

Dissecting the inflammatory response in melanoma

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Abstract: Dissecting the inflammatory response in melanoma
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While the immune system can control tumour growth, regulatory mechanisms, necessary in preventing immunopathology & autoimmunity, contribute to tumour progression impeding elimination of immunogenic tumours such as melanoma. To identify how the inflammatory response can influence tumour biology & be targeted in immunotherapy it is necessary to understand how expression of inflammatory molecules involved in regulatory mechanisms is controlled, which aspects of the inflammatory response are associated with accumulation of immune-regulatory populations & how these impact on frequency & activity of cells involved in tumour control.

Tumour-associated macrophages & regulatory T cells possess key tumour-promoting functions, including production of the suppressive cytokine, interleukin 10 (IL-10). I have analysed IL-10 regulation in myeloid cells by disrupting a putative enhancer.

The tumour inflammatory environment can influence neutrophil biology, leading to accumulation of low-density granulocytes, suggested to have immunosuppressive functions. I investigated the nature & frequency of low-density granulocytes in melanoma & their correlation with the inflammatory response & disease outcome. I identified low-density cells with an activated phenotype & cells with an immature phenotype enriched in the peripheral blood of stage IV melanoma patients. Marked inter-patient variation in the frequency of these subsets that correlated with accumulation of distinct cytokines, suggested the existence of distinct inflammatory axes. Analysis of patients undergoing anti-CTLA-4 treatment revealed that a lower frequency of activated neutrophils before treatment & an increase in frequency during treatment were associated with better response to anti-CTLA-4.

To establish whether accumulation of distinct neutrophil subsets was linked to wider inflammatory changes, the frequency of other immune cell subsets was determined. An enrichment of immature neutrophils was associated with Th2 cell enrichment, whereas an enrichment of activated neutrophils associated with inflammatory monocytes & activated TCR- $\gamma\delta$ T cells, further supporting the existence of distinct inflammatory axes. The frequency of activated neutrophils negatively correlated with CCR4⁺ CXCR3⁺ CD8⁺ effector memory T cells, perhaps reflecting greater tumour infiltration of this population. Given the association of this subset with a neutrophil population linked to anti-CTLA-4 response, the importance of CD8⁺ T cells in anti-tumour responses, & the fact this population had not previously been described, I attempted further characterisation, as well as characterisation of intratumoural CD8⁺ T cells.

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Abbreviations

ADAM 17	A disintegrin and metalloprotease 17
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADM	Adrenomedullin
APC	Antigen-presenting cell
Arg-1	Arginase 1
ATAC-seq	Assay for Transposase-accessible Chromatin with high-throughput sequencing
ATK	Protein kinase B
bFGF	Basic fibroblast growth factor
BTLA	B and T lymphocyte attenuator
C/EBP β	CCAAT/enhancer binding protein- β
CAR	Chimeric antigen receptor
CD137L	CD137 Ligand
CD40L	CD40 ligand
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
ChIP	Chromatin immunoprecipitation
CLA	Cutaneous lymphocyte antigen
CMV	Cytomegalovirus
CR3	Complement receptor 3
CREB	cAMP-responsive-element-binding protein
CRISPR	Clustered regularly-interspaced short palindromic repeats
CT	Cancer-testis
CTLA-4	Cytotoxic T lymphocyte-associated molecule-4
DC	Dendritic cell
DC-SIGN	DC-specific ICAM3-grabbing non-integrin
DMSO	Dimethyl sulfoxide
DNase-seq	DNase-sensitivity assay with high-throughput sequencing
dNTP	Deoxynucleotide triphosphate
DSB	Double-stranded DNA break
DTT	Dithiothreitol
EBV	Epstein-Barr virus
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced green fluorescent protein
ELISA	Enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EOMES	Eomesodermin
ERK	Extracellular signal-regulated kinase
ETS	E26 transcription-specific
FAIRE	Formaldehyde-assisted isolation of regulatory elements
FBS	Foetal bovine serum
FDA	Food and Drug Administration
fMLP	formyl-methionyl-leucyl-phenylalanine
FoxP3	Forkhead box P3
FPR2	Formyl peptide receptor 2
G-CSF	Granulocyte-colony stimulating factor
GAL9	Galectin 9
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GATA-3	GATA binding protein 3

gDNA	Genomic DNA
GEO	Gene Expression Omnibus
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GnT-V	β 1,6-N-acetylglucosaminyltransferase V
gp100	Glycoprotein 100
grMDSC	Granulocytic myeloid-derived suppressor cells
H3K27ac	Acetylation of Histone 3 on Lysine 27
H3K27me	Methylation of Histone 3 on Lysine 27
H3K4me3	Trimethylation of Histone 3 on Lysine 4
HCC	Hepatocellular carcinoma
HDM	House dust mite
HGF	Hepatocyte growth factor
HHV8	Human herpes virus 8
HLA-DR	Human Leukocyte Antigen (D-related antigen)
HMGB1	High mobility group box 1
HPRT	Hypoxanthine guanine phosphoribosyltransferase
HPV	Human papillomavirus
HRP	Horseradish peroxidase
HVEM	Herpes virus entry mediator
ICAM3	Intercellular adhesion molecule 3
ICOS	Inducible T-cell co-stimulator
ICOS-L	ICOS-ligand
IDO	Indoleamine 2, 3-dioxygenase
IFN- α	Interferon alpha
IFN- β	Interferon beta
IFN- γ	Interferon gamma
IL-10	Interleukin-10
IL-1R	IL-1 Receptor
IL-1ra	Interleukin 1 receptor antagonist
Indels	Insertions/deletions
iNKT	Invariant natural killer T
iNOS	Inducible nitric oxide synthase
IP-10	IFN- γ -inducible protein 10
irAEs	Immune-related adverse effects
IU	International Unit
KIR	Killer immunoglobulin-like receptor
KO	Knock-out
LAG3	Lymphocyte activation gene 3
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
M-CSF	Macrophage colony-stimulating factor
MAGE	Melanoma-associated antigen
MAPK	Mitogen-activated protein kinase
MART-1	Melanoma antigen recognized by T cells-1
MD	Melanoma differentiation
MET	Mesenchymal epithelial transition factor
MHC	Major histocompatibility complex
MICA/B	Major histocompatibility complex class I-related chain A and B
MIP-1 α/β	Macrophage inflammatory protein-1 alpha/beta
MMP	Matrix metalloproteinase
moMDSC	Monocytic myeloid-derived suppressor cells

mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary-response protein 88
NFIL	Nuclear factor, interleukin 3 regulated
NFκB	Nuclear factor-kappaB
NHEJ	Non-homologous DNA end joining
NICE	National Institute for Healthy and Care Excellence
NK	Natural Killer
NO	Nitric oxide
NOD2	Nucleotide-binding oligomerisation domain 2
NY-ESO-1	New York-esophageal cancer-1
OPD	o-Phenylenediamine dihydrochloride
ORB	Oxford Radcliffe Biobank
OX40L	OX40 Ligand
PAM	Protospacer-adjacent motif
PamCSK	Palmitoyl-3-cysteine-serine-lysine-4
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PD-1	Programmed cell death-1
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PDGF	Platelet-derived growth factor
PGE ₂	Prostaglandin E ₂
PHA	Phytohaemagglutinin
PI	Protease inhibitors
PI3K	Phosphoinositide 3-kinase
PMA	Phorbol 12-myristate 13-acetate
pMHC	peptide-Major Histocompatibility Complex
PMN	Polymorphonuclear leukocyte/neutrophil
PMSF	Phenylmethanesulfonyl fluoride
PPIB	Peptidylprolyl Isomerase B
PRDX5	Peroxiredoxin 5
PRR	Pattern recognition receptor
pTEFb	Positive transcription elongation factor b
PWM	Position weight matrix
qPCR	Quantitative polymerase chain reaction
RA	Rheumatoid arthritis
RAGE-1	Receptor for advanced glycation end-products
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
RECIST	Response Evaluation Criteria in Solid Tumours
RNS	Reactive nitrogen species
RORγT	Retinoid-related orphan receptor gamma T
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Reverse Transcription
SAA	Serum amyloid A
SCF	Stem cell factor
SDS	Sodium dodecyl sulphate
Siglec	Sialic acid-binding immunoglobulin-like lectin
SMAD4	Mothers against decapentaplegic homologue 4
SNP	Single nucleotide polymorphism
SoJIA	Systemic onset juvenile idiopathic arthritis
Sp1	Specific protein 1

SSX2	Synovial sarcoma
STAT	Signal transducer and activator of transcription
SYK	Spleen tyrosine kinase
T-bet	T-box transcription factor
TAA	Tumour-associated antigen
TAM	Tumour-associated macrophage
TB	Tuberculosis
Tc	Cytotoxic T cells
Tcm	Central memory T cell
TCR	T cell receptor
TEM	Tie2-expressing monocytes
Tem	Effector memory T cell
TEMRA	Terminally differentiated effector memory RA
TGF- β	Transforming growth factor beta
T _H	Helper T cells
TIM3	T cell membrane protein 3
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor alpha
Tregs	Regulatory T cells
TRIF	Toll-IL-1R homology domain-containing adapter-inducing IFN- β
TRP2	Tyrosinase-related protein 2
T _{SCM}	Stem cell memory T cells
TSS	Transcription start site
UCSC	University of California Santa Cruz
ULBPs	UL16-binding proteins
UV	Ultra-violet
VD3	Vitamin D3
VEGF	Vascular endothelial growth factor
VZV	Varicella zoster virus
α -GalCer	alpha-galactosylceramide
α -MEM	Minimum essential medium alpha

Chapter 1: Introduction

Solid tumours consist not only of cancerous cells themselves, but also of stromal cells that play an important role in determining tumour growth or regression. Stromal cells, such as endothelial cells that constitute blood vessels, supply tumours with nutrients required for growth as well offering a means by which cancerous cells may metastasise (Watnick, 2012). Fibroblasts, the main producers of extracellular matrix (ECM) components, may also act to promote tumour metastasis *via* production of pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and ECM-degrading enzymes, known as matrix metalloproteinases (MMPs) (Kalluri and Zeisberg, 2006). Another component of the tumour stroma is cells of the immune system and this introduction will focus on the role of different aspects of the immune system in controlling or promoting tumour growth.

Evidence that the immune system is able to control tumour growth comes from numerous immunodeficient mouse strains. There is increased development of spontaneous and chemically induced tumours in mice lacking B and T lymphocytes and increased incidence of lymphomas in mice lacking perforin, an important component of natural killer (NK) and cytotoxic T cell function (Shankaran et al., 2001) (Smyth et al., 2000). Furthermore, there is increased incidence of numerous types of cancer among transplant patients which is thought to be due in part to reduced tumour immune-surveillance during immunosuppression, although numerous cancers such as Kaposi Sarcoma and cervical cancer, likely result from a failure of the immune system to control oncogenic viral infections such as human herpes virus 8 (HHV8) and human papillomavirus (HPV) (Chapman et al., 2013) (Swann and Smyth, 2007).

The powerful nature of the immune response, critical in elimination of pathogens and cancerous cells, necessitates complex regulation in order to prevent immunopathology and autoimmunity. While the immune system can control tumour growth, there is increasing evidence that such regulatory

mechanisms contribute to the tumour-promoting function of immune cells. As well as focussing on a specific aspect of immune regulation, in the form of expression of the immunoregulatory cytokine interleukin 10 (IL-10), in this introduction I will discuss more generally the different aspects of the immune response to cancer and methods by which immune cells may control or promote tumour progression.

1.1 Neutrophils

Although neutrophils are best known for their role in anti-microbial immune responses, they have been shown to play beneficial or detrimental roles in a variety of inflammatory conditions and diseases.

Different studies have investigated the role of neutrophils in cancer, describing how these cells can have either anti-tumour or pro-tumour properties.

With regards to anti-tumour properties, it has been shown that recruitment of neutrophils, able to kill cancerous cells through nitric oxide (NO) production, is associated with decreased tumour growth, in models including melanoma, fibrosarcoma and hepatocellular carcinoma (Finisguerra et al., 2015). Recruitment of anti-tumour neutrophils in these models was dependent on upregulation of the mesenchymal epithelial transition factor (MET) gene in neutrophils, enabling migration into hepatocyte growth factor (HGF)-expressing tumours.

There is also evidence that neutrophils have the capacity to kill cancer cells by antibody-dependent cell-mediated cytotoxicity (ADCC). This is important in preventing relapse of skin tumours in mice treated with the topical chemotherapy compound, Ingerol-3-Angelate (Challacombe et al., 2006).

Despite the capacity of these cells to kill cancer cells, evidence exists that neutrophils can also promote tumour growth. It has been shown by numerous studies that an association exists between the degree of neutrophil infiltration in

tumours and disease prognosis; greater tumour infiltration is associated with poorer prognosis in a variety of cancers including melanoma (Antonio et al., 2015), renal cell carcinoma (Jensen et al., 2009) and colorectal cancer (CRC) (Rao et al., 2012). Indeed, of the 22 tumour-infiltrating leukocyte populations analysed in a recent study of 25 different malignancies, increased tumour infiltration by neutrophils was the most significant adverse prognostic factor (Gentles et al., 2015). Such an association is likely linked to the numerous pro-tumour properties of neutrophils. These include the ability of neutrophils to promote proliferation of cancerous cells, angiogenesis and metastasis, and the ability of neutrophils to suppress T cell responses.

In a zebrafish model of melanoma, it has been revealed that when neutrophils are recruited to the vicinity of pre-neoplastic cells through wounding, pre-neoplastic cells are able to attract neutrophils away from the wound (Antonio et al., 2015). Recruitment of neutrophils to such cells results in enhanced proliferation of pre-neoplastic cells, thought in part to be a consequence of neutrophil production of prostaglandin E₂ (PGE₂). Not only does this suggest that neutrophils can directly promote proliferation of pre-neoplastic cells but it also highlights the potential negative impact that surgery may have on any cancerous cells left behind.

As well as enhancing proliferation, neutrophils have been shown to promote metastasis. In a mouse model of melanoma, keratinocytes damaged by exposure to ultra-violet (UV) light, release a molecule known as high mobility group box 1 (HMGB1) (Bald et al., 2014). This molecule recruits neutrophils to the site of damage *via* Toll-like receptor 4 (TLR-4) signalling. Although repeated exposure of melanoma tumours to UV-light did not affect the growth of primary tumours, repeated exposure increased migration of tumour cells towards vascular endothelium and increased the number of lung metastases (Bald et al., 2014). As the number of lung metastases is reduced in TLR-4 knockout mice (TLR4^{-/-}) and in mice depleted of neutrophils (*via* anti-Ly6G antibodies), this implicates neutrophils in driving metastasis. Further experiments suggest a role for the pro-inflammatory cytokine, tumour necrosis factor alpha (TNF- α), in inducing

melanoma cell migration and melanoma-endothelial cell interaction, ultimately contributing to enhanced metastasis (Bald et al., 2014).

Furthermore, neutrophils have been shown to promote the process of angiogenesis. In a model of pancreatic cancer, neutrophils expressing MMP-9 localise in pancreatic lesions and their depletion is associated with a reduction in angiogenic switching of pre-malignant lesions (Nozawa et al., 2006). This is thought to be a result of MMP-9's ability to increase the availability of a proangiogenic factor, VEGF, which is sequestered in the ECM (Nozawa et al., 2006) (Belotti et al., 2003).

Neutrophils have also been implicated in tumour progression *via* their ability to suppress T cell responses. In a mouse model of breast cancer, $\gamma\delta$ T cell-derived IL-17 induces granulocyte-colony stimulating factor (G-CSF) expression which leads to the expansion of neutrophils with immature properties that are capable of suppressing CD8⁺ T cell responses (Coffelt et al., 2015). In this model, CD8⁺ T cell suppression results in increased metastasis in tumour-bearing mice. This suppression is thought to result from neutrophil expression of inducible nitric oxide synthase (iNOS), an enzyme that catalyses the synthesis of NO.

The notion that iNOS/NO can promote tumour progression somewhat contradicts the anti-tumour properties of tumour-infiltrating MET-expressing neutrophils that are able to kill cancer cells. It is possible that the action of this one enzyme is not the sole determinant of whether a neutrophil promotes or prevents tumour progression, but it may be that several factors influence whether overall a neutrophil promotes progression or is protective. The findings may also be dependent on the type of cancer studied and the model used to study that disease. It seems evident however that neutrophils play a diverse role in modulating different aspects of cancer and immune cell biology, that may promote cancer progression or regression.

Many of the functions of neutrophils in modulating cancer have been uncovered in animal models, in part due to the ability to follow tumour development *in vivo*

at an early stage. However, neutrophils are also relevant to human disease. As mentioned previously, the degree of tumour neutrophil infiltration is associated with poorer disease prognosis. Furthermore, neutrophil populations with immunosuppressive properties have been identified in patients with a variety of cancers. These cells have been termed granulocytic myeloid-derived suppressor cells (grMDSC).

1.2 Myeloid-derived suppressor cells (MDSCs)

Myeloid-derived suppressor cell (MDSC) is the term used to describe cells making up a heterogeneous population of immature myeloid cells, with a granulocytic or monocytic phenotype, that are expanded in patients with cancer and inflammatory conditions (Khaled et al., 2013). In mice, grMDSC can be defined as CD11b⁺ Ly6G⁺ Ly6C^{low} whereas monocytic MDSC (moMDSC) can be defined as CD11b⁺ Ly6G⁻ Ly6C^{hi}. Humans lack the Gr-1 analogue however myeloid cells with suppressive properties that are expanded in cancer and inflammation have been suggested to be CD11b⁺ CD15⁺ CD66b⁺ CD14⁻ (grMDSC) and CD11b⁺ CD14⁺ HLA-DR^{low/-} (moMDSC) (Khaled et al., 2013).

It should be noted that although grMDSC are frequently cited as immature cells that have failed to mature into differentiated neutrophils, the phenotype of suppressive granulocytic cells in patients with cancer is debated, and this is discussed further in section 3.1.

The accumulation of moMDSC and grMDSC in patients with cancer, including melanoma, is thought to contribute to tumour growth *via* their capacity to inhibit anti-tumour T cell responses (Filipazzi et al., 2007) (Schilling et al., 2013) (Poschke et al., 2010). Immunosuppressive mechanisms described in the literature are summarised in table 1.

Table 1: MDSCs suppress T cell responses by a variety of mechanisms

Abbreviations: ARG-1: Arginase-1, iNOS: inducible nitric oxide synthase, NO: nitric oxide, ROS: reactive oxygen species, CCR5: chemokine receptor type 5, MMP-9: matrix metalloproteinase-9, Tregs: Regulatory T cells, VEGF: vascular endothelial growth factor, TGF- β : transforming growth factor beta, TCR: T cell receptor, p-MHC: peptide-Major Histocompatibility Complex, FoxP3: forkhead box P3, IL-2: Interleukin 2, IFN- γ : Interferon gamma.

Characteristic	Suppressive mechanism	Reference
Up-regulation of ARG-1	Depletion of L-arginine by hydrolysis into L-ornithine and urea, thereby reducing T cell proliferation and function by preventing upregulation of cell cycle regulators and by down-regulating expression of CD3- ζ chain	Poschke et al., 2010 Schilling et al., 2013 Rodriguez et al., 2009
Production of NO and ROS	Upregulation of iNOS results in increased NO production. NO can suppress T cell proliferation and induce T cell apoptosis. NAPDH oxidase catalyses the conversion of oxygen to superoxide. Reaction of superoxide anions with NO results in the generation of peroxynitrite. Peroxynitrite can inhibit antigen-specific T cell responses by inducing nitration of tyrosine residues within the TCR-CD8 complex, disrupting binding to p-MHC (Nagaraj et al., 2007)	Schlecker et al., 2012 Poschke et al., 2010
Expression of CCL3, CCL4 and CCL5	Recruitment of Tregs <i>via</i> CCR5	Schlecker et al., 2012
Expression of MMP-9	MMP-9 increases bioavailability of VEGF, promoting angiogenesis	Yang et al., 2004
Production of TGF- β	Suppression of T cell proliferation. TGF- β can suppress proliferation by inhibiting cell cycle regulators and IL-2 production. It can inhibit IFN- γ production by inhibiting T-bet expression and can induce differentiation of Tregs by induction of the FoxP3 transcription factor (Yoshimura and Moto, 2011)	Filipazzi et al., 2007

1.3 Monocytes

Monocytic MDSC make up one of several monocytic subsets. The major monocytic subset consists of classical monocytes, defined by their cell surface phenotype of CD14⁺⁺ CD16⁻. Monocytes may also express the FcγRIII receptor, CD16 with CD16-expressing monocytes further subdivided into intermediate CD14⁺⁺ CD16⁺ and non-classical CD14⁺ CD16⁺⁺ monocytes (Wong et al., 2012). Although the process of monocyte development in humans is not completely understood, evidence from mice suggests that classical monocytes arise from bone marrow precursors, which upon entry into the circulation, can further differentiate into intermediate and patrolling monocytes (Heine et al., 2012).

Although able to migrate into tissues and differentiate into macrophages and dendritic cells (DC), monocytes themselves play an important role in immune defence against infectious agents and cancerous cells. Their capacity for phagocytosis (enriched in the classical population), their capacity for antigen processing and presentation (enriched in the intermediate population) and their ability to produce proinflammatory cytokines such as TNF-α (enriched in CD16⁺ cells) are important properties that may contribute to control of tumour growth (Wong et al., 2012) (Zawada et al., 2011). However, like neutrophils and other components of the immune system, monocytes may also act to promote tumour progression. A subset of intermediate monocytes, expressing the angiopoietin receptor, Tie2 (Tie2-expressing monocytes (TEM)), has been shown to promote tumour angiogenesis (De Palma et al., 2005) (Zawada et al., 2011). Furthermore, monocytes (particularly the classical subset) are a major source of IL-10, a cytokine with suppressive properties that is important in regulating immune responses (Wong et al., 2011).

1.4 IL-10 and its role in regulation of the inflammatory response

Upon exposure to pathogens, the immune system mounts a rapid and powerful response to protect the host from infection. If left unchecked, this response would cause host damage, and potentially death (Hedrich & Bream 2010). Thus

activation of the immune response not only drives production of pro-inflammatory cytokines and anti-microbial mediators, but also induces mechanisms that dampen down the immune response. IL-10 is a key anti-inflammatory cytokine, important in preventing immunopathology and autoimmunity (Saraiva & O'Garra, 2010). Its importance has been highlighted in IL-10 deficient mouse models. IL-10 knock-out (KO) mice, when infected with the protozoan, *Toxoplasma gondii*, show raised concentrations of pro-inflammatory cytokines, such as interleukin 12 (IL-12) and interferon gamma (IFN- γ), and die as a result of severe immunopathology (Gazzinelli et al., 1996). It has also been shown that mice lacking IL-10 are unable to control immune responses in the gut, and develop chronic enterocolitis (Kuhn et al., 1993).

Data from models such as these has been corroborated in human studies. Polymorphisms in the IL-10 gene have been associated with variations in IL-10 expression and have been linked to autoimmune and inflammatory conditions. For example, single nucleotide polymorphisms (SNPs) in the IL-10 promoter and intronic regions, associated with low IL-10 expression, have been linked to the inflammatory conditions systemic onset juvenile idiopathic arthritis (SoJIA) and Behçet's Disease (Moeller et al., 2010) (Remmers et al., 2010).

1.4.1 How does IL-10 regulate inflammation?

IL-10 acts on a range of cell types to exert its immunosuppressive function. It prevents the maturation of antigen-presenting cells (APCs) by down regulating the expression of MHC class II and costimulatory molecules and thus indirectly inhibits T cell activation (de Waal Malefyt et al., 1991) (Buelens et al., 1995). It can also affect T cell activation in a direct manner, by inhibiting signalling *via* CD28, and consequently inhibiting T cell proliferation and production of both T_H1 and T_H2 cytokines (Joss et al., 2000). IL-10 has also been shown to inhibit mast cells and eosinophils, and may therefore be important in regulating allergic disease (Royer et al., 2001) (Takanaski et al., 1994).

While IL-10 has key immunosuppressive properties, it is a pleiotropic cytokine, known to enhance the function of certain immune cells. For example, it has the capacity to promote proliferation and differentiation of B cells as well as to stimulate IL-10 production by T cells, promoting a positive feedback mechanism for IL-10 production (Kobayashi et al., 2002) (Barrat et al., 2002). Of interest in terms of tumour immunity is the fact IL-10 has been shown to stimulate NK cells and CD8⁺ T cells (Chen and Zlotnik, 1991) (Cai et al., 1999). IL-10 can increase the production of IFN- γ by NK cells in combination with IL-18, and in the presence of IL-2, IL-10 can increase the proliferation and cytotoxicity of CD8⁺ T cells (Cai et al., 1999) (Chen and Zlotnik, 1999). In keeping with this, IL-10 has been shown to enhance tumour infiltration of CD8⁺ T cells and increase IFN- γ and granzyme expression, with IL-10 treatment in certain mouse models associated with reduced tumour burden (Mumm et al., 2011).

IL-10 not only acts on, but is also produced by a range of cells. As well as monocytes, other innate cells such as macrophages, NK cells and myeloid DCs, and cells of the adaptive arm of the immune system, including CD4⁺ T cells and B cells, can produce IL-10 (Hedrich & Bream, 2010). More recently, De Santo et al. (2010) have shown that human neutrophils are also capable of IL-10 production.

