., GREENFIELD, E. A., BOURQUE, K., BOUSSIOTIS, V. A., CARTER, L. L., CARRENO, B. M., MALENKOVICH, N., NISHIMURA, H., OKAZAKI, T., HONJO, T., SHARPE, A. H. & FREEMAN, G. J. 2001. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol,* 2**,** 261-8.
LAU, J., CHEUNG, J., NAVARRO, A., LIANOGLOU, S., HALEY, B., TOTPAL, K., SANDERS, L., KOEPPEN, H., CAPLAZI, P., MCBRIDE, J., CHIU, H., HONG, R., GROGAN, J., JAVINAL, V., YAUCH, R., IRVING, B., BELVIN, M., MELLMAN, I., KIM, J. M. & SCHMIDT, M. 2017. Tumour and host cell PD-L1 is required to mediate suppression of anti-tumour immunity in mice. *Nat Commun,* 8**,** 14572.
LAWRENCE, T. 2009. The nuclear factor NF-kappaB pathway in inflammation. *Cold Spring Harb Perspect Biol,* 1**,** a001651.
LE BLANC, K., TAMMIK, L., SUNDBERG, B., HAYNESWORTH, S. E. & RINGDÉN, O. 2003. Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol,* 57**,** 11-20.
LE ROLLE, A. F., CHIU, T. K., FARA, M., SHIA, J., ZENG, Z., WEISER, M. R., PATY, P. B. & CHIU, V. K. 2015. The prognostic significance of CXCL1 hypersecretion by human colorectal cancer epithelia and myofibroblasts. *J Transl Med,* 13**,** 199.
LEE, H. J., KO, J. H., JEONG, H. J., KO, A. Y., KIM, M. K., WEE, W. R., YOON, S. O. & OH, J. Y. 2015a. Mesenchymal stem/stromal cells protect against autoimmunity via CCL2-dependent recruitment of myeloid-derived suppressor cells. *J Immunol,* 194**,** 3634-45.
LEE, H. W., PARK, Y. M., LEE, S. J., CHO, H. J., KIM, D. H., LEE, J. I., KANG, M. S., SEOL, H. J., SHIM, Y. M., NAM, D. H., KIM, H. H. & JOO, K. M. 2013. Alpha-smooth muscle actin (ACTA2) is required for metastatic potential of human lung adenocarcinoma. *Clin Cancer Res,* 19**,** 5879-89.
LEE, M. W., RYU, S., KIM, D. S., SUNG, K. W., KOO, H. H. & YOO, K. H. 2015b. Strategies to improve the immunosuppressive properties of human mesenchymal stem cells. *Stem Cell Res Ther,* 6**,** 179.
LIANG, S. C., LATCHMAN, Y. E., BUHLMANN, J. E., TOMCZAK, M. F., HORWITZ, B. H., FREEMAN, G. J. & SHARPE, A. H. 2003. Regulation of PD-1, PD-L1, and

PD-L2 expression during normal and autoimmune responses. *Eur J Immunol,* 33**,** 2706-16.

LIM, S. O., LI, C. W., XIA, W., CHA, J. H., CHAN, L. C., WU, Y., CHANG, S. S., LIN, W. C., HSU, J. M., HSU, Y. H., KIM, T., CHANG, W. C., HSU, J. L., YAMAGUCHI, H., DING, Q., WANG, Y., YANG, Y., CHEN, C. H., SAHIN, A. A., YU, D., HORTOBAGYI, G. N. & HUNG, M. C. 2016. Deubiquitination and Stabilization of PD-L1 by CSN5. *Cancer Cell,* 30**,** 925-939.

LIN, H. H., STACEY, M., STEIN-STREILEIN, J. & GORDON, S. 2010a. F4/80: the macrophage-specific adhesion-GPCR and its role in immunoregulation. *Adv Exp Med Biol,* 706**,** 149-56.

LIN, J. T., WANG, J. Y., CHEN, M. K., CHEN, H. C., CHANG, T. H., SU, B. W. & CHANG, P. J. 2013. Colon cancer mesenchymal stem cells modulate the tumorigenicity of colon cancer through interleukin 6. *Exp Cell Res,* 319**,** 2216-29.

LIN, Y., BAI, L., CHEN, W. & XU, S. 2010b. The NF-kappaB activation pathways, emerging molecular targets for cancer prevention and therapy. *Expert Opin Ther Targets,* 14**,** 45-55.

LIN, Y. M., SUNG, W. W., HSIEH, M. J., TSAI, S. C., LAI, H. W., YANG, S. M., SHEN, K. H., CHEN, M. K., LEE, H., YEH, K. T. & CHEN, C. J. 2015. High PD-L1 Expression Correlates with Metastasis and Poor Prognosis in Oral Squamous Cell Carcinoma. *PLoS One,* 10**,** e0142656.

LINCH, S. N., KASIEWICZ, M. J., MCNAMARA, M. J., HILGART-MARTISZUS, I. F., FARHAD, M. & REDMOND, W. L. 2016. Combination OX40 agonism/CTLA-4 blockade with HER2 vaccination reverses T-cell anergy and promotes survival in tumor-bearing mice. *Proc Natl Acad Sci U S A,* 113**,** E319-27.

LING, W., ZHANG, J., YUAN, Z., REN, G., ZHANG, L., CHEN, X., RABSON, A. B., ROBERTS, A. I., WANG, Y. & SHI, Y. 2014. Mesenchymal Stem Cells Use IDO to Regulate Immunity in Tumor Microenvironment. *Cancer Res*.

LIOTTA, L. A. & KOHN, E. C. 2001. The microenvironment of the tumour-host interface. *Nature,* 411**,** 375-9.

LIU, Y., HAN, Z. P., ZHANG, S. S., JING, Y. Y., BU, X. X., WANG, C. Y., SUN, K., JIANG, G. C., ZHAO, X., LI, R., GAO, L., ZHAO, Q. D., WU, M. C. & WEI, L. X. 2011. Effects of inflammatory factors on mesenchymal stem cells and their role in the promotion of tumor angiogenesis in colon cancer. *J Biol Chem,* 286**,** 25007-15.