1.4.2 Regulation of IL-10 expression

Regulation of IL-10 expression is complex, in part because it is expressed by a range of immune cells, but also because it is produced in response to a variety of stimuli. In innate cells, such as monocytes and myeloid DCs, IL-10 is induced upon activation of a variety of pattern recognition receptors (PRR), such as Toll-like receptors (TLR 2, 3, 4, 7 and 9) (Netea et al., 2004) (Boonstra et al., 2006), DC-specific ICAM3-grabbing non-integrin (DC-SIGN) (Geijtenbeek et al., 2003), Dectin-1 (Rogers et al., 2005) and nucleotide-binding oligomerisation domain 2 (NOD2) (Bansal & Balaji, 2011). It may also be induced in innate cells *via* PRR-independent mechanisms. Neutrophils produce IL-10 in response to stimulation *via* formyl peptide receptor 2 (FPR2) (De Santo et al., 2010) and macrophages and myeloid DCs *via* CD40 signalling (Boonstra et al., 2006). Signalling *via* CD40

has also been implicated in IL-10 production by B cells (Fillatreau et al., 2002), as has TLR 9 (Sun et al., 2005). IL-10 may also be induced in non-regulatory T cells including T_H1 and T_H2 cells in response to signalling *via* the T cell receptor (TCR); T_H1 cells requiring a strong TCR signal and IL-12 for induction of this cytokine (Saraiva et al., 2009).

As a consequence of the wide variety of cell types that can produce IL-10 in response to stimulation of a range of receptors, numerous signalling pathways that converge on a range of transcription factors have been implicated in regulation of the IL-10 gene. I am particularly interested in the regulation of the IL-10 gene in monocytic cells and signalling pathways involving the adapter proteins myeloid differentiation primary-response protein 88 (MyD88) and Toll/IL-1R homology domain-containing adapter-inducing IFN- β (TRIF) are important in induction of this gene (Saraiva & O'Garra, 2010). MyD88-dependent induction involves downstream activation of extracellular signal-regulated kinase (ERK), p38 and nuclear factor- κ B (NF κ B) pathways and examples of other signalling molecules and transcription factors involved in IL-10 induction in monocytes/macrophages as well as other cell types, are shown in figure 1 (Saraiva & O'Garra, 2010) (Chang et al., 2007).

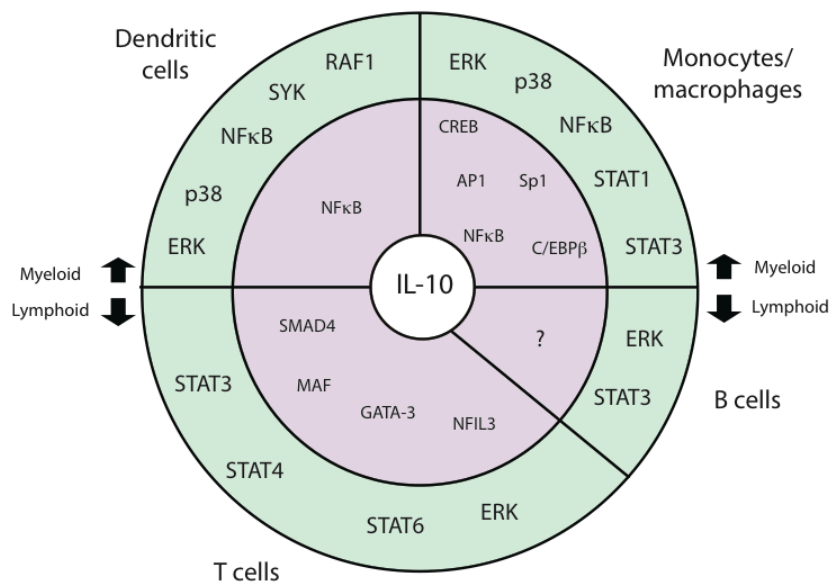


Figure 1: Regulation of IL-10 expression involves a range of signalling molecules and transcription factors in different cell types. A selection of signalling molecules and transcription factors involved in regulating IL-10 expression in myeloid and lymphoid cells are shown. Signalling molecules are in the outer green circle and transcription factors in the inner grey circle. Abbreviations - SYK: Spleen tyrosine kinase, Sp1: Specific protein 1, CREB: cAMP-responsive-element-binding protein, C/EBPβ: CCAAT/enhancer binding protein-β, GATA-3: GATA binding protein 3, SMAD4: Mothers against decapentaplegic homologue 4, STAT: signal transducer and activator of transcription, NFIL3: Nuclear factor, interleukin 3 regulated. This figure was adapted from Saraiva and O'Garra, 2010. The following references were also used: Iyer et al., 2010, Brightbill et al., 2000, Motomura et al., 2011, Huss et al., 2012.

Myeloid cells such as monocytes are a particularly important source of IL-10 during infection and inflammation. As mentioned, monocytes are not the only cells capable of IL-10 production, and production of this cytokine is an important property of tumour-promoting tumour-associated macrophages (TAMs) and regulatory T cells (Tregs).

1.5 Tumour-associated macrophages (TAMs)

Macrophages within the tumour microenvironment are referred to as tumour-associated macrophages (TAMs) and these cells can derive from circulating monocytes that differentiate into macrophages upon entry into the tumour, or from tissue-resident macrophages that migrate into the tumour (Chanmee et al., 2014). The macrophage population of phagocytic cells is functionally diverse. Classically activated macrophages (activated in the presence of TLR agonists such as lipopolysaccharide (LPS), and IFN- γ) acquire an M1 phenotype, characterised by high expression of IL-12 and low expression of IL-10, and are important in immunity against intracellular pathogens (Sica and Mantovani, 2012). Alternatively activated macrophages (activated in response to IL-4 and IL-13) acquire an M2 phenotype, characterised by high expression of IL-10 and low expression of IL-12, and are important in immunity against parasites as well as in wound healing (Sica and Mantovani, 2012).

Given the diversity in macrophage function, macrophages within the tumour microenvironment may contribute to control or promotion of tumour progression *via* numerous mechanisms.

Macrophages may contribute to control of tumour growth *via* presentation of tumour-associated antigens (with M1-like macrophages known to express high amounts of MHC class I and II), production of cytotoxic molecules such as NO, reactive nitrogen species (RNS) and TNF- α that are capable of killing cancer cells, as well as production of proinflammatory cytokines, including IL-12, important in activating an anti-tumour type 1 T cell response (discussed further in 1.6) (Rahat and Hemmerlein, 2013) (Keller et al., 1990) (Sica and Mantovani, 2012) (Chanmee et al., 2014).

There is evidence, at least in certain mouse models of cancer, that early in tumour development macrophages exhibit an M1-like phenotype that switches to an M2-like phenotype later in tumour development (Zaynagetdinov et al., 2011). Furthermore, there is evidence that the presence of tumour-associated M2 macrophages is associated with worse outcomes, and of the 22 leukocyte populations analysed in 25 different malignancies by Gentles et al. (2015), M2

macrophages within tumours were one of the most important adverse prognostic factors.

As well as reduced anti-tumour functions (such as reduced expression of MHC molecules), cells with an M2-like phenotype can contribute to tumour growth *via* numerous mechanisms. They are able to induce the growth of new blood vessels through production of pro-angiogenic factors including VEGF (Lewis et al., 2000). There is also evidence that macrophages within melanoma produce adrenomedullin (ADM), which not only stimulates angiogenesis *via* phosphorylating endothelial nitric oxide synthase (eNOS) but also promotes M2-like macrophage polarisation in an autocrine manner (Chen et al., 2011).

Solid tumours are often characterised by regions of hypoxia and there is evidence of TAM accumulation in these areas (Lewis et al., 2000). Furthermore, hypoxia can enhance the tumour-promoting capacity of TAMs, including induction of both VEGF and ADM expression (Lewis et al., 2000) (Chen et al., 2000) (Lewis and Murdoch, 2005). Hypoxia can also induce expression of enzymes, including MMP-7, that degrade ECM components and contribute to cancer cell migration and metastasis (Burke et al., 2003).

Macrophages have also been implicated in survival of cancerous cells at metastatic sites, promoting survival in response to tissue factor (TF)-dependent recruitment (Gil-Bernabé et al., 2012). Another important feature of TAMs is their ability to suppress anti-cancer immune responses. Their immunosuppressive activity includes production of the cytokines TGF- β and IL-10, the properties of which have been previously discussed (Chanmee et al., 2014). They may also recruit CCR4-expressing Tregs *via* production of the CCR4 ligand, CCL22 (the suppressive functions of this cell type are discussed later) (Curiel et al., 2004).

1.6 T cells

1.6.1 T cell recognition of cancerous cells

As mentioned at the start of the introduction, deficiencies in lymphocytes and cytotoxic function are associated with increased incidence of numerous types of cancer. While B cells and NK cells play an important anti-tumour role, I will focus specifically on the T cell response to cancer. Conventional CD4⁺ and CD8⁺ T cells play a significant role in anti-tumour immunity. CD8⁺ T cells are particularly important given their ability to directly recognise tumour-associated antigens (TAAs) presented on MHC class I on the surface of tumour cells, and to induce tumour cell apoptosis *via* the perforin/granzyme pathway (Breart et al., 2008). They may also contribute to tumour control through production of cytokines such as IFN- γ , recruiting other immune cells to the tumour. CD4⁺ T cells on the other hand recognise peptides presented on MHC class II, and as such can recognise TAAs taken up and processed by antigen-presenting cells such as DCs and macrophages. As well as providing help to B cells, CD4⁺ T cells are key in promoting CD8⁺ T cell activation and proliferation as well as generation of CD8⁺ memory cells (Lai et al., 2011). They can provide this help *via* CD40-CD40L interaction. The interaction of CD40L on the surface of activated CD4⁺ T cells with CD40 on the surface of APCs results in licensing of the APC and upregulation of costimulatory molecules such as CD80 and CD86, increased expression of IL-12 and an increased capacity for antigen-presentation (Schoenberger et al., 1998) (Nesbeth et al., 2010). This licensing has been associated with priming of anti-tumour CD8⁺ T cell responses (Nesbeth et al., 2010).

1.6.2 TAAs

Melanoma-associated antigens, and indeed TAAs from other types of cancer, fall into distinct categories. Focussing specifically on previously described HLA-A2 epitopes, there are three major classes of known melanoma-associated antigens (Kvistborg et al., 2012). These include melanoma differentiation (MD) antigens, such as MART-1/Melan-A (important in regulating pigmentation and melanosome structure (Hoashi et al., 2005)), cancer-testis (CT) antigens, expressed by a range of human tumours but only normally expressed in the testis and placenta, such as the MAGE family and NY-ESO-1 (MAGE-A1 for

example is thought to repress expression of genes required for differentiation during spermatogenesis (Fratta et al., 2011)), and overexpressed (OE) antigens, such as Meloe-1 and survivin (survivin with a known anti-apoptotic role, considered important in protecting cells in the skin from undergoing UV-induced apoptosis (Dallaglio et al., 2012)).

T cells can also recognise mutated antigens. Such mutations may arise in response to a failure of DNA repair mechanisms (such as with BRCA1/2 mutations), upon exposure to UV-light in the context of melanoma, and upon exposure to polycyclic hydrocarbons in cigarette smoke in the case of lung cancer (Yoshida and Miki, 2004) (Champiat et al., 2014). If presented on the surface of cancerous cells through interaction with MHC class I, epitopes from mutated antigens can elicit T cell responses (Champiat et al., 2014). Furthermore, there is evidence that greater mutational burden is associated with a higher response to treatment with immune-checkpoint inhibitors (such inhibitors are discussed in greater depth in section 1.6.4 but function by promoting the activation and effector functions of T cells). Immune-checkpoint inhibitors show higher efficacy when treating patients with tumours such as melanoma (with a median of 13.2 somatic mutations per megabase (Mb)) and non-small cell lung cancer (NSCLC) (median of 8.17 and 6.43 mutations per Mb for squamous and non-squamous respectively) than in treating patients with tumours of low mutational burden such as colorectal cancer (CRC) (median of 3.2 mutations per Mb) and prostate cancer (median of 0.73 mutations per Mb) (Champiat et al., 2014) (Topalian et al., 2012) (Brahmer et al., 2012). Furthermore comparison of NSCLC in patients who smoke/previously smoked compared to those who have never smoked reveals greater mutational burden in current/former smokers (median of 10.5 mutations per Mb compared to 0.6 in non-smokers) with a better response to immune-checkpoint inhibitors in current/former smokers (Champiat et al., 2014) (Herbst et al., 2014). It is also true that there is an association between long-term clinical benefit of immune-checkpoint inhibitors and mutational burden in melanoma (Snyder et al., 2014). It is thought that mutations promote immunogenicity and increase the repertoire of T cells capable of recognising tumour cells (Champiat et al., 2014).

1.6.3 CD4+ and CD8+ T cell subsets and function

Several subsets of CD4+ and CD8+ T cell exist which are defined by a distinct set of functional properties. The environment in which a naive T cell recognises its cognate antigen determines its subsequent differentiation/polarisation. In an environment rich in IL-12 and IFN- γ (such as during a viral infection in which an APC produces IL-12 and natural killer cells may act as a source of IFN- γ), CD4+ T cells will be polarised towards a T_H1 phenotype, and CD8+ T cells towards a Tc1 phenotype (Annunziato et al., 2015). T_H1-derived cytokines, such as IFN- γ , promote the activation of M1 macrophages and activation and expansion of Tc1 cells and higher intratumoural expression of T_H1 genes is associated with longer disease-free survival (Sica and Mantovani, 2012) (Tosolini et al., 2011) (Annunziato et al., 2015). Tc1 cells also produce IFN- γ and possess cytotoxic function, killing *via* the perforin/granzyme and Fas-Fas ligand pathways (Carter and Dutton, 1995).

T cell activation in the presence of IL-4 results in induction of a type 2 response, with differentiation of naïve T cells into T_H2 and Tc2 cells (Seder et al., 1992). Such cells are capable of IL-4, IL-5 and IL-13 production, important in inducing IgE production from B cells and in activating mast cells and eosinophils (Annunziato et al., 2015). Tc2 cells also possess cytotoxic function, but are restricted to the perforin/granzyme pathway and have been suggested to be less cytotoxic than Tc1 cells (Carter and Dutton, 1995) (Annunziato et al., 2015). Skewing of the immune system towards a type 2 response has been described in patients with melanoma, with a reduction in the concentration of type 1-polarising cytokines associated with relapse during IFN- α immunotherapy (Lauerova et al., 2002). Furthermore, and something which may also be relevant in a tumour setting, an association between MDSC accumulation and T_H2 cell polarisation has been observed in other types of inflammation (Delano et al., 2007) (Jeisy-Scott et al., 2011). In the context of sepsis, moMDSC have been shown to drive T_H2 polarisation and this may be another mechanism by which

MDSCs contribute to tumour progression, by reducing the number of anti-tumour T_H1 cells (Delano et al., 2007).

Activation of naïve T cells in an environment rich in IL-6, TGF- β , IL-1 β , IL-21 and IL-22 can result in the differentiation of T_H17 and Tc17 cells with the capacity to produce, among other factors, IL-17 (Annunziato et al., 2015). These cells, implicated in autoimmunity, have a conflicting role in tumour control. There is evidence suggesting a role for these cells in progression of cancers including prostate cancer and hepatocellular carcinoma (HCC) (Derhovanessian et al., 2009) (Kuang et al., 2010). However, T_H17 and Tc17 cells may be protective in human ovarian and lung cancer as well as in mouse models of melanoma (Kryczek et al., 2009) (Ye et al., 2010) (Muranski et al., 2008) (Garcia-Hernandez et al., 2010). In the context of melanoma, adoptive transfer of *in vitro*-generated tumour-specific T_H17 resulted in significant tumour regression and greater survival, compared to adoptive transfer of T_H1 or naïve T cells (Muranski et al., 2008). However, this anti-tumour effect was predominantly mediated by IFN- γ rather than IL-17 production. Indeed a subset of T_H17 cells (referred to as T_H17.1) has been identified that can produce both IL-17 and IFN- γ (Annunziato et al., 2007).

Another subset of T cells, Tregs, can be induced from CD4⁺ T cells in the presence of TGF- β and IL-2 (Fu et al., 2004) (Lazarevic et al., 2013). These cells are important in regulating immune responses and can function through production of IL-10 and TGF- β , the suppressive mechanisms of which have been previously discussed, as well as by inducing expression of indoleamine 2, 3-dioxygenase (IDO) in APCs (Fallarino et al., 2003). IDO is a tryptophan-catabolising enzyme and as tryptophan is essential for T cell proliferation, its depletion by Treg-conditioned APCs suppresses T cell responses (Intlekofer and Thompson, 2013). Indeed there is evidence that tumour-infiltration of Tregs is a negative prognostic factor in numerous types of cancer, including melanoma (Shang et al., 2015).

While I have mainly focussed on conventional $\alpha\beta$ CD4⁺ and CD8⁺ T cells that

recognise peptide antigens, it is important to note that other T cell subsets are capable of recognising tumour-associated antigens or skewing immune responses and therefore play an important role in tumour immunity. Such subsets include TCR- $\gamma\delta$ T cells and invariant natural killer T cells (iNKT) cells. Unlike conventional T cells, with a TCR composed of α and β chains that recognises peptide antigens, TCR- $\gamma\delta$ T cells possess a TCR composed of γ and δ chains (Pietschmann et al., 2009). In humans two subsets can be identified in the peripheral blood - V γ 9V δ 2 T cells and V δ 1 (paired with various V γ genes) T cells. $\gamma\delta$ T cells possess anti-tumour properties, such as the ability to lyse tumour cells *via* the perforin/granzyme pathway and to produce numerous pro-inflammatory cytokines (including IFN- γ) upon recognition of phosphoantigens, including a naturally occurring metabolite isopentenyl pyrophosphate (IPP) which can accumulate in cancer cells, as well as upon recognition of stress-inducible MHC class-1 related molecules (MICA/B and ULBPs) by the receptor NKG2D (Wrobel et al., 2007) (Toro et al., 2003) (Maccalli et al., 2007)(Silva-Santos et al., 2015). However, they may also possess pro-tumour functionality, such as the ability to induce the expansion of suppressive neutrophils *via* production of IL-17 as has been described in a mouse model of breast cancer (Coffelt et al. 2015) (Silva-Santos et al. 2015). iNKT cells in humans recognise lipid antigens presented on the MHC class I-like molecule, CD1d, and can influence the anti-tumour immune response *via* their ability to produce a range of cytokines, to mature antigen-presenting cells, and to directly kill cancer cells *via* perforin-mediated cytotoxicity (McEwen-Smith et al., 2015).

1.6.4 Promoting the anti-cancer immune response with immune-checkpoint inhibitors

Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) is an important regulator of T cell responses. In order to induce activation, proliferation and differentiation of effector T cells, a naïve T cell must recognise peptide-MHC *via* its TCR, in the presence of co-stimulation (Intlekofer and Thompson, 2013). In the presence of inflammatory signals, DCs upregulate cell surface expression of the B7

molecules, CD80 and CD86, and provide such co-stimulation (Mahnke et al., 2002). Recognition of peptide-MHC on immature DCs expressing low amounts of co-stimulatory molecules, can lead to anergy or apoptosis, which is important in inducing peripheral tolerance to self-reactive T cells (Mahnke et al., 2002).

CD80/CD86 are recognised by CD28 on the surface of the T cell, and TCR signalling in the presence of co-stimulatory signals drives expansion of activated T cells in part by activation of the phosphoinositide 3-kinase (PI3K) signalling pathway. This pathway is important for glucose uptake and production of IL-2 (Intlekofer and Thompson, 2013). Not only does CTLA-4 compete with CD28 for binding of CD80/CD86, it also sends inhibitory signals to the T cell. It inhibits protein kinase B (Akt), a downstream component of the PI3K pathway, inhibiting key processes, such as glucose uptake, that are necessary for activation and expansion (Intlekofer and Thompson, 2013) (Pardoll 2012).

The importance of CTLA-4 in regulating T cell proliferation and activation was revealed in CTLA-4 knockout mice. These mice develop severe lymphoproliferative disease with accumulation of activated lymphocytes in the spleen and lymph nodes as well as organs such as the heart, pancreas and liver (Waterhouse et al., 1995) (Tivol et al., 1995). Such accumulation is so severe that mice die by 3-4 weeks of age (Tivol et al., 1995).

CTLA-4 also plays a key role in the function of regulatory T cells (Tregs). Tregs constitutively express CTLA-4 and numerous suppressive functions are dependent on the expression of this molecule (Wing et al., 2008). For example, induction of IDO can occur *via* CTLA-4 signalling, with blocking of CTLA-4 reducing IDO expression (Fallarino et al., 2003) (Hryniewicz et al., 2006).

Not only can Tregs drive the expression of IDO, but they can also reduce the stimulatory capacity of APCs by downmodulating their expression of the co-stimulatory molecules, CD80 and CD86 (Oderup et al., 2006). Tregs do this by trans-endocytosis, whereby CTLA-4 on the surface of Tregs binds and captures B7 molecules on the surface of APCs, with subsequent degradation of B7

molecules within Tregs. This results in APCs with impaired stimulatory capacity however this can be prevented if APCs and Tregs are co-cultured in the presence of an anti-CTLA-4 antibody (Qureshi et al., 2011).

Therefore inhibiting CTLA-4 with blocking antibodies not only promotes effector T cell activation and proliferation by preventing inhibitory signalling to T cells *via* CTLA-4, and promoting CD28-mediated signalling, but also inhibits certain suppressive properties of Tregs. There is also evidence that anti-CTLA-4 may function *via* the action CD14⁺ CD16⁺⁺ non-classical ('patrolling') monocytes. CD16 is also known as FcγRIII and binds to the Fc region of antibodies, enabling cells expressing this receptor to kill antibody-coated cells, in the absence of phagocytosis. Although more well known as a mechanism employed by NK cells, other immune cells including monocytes can perform this process (Elavazhagan et al., 2015). It has been revealed that non-classical monocytes, in the presence of anti-CTLA-4, are capable of lysing Tregs *via* this mechanism (Romano et al., 2015). Furthermore, melanoma patients responding to ipilimumab have decreased Treg tumour infiltration after four cycles of treatment compared to baseline, possibly a result of non-classical monocyte-mediated Treg depletion (Romano et al., 2015).

Mounting an effective immune response requires a careful balance between co-stimulatory signals and inhibitory signals in order to prevent damage to self (Pardoll 2012). However, given the role of immune checkpoints, such as CTLA-4, as negative regulators of immunity, it was rationalised that inhibiting such regulators could promote immune responses, including anti-tumour immune responses. This ultimately led to the development of blocking antibodies against CTLA-4 for testing in humans.

In a phase III clinical trial involving 676 metastatic melanoma patients, ipilimumab, the anti-CTLA-4 antibody produced by Bristol-Myers Squibb, improved overall survival when compared to a gp100 vaccine alone (Hodi et al., 2010). Median overall survival in the ipilimumab-only treated group was 10.1 months and in the ipilimumab plus gp100 vaccine group 10 months, compared

to 6.4 months in the gp100 vaccine-only group. Ipilimumab was also shown to improve survival in a phase III clinical trial involving 502 patients with previously untreated and unresectable stage III and stage IV melanoma. In this trial, ipilimumab, when used in combination with dacarbazine, improved survival (11.2 months) compared to dacarbazine alone (9.1 months) (Robert et al., 2011).

It was in 2011 that the U.S. Food and Drug Administration (FDA) approved the use of ipilimumab for unresectable or metastatic melanoma and later in 2012 that it was approved by the National Institute for Health and Care Excellence (NICE) for previously-treatment advanced melanoma.

Analysis of the T cell compartment in patients treated with ipilimumab, has revealed that blocking CTLA-4 can lead to an increase in the absolute lymphocyte count, with upregulation of proliferation and activation markers on CD4+ and CD8+ T cells following treatment (Wang et al., 2012). With regards to the effects on tumour-specific T cell responses, analysis of 145 HLA-A2-associated melanoma epitopes before and after ipilimumab treatment has revealed that blocking CTLA-4 leads to a significant induction of newly detected CD8+ T cell responses, while rarely increasing the pre-existing response, suggesting that ipilimumab functions to broaden the T cell response and that priming of T cell responses is an important mechanism by which this drug works (Kvistborg et al., 2014).

Although complete responses have been observed with ipilimumab, such events are incredibly rare. While anti-CTLA-4 functions to promote the expansion of T cells, including tumour-specific T cells, the powerful nature of the T cell response (evidenced by around 20% patients suffering grade 3-5 immune-related adverse events (irAEs) during ipilimumab treatment) necessitates tight regulation in order to prevent immunopathology. Such regulation is complex and does not merely rely on balancing signalling *via* CD28 and CTLA-4. Numerous co-stimulatory and inhibitory interactions are important in regulating T cell responses and a selection are displayed in figure 2.

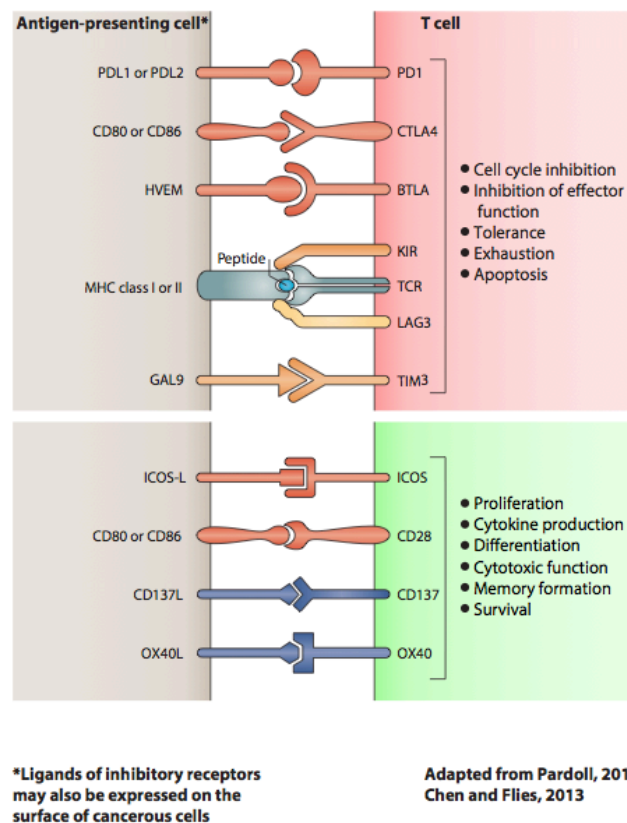


Figure 2: Regulation of T cell activation and function. T cell activation not only depends on recognition of peptide-MHC but also on the balance of numerous co-stimulatory and inhibitory signals. Co-stimulatory receptors include ICOS, CD28, CD137 and OX40. Inhibitory receptors include CTLA-4, PD-1, BTLA, KIR, LAG3 and TIM3. Abbreviations: PD-L1 and 2: Programmed death-ligand 1 and 2, HVEM: Herpes virus entry mediator, GAL9: Galectin 9, ICOS: Inducible T-cell co-stimulator, ICOS-L: ICOS-ligand, CD137L: CD137-ligand, OX40L: OX40-ligand, MHC: Major histocompatibility complex, PD-1: Programmed cell death-1, BTLA: B and T lymphocyte attenuator KIR: Killer immunoglobulin-like receptor, TCR: T cell receptor, LAG3: Lymphocyte activation gene 3, TIM3: T cell membrane protein 3.

Of note is the inhibitory receptor PD-1. PD-1 is also upregulated on activated T cells and functions *via* interaction with its ligands programmed death-ligand 1 (PD-L1) and PD-L2 (Intlekofer and Thompson, 2013). Although PD-L1 and PD-L2 are expressed by APCs, PD-L1 is also expressed on non-haematopoietic tissue including epithelial cells, and can be further induced in the presence of inflammatory cytokines such as TNF- α (Intlekofer and Thompson, 2013).

Furthermore PD-L1 and PD-L2 can be expressed by cancerous tissue, including melanoma. In contrast to an acute infection in which there is resolution of infection and generation of memory T cells, chronic stimulation as seen in a tumour setting, results in T cell exhaustion, often a failure to generate memory cells, and is associated with high expression of PD-1 and T cell dysfunction (Wherry et al., 2007). Based on the expression pattern of PD-1 ligands, it is thought that, in contrast to CTLA-4 which is important in regulating the early stages of T cell activation, PD-1 plays a major role in negatively regulating T cell effector function in peripheral tissues (Intlekofer and Thompson, 2013) (Pardoll 2012).

Nivolumab and pembrolizumab, developed by Bristol-Myers Squibb and Merck respectively, are blocking antibodies against PD-1 and have shown efficacy in cancer patients, including those with melanoma. Both antibodies were initially approved by the FDA in 2014 for use in melanoma patients that no longer respond to other available drugs. Survival benefit of anti-PD-1 treatment over chemotherapy in untreated melanoma patients was shown in a phase III clinical trial in which one year overall survival was 72.9% in patients treated with nivolumab, compared to 42.1% in patients treated with dacarbazine (Robert et al., 2015i). Efficacy of nivolumab was also tested in patients who had previously progressed on ipilimumab. Patients were treated with either nivolumab or chemotherapy, with a greater percentage of objective responses observed in the nivolumab-treated group (31.7% compared to 10.6% with chemotherapy) (Weber et al., 2015). When treatment with either ipilimumab or pembrolizumab was compared, six month progression-free survival was greater in pembrolizumab-treated patients (47.3%) compared to ipilimumab-treated patients (26.5%) (Robert et al., 2015ii). Furthermore, fewer adverse events were observed with PD-1 blockade, with grade 3-5 irAEs in 13% pembrolizumab-treated patients compared to 20% ipilimumab-treated patients. The response to a combination of anti-PD-1 and anti-CTLA-4 treatment has also been analysed in comparison to monotherapy with either blocking antibody. In untreated patients (with unresectable stage III or stage IV melanoma), those who received a combination of nivolumab and ipilimumab had median progression-free survival

of 11.5 months compared to 6.9 months in the nivolumab-only group and 2.9 months in the ipilimumab-only group (Larkin et al., 2015).

1.7. Summary and aims

While the immune system can control tumour growth, there is increasing evidence that regulatory mechanisms, necessary in preventing immunopathology and autoimmunity, contribute to tumour progression and result in a failure to eliminate immunogenic tumours such as melanoma. To identify how the inflammatory response can influence tumour biology and be targeted in immunotherapy, it is necessary to understand how expression of inflammatory molecules involved in these regulatory mechanisms is controlled, which aspects of the inflammatory response are associated with accumulation of immune-regulatory populations and how these inflammatory responses impact on the frequency and activity of cells involved in tumour control. It has been shown that the tumour inflammatory environment can influence neutrophil biology, leading to accumulation of low-density granulocytes, suggested to have immunosuppressive and tumour-promoting functions. In this thesis I investigate the nature and frequency of low-density granulocytes in melanoma and their correlation with the inflammatory response and response to immune checkpoint inhibitor treatment. I look at how changes in neutrophil biology are associated with wider changes in the inflammatory profile of patients, looking at changes in frequency and activation of monocyte and T cell subsets. I also focus more specifically on a particular aspect of immune regulation by investigating the role of a putative enhancer in regulating expression of the gene encoding IL-10.

Chapter 2: Materials and Methods

2.1 Isolation of human peripheral blood mononuclear cells (PBMCs)

Peripheral blood was collected from patients with stage IV melanoma and healthy controls. All blood samples obtained were in accordance with the Oxford Radcliffe Biobank (ORB) ethical approval (REC reference 09/H0606/5). A summary of the patients and healthy donors in which neutrophil frequency, T cell frequency or plasma concentration of cytokines/growth factors were determined is shown in table 1.

Table 1: Patient and healthy donor characteristics

Variable	Melanoma Patients	Healthy Donors
Number	54	10
Mean age (years)	63	60
Age range (years)	41-85	45-72
Males (%)	68	60
Females (%)	32	40
M stage : M1a (%)	9	-
M1b (%)	13	-
M1c (%)	78	-
Last previous treatment*: None (%)	56	-
Chemotherapy (%)	6	-
BRAF/MEK/pan-RAF inhibitor (%)	12	-
Immunotherapy (%)	20	-
Other (%)	6	-
Multiple previous treatments (%)	18	-

* Systemic

Blood was collected at least one month after previous therapy ended. Blood was diluted 1:2 in RPMI-1640 medium (Sigma-Aldrich). Diluted blood was applied to Lymphoprep (Axis-Shield) solution and subject to density gradient centrifugation at 2000rpm for 30mins. Following centrifugation, peripheral blood mononuclear cells (PBMCs) were isolated from the interface using a

Pasteur pipette and washed in RPMI. Plasma (5ml) was collected from each patient and healthy control and stored at -80°C.

2.2 Isolation of human neutrophils (PMNs)

Human PMNs were collected from on top of the erythrocyte layer and contaminating erythrocytes were lysed in RBC Lysis Buffer (Qiagen) for 15mins at 4°C. Pellet was washed in ice cold RPMI-1640 medium.

2.3 Staining of bone marrow cells

Frozen bone marrow samples from leukaemia patients in remission were kindly provided by Bilyana Stoilova (Vyas lab, WIMM, Oxford). Samples were thawed, subjected to density gradient centrifugation, filtered to remove dead neutrophils and stained for flow cytometry (see below).

2.4 Isolation of immune cells from tumours

Cells were isolated by Jane Maraka (Cerundolo lab, WIMM, Oxford) using the Tumour Dissociation Kit (Miltenyi).

2.5 Flow cytometry staining

2.5.1 Viability and surface staining

Typically 0.5×10^6 – 1×10^6 cells were stained in a 96 well round-bottom plate. Cells were washed once in phosphate-buffered saline (PBS) (Lonza) and incubated with 10µl LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life technologies) (diluted 1:50 in PBS) for 15 minutes at room temperature. Cells were then washed once in FACS buffer (PBS supplemented with 10% foetal bovine serum (FBS) (Sigma)) before addition of 20µl of antibody cocktail. Cocktails were made using the antibodies in table 2. Surface antibodies were diluted in FACS buffer and cells were incubated at 4°C for 20 minutes. Cells were washed twice in FACS buffer at 1500rpm for five minutes and resuspended in FACS buffer. If samples could not be analysed immediately, they were resuspended in IC fixation buffer (eBioscience) diluted 1:2 in PBS for up to three days.

2.5.2 Intracellular staining

Cells were first stained with viability dye and surface antibodies, and then washed in FACS buffer as above. Cells were then fixed in 100µl IC fixation buffer for 30 minutes at 4°C. Following fixation, wells were topped up with 100µl PBS and centrifuged at 1700rpm for three minutes. Cells were then washed in 1X Permeabilization Buffer (made from dilution of 10X Permeabilization Buffer (eBioscience) with distilled water). Antibodies against intracellular antigens (table 2) were diluted in 1X Permeabilization buffer and cells incubated with cocktail (20µl/well) for 30 minutes on ice. Cells were then washed twice in 1X Permeabilization Buffer before resuspension in FACS buffer.

2.5.3 Nuclear staining

For nuclear staining, the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used. Following viability and surface antibody staining, and subsequent washes in FACS buffer, cells were pulse vortexed before being resuspended in 200µl Foxp3 Fixation/Permeabilization working solution (made by diluting one part Foxp3 Fixation/Permeabilization Concentrate with three parts Foxp3 Fixation/Permeabilization Diluent). Samples were incubated at room temperature for 30 minutes before centrifugation at 300 x g for five minutes. Cells were then washed twice in 1x Permeabilization Buffer (made by diluting 10X Permeabilization Buffer in distilled water) at 300g for five minutes. Antibodies against nuclear antigens (table 2) were diluted in 1X Permeabilization Buffer and cells were incubated with the cocktail (20µl/well) for 30 minutes at room temperature. Samples were then washed twice in 1X Permeabilization buffer for five minutes at 300 x g and then resuspended in FACS buffer. All samples were protected from light during staining and until acquisition.

2.5.4 Human flow cytometry antibodies

Table 2: Antibodies used for flow cytometry

Specificity	Fluorophore	Clone	Supplier	Volume per well (µl)
Myeloid panel				
CD62L	FITC	DREG-56	eBioscience	1
CD15	PECy7	W6D3	Biolegend	0.2
CD14	BV605	M5E2	Biolegend	0.2
CD11b	PerCPCy5.5	ICRF44	Biolegend	0.2
CD10	BV421	HI10a	Biolegend	0.2
CD33	PE	WM-53	eBioscience	0.2
HLA-DR	APC-cy7	L243	Biolegend	0.2
CD16	APC	eBioCB16	eBioscience	0.1
CXCR1	FITC	8F1/CXCR1	Biolegend	1
CXCR2	APC	5E8/CXCR2	Biolegend	1
CXCR4	PE	12G5	Biolegend	1
T cell panels				
CD8	PerCP-Cy5.5	HIT8a	Biolegend	0.5
CD8	FITC	HIT8a	BD Biosciences	1
CD161	APC	eBioCB16	eBioscience	0.5
CD161	PerCP-Cy5.5	HP-3G10	Biolegend	2
CD4	AF700	OKT4	Biolegend	0.4
HLA-DR	APC-Cy7	L243	Biolegend	0.2
TCR γδ	BV421	B1	Biolegend	2
CD3	BV650	SK7	BD Biosciences	0.5
CD38	BV786	HIT2	BD Biosciences	0.5
CD14	BV510	M5E2	Biolegend	0.5
CD19	BV510	6D5	Biolegend	0.5
PD-1	PE-eFluor 610	eBioJ105	eBioscience	0.2
α-GalCer CD1d tetramer	PE			0.5*
CLA	FITC	HECA-452	Biolegend	2
CXCR3	Alexa Fluor 647	G025H7	Biolegend	1
CCR7	APC-Cy7	G043H7	Biolegend	1
CCR7	BV785	G043H7	Biolegend	1
CD25	BV421	BC96	Biolegend	0.2
CD25	PerCP-Cy5.5	BC96	Biolegend	0.5
CCR4	BV605	L291H4	Biolegend	0.4
CD45RA	BV605	HI100	Biolegend	0.2
CD45RA	BV711	HI100	Biolegend	0.3
CCR6	PE-Cy7	G034E3	Biolegend	0.5
ICOS	PE	C398.4A	Biolegend	0.2
CXCR5	BV421	J252D4	Biolegend	0.5
CD62L	PE	DREG-56	eBioscience	0.5
EOMES	FITC	WD1928	eBioscience	2
FoxP3	Alexa Fluor 647	150D	Biolegend	2
FoxP3	PE-Cy7	PCH101	eBioscience	2
GATA-3	PE-Cy7	TWAI	eBioscience	1
T-bet	PE	eBio4B10	eBioscience	1
CD107a	FITC	H4A3	BD Biosciences	2.5
Perforin	PE	B-D48	Biolegend	2.5
IFN- γ	FITC	4S.B3	eBioscience	0.4
IFN- γ	BV786	4S.B3	BD Biosciences	1

IFN- γ	APC	4S.B3	eBioscience	0.1
TNF- α	BV650	MAb11	BD Biosciences	1
TNF- α	PE-Cy7	MAb11	Biologend	0.2
IL-4	PE-Cy7	8D4-8	eBioscience	0.5
TCR V α 7.2	PE	3C10	Biologend	0.25
HLA-A2	PE	BB7.2	Biologend	0.5
CD40L	PE	24-31	Biologend	1
CD45	PE-Cy7	HI30	BD Biosciences	1
Ki67	PE	Ki-67	Biologend	1
CD34+ Differentiation Panel				
CD45	APC-eFluor 780	HI30	eBioscience	0.5
CD15	PerCP	W6D3	Biologend	1
CD14	PE	HCD14	Biologend	0.2
CD19	PE-Cy7	HIB19	Biologend	0.5
CD56	APC	MEM-188	Biologend	1

* alpha-galactosylceramide (α -GalCer) CD1d tetramer-PE was kindly provided by D. Shepherd (Cerundolo Lab, WIMM, Oxford). In panels containing the α -GalCer tetramer-PE, cells were first stained with 0.5 μ l tetramer in 20 μ l PBS for 30 minutes at room temperature in the dark. Cells were washed in PBS and then stained with aqua followed by cell surface antibodies as described above.

2.5.5 Analysis of stained samples

Samples were analysed on LSR Fortessa and LSR Fortessa X20 (BD).

2.6 Analysis of plasma cytokine/chemokine concentrations

2.6.1 Serum Amyloid A (SAA) ELISA

The Human SAA ELISA Kit (Life Technologies) was used to determine SAA levels in plasma collected in Materials and Methods 2.1. The assay was performed as per protocol and using the provided reagents. The assay works within a range of 9ng/ml – 600ng/ml. Plasma samples were therefore diluted in the Standard Diluent Buffer, typically 1:1000 dilutions were used, but up to 1:12,000 dilutions were necessary for certain samples. Serial dilutions of the standards were prepared using Standard Diluent Buffer. 100 μ l Standard Diluent was added to blank wells and 100 μ l sample and 100 μ l standard were added to appropriate wells of the microtiter wells of the plate (pre-coated with anti-human SAA antibody). 50 μ l biotinylated Human SAA Biotin Conjugate solution was then added to each well and the plate incubated for two hours at room temperature. Wells were washed four times with the Wash Buffer Concentrate and the plate was tapped dry. 100 μ l Streptavidin-HRP Working Solution was added to each

sample and standard well and the plate was incubated for 30 minutes at room temperature prior to washing as above. 100µl Stabilized Chromogen was added to each well, the plate incubated for 30 minutes in the dark at room temperature before addition of 100µl Stop Solution to each well. The plate was read at 450nm. The concentration of SAA in the plasma samples was determined using the standard curve and corrected for the dilution factor.

2.6.2 Bio-Plex Assay

A 27-Plex Panel Bio-Plex assay (Bio-Rad - M50-0KCAF0Y) was used to determine the levels of 27 different cytokines/chemokines in plasma samples. The standard mix was diluted in standard diluent and incubated on ice for 30 minutes. Serial dilutions of the standard were then made using standard diluent. Meanwhile, the cytokine beads were diluted in assay buffer and added to each well of the provided 96 well plate. The beads were washed twice with wash buffer and the use of a magnetic hand-held plate washer. Standards and samples were added to appropriate wells of the plate, in duplicate. The plate was sealed and incubated in the dark for 30 mins at room temperature. The wells of the plate were then washed three times as above. The detection antibody mix was then diluted and added to each well, followed by incubation in the dark for 30 mins at room temperature. The plate was again washed three times prior to addition of Streptavidin-PE to each well. The plate was incubated for 10 minutes at room temperature in the dark. The plate was washed three times and wells resuspended in assay buffer before being run on the Bioplex 200 machine.

2.7 Differentiation of CD34+ progenitor cells

The protocol for differentiation of CD34+ progenitor cells co-cultured with MS5 stromal cells, into B cells, NK cells and myeloid cells was kindly provided by Dimitris Karamitros (Vyas lab, WIMM, Oxford).

2.7.1 Maintenance of MS5 cells

MS5 stromal cells, kindly provided by Z. Aboukhalil (Vyas lab, WIMM, Oxford), were maintained in α -MEM complete medium (α -MEM (Invitrogen)

supplemented with 10% Hyclone foetal bovine serum (FBS) (Thermo Scientific), 100 IU Penicillin, 0.1mg/ml Streptomycin (Sigma) and 2mM L-glutamine (Sigma)). Once MS5 cells reached 80% confluence, they were passaged. Cells were washed once in PBS before incubation in Trypsin-EDTA (Invitrogen) for four minutes at 37°C. The cells were then washed in α -MEM complete medium.

2.7.2 Setting up co-culture assay

MS5 stromal cell layers were prepared 24 hours before addition of CD34+ progenitor cells. Firstly, 24 well tissue culture plates were coated with 0.1% Gelatin (Sigma). After two hours incubation at room temperature, Gelatin was removed and 12,500 MS5 cells were added to each coated well in 500 μ l of α -MEM complete medium. The following day, 250 μ l of medium was removed from each well, human bone marrow CD34+ cells (Lonza) were thawed, washed and 100 bone marrow cells were resuspended in 250 μ l 2X concentrated in vitro co-culture assay medium. The final 1x assay medium contained α -MEM supplemented with 10% FBS, 100 IU Penicillin, 0.1mg/ml Streptomycin, 2mM L-glutamine, 20ng/ml stem cell factor (SCF) (PeproTech), 10ng/ml G-CSF (PeproTech), 10ng/ml Flt3-Ligand (PeproTech), IL-15 (PeproTech), 10ng/ml IL-2 (PeproTech) and 1×10^{-7} M Dup697 (Cayman Chemicals). Half of the medium was removed and replaced with fresh 2X concentrated in vitro co-culture assay medium on day 7 and 14. Cells were differentiated for 18 days before analysis by flow cytometry.

In addition to the original protocol, at the beginning of the differentiation process, certain wells were also supplemented with 1 μ g/ml or 0.1 μ g/ml recombinant human Apo-SAA1 (PeproTech), 1 μ g/ml or 0.1 μ g/ml palmitoyl-3-cysteine-serine-lysine-4 (PamCSK) (Invivogen) or plasma (diluted 1:4, 1:8 or 1:16) from stage IV melanoma patients. Subsequent feeds were with the defined 2X concentrated in vitro co-culture assay medium or 2x concentrated in vitro co-culture assay medium supplemented with the same initial concentration of recombinant human Apo-SAA1 or PamCSK.

2.7.3 Analysis of co-culture assay

On day 18, cells from each 24 well plate were detached via pipetting and transferred to FACS tubes. Cells were first stained with aqua followed by antibodies described in table 2.

2.8 Testing immunosuppressive capacity of neutrophil populations

2.8.1 Sorting

PBMC were purified as in 2.1. Cells were stained with a selection of antibodies mentioned in table 2, in order to sort for populations based on CD15, CD10 and CD33 expression. Prior to sorting, cells were passed through a 30µm Cell Tric (Sysmex) and kept on ice. Cells were sorted on an AriaFusion (BD Biosciences) using the 100µm nozzle. They were sorted directly into RH10 complete medium (RPMI (Sigma-Aldrich) supplemented with 10mM HEPES (Life Technologies), 1mM Sodium Pyruvate (Life Technologies), 100 IU Penicillin, 0.1mg/ml Streptomycin (Sigma), 1X MEM Non-essential Amino Acids (Life Technologies), 10% heat inactivated human serum (Corning), 50µM 2-mercaptoethanol (Life Technologies), 2mM L-glutamine (Sigma)). Following sorting cells were washed and resuspended in fresh medium. For comparison, mature neutrophils were also sorted.

2.8.2 Suppression assays

For assays in which DCs were used to stimulate T cell proliferation, DCs were generated in vitro from healthy donor monocytes. CD14⁺ monocytes were purified by MACS using anti-CD14 microbeads (Miltenyi). PBMC were first washed in ice cold MACS buffer (PBS supplemented with 5% human serum and 2mM EDTA (Life Technologies)) and then incubated with 50-200µl CD14 MicroBeads (1 part CD4 MicroBeads to 4 parts MACS buffer) for 15 minutes at 4°C. Cells were washed in MACS buffer before being resuspended in 4ml MACS buffer and passed through a 30µm Cell Tric. Cells were then applied to a pre-washed LS column (attached to the MACS separator magnet) (Miltenyi). Columns were washed three times with 3ml MACS buffer and cells were eluted from the column in 5ml MACS buffer using the provided plunger. Monocytes were washed in R10 (as RH10 but with 10% FBS instead of human serum) and

cultured in the presence of 50ng/ml GM-CSF (PeproTech) and 50ng/ml IL-4 (PeproTech) for 5 days and then stimulated overnight with 1µg/ml Lipopolysaccharide (LPS) (Sigma) to induce maturation.

T cells from a different healthy donor were isolated from PBMCs by MACS with either a pan T cell isolation kit or CD4 microbeads (both Miltenyi). Cells were washed twice in ice cold PBS, resuspended in 1ml room temperature PBS to which 1ml 1:10000 Vybrant CFDA SE (carboxyfluorescein succinimidyl ester (CFSE)) (Invitrogen) was added for 8 minutes at room temperature in the dark. One volume of RH10 was then added and cells washed, resuspended in RH10 and rested for at least 30 minutes prior to stimulation. T cells alone, T cells plus allogeneic DCs and T cells plus allogeneic DCs in addition to neutrophil populations were cultured for 3-5 days before staining of cells with aqua, CD4 and CD8 (table 2), acquisition on the flow cytometer and determination of CFSE dilution. Typically, 25,000 mature DCs were used to stimulate 50,000 labelled T cells.

For assays in which T cells were stimulated with anti-CD3/28 antibodies, wells of MaxiSorp plates (Nunc) were coated with 1µg/ml purified NA/LE mouse anti-human CD3 and 1µg/ml purified NA/LE mouse anti-human CD28 (both BD Pharmingen) and incubated overnight at 4°C. Wells were washed twice in PBS prior to addition of cells. T cells, sorted as above, were cultured in the presence or absence of neutrophil populations (as well as in wells without coating as a control) and CFSE dilution was analysed on day 3 post-stimulation.

2.9 Sorting of T cell populations

PBMC from healthy donors were purified (as previously described) from leukocyte cones (NHS Blood and Transplant). Alternatively, PBMCs from melanoma patients with stage IV disease or a single cell suspension of cells isolated from melanoma metastases (kindly provided by J. Maraka) that had previously been frozen, were thawed, washed and resuspended in RH10 complete medium. PBMC were rested for 1.5-3hours at 37°C and 5% CO₂. CD8⁺ T cells were then purified by positive selection using CD8 MicroBeads (Miltenyi) (using the protocol described above for CD14 positive selection). Cells were

stained with aqua followed by a selection of antibodies mentioned in table 2, in order to sort for populations based on differential expression of CCR4 and CXCR3. Prior to sorting, cells were passed through a 30µm Cell Tric and kept on ice. Cells were sorted on an AriaFusion (BD Biosciences) using the 100µm nozzle. They were sorted directly into RH10 complete medium for cells to be cultured, or into TRIzol LS (Life Technologies) for gene expression analysis. For RNA, cells were sorted into 750µl TRIzol LS. Cells were lysed by pipetting up and down multiple times and then snap frozen and stored at -80°C until RNA isolation.

2.10 RNA isolation

RNA was isolated from TRIzol LS as per the Life Technologies protocol. Briefly, samples were thawed and left at room temperature for five minutes. 200µl of chloroform (Sigma) was added to each sample and samples were shaken by hand for 15 seconds. Samples were then incubated at room temperature for three minutes before centrifugation for 15 minutes at 12,000 x g at 4°C. The aqueous phase was then removed and placed in a new tube. In order to precipitate the RNA, 500 µl 100% isopropanol (Sigma) was added to each aqueous phase, along with 10µg of GlycoBlue Coprecipitant (Life Technologies). Samples were incubated for ten minutes at room temperature before centrifugation at 12,000 x g for ten minutes at 4°C. The supernatant was removed from each tube and the RNA pellets washed with 1ml of 75% ethanol for five minutes at 7500 x g and 4°C. Pellets were air-dried and then resuspended in 25µl RNase-free water, heated to 60°C for ten minutes and then stored at -80°C until RNA analysis. RNA quality and quantity was determined using the Agilent Bioanalyser and RNA 6000 Nano Kit (Agilent) or the RNA 6000 Pico Kit (Agilent).