LJUJIC, B., MILOVANOVIC, M., VOLAREVIC, V., MURRAY, B., BUGARSKI, D., PRZYBORSKI, S., ARSENIJEVIC, N., LUKIC, M. L. & STOJKOVIC, M. 2013. Human mesenchymal stem cells creating an immunosuppressive environment and promote breast cancer in mice. *Sci Rep,* 3**,** 2298.

LOEBINGER, M. R., EDDAOUDI, A., DAVIES, D. & JANES, S. M. 2009. Mesenchymal stem cell delivery of TRAIL can eliminate metastatic cancer. *Cancer Res,* 69**,** 4134-42.

LUCHINI, C., WOOD, L. D., CHENG, L., NOTTEGAR, A., STUBBS, B., SOLMI, M., CAPELLI, P., PEA, A., SERGI, G., MANZATO, E., FASSAN, M., BAGANTE, F., BOLLSCHWEILER, E., GIACOPUZZI, S., KANEKO, T., DE MANZONI, G., BARBARESCHI, M., SCARPA, A. & VERONESE, N. 2016. Extranodal extension of lymph node metastasis is a marker of poor prognosis in oesophageal cancer: a systematic review with meta-analysis. *J Clin Pathol*.

LUO, J. L., MAEDA, S., HSU, L. C., YAGITA, H. & KARIN, M. 2004. Inhibition of NF-kappaB in cancer cells converts inflammation- induced tumor growth

mediated by TNFalpha to TRAIL-mediated tumor regression. *Cancer Cell,* 6**,** 297-305.
LYNCH, D. & MURPHY, A. 2016. The emerging role of immunotherapy in colorectal cancer. *Ann Transl Med,* 4**,** 305.
MA, W., GILLIGAN, B. M., YUAN, J. & LI, T. 2016. Current status and perspectives in translational biomarker research for PD-1/PD-L1 immune checkpoint blockade therapy. *J Hematol Oncol,* 9**,** 47.
MADAR, S., GOLDSTEIN, I. & ROTTER, V. 2013. 'Cancer associated fibroblasts'--more than meets the eye. *Trends Mol Med,* 19**,** 447-53.
MADRIGAL, M., RAO, K. S. & RIORDAN, N. H. 2014. A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. *J Transl Med,* 12**,** 260.
MALYGIN, A. M., MERI, S. & TIMONEN, T. 1993. Regulation of natural killer cell activity by transforming growth factor-beta and prostaglandin E2. *Scand J Immunol,* 37**,** 71-6.
MANTOVANI, A. 2012. MSCs, macrophages, and cancer: a dangerous ménage-à-trois. *Cell Stem Cell,* 11**,** 730-2.
MANTOVANI, A., BISWAS, S. K., GALDIERO, M. R., SICA, A. & LOCATI, M. 2013. Macrophage plasticity and polarization in tissue repair and remodelling. *J Pathol,* 229**,** 176-85.
MAQBOOL, M., VIDYADARAN, S., GEORGE, E. & RAMASAMY, R. 2011. Human mesenchymal stem cells protect neutrophils from serum-deprived cell death. *Cell Biol Int,* 35**,** 1247-51.
MARONI, P., BENDINELLI, P., MATTEUCCI, E. & DESIDERIO, M. A. 2007. HGF induces CXCR4 and CXCL12-mediated tumor invasion through Ets1 and NF-kappaB. *Carcinogenesis,* 28**,** 267-79.
MARVEL, D. & GABRILOVICH, D. I. 2015. Myeloid-derived suppressor cells in the tumor microenvironment: expect the unexpected. *J Clin Invest,* 125**,** 3356-64.
MASUGI, Y., NISHIHARA, R., YANG, J., MIMA, K., DA SILVA, A., SHI, Y., INAMURA, K., CAO, Y., SONG, M., NOWAK, J. A., LIAO, X., NOSHO, K., CHAN, A. T., GIANNAKIS, M., BASS, A. J., HODI, F. S., FREEMAN, G. J., RODIG, S., FUCHS, C. S., QIAN, Z. R. & OGINO, S. 2016. Tumour CD274 (PD-L1) expression and T cells in colorectal cancer. *Gut*.
MATSUSHITA, H., VESELY, M. D., KOBOLDT, D. C., RICKERT, C. G., UPPALURI, R., MAGRINI, V. J., ARTHUR, C. D., WHITE, J. M., CHEN, Y. S., SHEA, L. K., HUNDAL, J., WENDL, M. C., DEMETER, R., WYLIE, T., ALLISON, J. P., SMYTH, M. J., OLD, L. J., MARDIS, E. R. & SCHREIBER, R. D. 2012. Cancer exome analysis reveals a T-cell-dependent mechanism of cancer immunoediting. *Nature,* 482**,** 400-4.
MBEUNKUI, F. & JOHANN, D. J. 2009. Cancer and the tumor microenvironment: a review of an essential relationship. *Cancer Chemother Pharmacol,* 63**,** 571-82.
MEISEL, R., ZIBERT, A., LARYEA, M., GOBEL, U., DAUBENER, W. & DILLOO, D. 2004. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood,* 103**,** 4619-21.
MEISSL, K., MACHO-MASCHLER, S., MULLER, M. & STROBL, B. 2017. The good and the bad faces of STAT1 in solid tumours. *Cytokine,* 89**,** 12-20.

MELERO, I., HERVAS-STUBBS, S., GLENNIE, M., PARDOLL, D. M. & CHEN, L. 2007. Immunostimulatory monoclonal antibodies for cancer therapy. *Nat Rev Cancer,* 7**,** 95-106.

MICKE, P. & OSTMAN, A. 2004. Tumour-stroma interaction: cancer-associated fibroblasts as novel targets in anti-cancer therapy? *Lung Cancer,* 45 Suppl 2**,** S163-75.

MIN, L., CHEN, D., QU, L. & SHOU, C. 2014. Tumor necrosis factor-a polymorphisms and colorectal cancer risk: a meta-analysis. *PLoS One,* 9**,** e85187.

MISHRA, P. J., MISHRA, P. J., HUMENIUK, R., MEDINA, D. J., ALEXE, G., MESIROV, J. P., GANESAN, S., GLOD, J. W. & BANERJEE, D. 2008. Carcinoma-associated fibroblast-like differentiation of human mesenchymal stem cells. *Cancer Res,* 68**,** 4331-9.

MIYAMOTO, H., KATSUYAMA, E., MIYAUCHI, Y., HOSHI, H., MIYAMOTO, K., SATO, Y., KOBAYASHI, T., IWASAKI, R., YOSHIDA, S., MORI, T., KANAGAWA, H., FUJIE, A., HAO, W., MORIOKA, H., MATSUMOTO, M., TOYAMA, Y. & MIYAMOTO, T. 2012. An essential role for STAT6-STAT1 protein signaling in promoting macrophage cell-cell fusion. *J Biol Chem,* 287**,** 32479-84.

MOO-YOUNG, T. A., LARSON, J. W., BELT, B. A., TAN, M. C., HAWKINS, W. G., EBERLEIN, T. J., GOEDEGEBUURE, P. S. & LINEHAN, D. C. 2009. Tumor-derived TGF-beta mediates conversion of CD4+Foxp3+ regulatory T cells in a murine model of pancreas cancer. *J Immunother,* 32**,** 12-21.

MORRISSEY, K. M., YURASZECK, T. M., LI, C. C., ZHANG, Y. & KASICHAYANULA, S. 2016. Immunotherapy and Novel Combinations in Oncology: Current Landscape, Challenges, and Opportunities. *Clin Transl Sci,* 9**,** 89-104.

MOSSER, D. M. & EDWARDS, J. P. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol,* 8**,** 958-69.

MUELLER, L. P., LUETZKENDORF, J., WIDDER, M., NERGER, K., CAYSA, H. & MUELLER, T. 2011. TRAIL-transduced multipotent mesenchymal stromal cells (TRAIL-MSC) overcome TRAIL resistance in selected CRC cell lines in vitro and in vivo. *Cancer Gene Ther,* 18**,** 229-39.

MURPHY, K. 2012. *Janeway's Immunobiology*, Garland Science.

NAGASAKI, T., HARA, M., NAKANISHI, H., TAKAHASHI, H., SATO, M. & TAKEYAMA, H. 2014. Interleukin-6 released by colon cancer-associated fibroblasts is critical for tumour angiogenesis: anti-interleukin-6 receptor antibody suppressed angiogenesis and inhibited tumour-stroma interaction. *Br J Cancer,* 110**,** 469-78.

NAKAGAWA, H., LIYANARACHCHI, S., DAVULURI, R. V., AUER, H., MARTIN, E. W., DE LA CHAPELLE, A. & FRANKEL, W. L. 2004. Role of cancer-associated stromal fibroblasts in metastatic colon cancer to the liver and their expression profiles. *Oncogene,* 23**,** 7366-77.

NAKAMIZO, A., MARINI, F., AMANO, T., KHAN, A., STUDENY, M., GUMIN, J., CHEN, J., HENTSCHEL, S., VECIL, G., DEMBINSKI, J., ANDREEFF, M. & LANG, F. F. 2005. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res,* 65**,** 3307-18.

NASUNO, M., ARIMURA, Y., NAGAISHI, K., ISSHIKI, H., ONODERA, K., NAKAGAKI, S., WATANABE, S., IDOGAWA, M., YAMASHITA, K., NAISHIRO, Y., ADACHI, Y., SUZUKI, H., FUJIMIYA, M., IMAI, K. & SHINOMURA, Y. 2014. Mesenchymal stem cells cancel azoxymethane-induced tumor initiation. *Stem Cells,* 32**,** 913-25.

NGUYEN, L. T. & OHASHI, P. S. 2015. Clinical blockade of PD1 and LAG3--potential mechanisms of action. *Nat Rev Immunol,* 15**,** 45-56.

NIESS, H., VON EINEM, J. C., THOMAS, M. N., MICHL, M., ANGELE, M. K., HUSS, R., GUNTHER, C., NELSON, P. J., BRUNS, C. J. & HEINEMANN, V. 2015. Treatment of advanced gastrointestinal tumors with genetically modified autologous mesenchymal stromal cells (TREAT-ME1): study protocol of a phase I/II clinical trial. *BMC Cancer,* 15**,** 237.

NIH. 2014. *Clinical trials registry* [Online]. Available: http://clinicaltrials.gov/ct2/results?term=mesenchymal+stem+cells&Search=Search.

NISHIMURA, T., IWAKABE, K., SEKIMOTO, M., OHMI, Y., YAHATA, T., NAKUI, M., SATO, T., HABU, S., TASHIRO, H., SATO, M. & OHTA, A. 1999. Distinct role of antigen-specific T helper type 1 (Th1) and Th2 cells in tumor eradication in vivo. *J Exp Med,* 190**,** 617-27.

NISHIMURA, T., NAKUI, M., SATO, M., IWAKABE, K., KITAMURA, H., SEKIMOTO, M., OHTA, A., KODA, T. & NISHIMURA, S. 2000. The critical role of Th1-dominant immunity in tumor immunology. *Cancer Chemother Pharmacol,* 46 Suppl**,** S52-61.

NOH, H., HU, J., WANG, X., XIA, X., SATELLI, A. & LI, S. 2015. Immune checkpoint regulator PD-L1 expression on tumor cells by contacting CD11b positive bone marrow derived stromal cells. *Cell Commun Signal,* 13**,** 14.

NOMAN, M. Z., DESANTIS, G., JANJI, B., HASMIM, M., KARRAY, S., DESSEN, P., BRONTE, V. & CHOUAIB, S. 2014. PD-L1 is a novel direct target of HIF-1alpha, and its blockade under hypoxia enhanced MDSC-mediated T cell activation. *J Exp Med,* 211**,** 781-90.

NOSHO, K., BABA, Y., TANAKA, N., SHIMA, K., HAYASHI, M., MEYERHARDT, J. A., GIOVANNUCCI, E., DRANOFF, G., FUCHS, C. S. & OGINO, S. 2010. Tumour-infiltrating T-cell subsets, molecular changes in colorectal cancer, and prognosis: cohort study and literature review. *J Pathol,* 222**,** 350-66.

NOY, R. & POLLARD, J. W. 2014. Tumor-associated macrophages: from mechanisms to therapy. *Immunity,* 41**,** 49-61.