2.11 NanoString Gene Expression Analysis

100,000 of each sorted T cell population were cultured in RH10 complete medium supplemented with 10ng/ml PMA (Sigma) and 0.5 µg/ml ionomycin (Sigma) for two hours at 37°C. Sorted populations were also left unstimulated for two hours. Following incubation, cells were lysed in TRIzol LS and RNA isolated as above. 5µg of RNA was used for NanoString gene expression analysis, using an

Epigenetic panel. Firstly, a hybridisation reaction was set up containing the following reagents:

Final hybridisation reaction contains:

10µl Reporter CodeSet

10µl hybridisation buffer

5ul RNA sample

5µl Capture ProbeSet

The Reporter CodeSet and Capture CodeSet were thawed on ice, inverted multiple times to mix and spun down at a maximum speed of 1000rpm. To analyse 12 samples on the NanoString, 130µl of hybridisation buffer was added to the Reporter CodeSet tube. This was mixed by inverting and spinning down. 20µl of this mastermix was added to the tubes of a 12-tube strip, using a new tip for each step. 5µl of RNA was added to each tube followed by 5µl of Capture ProbeSet. Samples were immediately transferred to 65°C for at least 12 hours (maximum of 30 hours). Following incubation, samples were transferred to the nCounter Prep-station. The high-sensitivity protocol was selected. At the end of the run, the Sample Cartridge was sealed and scanned immediately. Data was analysed as per the nCounter Expression Data Analysis Guide.

2.12 Analysing cytokine production of sorted T cell populations

200,000 sorted T cells were washed and transferred to a 96 well round bottom plate (Corning) in 100µl RH10 complete medium supplemented with 10ng/ml phorbol 12-myristate 13-acetate (PMA) and 0.5 µg/ml ionomycin. Control wells contained cells in medium lacking PMA/ionomycin. After one hour stimulation at 37°C, 20µg/ml Brefeldin A (eBioscience) was added to each well. Cells were cultured at 37°C for a further four hours. Cells were centrifuged at 1500rpm for five minutes, washed once in PBS and then stained with aqua, cell surface antibodies followed by fixation/permeabilisation and staining with antibodies against intracellular cytokines, as previously described.

2.13 Expansion of sorted CD8+ T cell populations and phenotypic analysis

Sorted CD8⁺ T cell populations to be cultured were washed and resuspended in RH10 and stimulated with 1µg/ml phytohaemagglutinin (PHA) (Sigma) or Human T-Activator CD3/28 Dynabeads (Invitrogen) at a bead to cell ratio of 1:1. Prior to use, Dynabeads were washed in 1ml PBS before being resuspended in RH10 complete medium. Cells were expanded in the presence of 500 IU IL-2 (Novartis). After expansion, cells were re-stained using a combination of antibodies in table 2.

2.14 Expansion of sorted CD8⁺ T cell populations - using PHA/IL-2 for antigen-specific T cells

CD8⁺ T cell populations were FACS sorted from matched peripheral blood and tumour samples from HLA-A2 positive stage IV melanoma patients. Cells were expanded on top of irradiated (5000 rad) feeder cells (200,000 cells per 96 well round bottom plate using PHA/IL-2 as described in 2.13). Following expansion for three weeks (during which cells were fed with 500IU IL-2), cells were stimulated with a pool of HLA-A2 peptides from tumour proteins or microbial proteins (Sigma). Peptides were chosen from a panel of 145 melanoma-associated CD8⁺ epitopes described by Kvistborg et al., 2014 (table 3). The chosen peptides had a detectable response in at least one of the melanoma patients previously tested.

Table 3: HLA-A2-restricted peptides used to detect tumour-specific CD8+ T cells in stage IV melanoma patients

Tumour Antigen	Peptide Sequence
gp100	IMDQVPFSV
gp100	ITDQVPFSV
gp100	KTWGQYWQV
gp100	YLEPGPVT A
gp100	VLYRYGSFSV
gp100	YLEPGPVT A
gp100	IMDQVPFSV
LAGE-1	MLMAQEALAF L
LAGE-1	SLLMWITQC
NY-ESO-1	SLLMWITQA
MAGE-A1	KVLEYVIK V
MAGE-A10	GLYDGMEHL
MAGE-A2	YLQLVFGIEV
MAGE-A3	FLWGPRALV
MAGE-A3	KVAELVHFL
MAGE-A3	FLWGPRALV
MAGE-C2	ALKDVEERV
MAGE-C2	KVLEFLAKL
MAGE-C2	LLFGLALIEV
MART-1	ELAGIGILTV
Meloe-1	TLNDECWPA
PRDX5	AMAPIKVRL
RAGE-1	LKLSGVVRL
SSX2	KASEKIFYV
TRP2	SVYDFFVWL
Tyrosinase	YMDGTMSQV
BING4	CQWGRLWQL
GnT-V	VLPDVFIRCV

Abbreviations – gp100: glycoprotein 100, MAGE: melanoma-associated antigen, NY-ESO-1: New York-esophageal cancer-1, MART-1: melanoma antigen recognized by T cells-1, PRDX5: peroxiredoxin 5, RAGE-1: receptor for advanced glycation end-products, TRP2: tyrosinase-related protein 2, SSX2: synovial sarcoma, X breakpoint 2, GnT-V: β 1,6-N-acetylglucosaminyltransferase V.

Table 4: HLA-A2-restricted peptides used to detect microbe-specific CD8+ T cells in stage IV melanoma patients

Microbial Antigen	Peptide Sequence
M1 (Influenza A)	GILGFVFTL
IE62 (VZV)	ALWALPHAA
Ad11 Hexon (Adenovirus)	LLFEVFDVV
AdV Hexon (Adenovirus)	YLLFEVFDV
CMV pp65 (CMV)	NLVPMVATV
IE1 (CMV)	VLEETSVML
LMP2 (EBV)	TVCGGIMFL
LMP1 (EBV)	YLQQNWWTL
E6 (HPV)	KLPQLCTEL
E7 (HPV)	YMLDLQPET
16kDa (TB)	GILTVSVAV
Ag85a (TB)	GLPVEYLQV
Ag85b (TB)	KLVANNTRL
Der p 1 (HDM)	MMIEEYPYV
Der p 1 (HDM)	SLDLAEQEL
Der p 1 (HDM)	YLAYRNQSL

Abbreviations - VZV: varicella zoster virus, CMV: cytomegalovirus, EBV: Epstein-Barr virus, HPV: human papilloma virus, TB: tuberculosis, HDM: house dust mite.

After expansion, cells were washed and left overnight in RH10 complete medium. The following day 200,000 cells were transferred to a 96 well round bottom plate. Each population was individually stimulated with dimethyl sulfoxide (DMSO) (in which the peptides are dissolved), 10 μ M tumour pool 10 μ M the microbe pool and 10ng/ml PMA and 0.5 μ g/ml ionomycin. After one hour stimulation at 37°C, 20 μ g/ml Brefeldin A was added to each well. Cells were cultured at 37°C for a further four hours. Cells were centrifuged at 1500rpm for five minutes, washed once in PBS and then stained with aqua, cell surface antibodies followed by fixation/permeabilisation and staining with antibodies against intracellular cytokines, as previously described.

2.15 Testing antigen-specificity of sorted CD8+ T cell populations - expansion using peptide pools and IL-2

PBMC were purified from HLA-A2 positive melanoma patients. If frozen samples were used, samples were rested for 1.5-3 hrs following thawing. CD8+ T cells were selected using CD8 microbeads and sorted as in 2.9. Between 20,000 and 100,000 sorted cells were transferred to a 48 well plate (Corning) along with 3x10⁶ autologous irradiated (5000 rad) PBMC (CD8 negative fraction). Cells were

stimulated with either 10 μ M of the tumour or microbe pool and 500IU IL-2. Cells were expanded for two weeks.

2.16 Isolation of CD14+ monocytes

Monocytes were isolated from PBMCs of healthy donors using CD14 positive selection beads (Miltenyi). Monocytes were cultured in RPMI (Sigma-Aldrich) supplemented with 10mM HEPES (Life Technologies), 1mM Sodium Pyruvate (Life Technologies), 100 IU Penicillin, 0.1mg/ml Streptomycin (Sigma), 1X MEM Non-essential Amino Acids (Life Technologies), 10% heat inactivated foetal calf serum, 50 μ M 2-mercaptoethanol (Life Technologies), 2mM L-glutamine (Sigma). Cells were kept at 37°C and 5% CO₂. Expression of IL-10 was determined in the presence of Lipopolysaccharide (LPS) (Sigma).

2.17 Culture and differentiation of THP-1 cells and induction of IL-10

The THP-1 cell line was cultured in complete medium (as previously described). Cells were split approximately every three days, with the density of cells no greater than 1x10⁶/ml. THP-1 cells were induced to differentiate using 10nM PMA (Sigma) for 48 hours, before cells were washed in PBS and then stimulated with LPS.

2.18 Analysis of gene expression by Quantitative PCR (qPCR)

RNA isolation - The Qiagen RNeasy Mini Kit was used to isolate RNA from monocytes/THP-1 cells following LPS stimulation. Cells were lysed in 350 μ l RLT lysis buffer supplemented with β -mercaptoethanol. Lysate was passed through a QIAshredder homogeniser (Qiagen) prior to addition to an RNeasy spin column. Columns were washed with RW1 and RPE buffers as per available protocol, before RNA was eluted in 30 μ l RNase-free water.

Reverse Transcription - RNA samples were converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). A reverse transcription 2X mastermix was prepared as below.

Table 5: Reverse transcription reaction components

Component	Volume per reaction (µl)
10X RT Buffer	2
25X dNTP Mix (100mM)	0.8
10X RT Random Primers	2
Reverse Transcriptase	1
RNAse Inhibitor	1
Nuclease-free water	3.2
Total	10

The reverse transcription/RNA mix was placed in a thermocycler set to the following conditions:

Step 1: 10 minutes at 25°C

Step 2: 120 minutes at 37°C

Step 3: 5 minutes at 85°C

Following reverse transcription, cDNA was kept on ice.

qPCR using TaqMan - A mastermix containing TaqMan Fast Advanced Mastermix (Applied Biosystems) and appropriate TaqMan Probe (Applied Biosystems) (shown below) was prepared (IL-10 probe: Hs00961622_m1, hypoxanthine guanine phosphoribosyltransferase (HPRT1) probe: Hs02800695_m1 Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) probe: Hs02758991_g1). 5.5µl of this mastermix was added to appropriate wells of a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems) and 4.5µl of cDNA (diluted in nuclease-free water) was added to appropriate wells. The plate was covered with optical adhesive film (Applied Biosystems) and the plate briefly centrifuged before running in 7500 Fast qPCR machine (Applied Biosystems).

Table 6: qPCR reaction components

Component	Volume per reaction (µl)
TaqMan Fast Advanced Mastermix (2X)	5
TaqMan Gene Expression Assay Probe	0.5
Total	5.5

Quantitative PCR using SYBR green - A mastermix containing 5µl Fast SYBR green mastermix (Applied Biosystems) and 0.5µl forward and 0.5µl reverse primers (Eurofins MWG operon) was prepared. To this, 4µl cDNA (diluted in nuclease-

free water) was added. The qPCR reaction was prepared on the reaction plates described above and run on the 7500 Fast qPCR machine.

Table 7: qPCR primers for gene expression

	Forward primer	Reverse Primer
IL-10	ACATCAAGGCGCATGTGAAC	CAGGGAAGAAATCGATGACAGC
IFN- β	CATTACCTGAAGGCAAGGA	AGCAATTGTCCAGTCCCAGA
IL-27 p28	CTCCCTGATGTTTCCCTGAC	TCCTCTCCATGTTGGTCCAG
IL-27 EBI3	AGCTTCGTGCCTTTCATAACAG	AGTGAGAAGATCTCTGGGAAGG

2.19 Analysis of IL-10 protein production by ELISA

Following stimulation of CD14⁺ monocytes or THP-1 cells with LPS, cells were spun down and supernatant collected for ELISA and stored at -20°C until the ELISA was performed. The wells of a Costar Assay Plate (96 well, half area, flat bottom, high binding) (Corning) were coated with Purified Rat Anti-Human and Viral IL-10 antibody (BD Pharmingen) (diluted 1:250 in coating buffer – 0.1M Carbonate buffer, pH 8.2) and incubated overnight at 4°C. Wells of the plate were then washed five times by immersing in PBS + 0.1% Tween 20 (Acros Organics). The plate was tapped dry and 100µl blocking buffer (PBS + 10% FBS) was added to each well for 2hrs at room temperature. The wells were washed five times as above. The supernatant was thawed and 25µl added to appropriate wells of the plate. IL-10 standard (PeproTech) was prepared for the standard curve. A two-fold serial dilution, starting from 20ng/ml, was used with dilution of the standard in blocking buffer. 25µl of each standard concentration was added to the plate. 25µl of blocking buffer alone was also added to three wells of the plate, to serve as a blank for the plate reader. Supernatant and standard were incubated on the plate overnight at 4°C. The plate was again washed five times and dried prior to addition of the detection antibody - Biotin Anti-Human and Viral IL-10 (BD Pharmingen) diluted 1:500 in blocking buffer – and Avidin-HRP (horseradish peroxidase) (eBioscience) diluted 1:1000 in blocking buffer. The plate was incubated for one hour at room temperature. The plate was washed seven times as above and dried before addition of the HRP substrate, o-Phenylenediamine dihydrochloride (OPD) (Sigma). One OPD tablet (10mg) was dissolved in 10ml citrate-phosphate buffer containing 0.1% hydrogen peroxide (Sigma). Following a five minute incubation at room temperature with OPD,

2.5M sulphuric acid was added to each well to stop the reaction. The plate was then read at 492 nm).

2.20 Formaldehyde-assisted isolation of regulatory elements (FAIRE)

Nuclear extraction/cell lysis/sonication - Fixed cells (typically 5×10^6) were resuspended in Nuclear Extraction Buffer containing 0.5% IGEPAL (Sigma), 0.25% Triton X-100 (Sigma), 10mM Tris-HCl pH 7.5 (Sigma), 3mM calcium chloride (Sigma), 0.25M Sucrose (sigma), 1mM Dithiothreitol (DTT) (Sigma), 0.2mM phenylmethanesulfonyl fluoride (PMSF) (Sigma) and cOmplete protease inhibitors (PIs) (Roche). Cells were homogenised using a dounce homogeniser, the pellet resuspended in sodium dodecyl sulphate (SDS) lysis buffer (1% SDS, 50mM Tris, 10mM EDTA (Sigma), 0.2% Triton X-100 and PIs) and incubated on ice for 20mins. SDS was diluted prior to sonication of cells in a Diagenode Bioruptor for 7mins (30secs on, 30secs off).

Preparation of Input Control and FAIRE DNA (adapted from Simon et al., 2012) - 100 μ l of cell lysate was taken for input DNA. The remaining 300 μ l, from which FAIRE DNA was later prepared, was kept overnight at 4°C. Input DNA was subjected to DNase-free RNase A (Roche) and Proteinase K (Ambion) digestion and incubated overnight at 65°C to reverse crosslinks. The following day, input and FAIRE DNA was purified using phenol-chloroform-isoamyl alcohol (invitrogen) extraction followed by ethanol precipitation. Input DNA was resuspended in 30 μ l 10mM Tris-HCl, pH 7.5 and a sample of input DNA was used to test sonication efficiency by gel electrophoresis. FAIRE DNA was resuspended in 50 μ l 10mM Tris-HCl, pH 7.5 and subjected to DNase-free RNaseA and Proteinase K digestion, followed by overnight incubation at 65°C. DNA was purified using Zymo-I spin columns (Zymo), with DNA eluted in 20 μ l 10mM Tris-HCl, pH 7.5. DNA was quantified using NanoDrop, an acceptable FAIRE yield (adjusted for volume) being 1-3% of the input (Simon et al., 2012).

Detection of FAIRE enrichment by qPCR - FAIRE enrichment was determined by qPCR, using FAST SYBR Green Mastermix (Applied Biosystems) and the 7500 Fast Instrument (Applied Biosystems). The standard curve method was used for

analysis. Primers were ordered from Eurofins MWG Operon and used at a final concentration of 250nM.

Table 8: qPCR primers for FAIRE

Primer Name	Forward Primer	Reverse Primer
GAPDH*	TACTAGCGGTTTTACGGGCG	TCGAACAGGAGGAGCAGAGAGCGA
PPIBTSS	CGCCGGATAGTAGGTCCC	ATGAGGGAGCAATAACGTGG
PPIBD05	TGCTTTGTAATTGGAAGTGGC	GATGGTGAAAGATCACGCCT
U81B	CCACTGTCCTTACAACTATATGCC	CCGCCTGGAAGAGCACTAAT
U176	CAGGAAGTGAGAAAGAGCCTG	TCTAAGTCTGGCAGGACCAGT
PROM2	AGCTGTGCATGCCTTCTTTT	TACACATCAGGGGCTTGCTC
D05	TTCAGGTCTGGAAACGCTCT	ATTGAGGTGAAGATGCTCC
D12	CAGCTTGGTTCTAGCGATCC	AGCAGCCAGAGGGTTTACAA
D295C	CCCCAAAGACACTTAACAGAAA	TTGGAGAGAGTGTGGGAACC
D59C	GCAGTCAGAAGCCAATCTCA	TTGAGAGGATAGCCAAGGACA
D90	TCTGTCAGTGGCTGCATAGG	GGCTTTGGAGGTAAGGAAGG

* GAPDH primers recommended by Millipore.

2.21 Chromatin immunoprecipitation (ChIP)

Crosslinking - Cells were fixed in medium with a final concentration of 1% formaldehyde (Sigma) for 10 minutes on a rocking platform at 37°C. Crosslinking was stopped by addition of 125mM Glycine (Sigma). Cells were washed twice in ice cold PBS supplemented with PIs, 1mM DTT (Sigma) and 0.2mM PMSF (Sigma). PIs were added to PBS just prior to use. For ChIP involving antibodies against acetylated histones, PBS was also supplemented with the histone deacetylase inhibitor, 5mM sodium butyrate (Sigma). Fixed cells were snap frozen and stored at -80°C until lysis was performed.

Lysis and Sonication - The Chromatin Immunoprecipitation Kit from Millipore was used. Unless stated, all reagents were provided with this kit. Cells were resuspended in SDS Lysis Buffer (1x10⁶ cells per 200µl buffer). An extra 50µl of SDS lysis buffer was added to enable aliquot for the Input Control to be taken at a later point. Samples were incubated on ice for 10 minutes. Samples were sonicated for 7 cycles (a cycle being 30 seconds on and 30 seconds off) at 4°C using a Diagenode Bioruptor in order to obtain DNA fragments between 200-1000bp. Samples were then centrifuged at 13,000 rpm for 10 minutes at 4°C and 200µl supernatant was transferred to a new tube per ChIP and 50µl was frozen for Input Control. The 200µl supernatant was diluted 10X in ChIP Dilution Buffer

(supplemented with PIs, DTT, PMSF and sodium butyrate at the above concentrations) and pre-cleared by incubation with 75µl Salmon Sperm DNA/Protein A Agarose-50% Slurry for 30 minutes at 4°C on a rotator. Agarose was pelleted by centrifugation at 1000 x g for one minute and the supernatant transferred to a new tube.

Incubation with antibody and washes - Below are the antibodies used for ChIP.

Table 9: ChIP antibodies

Specificity	Supplier	Host	Antibody Type	Amount/Volume
H3K4me3	Millipore	Rabbit	Polyclonal	3µl
H3K27ac	Abcam	Rabbit	Polyclonal	5µg
IgG Isotype control	Millipore	Rabbit	Polyclonal	3 µl
PU.1	Santa Cruz	Rabbit	Polyclonal	1 µg

The ChIP antibody was added to the pre-cleared supernatant and incubated overnight at 4°C on a rotator. 60µl Salmon Sperm DNA/Protein A Agarose-50% Slurry was then added for one hour at 4°C while samples were rotated. The protein A agarose/antibody/chromatin complex was then pelleted by centrifugation at 1000 x g for one minute and washed in 1ml each of the provided buffers for five minutes whilst on a rotator (the agarose was pelleted at 1000 x g for one minute and wash buffer removed in between wash steps).

Elution - The protein A agarose/antibody/chromatin complex was resuspended in 250µl elution buffer – recipe kindly provided by T. S. Spengler (50mM Tris-HCl, pH 8 (Sigma), 10mM EDTA (Sigma), 1% SDS (Sigma) and nuclease free water (Ambion)) and incubated for 15 mins at room temperature on a rotator. The agarose was pelleted and the supernatant (with chromatin) transferred to another tube. This step was repeated. At the same time the input control sample was thawed and mixed with 450µl elution buffer. The crosslinks were reversed in the ChIP and Input Control samples by heating at 65°C for four hours-overnight. 10µl 0.5M EDTA, 20µl 1M Tris-HCL (pH 6.5) and 2µl 10mg/ml Proteinase A (Life Technologies) were added and samples were incubated at 45°C for one hour.

DNA purification - DNA was purified using phenol-chloroform-isoamyl alcohol (invitrogen) extraction followed by ethanol precipitation. DNA was resuspended

in 30µl 10mM Tris-HCl, pH 7.5 and a sample of input DNA was used to test sonication efficiency by gel electrophoresis. ChIP DNA was resuspended in 20µl 10mM Tris-HCl, pH 7.5

Detection by qPCR - qPCR was used, as for FAIRE, to detect regions associated with modified histones.

2.22 Generation of clustered regularly-interspaced short palindromic repeats (CRISPR)-modified clones

2.22.1 Transfection of THP-1 cells

The transfection of THP-1 cells was optimised using Lipofectamine LTX (Life Technologies), following the protocol suggested by the manufacturer. Briefly, freshly thawed THP-1 cells were plated in a 24-well plate (Corning) at a density of 1×10^5 cells per well in 0.5ml of R10 medium lacking antibiotics. Plasmid DNA, PLUS reagent and Lipofectamine LTX mixes were prepared in serum-free Opti-MEM I medium (Life Technologies) and 100µl of each mix was added to one well of the plated THP-1 cells.

It is recommended to use between 3-18µl of Lipofectamine LTX Reagent and 250ng-1000ng DNA per transfection. As seen in figure 1, a titration of the amount of Lipofectamine LTX reagent and the amount of DNA was carried out in order to determine the optimal amounts for use with THP-1 cells. Whilst increasing the amount of Lipofectamine LTX Reagent or the amount of DNA increases the efficiency of transfection, reflected by a higher percentage of GFP+ cells, higher amounts of Lipofectamine LTX Reagent and higher amounts of DNA per transfection lead to an increase in THP-1 cell death. By looking at a combination of percentage of live cells after transfection, absolute number of green cells (per 15,000 events recorded) and the percentage of green fluorescent protein (GFP)+ cells within the live gate, a combination of 6µl Lipofectamine LTX Reagent and 750ng DNA was chosen for use with THP-1 cells (figure 1).

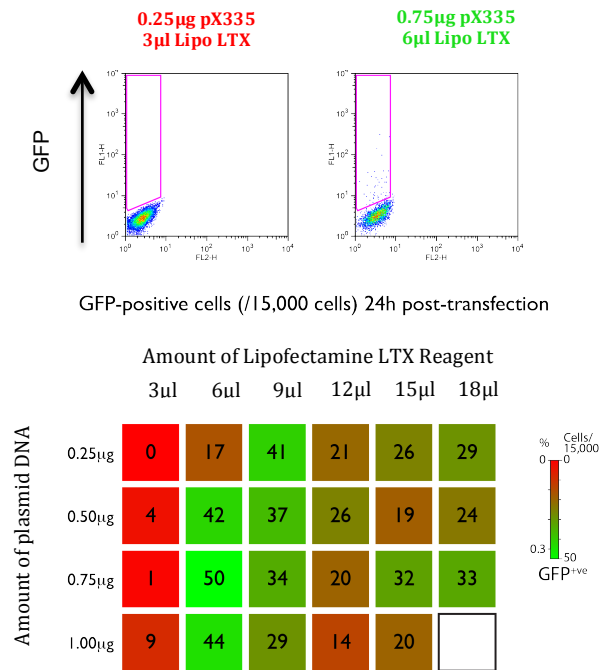


Figure 1: Optimisation of transient transfection of THP-1 cells using Lipofectamine LTX Reagent (Lipo LTX). A: Gating on live cells reveals the absence of GFP+ cells when a combination of 0.25µg DNA/3µl Lipofectamine LTX is used. However, when the concentration of DNA is increased to 0.75µg and used in combination with 6µl Lipofectamine LTX, GFP+ cells are now detected. B: A table to summarise the results from the optimisation of THP-1 transfection. The numbers in the table reflect the number of GFP+ cells obtained (per 15,000 live cells recorded) using various combinations of amount of DNA and Lipofectamine LTX.

2.22.2 Plasmids and constructs

Plasmids pX330EGFP-P2A-Neo and pX335-EGFP-T2A-Neo, were kindly provided by J. Riepsaame (WIMM, Oxford). These plasmids were derived from pX330 and pX335, respectively (Cong et al. 2013; Ran et al. 2013), where the parent plasmids were modified by the introduction of a pGK-EGFP-T2A-Neo cassette allowing for positive selection with G418/Neomycin and/or enrichment of transfected cells through FACS-sorting of eGFP-positive cells. Parent plasmids pX330 (or pX335) contain two expression cassettes, one cassette expressing codon-optimized *S. pyogenes Cas9* nuclease (*hSpCas9*) (or D10A *Cas9* nickase mutant, in the case of the pX335) under the control of the hybrid chicken β-actin (CBh) promoter; and a second cassette expressing the chimeric guide RNA (+85 nucleotides of trans-activating CRISPR RNA, tracrRNA) controlled by the U6 promoter (figure 2).