OKAZAKI, T., CHIKUMA, S., IWAI, Y., FAGARASAN, S. & HONJO, T. 2013. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. *Nat Immunol,* 14**,** 1212-8.

OLUMI, A. F., GROSSFELD, G. D., HAYWARD, S. W., CARROLL, P. R., TLSTY, T. D. & CUNHA, G. R. 1999. Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res,* 59**,** 5002-11.

OU, J. N., WIEDEMAN, A. E. & STEVENS, A. M. 2012. TNF-alpha and TGF-beta counter-regulate PD-L1 expression on monocytes in systemic lupus erythematosus. *Sci Rep,* 2**,** 295.

OWENS, B. M. 2015. Inflammation, Innate Immunity, and the Intestinal Stromal Cell Niche: Opportunities and Challenges. *Front Immunol,* 6**,** 319.

OWENS, B. M., STEEVELS, T. A., DUDEK, M., WALCOTT, D., SUN, M. Y., MAYER, A., ALLAN, P. & SIMMONS, A. 2013. CD90(+) Stromal Cells are Non-Professional Innate Immune Effectors of the Human Colonic Mucosa. *Front Immunol,* 4**,** 307.

PAGET, S. 1989. The distribution of secondary growths in cancer of the breast. 1889. *Cancer Metastasis Rev,* 8**,** 98-101.

PALLANGYO, C. K., ZIEGLER, P. K. & GRETEN, F. R. 2015. IKKbeta acts as a tumor suppressor in cancer-associated fibroblasts during intestinal tumorigenesis. *J Exp Med,* 212**,** 2253-66.
PARDOLL, D. M. 2012. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer,* 12**,** 252-64.
PATEL, S. A., MEYER, J. R., GRECO, S. J., CORCORAN, K. E., BRYAN, M. & RAMESHWAR, P. 2010. Mesenchymal stem cells protect breast cancer cells through regulatory T cells: role of mesenchymal stem cell-derived TGF-beta. *J Immunol,* 184**,** 5885-94.
PATEL, S. P. & KURZROCK, R. 2015. PD-L1 Expression as a Predictive Biomarker in Cancer Immunotherapy. *Mol Cancer Ther,* 14**,** 847-56.
PEDDAREDDIGARI, V. G., WANG, D. & DUBOIS, R. N. 2010. The tumor microenvironment in colorectal carcinogenesis. *Cancer Microenviron,* 3**,** 149-66.
PEISTER, A., MELLAD, J. A., LARSON, B. L., HALL, B. M., GIBSON, L. F. & PROCKOP, D. J. 2004. Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. *Blood,* 103**,** 1662-8.
PENG, Y., LI, Z., YANG, P., NEWTON, I. P., REN, H., ZHANG, L. & WU, H. 2014. Direct contact with colon cancer cells regulates differentiation of bone marrow mesenchymal stem cells into tumor associated fibroblasts. *Biochem Biophys Res Commun*.
PHILIPS, G. K. & ATKINS, M. 2015. Therapeutic uses of anti-PD-1 and anti-PD-L1 antibodies. *Int Immunol,* 27**,** 39-46.
PINCHUK, I. V., SAADA, J. I., BESWICK, E. J., BOYA, G., QIU, S. M., MIFFLIN, R. C., RAJU, G. S., REYES, V. E. & POWELL, D. W. 2008. PD-1 ligand expression by human colonic myofibroblasts/fibroblasts regulates CD4+ T-cell activity. *Gastroenterology,* 135**,** 1228-1237, 1237 e1-2.
PIOLI, C., GATTA, L., UBALDI, V. & DORIA, G. 2000. Inhibition of IgG1 and IgE production by stimulation of the B cell CTLA-4 receptor. *J Immunol,* 165**,** 5530-6.
POGGI, A., MUSSO, A., DAPINO, I. & ZOCCHI, M. R. 2014. Mechanisms of tumor escape from immune system: role of mesenchymal stromal cells. *Immunol Lett,* 159**,** 55-72.
PREVOST-BLONDEL, A., ROTH, E., ROSENTHAL, F. M. & PIRCHER, H. 2000. Crucial role of TNF-alpha in CD8 T cell-mediated elimination of 3LL-A9 Lewis lung carcinoma cells in vivo. *J Immunol,* 164**,** 3645-51.
PRIZMENT, A. E., VIERKANT, R. A., SMYRK, T. C., TILLMANS, L. S., NELSON, H. H., LYNCH, C. F., PENGO, T., THIBODEAU, S. N., CHURCH, T. R., CERHAN, J. R., ANDERSON, K. E. & LIMBURG, P. J. 2016. Cytotoxic T-cells and granzyme B associated with improved colorectal cancer survival in a prospective cohort of older women. *Cancer Epidemiol Biomarkers Prev*.
QIAO, L., XU, Z., ZHAO, T., ZHAO, Z., SHI, M., ZHAO, R. C., YE, L. & ZHANG, X. 2008. Suppression of tumorigenesis by human mesenchymal stem cells in a hepatoma model. *Cell Res,* 18**,** 500-7.
QUAIL, D. F., OLSON, O. C., BHARDWAJ, P., WALSH, L. A., AKKARI, L., QUICK, M. L., CHEN, I. C., WENDEL, N., BEN-CHETRIT, N., WALKER, J., HOLT, P. R., DANNENBERG, A. J. & JOYCE, J. A. 2017. Obesity alters the lung myeloid cell landscape to enhance breast cancer metastasis through IL5 and GM-CSF. *Nat Cell Biol,* 19**,** 974-987.

REN, G., SU, J., ZHANG, L., ZHAO, X., LING, W., L'HUILLIE, A., ZHANG, J., LU, Y., ROBERTS, A. I., JI, W., ZHANG, H., RABSON, A. B. & SHI, Y. 2009. Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. *Stem Cells,* 27**,** 1954-62.

REN, G., ZHANG, L., ZHAO, X., XU, G., ZHANG, Y., ROBERTS, A. I., ZHAO, R. C. & SHI, Y. 2008. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell,* 2**,** 141-50.

REN, G., ZHAO, X., WANG, Y., ZHANG, X., CHEN, X., XU, C., YUAN, Z. R., ROBERTS, A. I., ZHANG, L., ZHENG, B., WEN, T., HAN, Y., RABSON, A. B., TISCHFIELD, J. A., SHAO, C. & SHI, Y. 2012a. CCR2-dependent recruitment of macrophages by tumor-educated mesenchymal stromal cells promotes tumor development and is mimicked by TNFalpha. *Cell Stem Cell,* 11**,** 812-24.

REN, G., ZHAO, X., WANG, Y., ZHANG, X., CHEN, X., XU, C., YUAN, Z. R., ROBERTS, A. I., ZHANG, L., ZHENG, B., WEN, T., HAN, Y., RABSON, A. B., TISCHFIELD, J. A., SHAO, C. & SHI, Y. 2012b. CCR2-dependent recruitment of macrophages by tumor-educated mesenchymal stromal cells promotes tumor development and is mimicked by TNFα. *Cell Stem Cell,* 11**,** 812-24.

RESTIFO, N. P., ESQUIVEL, F., KAWAKAMI, Y., YEWDELL, J. W., MULÉ, J. J., ROSENBERG, S. A. & BENNINK, J. R. 1993. Identification of human cancers deficient in antigen processing. *J Exp Med,* 177**,** 265-72.

RIBAS, A. & HU-LIESKOVAN, S. 2016. What does PD-L1 positive or negative mean? *J Exp Med,* 213**,** 2835-2840.

RIIHIMAKI, M., HEMMINKI, A., SUNDQUIST, J. & HEMMINKI, K. 2016. Patterns of metastasis in colon and rectal cancer. *Sci Rep,* 6**,** 29765.

ROBERT, C., RIBAS, A., WOLCHOK, J. D., HODI, F. S., HAMID, O., KEFFORD, R., WEBER, J. S., JOSHUA, A. M., HWU, W. J., GANGADHAR, T. C., PATNAIK, A., DRONCA, R., ZAROUR, H., JOSEPH, R. W., BOASBERG, P., CHMIELOWSKI, B., MATEUS, C., POSTOW, M. A., GERGICH, K., ELASSAISS-SCHAAP, J., LI, X. N., IANNONE, R., EBBINGHAUS, S. W., KANG, S. P. & DAUD, A. 2014. Anti-programmed-death-receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. *Lancet,* 384**,** 1109-17.

ROSENBAUM, M. W., BLEDSOE, J. R., MORALES-OYARVIDE, V., HUYNH, T. G. & MINO-KENUDSON, M. 2016. PD-L1 expression in colorectal cancer is associated with microsatellite instability, BRAF mutation, medullary morphology and cytotoxic tumor-infiltrating lymphocytes. *Mod Pathol,* 29**,** 1104-12.

ROUSALOVA, I. & KREPELA, E. 2010. Granzyme B-induced apoptosis in cancer cells and its regulation (review). *Int J Oncol,* 37**,** 1361-78.

ROVIN, B. H., DICKERSON, J. A., TAN, L. C. & HEBERT, C. A. 1995. Activation of nuclear factor-kappa B correlates with MCP-1 expression by human mesangial cells. *Kidney Int,* 48**,** 1263-71.

RYAN, A. E., COLLERAN, A., O'GORMAN, A., O'FLYNN, L., PINDJACOVA, J., LOHAN, P., O'MALLEY, G., NOSOV, M., MUREAU, C. & EGAN, L. J. 2014. Targeting colon cancer cell NF-κB promotes an anti-tumour M1-like macrophage phenotype and inhibits peritoneal metastasis. *Oncogene,* 0.

RYAN, A. E., COLLERAN, A., O'GORMAN, A., O'FLYNN, L., PINDJACOVA, J., LOHAN, P., O'MALLEY, G., NOSOV, M., MUREAU, C. & EGAN, L. J. 2015. Targeting colon cancer cell NF-kappaB promotes an anti-tumour M1-like

macrophage phenotype and inhibits peritoneal metastasis. *Oncogene,* 34**,** 1563-74.
RYAN, J. M., BARRY, F. P., MURPHY, J. M. & MAHON, B. P. 2005. Mesenchymal stem cells avoid allogeneic rejection. *J Inflamm (Lond),* 2**,** 8.
SAKAMOTO, K., MAEDA, S., HIKIBA, Y., NAKAGAWA, H., HAYAKAWA, Y., SHIBATA, W., YANAI, A., OGURA, K. & OMATA, M. 2009. Constitutive NF-kappaB activation in colorectal carcinoma plays a key role in angiogenesis, promoting tumor growth. *Clin Cancer Res,* 15**,** 2248-58.
SANTARPIA, M. & KARACHALIOU, N. 2015. Tumor immune microenvironment characterization and response to anti-PD-1 therapy. *Cancer Biol Med,* 12**,** 74-8.
SARKAR, P., RANDALL, S. M., MUDDIMAN, D. C. & RAO, B. M. 2012. Targeted proteomics of the secretory pathway reveals the secretome of mouse embryonic fibroblasts and human embryonic stem cells. *Mol Cell Proteomics,* 11**,** 1829-39.
SCHUTZE, S., WIEGMANN, K., MACHLEIDT, T. & KRONKE, M. 1995. TNF-induced activation of NF-kappa B. *Immunobiology,* 193**,** 193-203.
SEMENZA, G. L. 2011. Oxygen sensing, homeostasis, and disease. *N Engl J Med,* 365**,** 537-47.
SHANKARAN, V., IKEDA, H., BRUCE, A. T., WHITE, J. M., SWANSON, P. E., OLD, L. J. & SCHREIBER, R. D. 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature,* 410**,** 1107-11.
SHARMA, P. & ALLISON, J. P. 2015. The future of immune checkpoint therapy. *Science,* 348**,** 56-61.
SHENG, H., WANG, Y., JIN, Y., ZHANG, Q., ZHANG, Y., WANG, L., SHEN, B., YIN, S., LIU, W., CUI, L. & LI, N. 2008. A critical role of IFNgamma in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. *Cell Res,* 18**,** 846-57.
SHI, M., LI, J., LIAO, L., CHEN, B., LI, B., CHEN, L., JIA, H. & ZHAO, R. C. 2007. Regulation of CXCR4 expression in human mesenchymal stem cells by cytokine treatment: role in homing efficiency in NOD/SCID mice. *Haematologica,* 92**,** 897-904.
SHINAGAWA, K., KITADAI, Y., TANAKA, M., SUMIDA, T., KODAMA, M., HIGASHI, Y., TANAKA, S., YASUI, W. & CHAYAMA, K. 2010. Mesenchymal stem cells enhance growth and metastasis of colon cancer. *Int J Cancer,* 127**,** 2323-33.
SHOU, P., CHEN, Q., JIANG, J., XU, C., ZHANG, J., ZHENG, C., JIANG, M., VELLETRI, T., CAO, W., HUANG, Y., YANG, Q., HAN, X., ZHANG, L., WEI, L., RABSON, A. B., CHIN, Y. E., WANG, Y. & SHI, Y. 2016. Type I interferons exert anti-tumor effect via reversing immunosuppression mediated by mesenchymal stromal cells. *Oncogene,* 35**,** 5953-5962.
SIEBERT, H., SACHSE, A., KUZIEL, W. A., MAEDA, N. & BRÜCK, W. 2000. The chemokine receptor CCR2 is involved in macrophage recruitment to the injured peripheral nervous system. *J Neuroimmunol,* 110**,** 177-85.
SIEGEL, R., DESANTIS, C. & JEMAL, A. 2014. Colorectal cancer statistics, 2014. *CA Cancer J Clin,* 64**,** 104-17.
SINGH, P. P., SHARMA, P. K., KRISHNAN, G. & LOCKHART, A. C. 2015. Immune checkpoints and immunotherapy for colorectal cancer. *Gastroenterol Rep (Oxf),* 3**,** 289-97.