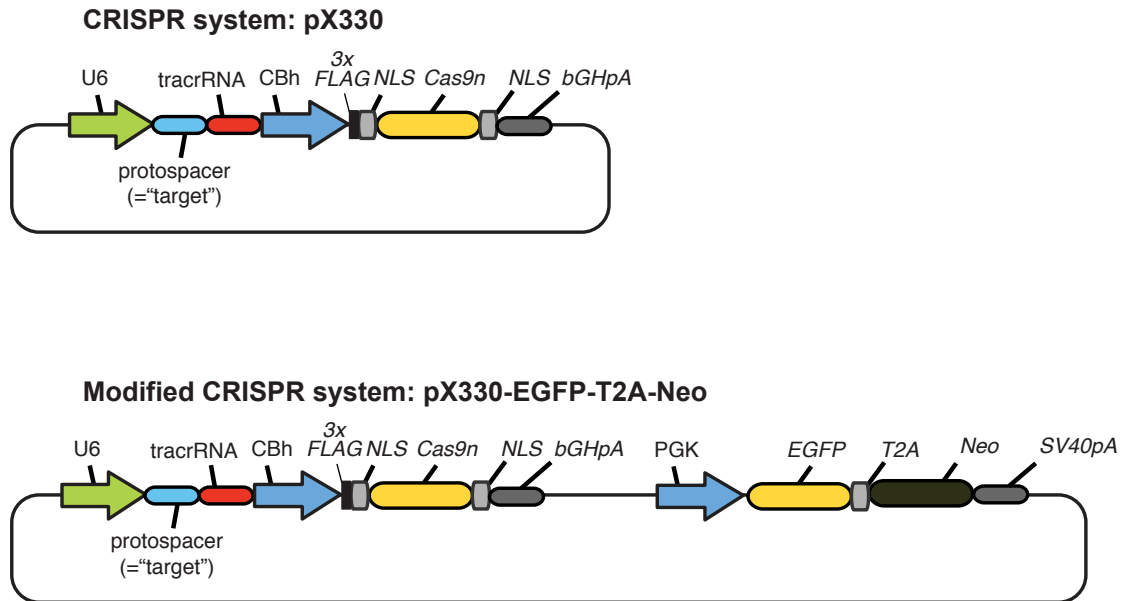


Figure 2: The pX330 and modified pX330-EGFP-T2A-Neo constructs. The pX330 CRISPR/Cas9 system consists of a hybrid crRNA/tracrRNA (a.k.a "guideRNA" or sgRNA) and a human codon-optimized Cas9 gene (*S. pyogenes*). This system is suitable for multiplex genome editing in human cells. The original plasmid was modified (by Joey Riepsaame, WIMM, Oxford) by introducing a pGK-EGFP-T2A-Neo cassette allowing for positive selection with G418 and/or enrichment of transfected cells through FACS sorting of eGFP positive cells.

The vectors were digested using BbsI, and a pair of annealed oligos (Eurofins MWG Operon) (corresponding to the chosen 20 nucleotides of the target guide sequence) were then cloned into the plasmid (figure 3). Figure 3 shows the section of the IL-10 gene with six potential PU.1 binding sites. Three guide RNAs were individually cloned into pX330 and pX335 and different combinations were used to transfect THP-1 cells.

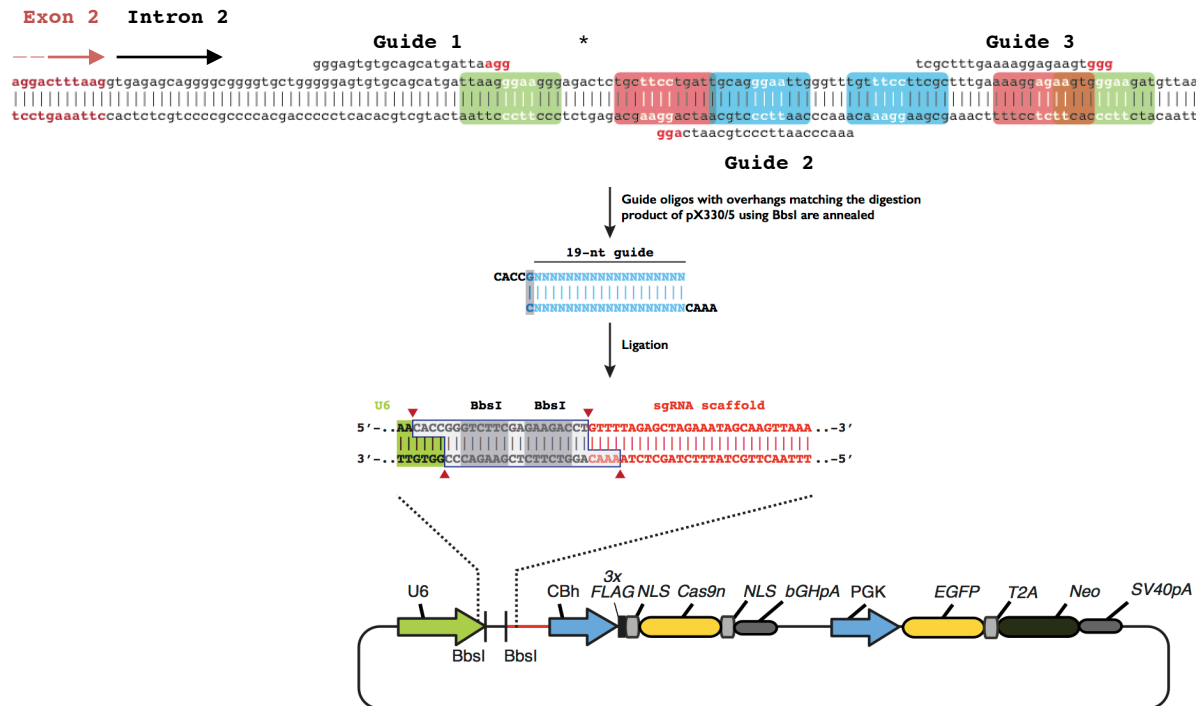


Figure 3: Target guide selection for the human IL-10 gene and Cas9 plasmid preparation.

Top: a genome view of the region of interest (left to right: Exon2-Intron2) with the putative PU.1 binding sites highlighted in blue, green and red (with position weight matrix (PWM) values from low to high – see chapter 5 for explanation). Guides for target sites were identified (explained below) and oligos designed and annealed to generate overhangs to insert into the pX330 or pX335 plasmids. These were then used to target the region of interest in THP-1 cells (Adapted from Ran et al. 2013 Nat. Protocols).

Target site sequences are 20nt-long, they can have a sense or anti-sense direction, and are flanked on the 3' end by a 3 base pair NGG protospacer-adjacent motif (PAM) sequence. A BLAST search is performed on the designed target sequences (via crispr.mit.edu server), and a score is assigned to each sequence indicating the likelihood of off-target effects on similar or identical sequences if that guide is used (Hsu et al. 2013 Nat. Biotech.).

2.22.3 Screening of THP-1 clones

As Cas-9 induced double-stranded DNA breaks (DSBs) in genomic DNA (gDNA) tend to be repaired in the absence of a repair template, by the non-homologous DNA end joining (NHEJ) repair mechanism, this can result in imprecise insertions/deletions (indels), which may not be the same on both alleles (Lieber

et al., 2003). This complicates screening of diploid (or polyploid) genomes edited by CRISPR-Cas9.

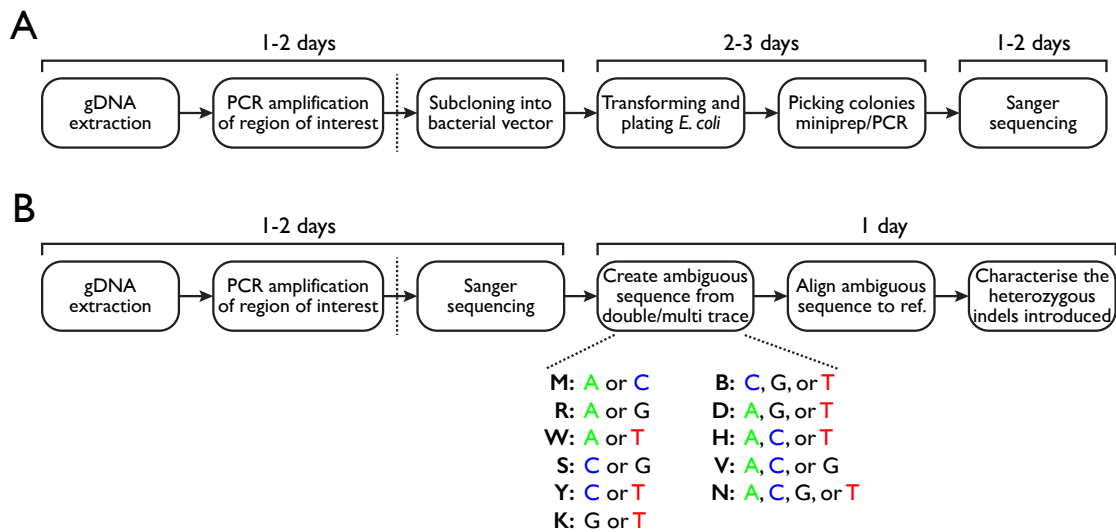


Figure 4: Schematic describing the protocol for genotyping THP-1 clones modified by CRISPR/Cas9. A: The method described by Ran et al., 2013 involves PCR-amplifying the region of interest from genomic DNA and sub-cloning each amplicon into *E. coli*. This is followed by sequencing of individual colonies. B: A new method developed in our lab by Hemza Ghadbane, involves Sanger sequencing the PCR-amplified region of interest followed by deconvolution of ambiguous sequences.

Ran et al. (2013) suggest that the gDNA of each clone be extracted and used as a template to PCR-amplify the targeted region. Each amplicon should then subcloned into a bacterial plasmid, which in turn is used to transform *E. coli*, and individual colonies sequenced to identify the indels introduced and uncover the genotype of each clone (Figure 4A). However, this approach is not cost- or time-effective. A post-doctoral scientist Hemza Ghadbane (Cerundolo lab, WIMM, Oxford) developed a more efficient approach to genotype each clone by characterising the indels introduced into alleles of diploid/polypolyploid cells by NHEJ (Figure 4B). Similar methods have been described previously and involve the amplification of the region of interest, Sanger sequencing of this region, followed by deconvolution of ambiguous sequences (figure 5) (Chang et al., 2012)

This approach was used to genotype the clones generated by my CRISPR-Cas9 constructs. DNA was purified from a minimum of 1000 cells using DNeasy Blood

and Tissue Kit (Qiagen). Exon 2-Intron 2-Exon 3 of the IL-10 gene was amplified using primers in table 10.

Table 10: qPCR primers

Name	Forward	Reverse
Ex2-Ex3	AATAACTTGACTCTGAGGAGGAGG	CCAACGCCTGCTCAAAGAGAAATG

The PCR reaction and conditions used are shown in table 11.

Table 11: PCR conditions

Component	Volume (μl)
Mango Buffer	5
MgCl ₂	1
dNTP	0.5
Forward primer	2
Reverse primer	2
MangoTaq DNA polymerase	0.5
Nuclease-free water	15.1
Genomic DNA	2.5

Temperature (°C)	Duration
95	5 minutes
95	20 seconds
60	20 seconds
72	40 seconds
72	7 minutes
4	∞

37 cycles

The PCR reaction was cleaned up using the QIAquick PCR Purification Kit (Qiagen) and samples were sent for sequencing. Ambiguous sequences were deconvoluted as in figure 5.

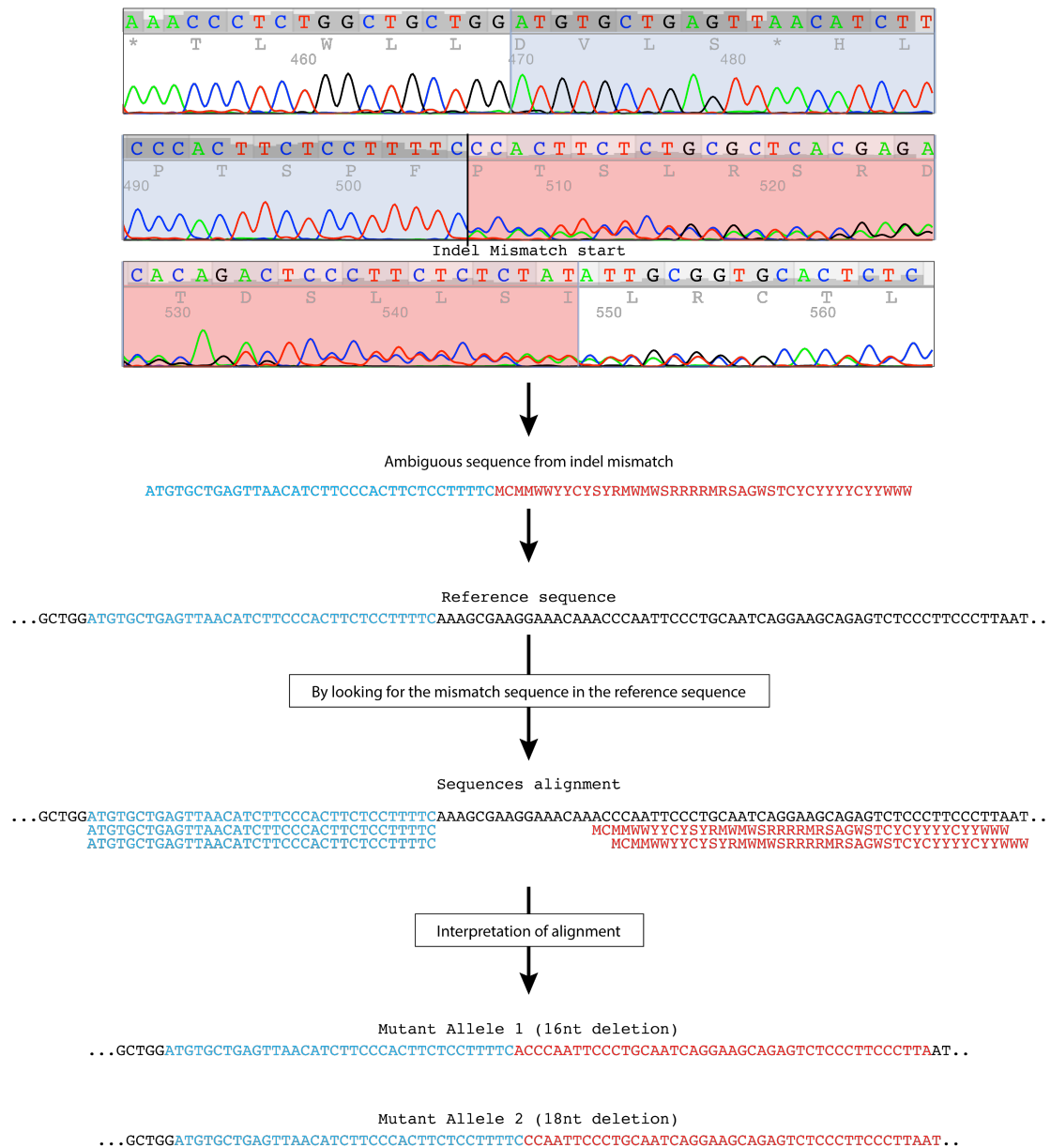


Figure 5: Deconvolution of ambiguous sequences. An example of a CRISPR/Cas9 generated clone with two different mutations on each chromosome. Initially the sequence is the same on both chromosomes (highlighted in blue on the upper sequencing panel). This is followed by an ambiguous sequence (highlighted in red), in which it appears that there are different nucleotides at the same position (corresponding to mismatch between the sequence of the two chromosomes). If the mismatched sequence is written using the alphabet described in figure 4B, and then aligned with the wild-type sequence, it is possible to determine where this aligns to the wild-type sequence and thus determine the DNA sequence on the different alleles.

2.22.4 PCR-based verification of deletion generated by pX330 nuclease

To confirm the sequencing results that show a deletion of a section of intron 2 by CRISPR-Cas9, PCR using a forward primer specific for the missing region (“fwd mismatch primer”) and a reverse primer specific for a region of intron 3 that should be present in both wild-type and modified cells (“LB012s”) was carried out. PCR conditions were as mentioned in 2.22.

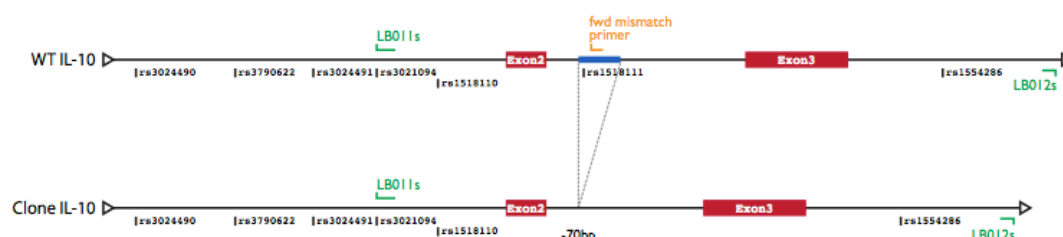


Figure 6: PCR-based detection of a deletion in CRISPR-Cas9 modified clones. A section of DNA is shown covering a section of intron 1, exon 2, intron 2, exon 3 and intron 3 of the IL-10 gene. The upper sequence represents wild-type IL-10 (WT IL-10) and the lower sequence represents that of modified clones (Clone IL-10). The position of the PCR primers is shown, as is the section of DNA missing in modified clones (highlighted in blue in the wild-type sequence). A PCR product is expected when genomic DNA is amplified with “fwd mismatch primer” and “LB012s” in wild-type cells. As “fwd mismatch primer” recognises the region thought to be deleted in modified cells, a PCR product is not expected when genomic DNA from modified cells is amplified with these primers. A control PCR, in which the forward primer (LB011s) and the reverse primer (LB012s) are used to amplify DNA, should result in a product when genomic DNA from either wild-type or modified clones is used.

2.23 Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq)

E. Ballabio and M. Tapia in Prof. T. Milne’s lab (WIMM, Oxford) kindly provided a protocol and advice.

Cell lysis - 50,000 cells of the three modified CRISPR THP-1 cell lines (modified/mutant clones), three unmodified CRISPR THP-1 cell lines (wild-type clones) as well as 50,000 parent THP-1 cells (original wild-type THP-1) were centrifuged at 500 x g for 5 minutes at 4°C followed by one wash in 50µl cold PBS. The cells were gently resuspended in 50µl cold lysis buffer (10mM Tris-HCl, pH 7.4 (Life Technologies), 10mM NaCl (Ambion), 3mM MgCl₂ (Ambion), 0.1% IGEPAL CA-630 (Sigma)) and immediately centrifuged at 500 x g for 10 mins at 4°C. The supernatant was removed and the pellets placed on ice.

Transposition and DNA purification - The nuclei were resuspended in 50µl transposition reaction mix (25µl 2X TD Buffer, 2.5µl Tn5 Transposase (both from the Nextera DNA Sample Prep Kit (Illumina)) and 22.5µl nuclease-free water) and the reaction was incubated for 40 minutes at 37°C before immediate purification of DNA using the MinElute Reaction Cleanup Kit (Qiagen). DNA was eluted in 10µl of the provided elution buffer and stored at -20°C until the following step.

PCR Amplification - 10µl of transposed DNA was added to the following PCR mix (25µl NEBNext High-Fidelity 2X PCR Master Mix (New England BioLabs), 2.5µl 25µM Customised Nextera PCR Primer 1 (Universal primer), 2.5µl 25µM Customised Nextera PCR Primer 2 (Barcode) (primers provided by E. Ballabio) and 10µl nuclease-free water)). The PCR conditions in table 12 were used.

Table 12: PCR conditions

Temperature (°C)	Duration
72	5 minutes
98	30 seconds
98	10 seconds
63	30 seconds
72	1 minute
4	∞

11 cycles

PCR Cleanup - The PCR reaction was cleaned up using QIAquick PCR Purification Kit (Qiagen), with the library eluted in 20µl of the provided elution buffer.

Library Quantification and Sequencing - The library was quantified using a protocol from Tatjana Sauka-Spengler's lab (WIMM, Oxford). Each library was quantified using Qubit dsDNA HS Assay kit (Invitrogen). Each library was run on the Agilent Tapestation and the area of each sized peak was quantified. A formula was used to calculate the volume of each library that would need to go into the pooled library in order to create a 4nM library. The total volume of the pooled library was made up to 20µl. The pooled library was then quantified using the KAPA Library Quant Kit (KAPA Biosystems). The pooled library was run on a NextSeq500, giving approximately 60 million reads per sample. R. Thorne (Milne Lab, WIMM, Oxford) kindly analysed the sequencing data generated.

Chapter 3: Analysis of neutrophils in patients with melanoma

3.1 Introduction

As mentioned earlier in the main introduction, while neutrophils may offer protection against tumour growth, *via* their capacity to kill cancer cells with NOS production and ADCC (Finnisguerra et al., 2013) (Challacombe et al., 2006), there is accumulating evidence that neutrophils can promote the growth of tumours. Indeed, of the 22 tumour-infiltrating leukocyte populations analysed in a recent study of 25 different malignancies, increased tumour infiltration by neutrophils was the most significant adverse prognostic factor (Gentles et al., 2015). Such an association is likely linked to the numerous pro-tumour functions of this cell type, which includes the ability to promote proliferation of cancer cells and to promote angiogenesis and metastasis (Antonio et al., 2015) (Bald et al., 2014) (Nozawa et al., 2006). Furthermore, neutrophil populations with immune suppressive properties have been identified in patients with a variety of cancers and these cells have been termed granulocytic myeloid-derived suppressor cells (grMDSC).

Numerous studies have attempted to define the phenotype of grMDSC in cancer patients, including those with melanoma, typically by analysing a variety of different markers by flow cytometry and their suppressive capability.

A selection of neutrophil markers that are differentially expressed during neutrophil development are highlighted in figure 1.

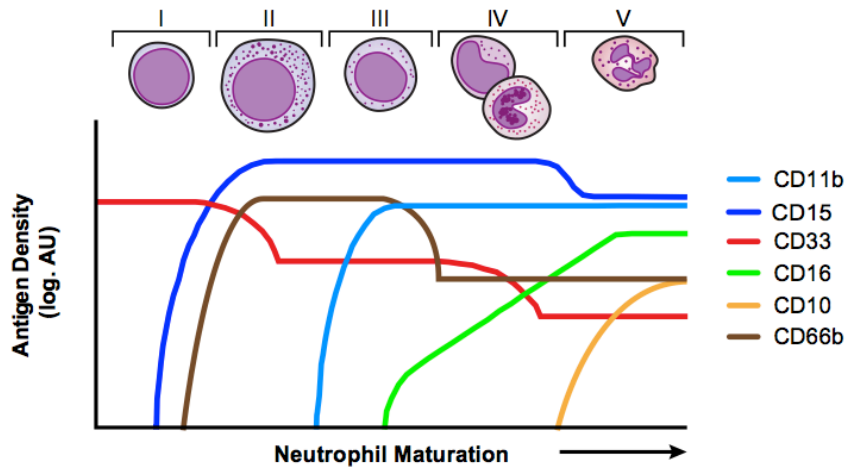


Figure 1: Expression of neutrophil surface markers during differentiation. I: Myeloblasts, II: promyelocytes, III: myelocytes, IV: metamyelocytes/bands, V: mature, segmented neutrophils. Figure adapted from Loken et al., 2008.

CD15 is a fucosylated carbohydrate molecule and while it is also expressed by other cells including intestinal epithelial cells and monocytes, its high expression on the surface of neutrophils means it is commonly used as a marker to identify the neutrophil population (Kerr and Stocks, 1992) (Nakayama et al., 2001). It is expressed from the promyelocyte stage onwards (figure 1) and through its interaction with E-selectin (a molecule expressed on activated endothelium) functions in recruitment of neutrophils to sites of inflammation (Elghetany 2002) (Nakayama et al., 2001) (Kerr and Stocks, 1992).

Unlike CD15, expression of CD10 is restricted to mature, segmented neutrophils (Elghetany 2002). CD10 is an endopeptidase thought to function in regulating the local concentration of inflammatory mediators, including fMLP (formyl-methionyl-leucyl-phenylalanine), a chemotactic tripeptide produced by certain bacteria (Shipp et al., 1991).

In contrast to CD10, the highest amounts of CD33 are observed on neutrophil progenitors such as myeloblasts and promyelocytes, with expression decreasing as cells become more differentiated (Lochem et al., 2004) (Elghetany 2002). CD33, which is also expressed by other myeloid cells including monocytes, is a member of the sialic acid-binding immunoglobulin-like lectin (siglec) family

which function as adhesion receptors through binding of sialylated glycoproteins (Paul et al., 2000). Furthermore, there is evidence that CD33 can function as an inhibitory receptor, decreasing signalling *via* the IgG receptor, CD64 (Paul et al., 2000). It has been suggested that decreased expression of CD33 during differentiation may enable cells to become more responsive to activating stimuli.

Another marker differentially expressed during neutrophil differentiation is CD16. CD16, also known as the Fc γ receptor III, is involved in phagocytosis (Hart et al., 2000). It is expressed from the metamyelocyte/band stage and increases during development (Lochem et al., 2004).