SMITH-GARVIN, J. E., KORETZKY, G. A. & JORDAN, M. S. 2009. T cell activation. *Annu Rev Immunol,* 27**,** 591-619.

SPAETH, E. L., DEMBINSKI, J. L., SASSER, A. K., WATSON, K., KLOPP, A., HALL, B., ANDREEFF, M. & MARINI, F. 2009. Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PLoS One,* 4**,** e4992.

SPAETH, E. L., LABAFF, A. M., TOOLE, B. P., KLOPP, A., ANDREEFF, M. & MARINI, F. C. 2013. Mesenchymal CD44 expression contributes to the acquisition of an activated fibroblast phenotype via TWIST activation in the tumor microenvironment. *Cancer Res,* 73**,** 5347-59.

SPAGGIARI, G. M., ABDELRAZIK, H., BECCHETTI, F. & MORETTA, L. 2009. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin E2. *Blood,* 113**,** 6576-83.

SPAGGIARI, G. M., CAPOBIANCO, A., ABDELRAZIK, H., BECCHETTI, F., MINGARI, M. C. & MORETTA, L. 2008. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood,* 111**,** 1327-33.

STAGG, J., POMMEY, S., ELIOPOULOS, N. & GALIPEAU, J. 2006. Interferon-gamma-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood,* 107**,** 2570-7.

STANILOV, N., MITEVA, L., DOBREVA, Z. & STANILOVA, S. 2014. Colorectal cancer severity and survival in correlation with tumour necrosis factor-alpha. *Biotechnol Biotechnol Equip,* 28**,** 911-917.

STARKE, A., WUTHRICH, R. P. & WAECKERLE-MEN, Y. 2007. TGF-beta treatment modulates PD-L1 and CD40 expression in proximal renal tubular epithelial cells and enhances CD8 cytotoxic T-cell responses. *Nephron Exp Nephrol,* 107**,** e22-9.

STEIN, A. & BOKEMEYER, C. 2014. How to select the optimal treatment for first line metastatic colorectal cancer. *World J Gastroenterol,* 20**,** 899-907.

STEWART, T. J. & ABRAMS, S. I. 2008. How tumours escape mass destruction. *Oncogene,* 27**,** 5894-903.

STUDENY, M., MARINI, F. C., CHAMPLIN, R. E., ZOMPETTA, C., FIDLER, I. J. & ANDREEFF, M. 2002. Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res,* 62**,** 3603-8.

STUDENY, M., MARINI, F. C., DEMBINSKI, J. L., ZOMPETTA, C., CABREIRA-HANSEN, M., BEKELE, B. N., CHAMPLIN, R. E. & ANDREEFF, M. 2004. Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. *J Natl Cancer Inst,* 96**,** 1593-603.

SUN, Z., WANG, S. & ZHAO, R. C. 2014. The roles of mesenchymal stem cells in tumor inflammatory microenvironment. *J Hematol Oncol,* 7**,** 14.

TAKATSU, K. 2011. Interleukin-5 and IL-5 receptor in health and diseases. *Proc Jpn Acad Ser B Phys Biol Sci,* 87**,** 463-85.

TAN, C. & DU, X. 2012. KRAS mutation testing in metastatic colorectal cancer. *World J Gastroenterol,* 18**,** 5171-80.

TANAKA, T., KOHNO, H., SUZUKI, R., YAMADA, Y., SUGIE, S. & MORI, H. 2003. A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci,* 94**,** 965-73.

TERZIC, J., GRIVENNIKOV, S., KARIN, E. & KARIN, M. 2010. Inflammation and colon cancer. *Gastroenterology,* 138**,** 2101-2114 e5.

THIBULT, M. L., MAMESSIER, E., GERTNER-DARDENNE, J., PASTOR, S., JUST-LANDI, S., XERRI, L., CHETAILLE, B. & OLIVE, D. 2013. PD-1 is a novel regulator of human B-cell activation. *Int Immunol,* 25**,** 129-37.

THOMAS, A., TEICHER, B. A. & HASSAN, R. 2016. Antibody-drug conjugates for cancer therapy. *Lancet Oncol,* 17**,** e254-62.

TÖGEL, F., HU, Z., WEISS, K., ISAAC, J., LANGE, C. & WESTENFELDER, C. 2005. Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. *Am J Physiol Renal Physiol,* 289**,** F31-42.

TOH, J. W., DE SOUZA, P., LIM, S. H., SINGH, P., CHUA, W., NG, W. & SPRING, K. J. 2016. The Potential Value of Immunotherapy in Colorectal Cancers: Review of the Evidence for Programmed Death-1 Inhibitor Therapy. *Clin Colorectal Cancer,* 15**,** 285-291.