CD11b, a component of the β -2 integrin CD11b/CD18 (Mac1), is expressed from the myelocyte stage, continues to increase during differentiation and is important for cell attachment to the endothelium *via* interaction with intercellular adhesion molecule-1 (ICAM-1) (Hentzen et al., 2000) (Lochem et al., 2004) (Anderson et al., 2000). Different domains of CD11b also enable Mac-1 to function as complement receptor 3 (CR3), facilitating phagocytosis of complement-opsonised particles (Le Cabec et al., 2002). As well as being present in the membrane of secretory vesicles, the main sources of CD11b in neutrophils are the gelatinase and specific granules (Borregaard & Cowland, 1997). Upon neutrophil activation, mobilisation of granules towards the plasma membrane results in incorporation of CD11b and thus increased cell surface expression of this molecule (Borregaard & Cowland, 1997). Therefore, as well as a lack of CD11b being associated with an immature neutrophil phenotype, increased expression, when compared to the mature, unstimulated neutrophil pool, is associated with an activated phenotype.

Like CD11b, cell surface expression of CD62L differs during neutrophil activation. Along with other adhesion molecules, it functions in rolling and attachment of neutrophils (and other leukocytes) along activated endothelium at sites of inflammation (Venturi et al., 2003). Upon neutrophil activation, in response to a variety of stimuli such as the TLR ligand LPS and cytokines such as TNF- α and IL-1 β , CD62L is shed from the surface, predominantly the result of

cleavage by a disintegrin and metalloprotease, ADAM17 (Li et al., 2006). Cleavage of CD62L has been suggested to enable cells to detach from endothelial cells and extravasate into inflamed tissue (Venturi et al., 2013).

Markers that are differentially expressed by neutrophils during differentiation or activation, including those described above, have been used in numerous studies to describe the phenotype of low-density granulocytes detected in the peripheral blood of cancer patients.

There is however conflicting data regarding the phenotype of these cells. Certain studies define these suppressive cells as immature neutrophils, whereas others suggest these cells are activated neutrophils. A recent paper by Stanojevic et al. (2015) attempts to clarify the phenotype of grMDSC in melanoma patients. They describe the phenotype of these CD15⁺ cells as immature cells, expressing lower amounts of CD16, CD11b, CD33 and CD45 compared to mature granulocytes. When patients are stratified according to progressive or non-progressive disease, elevated frequencies of these cells were observed in the progressive disease group.

One of the first reports of grMDSC in melanoma patients was by Schilling et al. (2013). They describe the enrichment of CD66b⁺ CD11b⁺ CD33⁺ CD16^{-/low} in patients with stage IV melanoma. From figure 1, it is evident that CD16 is a marker associated with more mature neutrophils and so the presence of CD66b⁺ CD16^{-/low} cells suggests the presence of neutrophils with an immature phenotype that are capable of immune suppression. However, from their FACS staining, it seems that a population of CD66b⁺ CD16⁺ exist within the low-density granulocyte population. Such cells could represent more differentiated neutrophils or activated neutrophils, lower in density due to release of granular contents. Indeed Rodriguez et al. (2009) identify a population of suppressive granulocytes in the peripheral blood of patients with renal cell carcinoma (RCC) that have an activated phenotype. It has also been shown (although in the context of acute inflammation) that following administration of LPS to human volunteers, that there is both an enrichment of neutrophils with an activated

phenotype as well as neutrophils with an immature phenotype (Pillay et al., 2012). In this study, the suppressive capacity of both populations was compared and it was in fact the cells with an activated phenotype that were capable of T cell suppression.

Although different combinations of markers are used in immunoprofiling of grMDSC, all studies published so far have considered low-density granulocytes in cancer patients as a whole, and if multiple populations exist, have not tried to determine which population is responsible for suppression. For example, the immune suppression assays by Schilling et al (2013) were carried out using total low-density granulocytes, rather than sorted CD66b+ CD16- cells, therefore it is unclear whether it is in fact cells with an immature neutrophil phenotype that are responsible for T cell suppression. If this low-density population is in fact heterogeneous, it may be that it is a specific sub-population that is responsible for suppression, and other populations may even have distinct but potentially clinically relevant functions. Assessing whether the low-density population of granulocytes associated with disease progression is composed of immature or activated neutrophils, is essential to understanding how cancer affects neutrophil biology, and to understanding which pathways might represent relevant therapeutic targets in order to interfere with the activation/differentiation of granulocytic MDSC. Other populations may have functional relevance or may be useful as markers of disease progression or treatment response, for example.

Furthermore, the enrichment of a neutrophil population/populations may be a feature of greater changes in the inflammatory status of patients. Instead of thinking of these cells in the context of isolated *in vitro* experiments in which they may have the capacity to suppress T cell responses, perhaps these cells reflect (directly or indirectly) greater changes to the immune status of these patients, which may be targetable by therapy.

In this chapter, I immunophenotype low-density granulocytes in the peripheral blood of patients with stage IV melanoma. Furthermore, I assess whether the

phenotype or frequency of low-density granulocytes changes during treatment of melanoma patients with the immune-modulatory drugs, anti-CTLA-4 or anti-PD-1.

3.2 Identification of distinct populations of low-density granulocytes that co-purify with peripheral blood mononuclear cells (PBMCs) in patients with stage IV melanoma

In order to assess the immunophenotype of low-density granulocytes, I collected blood from patients with stage IV melanoma (see Chapter 2: Materials and Methods). Blood was subjected to density-based centrifugation and the PBMCs isolated and stained with a panel containing neutrophil-specific antibodies. FACS analysis of the PBMCs revealed the presence of CD15⁺ cells within the myeloid compartment (as defined by expression of the myeloid marker CD33) (figure 2). CD15 is expressed by mature neutrophils as well as those at earlier stages of differentiation (Nakayama et al., 2001) (SSemaganda et al., 2014). As CD15 can be expressed by certain monocyte populations, monocytes were excluded by their lower side-scatter and higher CD14 expression.

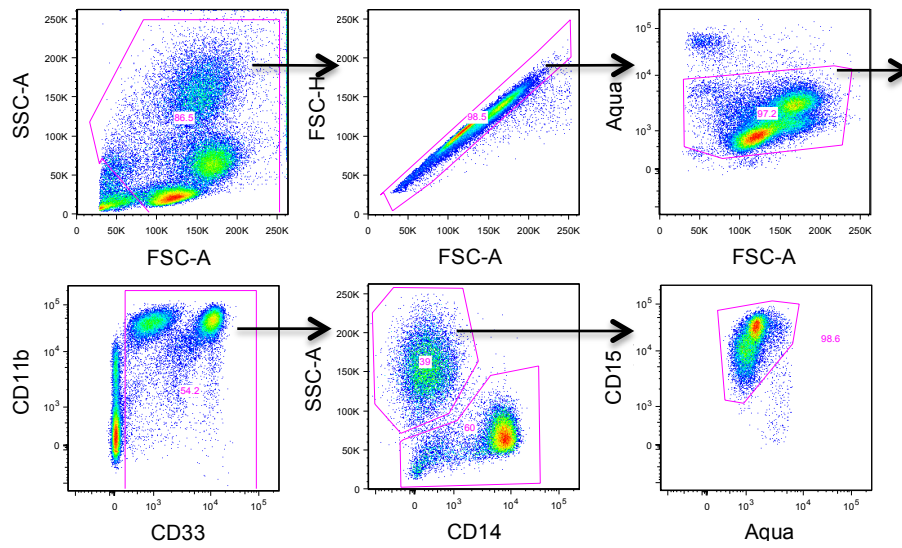


Figure 2: FACS analysis of PBMCs from stage IV melanoma patients reveals the presence of CD15+ low-density granulocytes. Firstly, debris was excluded followed by gating on singlets and live cells using the viability dye, aqua. Myeloid cells, defined as CD33+, were selected followed by gating on cells with high side scatter and low expression of the monocytic marker, CD14. The majority of these cells are CD15+.

Ordinarily, neutrophils do not purify with PBMCs, rather they are higher in density and purify on top of the erythrocyte layer, lower in the density gradient. The detection of cells within the PBMCs that have features of neutrophils, such as CD15 and CD33 expression, and high side scatter, suggests that low-density granulocytes resembling neutrophils are present in the peripheral blood of patients with stage IV melanoma. These cells however are rare in the peripheral blood of healthy controls (figure 3).

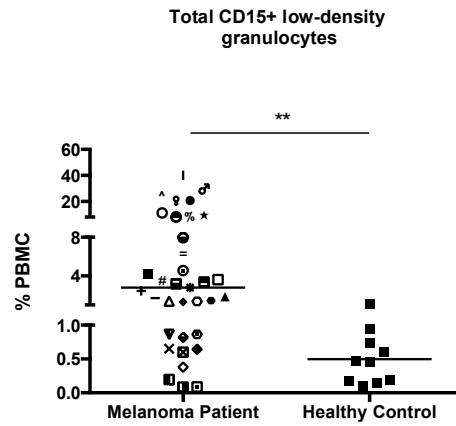


Figure 3: Low-density CD15+ granulocytes are enriched in the peripheral blood of patients with stage IV melanoma compared to healthy controls. Frequency is expressed as percentage of PBMCs. The median is highlighted and the difference in distribution was determined using the Mann-Whitney U test. ** $p < 0.01$.

Previous findings suggest that low-density granulocytes enriched in cancer patients have immunosuppressive properties (grMDSC), however the exact nature of these cells in patients is unclear; certain studies have described these cells as immature neutrophils, others as activated neutrophils (Rodriguez et al., 2009) (Schilling et al., 2013). It is known that the expression of certain molecules on the surface of neutrophils differs depending on their stage of differentiation and activation (see section 3.1) In an attempt to better understand the phenotype of the low-density granular CD15+ cells in patients, I included in my panel antibodies against markers such as CD10 and CD33. As mentioned, surface expression of CD10 is restricted to mature, segmented neutrophils and CD33 is highly expressed on the surface of neutrophil progenitors such as myeloblasts and promyelocytes, but expression decreases as neutrophils become more differentiated (Lochem et al., 2004) (Elghetany 2002).

Analysis of cell surface expression of CD10 and CD33 revealed that the high side-scatter CD15+ cells contain two distinct populations of cells (figure 4); one population expresses CD10 and intermediate amounts of CD33 (CD10+ CD33int), the other lacks expression of CD10 and expresses higher amounts of CD33 (CD10- CD33hi). Based on the differential expression of CD10 and CD33 during neutrophil differentiation, the CD10- CD33hi population of CD15+ cells may

represent a pool of immature neutrophils present within the peripheral blood of stage IV patients. CD10+ CD33int CD15+ cells identified in the PBMCs of patients with melanoma express surface markers associated with mature neutrophils. However, mature neutrophils do not typically co-purify with PBMCs during density centrifugation, suggesting they differ from the mature neutrophil pool. As neutrophils are known to release granules upon activation one possibility is that these low-density neutrophils represent activated cells (Greer et al., 2009).

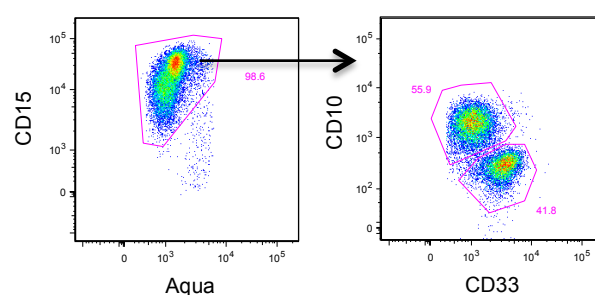


Figure 4: Further analysis of low-density granulocytes in the PBMCs of stage IV melanoma patients reveals the presence of distinct populations. CD15+ low-density granulocytes consist of two distinct populations in terms of CD10 and CD33 expression; CD10+ CD33int and CD10- CD33hi.

3.3 Detection of CD62L negative granulocytes in the whole blood of patients with stage IV melanoma patients that are absent from the peripheral blood of healthy donors

In order to test whether the low-density CD10+ CD33int CD15+ cells represent a subset of activated neutrophils, I included in my staining panel an antibody against CD62L, a protein on the surface of mature neutrophils (amongst other immune cells), which is shed during activation of neutrophils by various stimuli (Kishimoto et al., 1989) (Li et al., 2006). I carried out whole blood staining and compared the phenotype of CD15+ cells in healthy controls with those in stage IV melanoma patients.

In whole blood staining of a sample from a healthy donor the majority of granulocytes (high side scatter cells) are CD15+, in keeping with the fact that neutrophils are the most abundant leukocyte in human circulation (figure 5)

(Summers et al., 2010). When CD15+ granulocytes are gated on, these cells are CD62L+ CD10+ CD33int, in keeping with the phenotype of mature neutrophils.

Analysis of CD15+ granulocytes in the whole blood of stage IV melanoma patients however, reveals the presence of a subset of granulocytes lacking expression of cell surface CD62L. Further gating shows that CD62L- cells consist of two distinct populations, CD10+ CD33int and CD10- CD33hi cells. This mirrors what is observed in the PBMCs of stage IV melanoma patients. The lack of expression of CD62L on CD15+ cells with a mature phenotype in terms of CD10 and CD33 expression, supports the idea that there is a population of activated neutrophils present in the peripheral blood of stage IV melanoma patients and these may represent the low-density CD10+ granulocytes observed in the PBMCs.

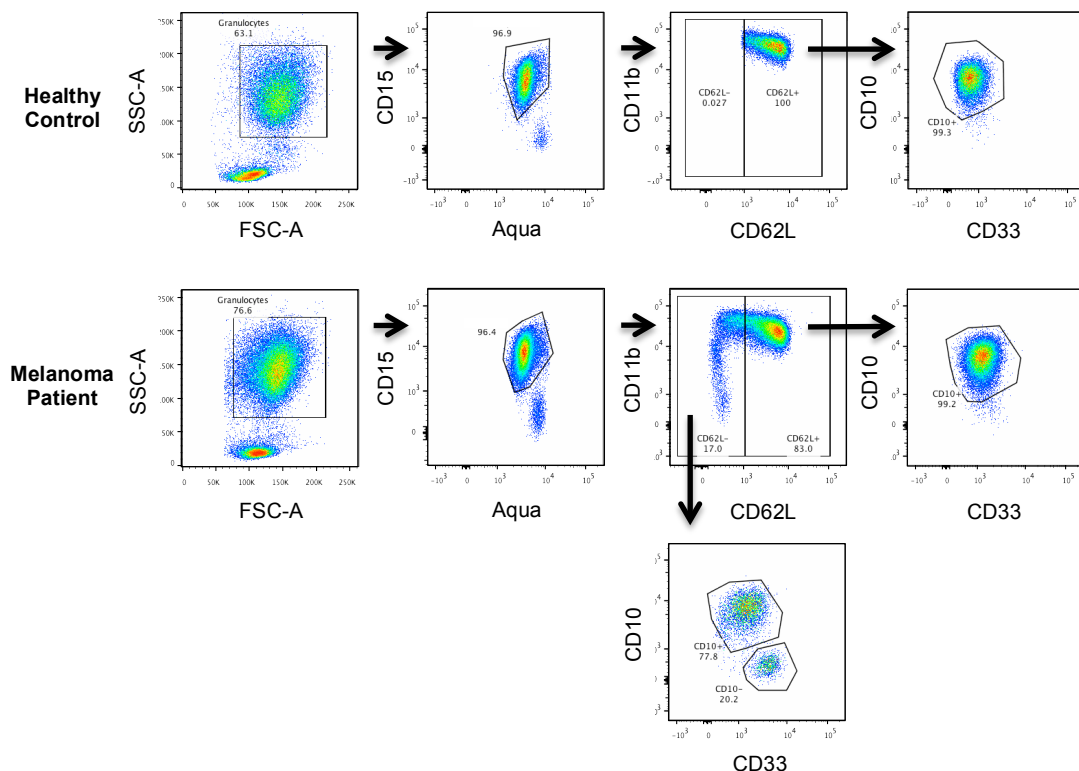


Figure 5: Comparison of whole blood staining in stage IV melanoma patients compared to healthy donors. In the peripheral blood of stage IV melanoma patients, CD15+ granulocytes that lack surface expression of CD62L are present. These cells consist of two distinct populations: CD10+ CD33int and CD10- CD33hi. In healthy controls, these cells are absent, only CD62L+ CD15+ granulocytes are present which are CD10+ CD33int. These represent the mature neutrophil population.

3.4 Further phenotypic analysis suggests that CD10+CD33int and CD10-CD33hi cells present in stage IV melanoma patients and not healthy controls, represent activated and immature granulocyte populations respectively

Thus far, I have defined low-density neutrophils in stage IV melanoma as immature or activated neutrophils based on a minimal number of surface markers and density. However, to demonstrate that low-density CD10+ and CD10- cells represent distinct populations of activated and immature granulocytes respectively, I looked for other measurable properties associated with these cells which might differentiate these two populations from each other and from the mature neutrophil pool, and attempted to better characterise their developmental stage and functional properties.

3.4.1 Comparison of cell surface expression of CD16 and CD11b expression

Both CD11b and CD16 expression increase during granulocytic differentiation, CD11b expressed from the myelocyte stage and continues to increase during differentiation and CD16 expressed from the metamyelocyte/band stage (Lochem et al., 2004) (Anderson et al., 2000).

It is evident from the staining in figure 6 that cells defined as activated neutrophils (by their CD62L- CD10+ CD33int phenotype) and immature neutrophils (by their CD62L- CD10- CD33hi phenotype) differ in terms of CD16 and CD11b expression.

In both the whole blood and PBMC, the CD15+ CD62L- cells that are CD10+ CD33int (highlighted in red on figure 6) express CD16 and CD11b, whereas the CD10- CD33hi cells (highlighted in blue on figure 6) lack CD16 expression and consist of both CD11b+ and CD11b- cells. The presence of two different subsets in the CD10- CD33hi population defined by lower or absent expression of CD11b suggests that this subsets contain immature cells at different stages of development. In whole blood staining, it is also possible to compare the phenotype of mature neutrophils. The CD62L+ CD10+ CD33int cells (highlighted in orange on figure 6), defined as mature neutrophils, express both CD16 and

CD11b. Not only is CD11b a molecule that increases in expression during differentiation, but it is also a molecule whose surface expression changes during neutrophil activation. It has been shown that neutrophils activated in the presence of cytokines, such as TNF- α , granulocyte-macrophage colony-stimulating factor (GM-CSF) or IL-8, upregulate cell surface expression of CD11b (as well as downregulating cell surface CD62L) (Wittmann et al., 2004). CD11b is present on the membrane of certain neutrophil granules and upon activation, mobilisation of granules towards the plasma membrane results in incorporation of CD11b and thus increased cell surface expression of this molecule (Borregaard & Cowland, 1997).

It can be seen in the histogram in figure 6, that CD10+ cells lacking cell surface CD62L stain more intensely for CD11b than the CD10+ CD62L+ cells, supporting the notion that CD15+ CD62L- CD10+ CD33int cells represent activated neutrophils in the blood of stage IV melanoma patients.

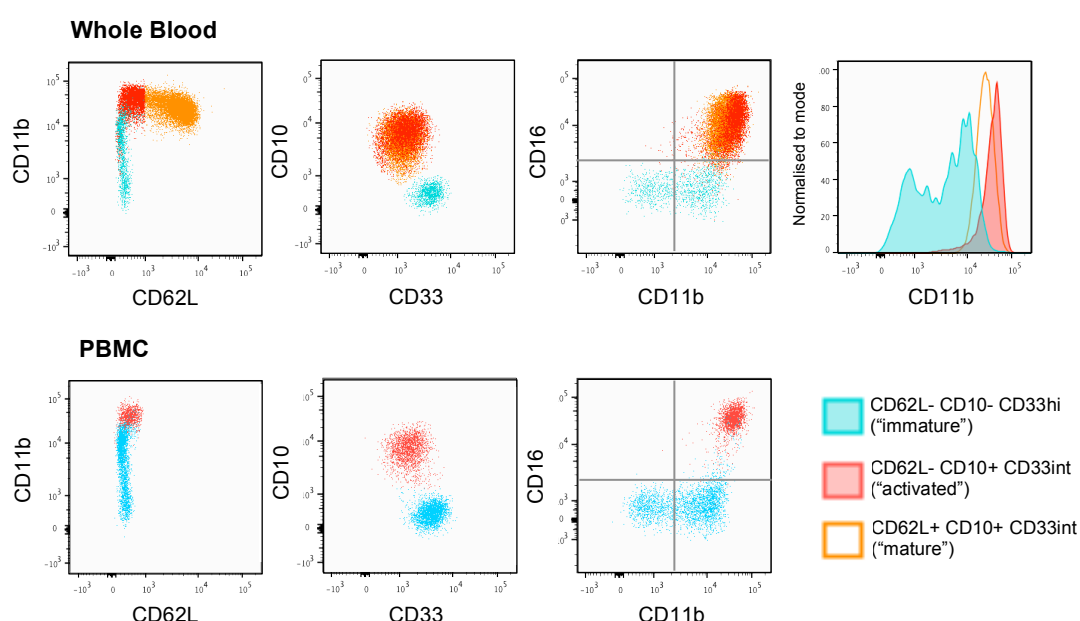


Figure 6: Analysis of CD11b and CD16 expression on granulocytes in whole blood and PBMCs of a patient with stage IV melanoma. Gated on live cells, singlets and CD15+ cells (gating not shown). Mature neutrophils were defined as CD62L+ CD10+ CD33int (orange), activated neutrophils as CD62L- CD10+ CD33int (red) and immature neutrophils as CD62L- CD10- CD33hi (blue). An overlay of the three populations (in blood) or two populations (in PBMC) enables the expression of CD16 and CD11b to be compared. Mature and activated neutrophils express both CD16 and CD11b, although as the histogram reveals, CD11b expression is higher in the activated

neutrophil population. Immature neutrophils do not express CD16 and consist of both CD11b+ and CD11b- cells.

3.4.2 Differential cell surface expression of chemokine receptors

The two major chemokine receptors expressed by mature peripheral blood neutrophils are CXCR1 and CXCR2 (Sabroe et al., 2005). These receptors bind to molecules such as CXCL8 (IL-8), CXCL6 and CXCL1 and are important for neutrophil recruitment to sites of inflammation (Sabroe et al., 2005). Downregulation of these receptors on neutrophils has been previously described upon stimulation with TLR agonists for example (Sabroe et al., 2005) (Schmausser et al., 2004). CXCR4 on the other hand, is a chemokine receptor expressed by neutrophils in the bone marrow. During steady-state granulopoiesis neutrophils are retained in the bone marrow through interaction of CXCR4 with CXCL12 (also known as stromal-derived factor 1 (SDF-1)) (Borregaard 2010). During emergency granulopoiesis, immature neutrophils are observed in peripheral blood. It is known that factors such as G-CSF, which drive emergency granulopoiesis, can downregulate CXCL12 in the bone marrow and this is thought to contribute to the release of immature neutrophils into the periphery (Eash et al., 2009).

From the histograms in figure 7 it can be seen that CD62L+ CD10+ CD33int 'mature' neutrophils (orange line) and CD62L- CD10+ CD33int 'activated' neutrophils (red line) express comparatively higher amounts of CXCR1 and CXCR2 on their surface than CD62L- CD10- CD33hi 'immature' neutrophils. The converse is true for cell surface expression of bone marrow-associated CXCR4, with higher amounts being observed on the surface of 'immature' neutrophils.

Comparison of 'mature' (orange line) with 'activated' (red line) neutrophils, reveals that 'activated' cells have lower amounts of CXCR1 and CXCR2 on their surface. As activation of neutrophils with TLR agonists for example has been shown to result in downregulation of CXCR1 and CXCR2 (Sabroe et al., 2005) (Schmausser et al., 2004), this further supports the notion that CD62L- CD10+

CD33int cells in melanoma patients represent a population of neutrophils that have undergone activation.

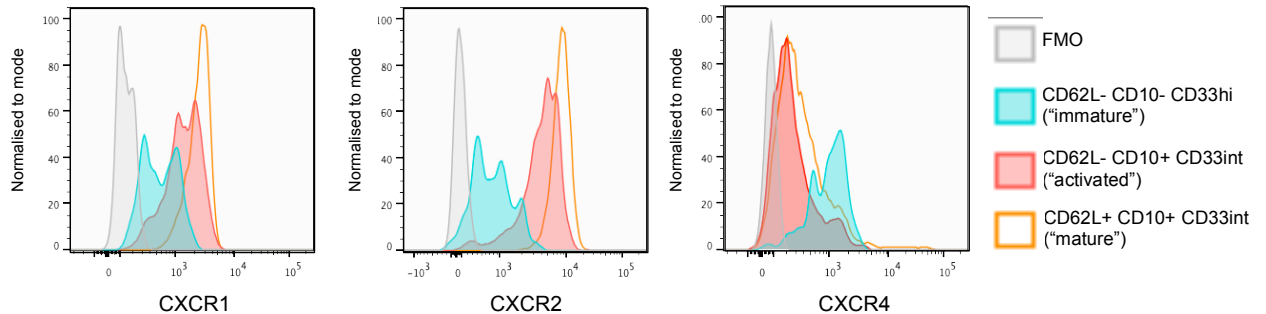


Figure 7: Differential chemokine receptor expression by peripheral blood neutrophil populations. Whole blood staining from stage IV melanoma patients reveals higher expression of CXCR1 and CXCR2 and lower expression of CXCR4 by CD62L+ CD10+ CD33int (orange) and CD62L- CD10+ CD33int (red) neutrophils when compared to CD62L- CD10- CD33hi (blue) cells.

3.4.3 Differential expression of the IL-8 and Arginase-I genes

In terms of cell surface markers, such as CD10, CD33, CD11b, CD16, CD62L and chemokine receptor expression, it seems that cells with the phenotype of activated neutrophils, and cells with the phenotype of immature neutrophils, are present in the peripheral blood of stage IV melanoma patients.

To further support this, expression of candidate genes was tested by qPCR on cDNA derived from FACS-sorted populations of ‘activated’ and ‘immature’ neutrophils from patient PBMCs.

Low-density CD10+ neutrophils (orange bars) express the pro-inflammatory gene IL-8, which is known to be expressed by neutrophils when activated by a variety of stimuli, including the TLR agonist LPS and the cytokine TNF- α (figure 8) (Strieter et al., 1992). Low-density CD10- neutrophils (blue bars) express barely detectable IL-8 mRNA. Conversely, CD10- cells (blue bars) express more arginase 1 (ARG-1) mRNA than CD10+ cells. It is known that ARG-1 is more highly expressed (in terms of mRNA) in neutrophils in the bone marrow

(particularly by myelocytes and metamyelocytes and to a lesser extent more mature bone marrow neutrophils) compared to peripheral blood neutrophils (Theilgaard-Monch et al., 2005, Jacobsen et al., 2007). The differential expression of the proinflammatory cytokine IL-8 and ARG-1 further supports the defining of these low-density granulocyte populations as activated and immature neutrophils.

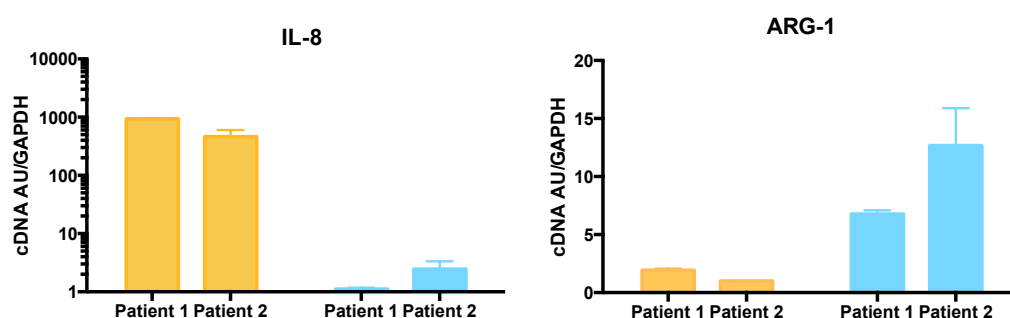


Figure 8: Quantitative PCR analysis of IL-8 and ARG-1 expression in low-density granulocytes from patients with stage IV melanoma. CD10⁺ CD33^{int} (orange bars) and CD10⁻ CD33^{hi} granulocytes (blue bars) were FACS sorted from patient PBMCs. Expression of IL-8 and ARG-1 was determined by qPCR and is shown relative to the house-keeping gene, GAPDH. The mean and standard deviation of triplicate values are shown.

3.5 Staining of bone marrow reveals presence of cells with a similar phenotype to peripheral immature cells

CD15⁺ CD10⁻ CD33^{hi} cells in the peripheral blood of stage IV melanoma patients have a phenotype that suggests they are immature neutrophils. The bone marrow is the site of granulopoiesis and it contains immature neutrophils at various stages of differentiation (as well as a pool of mature neutrophils). I therefore stained bone marrow samples in order to compare the phenotype of immature-like neutrophils in the peripheral blood with the phenotype of those in the bone marrow. Bone marrow samples (previously frozen and kindly provided by B. Stoilova in Prof. Vyas' lab, WIMM) were thawed and filtered (to remove large clumps thought to represent the mature neutrophil population which does not withstand freezing). Cells were then subject to lymphoprep density-based

centrifugation and the PBMC equivalent was purified and stained with the same antibody cocktail used to analyse the peripheral blood populations. When gating on the CD15+ cells, the majority of cells are CD10- CD33hi and as in the blood, they lack expression of CD16 (figure 9). Although my panel of antibodies is insufficient to pin-point the exact stages of neutrophil differentiation, it appears that a cell population observed in the periphery of patients with melanoma is consistent with a population detectable within bone marrow.

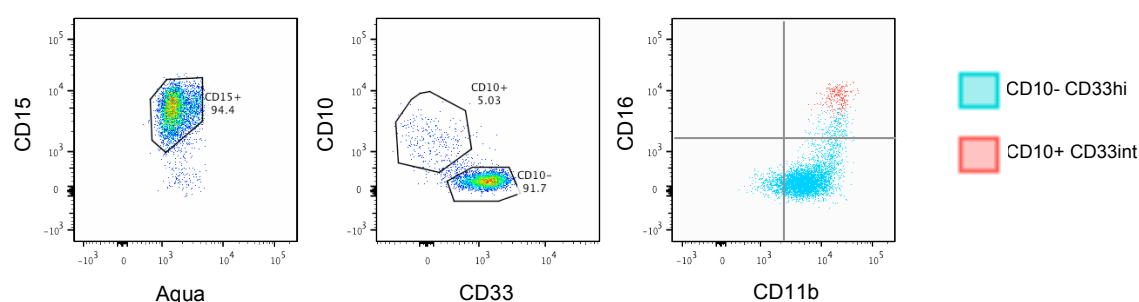


Figure 9: FACS staining of low-density granulocytes isolated from a thawed bone marrow sample. Live, singlet cells high in side scatter and CD14lo (gating not shown) are predominantly CD15+. The majority of these cells are CD10- CD33hi and CD16- (shown in blue on the overlay).

3.6 Enrichment of low-density granulocyte populations in stage IV melanoma patients compared to age-matched healthy controls

Comparison of peripheral blood samples from stage IV melanoma patients and age-matched healthy controls, reveals a higher frequency of activated neutrophils in the PBMCs of patients with melanoma (figure 10). When considering the frequency of immature neutrophils, whilst the difference in the distribution of frequencies in patient and healthy control samples does not reach significance, it is clear that at least a subset of patients have a higher frequency of these cells compared to healthy controls (figure 10). This is in fact also true for the frequency of activated neutrophils in patients, in that there is a range of frequencies; certain patients have a low frequency of cells which is no different to the frequency observed in healthy controls, while other patients have a higher frequency.

In figure 10, each patient is represented by a unique symbol. Analysis of individual patients reveals that whilst certain patients have a high frequency of both activated and immature neutrophils in their PBMCs and certain patients have a low frequency of both activated and immature neutrophils in their PBMCs, some patients have only an enrichment of one of these neutrophil populations.

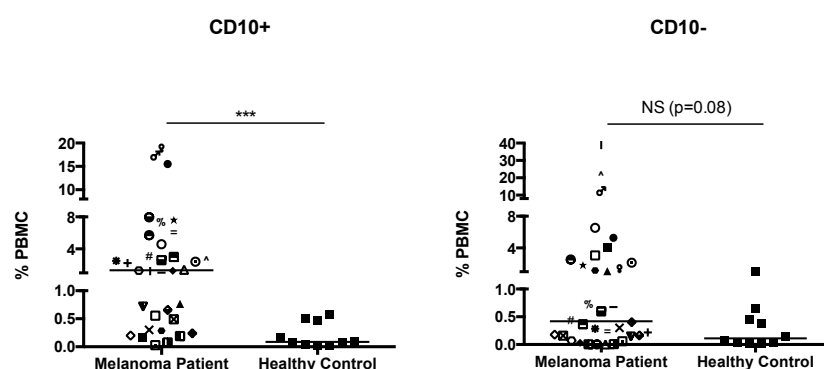


Figure 10: Frequency of low-density granulocytes in the peripheral blood of stage IV melanoma patients versus healthy controls. FACS staining of PBMCs was used to determine the frequency of CD10+ (CD33int) and CD10- (CD33hi) CD15+ cells. Values are expressed as percentage of PBMCs. Each patient is represented by a unique symbol, revealing inter-patient variation in the frequency of these distinct populations. The median is highlighted and the difference in distribution was determined using the Mann-Whitney U test. *** $p < 0.001$, NS: non-significant.

Staining of further patients (without the use of unique symbols) and stratification of patients according to last previous treatment are shown in appendix A1.1.

3.7 Are these neutrophil populations present in tumours of melanoma patients?

CXCR1 and CXCR2 recognise ligands including CXCL8 (IL-8). IL-8 can be detected in the tumour microenvironment and may originate from innate immune cells such as monocytes and neutrophils themselves or from melanoma cells (Ryu et al., 2007) (Peng et al., 2007). As well as being produced in the bone marrow, it has been shown that CXCL12, the ligand for CXCR4, is produced by tumour cells

as well as tumour-associated stromal cells (Domanska et al., 2013) (Vianello et al., 2006). Therefore these two low-density granulocytic populations have the potential to be recruited to melanoma tumours. As low-density granulocytes have been associated with suppression of T cell responses, this may have important implications for tumour-specific T cells within the tumour microenvironment. I therefore attempted to determine whether such neutrophil populations could be found within melanoma tumours. CD15+ cells can be found in melanoma tumours (two examples shown in figure 11). When these cells were analysed for expression of CD10 and CD33, the cells were CD10+ CD33int. However, when tumour and matched blood samples were stained, the low-density granulocytes in the PBMCs were predominantly activated CD10+ CD33int neutrophils. Unfortunately, due to sensitivity to cryopreservation, only a limited number of fresh tumour and blood samples could be analysed for the presence of neutrophil populations. As I did not have access to a matched fresh blood and tumour sample from a patient with a high frequency of immature neutrophils in the peripheral blood, it is unclear whether the inability to detect such cells in the tumour samples is because they are unable to migrate into tumours or because the patients did not have any of these cells.

As mentioned, a recent study revealed that out of 22 different tumour-infiltrating leukocyte populations analysed, increased tumour infiltration by neutrophils was the most significant adverse prognostic factor (Gentles et al., 2015). It would be interesting to further investigate whether different subsets make up the tumour-infiltrating neutrophil population.

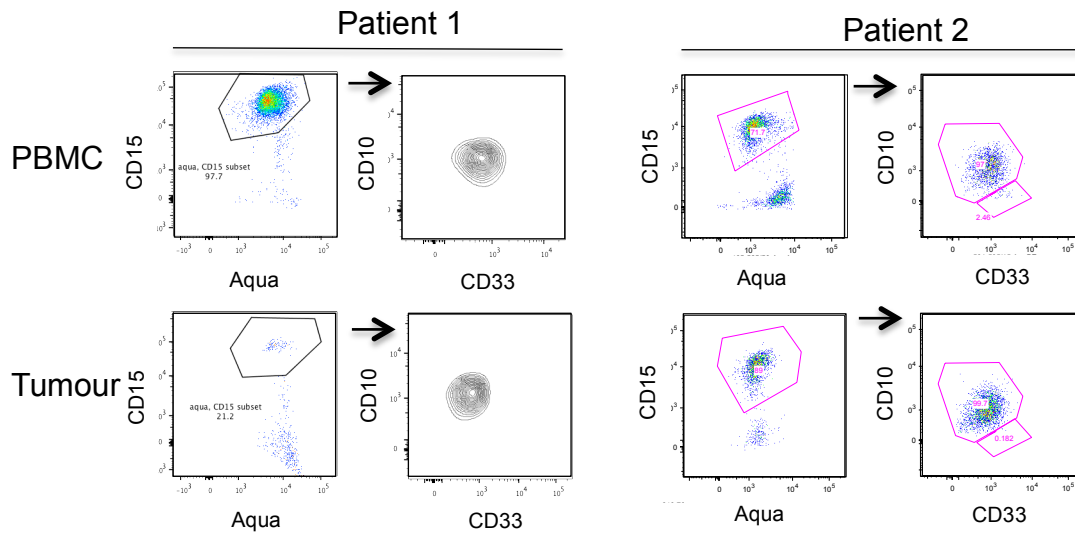


Figure 11: Detection of CD15+ cells in melanoma tumour samples. FACS staining of matched blood and tumour samples from two melanoma patients. In both blood and tumour, and in both patients, the CD15+ cells are CD10+ CD33int. Previous gating on live, singlets and CD45+ cells not shown.

3.8. Functional characterisation of the low-density granulocytes found in the peripheral blood of stage IV melanoma patients

Attempts were made to functionally characterise the activated and immature CD15+ cells found in the peripheral blood. Attempts were confounded by the inability to sort large number of cells and the unpredictability of cell frequency within blood samples. Data can be found in the appendix A1.2.

3.9 Comparison of the frequency of low-density granulocytes between different subclasses of stage IV melanoma

Due to the difficulty in functionally characterising the two subsets of neutrophils in melanoma patients, I asked whether elevated frequencies of immature or activated neutrophils correlate with disease progression. Indeed stage IV melanoma patients are fairly heterogeneous in their frequency of activated and/or immature neutrophils in the peripheral blood, with a proportion of patients showing low frequency of these cells (and no different to healthy

individuals) and a proportion of patients where these subsets are elevated in comparison to age-matched healthy controls.

Although all blood samples were taken from patients diagnosed with stage IV melanoma, different subclasses of stage IV disease exist, reflecting different extents of metastasis. The subclasses are M1a, M1b and M1c. M1a defines disease which has metastasised to distant skin, the subcutaneous layer or distant lymph nodes but in which patients have a normal serum lactate dehydrogenase (LDH) concentration (Balch et al., 2004). M1b defines disease involving lung metastases and normal serum LDH, whereas M1c defines disease that has metastasised to other visceral sites or to any distant site, in patients with raised serum LDH (Balch et al., 2004).

Although the difference in the percentages of activated CD10+ or immature CD10-neutrophils in M1a/b versus M1c patients do not reach statistical significance, it is evident (especially for the activated neutrophil population) that the patients with the highest frequencies, fall within the M1c subclass (figure 12). This may reflect, to some degree, a relationship between the extent of metastasis and altered neutrophil biology. However, the frequency of CD10+ or CD10- neutrophils in many patients with M1c disease is no different to the frequency observed in patients with M1a or M1b disease. This suggests that other factors exist that distinguish patients with an abundance of distinct low-density granulocytes from those lacking these cells.

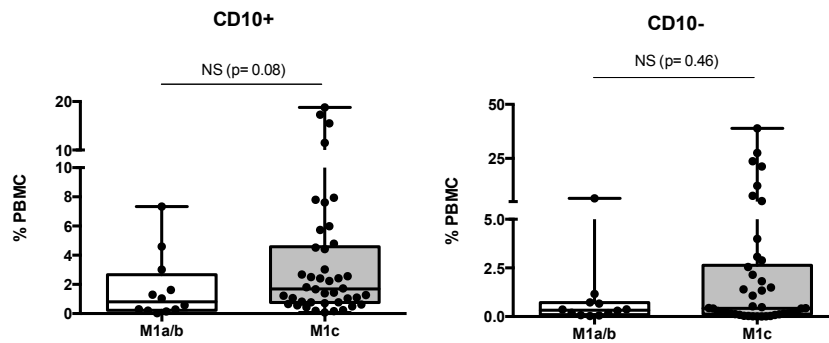


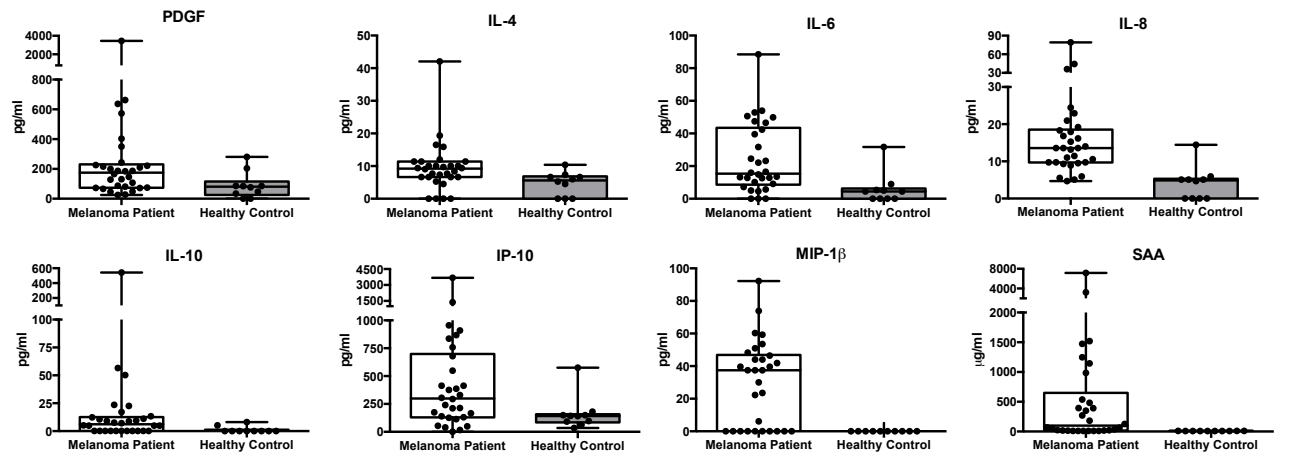
Figure 12: Stratification of stage IV melanoma patients according to subclasses of stage IV melanoma. The frequency of CD10+ (CD33int) activated neutrophils and CD10- (CD33hi) immature neutrophils in patients with M1a/b disease versus M1c disease. The median and interquartile range are highlighted and the difference in distribution was determined using the Mann-Whitney U test.

3.10 There is an increase in the concentration of different cytokines/growth factors in the plasma of patients with stage IV melanoma compared to healthy controls

The interplay between cancer and the immune system leads to an inflammatory response which may have different effects on tumour growth and tumour control. Cytokines, chemokines and growth factors can be released into the circulation and strongly influence the activation of immune cells as well as their differentiation and egression from the bone marrow. Increases in the concentration of specific cytokines may reflect the type of inflammation at the site of tumour growth.

In order to explore which factors might contribute mechanistically to the heterogeneity in the frequency of the distinct low-density neutrophil populations, I asked whether the abundance of distinct neutrophils might be associated with a particular inflammatory axis in the patients analysed. To answer this question, I profiled the plasma from a subset of patients for the concentration of 28 cytokines/growth factors using a BioPlex assay and ELISA.

A



B

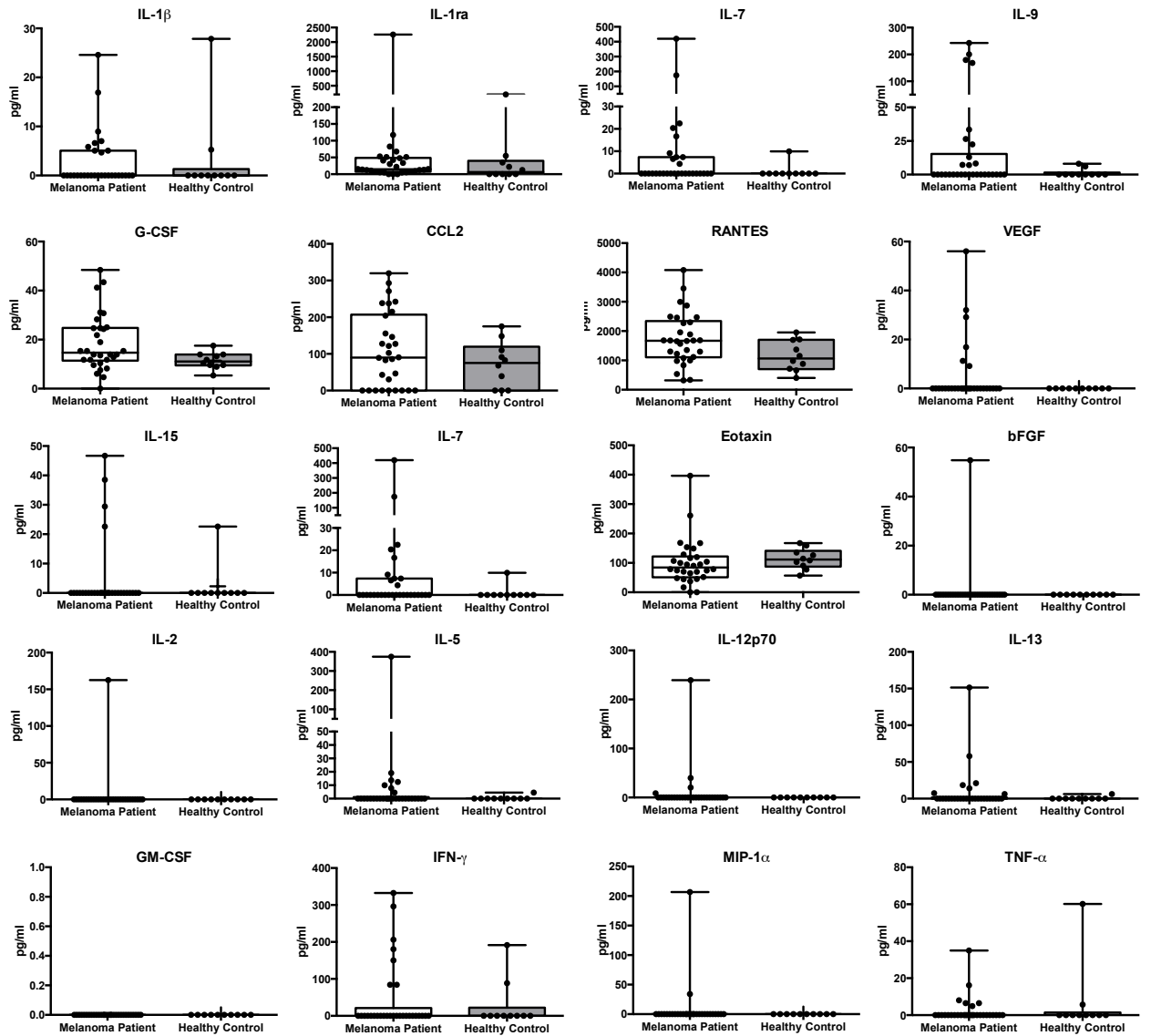


Figure 13: The concentration of cytokines/growth factors in the plasma of stage IV melanoma patients compared to healthy controls. A: Factors that show a statistically different distribution in patient samples compared to healthy controls, as determined by the Mann Whitney U Test. The median and interquartile range are shown (p values: PDGF *, IL-4 *, IL-6 ***, IL-8 ****, IL-10 *, IP-10 *, MIP1-1 β ***, SAA *** where * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). B: Factors whose distributions do not reach statistical significance. Abbreviations - PDGF: platelet-derived growth factor, IFN- γ : interferon gamma, IP-10: IFN- γ -inducible protein 10 (also known as CXCL10), MIP-1 α/β : macrophage inflammatory protein-1 alpha/beta, bFGF: basic fibroblast growth factor, G-CSF: granulocyte colony-stimulating factor, TNF- α : tumour necrosis factor alpha, IL-1ra: interleukin 1 receptor antagonist, RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted, VEGF: Vascular endothelial growth factor, SAA: Serum amyloid A.

To identify which factors are elevated in melanoma patients compared to healthy controls, I applied a Mann–Whitney U Test. Comparison of the distribution of concentrations in the two cohorts reveals that eight cytokines/growth factors showed statistically significant differences between healthy controls and melanoma patients (figure 13A). For certain cytokines/growth factors, for example IL-9, VEGF and CCL2, although the difference between the two cohorts did not reach statistical significance, in a subset of patients the concentration of these factors was increased (figure 13B). Given the previous observation that the peripheral blood of certain patients is enriched with activated or immature neutrophils, whereas in other patients the frequency of these populations is low and comparable to healthy controls, it may be premature to rule out results that do not reach statistical significance. It is also true that for the statistically enriched cytokines/growth factors, there are certain patients with clearly higher concentrations compared to healthy controls but there are also patients who have no detectable or similar concentrations to healthy controls. It may be that the range of cytokine/growth factor concentrations among patients is linked to the range of low-density granulocyte frequencies that are also observed in the PBMCs of patients.

3.11 There are correlations between the concentration of certain cytokines/growth factors in plasma and the abundance of distinct granulocyte subsets in stage IV melanoma patients

Increases in the frequency of activated neutrophils in peripheral blood in the context of inflammation have previously been described (Pillay et al., 2012). It is also known that during inflammation, immature neutrophils can be prematurely released from the bone marrow in processes referred to as emergency and reactive granulopoiesis (Manz & Boettcher, 2014). One could therefore assume that the observed enrichment of activated and immature neutrophils in stage IV melanoma patients compared to healthy controls merely reflects generic inflammation that exists in patients with advanced cancer. However, although certain patients have elevated frequencies of both granulocyte populations, in others there is marked inter-patient variation in the frequency of immature and activated granulocytes. On the basis of this, I sought to ask whether the presence of distinct cytokines/growth factors in the plasma correlate with the presence of distinct low-density granulocytes in the peripheral blood.

I calculated the Spearman's Rank correlation coefficients for the different neutrophil populations and the concentration of cytokines/growth factors detected in patient plasma. I included in the correlation matrix both those cytokines/growth factors which showed a statistically significant difference in distribution in patient plasma compared to healthy control plasma as well as those that correlated with statistically elevated cytokines/growth factors. A visualisation of the correlation matrix (correlogram) was created using RStudio software and is represented in figure 14.