TOPALIAN, S. L., SZNOL, M., MCDERMOTT, D. F., KLUGER, H. M., CARVAJAL, R. D., SHARFMAN, W. H., BRAHMER, J. R., LAWRENCE, D. P., ATKINS, M. B., POWDERLY, J. D., LEMING, P. D., LIPSON, E. J., PUZANOV, I., SMITH, D. C., TAUBE, J. M., WIGGINTON, J. M., KOLLIA, G. D., GUPTA, A., PARDOLL, D. M., SOSMAN, J. A. & HODI, F. S. 2014. Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. *J Clin Oncol,* 32**,** 1020-30.

TSENG, W., LEONG, X. & ENGLEMAN, E. 2007. Orthotopic mouse model of colorectal cancer. *J Vis Exp***,** 484.

TUMEH, P. C., HARVIEW, C. L., YEARLEY, J. H., SHINTAKU, I. P., TAYLOR, E. J., ROBERT, L., CHMIELOWSKI, B., SPASIC, M., HENRY, G., CIOBANU, V., WEST, A. N., CARMONA, M., KIVORK, C., SEJA, E., CHERRY, G., GUTIERREZ, A. J., GROGAN, T. R., MATEUS, C., TOMASIC, G., GLASPY, J. A., EMERSON, R. O., ROBINS, H., PIERCE, R. H., ELASHOFF, D. A., ROBERT, C. & RIBAS, A. 2014. PD-1 blockade induces responses by inhibiting adaptive immune resistance. *Nature,* 515**,** 568-71.

TURLEY, S. J., CREMASCO, V. & ASTARITA, J. L. 2015. Immunological hallmarks of stromal cells in the tumour microenvironment. *Nat Rev Immunol,* 15**,** 669-82.

UCHIBORI, R., TSUKAHARA, T., MIZUGUCHI, H., SAGA, Y., URABE, M., MIZUKAMI, H., KUME, A. & OZAWA, K. 2013. NF-kappaB activity regulates mesenchymal stem cell accumulation at tumor sites. *Cancer Res,* 73**,** 364-72.

UHLEN, M., FAGERBERG, L., HALLSTROM, B. M., LINDSKOG, C., OKSVOLD, P., MARDINOGLU, A., SIVERTSSON, A., KAMPF, C., SJOSTEDT, E., ASPLUND, A., OLSSON, I., EDLUND, K., LUNDBERG, E., NAVANI, S., SZIGYARTO, C. A., ODEBERG, J., DJUREINOVIC, D., TAKANEN, J. O., HOBER, S., ALM, T., EDQVIST, P. H., BERLING, H., TEGEL, H., MULDER, J., ROCKBERG, J., NILSSON, P., SCHWENK, J. M., HAMSTEN, M., VON FEILITZEN, K., FORSBERG, M., PERSSON, L., JOHANSSON, F., ZWAHLEN, M., VON HEIJNE, G., NIELSEN, J. & PONTEN, F. 2015. Proteomics. Tissue-based map of the human proteome. *Science,* 347**,** 1260419.

VAN DER BRUGGEN, T., CALDENHOVEN, E., KANTERS, D., COFFER, P., RAAIJMAKERS, J. A., LAMMERS, J. W. & KOENDERMAN, L. 1995. Interleukin-5 signaling in human eosinophils involves JAK2 tyrosine kinase and Stat1 alpha. *Blood,* 85**,** 1442-8.

VEGRAN, F., BERGER, H., BOIDOT, R., MIGNOT, G., BRUCHARD, M., DOSSET, M., CHALMIN, F., REBE, C., DERANGERE, V., RYFFEL, B., KATO, M., PREVOST-

BLONDEL, A., GHIRINGHELLI, F. & APETOH, L. 2014. The transcription factor IRF1 dictates the IL-21-dependent anticancer functions of TH9 cells. *Nat Immunol,* 15**,** 758-66.

VELLINGA, T. T., DEN UIL, S., RINKES, I. H., MARVIN, D., PONSIOEN, B., ALVAREZ-VARELA, A., FATRAI, S., SCHEELE, C., ZWIJNENBURG, D. A., SNIPPERT, H., VERMEULEN, L., MEDEMA, J. P., STOCKMANN, H. B., KOSTER, J., FIJNEMAN, R. J., DE ROOIJ, J. & KRANENBURG, O. 2016. Collagen-rich stroma in aggressive colon tumors induces mesenchymal gene expression and tumor cell invasion. *Oncogene,* 35**,** 5263-5271.

VIGO, T., PROCACCINI, C., FERRARA, G., BARANZINI, S., OKSENBERG, J. R., MATARESE, G., DIASPRO, A., KERLERO DE ROSBO, N. & UCCELLI, A. 2017. IFN-gamma orchestrates mesenchymal stem cell plasticity through the signal transducer and activator of transcription 1 and 3 and mammalian target of rapamycin pathways. *J Allergy Clin Immunol,* 139**,** 1667-1676.

VIVIER, E., UGOLINI, S., BLAISE, D., CHABANNON, C. & BROSSAY, L. 2012. Targeting natural killer cells and natural killer T cells in cancer. *Nat Rev Immunol,* 12**,** 239-52.

WALLACH, D., KANG, T. B. & KOVALENKO, A. 2014. Concepts of tissue injury and cell death in inflammation: a historical perspective. *Nat Rev Immunol,* 14**,** 51-9.

WANG, B., LI, Q., QIN, L., ZHAO, S., WANG, J. & CHEN, X. 2011. Transition of tumor-associated macrophages from MHC class II(hi) to MHC class II(low) mediates tumor progression in mice. *BMC Immunol,* 12**,** 43.

WANG, C., THUDIUM, K. B., HAN, M., WANG, X. T., HUANG, H., FEINGERSH, D., GARCIA, C., WU, Y., KUHNE, M., SRINIVASAN, M., SINGH, S., WONG, S., GARNER, N., LEBLANC, H., BUNCH, R. T., BLANSET, D., SELBY, M. J. & KORMAN, A. J. 2014a. In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human primates. *Cancer Immunol Res,* 2**,** 846-56.

WANG, D. & DUBOIS, R. N. 2010. The role of COX-2 in intestinal inflammation and colorectal cancer. *Oncogene,* 29**,** 781-8.

WANG, I. M., LIN, H., GOLDMAN, S. J. & KOBAYASHI, M. 2004. STAT-1 is activated by IL-4 and IL-13 in multiple cell types. *Mol Immunol,* 41**,** 873-84.

WANG, X., TENG, F., KONG, L. & YU, J. 2016. PD-L1 expression in human cancers and its association with clinical outcomes. *Onco Targets Ther,* 9**,** 5023-39.

WANG, Y., CHEN, X., CAO, W. & SHI, Y. 2014b. Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nat Immunol,* 15**,** 1009-1016.

WEBER, C. E. & KUO, P. C. 2012. The tumor microenvironment. *Surg Oncol,* 21**,** 172-7.

WEI, F., ZHONG, S., MA, Z., KONG, H., MEDVEC, A., AHMED, R., FREEMAN, G. J., KROGSGAARD, M. & RILEY, J. L. 2013. Strength of PD-1 signaling differentially affects T-cell effector functions. *Proc Natl Acad Sci U S A,* 110**,** E2480-9.

WEISS, L., ABDUL-HAI, A., OR, R., AMIR, G. & POLLIACK, A. 2003. Fludarabine in combination with cyclophosphamide decreases incidence of GVHD and maintains effective graft-versus-leukemia effect after allogeneic stem cell transplantation in murine lymphocytic leukemia. *Bone Marrow Transplant,* 31**,** 11-5.

WEST, N. P., DATTANI, M., MCSHANE, P., HUTCHINS, G., GRABSCH, J., MUELLER, W., TREANOR, D., QUIRKE, P. & GRABSCH, H. 2010. The proportion of

tumour cells is an independent predictor for survival in colorectal cancer patients. *Br J Cancer,* 102**,** 1519-23.
WONG, C. K., WANG, C. B., IP, W. K., TIAN, Y. P. & LAM, C. W. 2005. Role of p38 MAPK and NF-kB for chemokine release in coculture of human eosinophils and bronchial epithelial cells. *Clin Exp Immunol,* 139**,** 90-100.
WONG, M. & FISH, E. N. 1998. RANTES and MIP-1alpha activate stats in T cells. *J Biol Chem,* 273**,** 309-14.
WRIGHT, H. L., MOOTS, R. J., BUCKNALL, R. C. & EDWARDS, S. W. 2010. Neutrophil function in inflammation and inflammatory diseases. *Rheumatology (Oxford),* 49**,** 1618-31.
WU, H., YUAN, TSENG, LIN, YEH Durable response to programmed death-1 (PD-1) blockade in a metastatic gastric cancer patient with mismatch repair deficiency and microsatellite instability. Journal of Cancer Research and Practice, 2016.
XIN, H., KANEHIRA, M., MIZUGUCHI, H., HAYAKAWA, T., KIKUCHI, T., NUKIWA, T. & SAIJO, Y. 2007. Targeted delivery of CX3CL1 to multiple lung tumors by mesenchymal stem cells. *Stem Cells,* 25**,** 1618-26.
XIN, H., SUN, R., KANEHIRA, M., TAKAHATA, T., ITOH, J., MIZUGUCHI, H. & SAIJO, Y. 2009. Intratracheal delivery of CX3CL1-expressing mesenchymal stem cells to multiple lung tumors. *Mol Med,* 15**,** 321-7.
YEN, B. L., YEN, M. L., HSU, P. J., LIU, K. J., WANG, C. J., BAI, C. H. & SYTWU, H. K. 2013. Multipotent human mesenchymal stromal cells mediate expansion of myeloid-derived suppressor cells via hepatocyte growth factor/c-met and STAT3. *Stem Cell Reports,* 1**,** 139-51.
YLIKOSKI, E., LUND, R., KYLANIEMI, M., FILEN, S., KILPELAINEN, M., SAVOLAINEN, J. & LAHESMAA, R. 2005. IL-12 up-regulates T-bet independently of IFN-gamma in human CD4+ T cells. *Eur J Immunol,* 35**,** 3297-306.
YU, H., PARDOLL, D. & JOVE, R. 2009. STATs in cancer inflammation and immunity: a leading role for STAT3. *Nat Rev Cancer,* 9**,** 798-809.
ZAPPASODI, R., DE BRAUD, F. & DI NICOLA, M. 2015. Lymphoma Immunotherapy: Current Status. *Front Immunol,* 6**,** 448.
ZHANG, J. Y., LI, Y., REN, Y. F. & SHEN, B. F. 2003. Comparison of IL-6-induced STAT1 activation in different responsive cells. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi,* 19**,** 106-8.
ZHANG, Q. Z., SU, W. R., SHI, S. H., WILDER-SMITH, P., XIANG, A. P., WONG, A., NGUYEN, A. L., KWON, C. W. & LE, A. D. 2010. Human gingiva-derived mesenchymal stem cells elicit polarization of m2 macrophages and enhance cutaneous wound healing. *Stem Cells,* 28**,** 1856-68.
ZHANG, X., ZHU, Q., YUAN, X., QIAN, H. & XU, W. 2014. Mesenchymal stem cells in cancer: a new link to neutrophils. *Cancer Cell & Microenvironment,* 1.
ZHANG, Y., TANG, H., CAI, J., ZHANG, T., GUO, J., FENG, D. & WANG, Z. 2011. Ovarian cancer-associated fibroblasts contribute to epithelial ovarian carcinoma metastasis by promoting angiogenesis, lymphangiogenesis and tumor cell invasion. *Cancer Lett,* 303**,** 47-55.
ZHOU, A., SCOGGIN, S., GAYNOR, R. B. & WILLIAMS, N. S. 2003. Identification of NF-kappa B-regulated genes induced by TNFalpha utilizing expression profiling and RNA interference. *Oncogene,* 22**,** 2054-64.
ZUK, P. A., ZHU, M., ASHJIAN, P., DE UGARTE, D. A., HUANG, J. I., MIZUNO, H., ALFONSO, Z. C., FRASER, J. K., BENHAIM, P. & HEDRICK, M. H. 2002. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell,* 13**,** 4279-95.