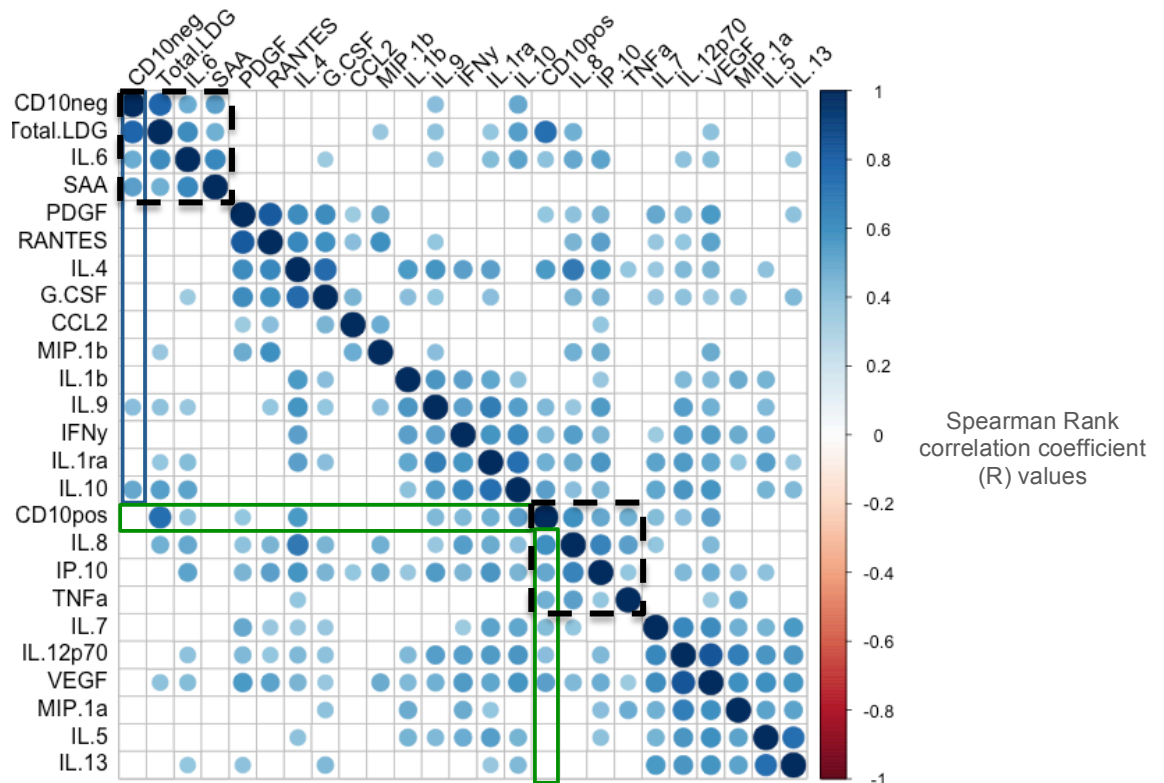


Figure 14: Correlogram highlighting correlations between plasma cytokine/growth factor concentrations and frequency of low-density neutrophil populations in the peripheral blood of stage IV melanoma patients. The colour and size of the circles represents the Spearman's Rank (R) correlation coefficient (larger circles/more intense colour representing values closest to +1/-1). Only correlations with p values ≤ 0.05 are shown. Factors that correlate with immature neutrophils (CD10-) are highlighted in the blue box and those that correlate with activated neutrophils (CD10+) are highlighted in the green boxes. Correlations with the frequency of total low-density granulocytes (Total LDG) are also shown. Abbreviations - CD10pos: CD10+ CD33int neutrophils, CD10neg: CD10- CD33hi neutrophils, MIP.1a and MIP.1b: MIP-1 α/β , IL.1b: IL-1 β , TNFa: TNF α , IFNy: IFN γ . Variables were subject to hierarchical clustering using the hclust function on RStudio software.

The frequency of immature neutrophils correlates with the concentration of SAA, IL-6, IL-9 and IL-10 in the plasma (figure 14). While there is overlap between immature and activated neutrophil correlations, in that the frequency of activated neutrophils also positively correlates with the concentration of plasma IL-6, IL-9 and IL-10, activated neutrophils positively correlate with ten distinct factors (IL-1ra, IFN γ , IL-7, IL-12p70, VEGF, IL-8, IP-10, TNF α , IL-4 and PDGF).

Immature neutrophils cluster with SAA, IL-6 and total low-density granulocyte frequency, whereas activated neutrophils cluster with IL-8, IP-10 and TNF α (highlighted by the black dashed outline in figure 14). The fact that, in a correlation matrix containing CD10+ and CD10- neutrophils and different soluble factors, these two neutrophil populations do not correlate with one another implies that the frequency of these populations might be associated with distinct inflammatory axes. This notion is supported by the fact that there are certain stage IV melanoma patients that have an enrichment of only activated neutrophils or only immature neutrophils.

3.12 SAA and IL-8 independently correlate with the enrichment of a distinct neutrophil population

The acute phase protein SAA has been found to induce granulopoiesis in mice but *in vitro* it has also been shown to induce production of IL-8 by neutrophils and monocytes (De Santo et al., 2010) (Ribeiro et al., 2003). In the context of this cohort of patients, these two factors appear to be independent of one another. There is no correlation between the concentration of plasma SAA and the concentration of IL-8 (figure 15B left panel) and each molecule correlates with enrichment of a distinct low-density neutrophil population – SAA with immature CD10- neutrophils and IL-8 with activated CD10+ neutrophils (figure 14 and 15A). Neither molecule correlates with the other neutrophil population (figure 15B).

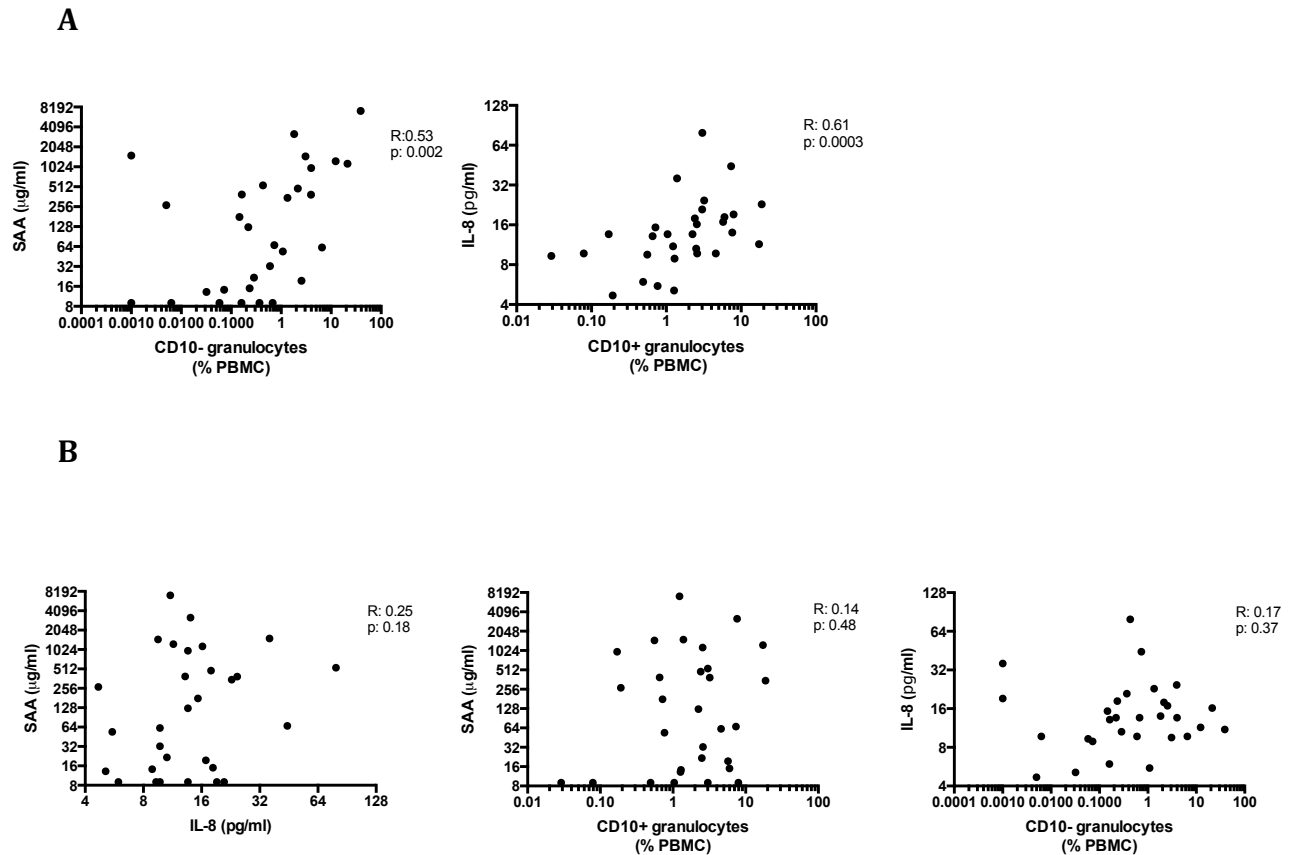


Figure 15: SAA and IL-8 independently correlate with the enrichment of a distinct neutrophil population. A: The concentration of plasma SAA positively correlates with the frequency of CD10- immature neutrophils, whereas the concentration of plasma IL-8 correlates with the frequency of CD10+ activated neutrophils. B: The concentration of SAA does not correlate with the concentration IL-8 in the plasma or the frequency of low-density CD10+ activated neutrophils and neither does the concentration of plasma IL-8 correlate with the frequency of immature CD10- granulocytes. Spearman's Rank correlation coefficients were determined.

3.13 Stratification of patients according to BRAF status

As well as immune cells being a potential source of cytokines/growth factors within patient plasma, melanoma cells themselves can produce such factors. A common mutation resulting in immortalisation of melanocytes occurs within the BRAF protein kinase, with around 90% of BRAF mutations being a substitution of valine to glutamic acid at position 600 (V600E) (Packer et al., 2009). BRAF is part of the mitogen-activated protein kinase (MAPK) signalling pathway,

important for survival and proliferation of melanocytes, but which also plays a role in the production of cytokines (Sumimoto et al., 2006). Cells with the activating BRAF V600E mutation have been shown to produce cytokines/growth factors such as IL-6, VEGF and IL-10, with inhibition of BRAF signalling resulting in suppression of such cytokine production (Sumimoto et al., 2006). It may be that melanoma cells themselves, through acquisition of a particular mutation, contribute to an inflammatory environment which may have an effect on neutrophil biology (such as driving the premature release of neutrophils from the bone marrow or driving neutrophil activation).

Patients with BRAF mutant melanoma are treated with BRAF inhibitors, such as Vemurafenib, and thus testing for the BRAF V600E mutation is routinely carried out in the clinic (Spagnolo et al., 2015). In order to assess whether the correlation of granulocyte populations with particular factors is linked to BRAF status, I stratified patients according to the presence of the V600E mutation. There is no difference in the distribution of activated or immature granulocytes between the two groups of patients (figure 16). Indeed there is no difference in the concentration of factors such as IL-6, IL-10 and VEGF in the plasma of patients with or without the BRAF V600E mutation (appendix A1.3). Of the 28 tested, the only differences observed are in the concentrations of IL-15 and eotaxin, with a statistically significant increase in the concentration in the group of patients with this mutation (appendix A1.3).

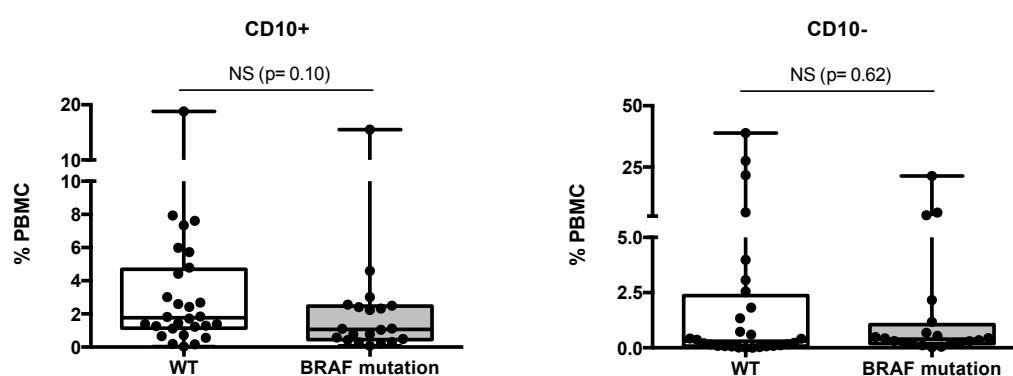


Figure 16: Stratification of patients according to the presence of the BRAF V600E mutation. There is no statistical difference in the frequency of activated (CD10+) or immature (CD10-) neutrophils in patients with or without the BRAF V600E mutation. The median and interquartile range are highlighted and the difference in distribution was analysed using the Mann-Whitney U test. NS: non-significant.

3.14 A high frequency of mature neutrophils and a high concentration of SAA in plasma correlate with a higher frequency of immature granulocytes in peripheral blood

It is evident from the correlogram (figure 14) and the raw data in figure 17C, that there is a positive correlation between the concentration of plasma SAA and the abundance of immature CD10- neutrophils. It is also true that there is a positive correlation between the frequency of mature neutrophils in the peripheral blood and SAA concentration (figure 17B) as well as a positive correlation between the frequency of immature CD10- neutrophils and mature neutrophils in the peripheral blood (figure 17A). Such an increase in immature neutrophils peripherally which is linked to an increase in the mature neutrophil pool, is suggestive of increased granulopoiesis in certain patients. Emergency and reactive granulopoiesis occur during inflammation, such as during an infection or inflammatory disease. The association of SAA with such increases suggests a role for this acute phase molecule in granulopoiesis.

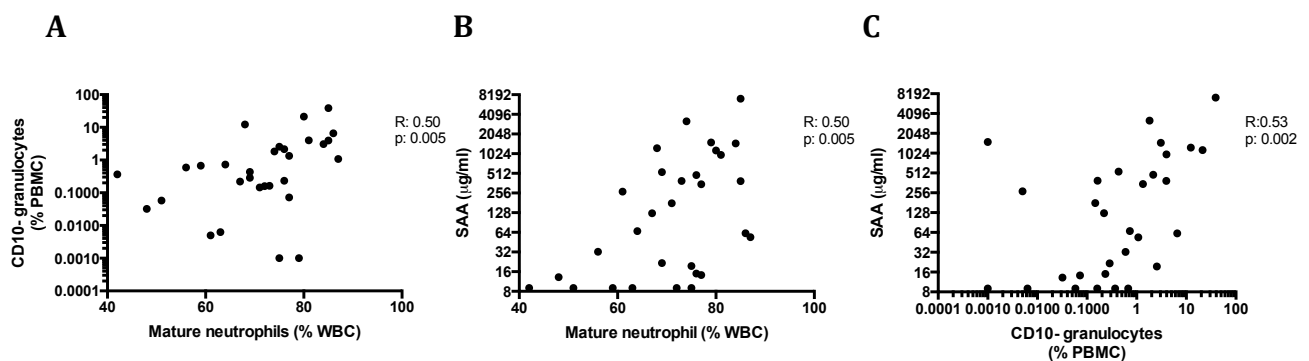


Figure 17: Positive correlations between CD10- immature granulocytes, mature neutrophils and plasma concentration of SAA. Spearman Rank correlation coefficients (R) were determined. A: A positive correlation is observed between the frequency of CD10- immature granulocytes and mature neutrophil count (as percentage of white blood cells (WBC)). B: A positive correlation is observed between plasma SAA concentration and mature neutrophil count. C: A positive correlation is observed between plasma SAA concentration and the frequency of CD10- immature granulocytes.

3.15 Can SAA promote neutrophil differentiation?

SAA has previously been associated with an accumulation of MDSC and administration of recombinant SAA in mice results in an increase in neutrophil frequency in the peripheral blood (De Santo et al., 2010) (Sander et al., 2010). However, a direct effect of SAA on haematopoietic progenitors has not been shown. Furthermore, it has been suggested that recombinant SAA is a more potent proinflammatory stimulus than physiological SAA (Christenson et al., 2013). In an attempt to assess the role of SAA in the differentiation of haematopoietic progenitors towards a neutrophil lineage, I used a protocol kindly provided by D. Karamitros (Vyas lab, WIMM), which uses a cocktail of factors known to induce neutrophil, monocyte, B and NK cell differentiation from CD34+ progenitors. I supplemented this cocktail with either recombinant SAA or physiological SAA in the form of patient plasma.

Figure 18A shows representative FACS plots corresponding to differentiation in the presence of the defined cocktail as well as differentiation in the presence of this cocktail supplemented with patient plasma. Figure 18B shows the percentage of CD15+ cells (of total haematopoietic CD45+ cells) in the different conditions tested. It is evident from figure 18Bi, that supplementation of the defined cocktail with recombinant SAA for the initial feed does not result in an increase in the percentage of CD15+ cells at the end of the 18 day culture. Supplementation with PamCSK, a TLR-2 agonist previously implicated in enhancing myeloid differentiation (Luca et al., 2009), increases the percentage of CD15+ cells when used at a concentration of 1µg/ml, however this effect is lost when the concentration is reduced to 0.1µg/ml. As differentiating progenitors must be fed a further two times over the course of the 18 day culture, I also compared differentiation of CD15+ cells when the defined cocktail was supplemented with recombinant SAA or PamCSK at each of the feeds (Figure 18Bii). However, it remains that the only statistically significant increase in CD15+ percentage occurs in cultures supplemented with 1µg/ml PamCSK.

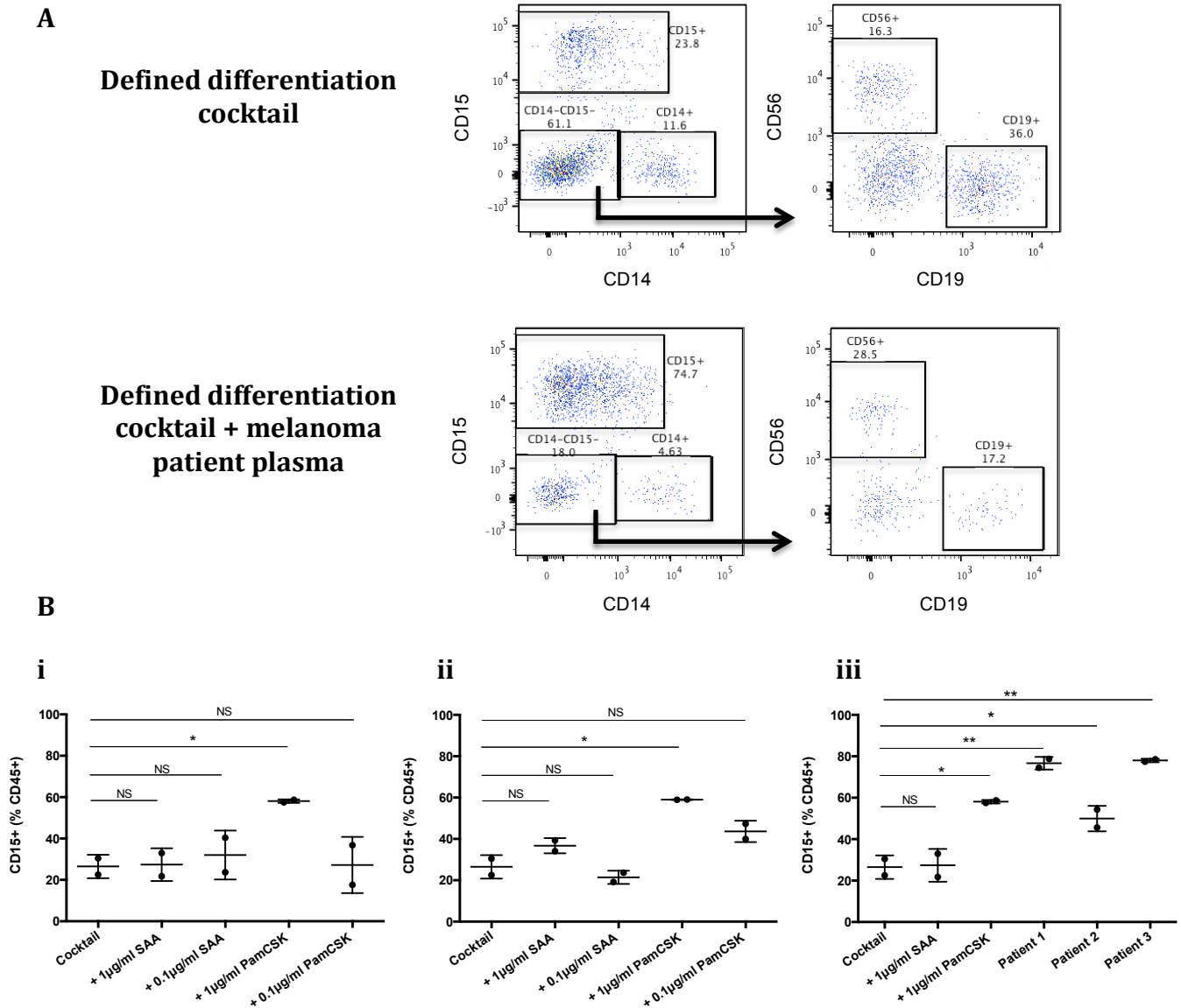


Figure 18: Example FACS plots of CD34⁺ cells at the end of 18 days culture and quantification of CD15⁺ cells. A: The upper panel represents cells cultured in the presence of a defined differentiation cocktail. The lower panel represents cells cultured in the defined differentiation cocktail supplemented with plasma from a stage IV melanoma patient. The cells were previously gated on live, singlets and CD45⁺ cells. B: CD15⁺ cells are expressed as a percentage of CD45⁺ cells. Recombinant SAA at a concentration of 1µg/ml or 0.1µg/ml, when added at the start of CD34⁺ differentiation (i) or at the start as well as at subsequent feeds (ii), does not increase the percentage of CD15⁺ cells after 18 days of in vitro culture. There is an increase in the percentage of CD15⁺ cells when the cocktail is supplemented with 1µg/ml PamCSK when added at the start of differentiation (i) or at the start as well as at subsequent feeds (ii). iii: When patient plasma was used to supplement the defined differentiation cocktail, there was an increase in the percentage of CD15⁺ cells at the end of the culture, regardless of whether that plasma was taken from patients with a high SAA concentration (patient 1 and patient 2) or a patient with barely detectable SAA (patient 3). Plasma samples were diluted 1:4. Original plasma concentrations of SAA, among other cytokines, for patient 1, 2 and 3 are shown in figure 19. The difference between the mean frequency of CD15⁺

*cells in different conditions was determined using a T-test. * $p < 0.05$, ** $p < 0.01$, NS: non-significant.*

As it has been suggested that the function of recombinant SAA does not necessarily reflect the function of physiological SAA (Christenson et al., 2013), and as I had plasma samples from patients with a high concentration of SAA, I attempted to determine whether physiological SAA could influence differentiation of CD15+ cells. Supplementing the defined differentiation cocktail with plasma from patients with a high concentration of SAA (patient 1 and 2) or undetectable SAA (patient 3) resulted in an increase in CD15+ differentiation when compared to the original cocktail (Figure 18Biii). This increase however occurs irrespective of SAA concentration.

The plasma samples chosen for differentiation were from two patients with a high SAA concentration (greater than 1000 μ g/ml) (patients 1 and 2) and from a patient with an SAA concentration below the detectable limit of 9ng/ml (patient 3). All three patients had low but comparable plasma concentrations of IL-8 (less than 10pg/ml), IL-8 being the opposing cytokine that I have previously shown to most strongly positively correlate with the frequency of activated neutrophils in PBMCs, but not with the frequency of immature neutrophils.

The expectation, if physiological SAA does positively regulate neutrophil differentiation, was that an increase in neutrophil percentage would be observed when progenitors were cultured in medium supplemented with plasma from patient 1 and 2 but not when cultured in medium supplemented with the plasma of patient 3. Interestingly, plasma from patient 3 results in a higher frequency of CD15+ cells than plasma from patient 2, and is comparable to the frequency observed with the plasma from patient 1. These plasma samples however will contain many other factors, which may promote neutrophil differentiation, or indeed skew differentiation in favour of another cell type. Samples were therefore analysed for the presence of other cytokines/growth factors. Of the 28 tested, 14 were detectable in these samples. Differences in the concentration of factors can be seen in figure 19. Of the three samples, patient 3 has the highest

concentration of G-CSF, a key granulopoietic factor (Manz & Boettcher, 2014). This concentration however is only marginally higher than the concentration in the samples of patient 1 and 2. IL-6, another factor implicated in positively regulating granulopoiesis is marginally higher in the samples in patient 1 and 2 (Manz & Boettcher, 2014). Interestingly, all samples contain a high concentration of RANTES (CCL5), with plasma samples containing the highest concentrations (patients 1 and 3) associated with the greatest CD15+ differentiation. RANTES is a cytokine produced by damaged stromal cells as well as activated T cells and is important for recruitment of cells such as monocytes and T cells to sites of inflammation (Ergen et al., 2012). RANTES has also been shown to influence the differentiation of haematopoietic cells. In ageing mice, RANTES skews differentiation towards a myeloid phenotype and may explain why progenitors cultured with this plasma sample show enhanced neutrophil differentiation (Ergen et al., 2012).

It may be that SAA does not influence neutrophil differentiation, and other factors such as RANTES are responsible for the observable differences in figure 18Biii. It could also be that SAA does positively influence neutrophil differentiation but that there are other factors, besides those tested, that skew differentiation towards a different cell type.

It should be noted that there is however no correlation between the concentration of RANTES in the plasma and the frequency of immature neutrophils in the peripheral blood (figure 14). It may be that although RANTES can influence differentiation of progenitors in the bone marrow, perhaps the concentration in the plasma does not reflect the concentration within the bone marrow. In the same way it is possible that the concentration of SAA in the bone marrow is more elevated than in the plasma, or that other factors present in the bone marrow niche can synergize with SAA to favour granulopoiesis.

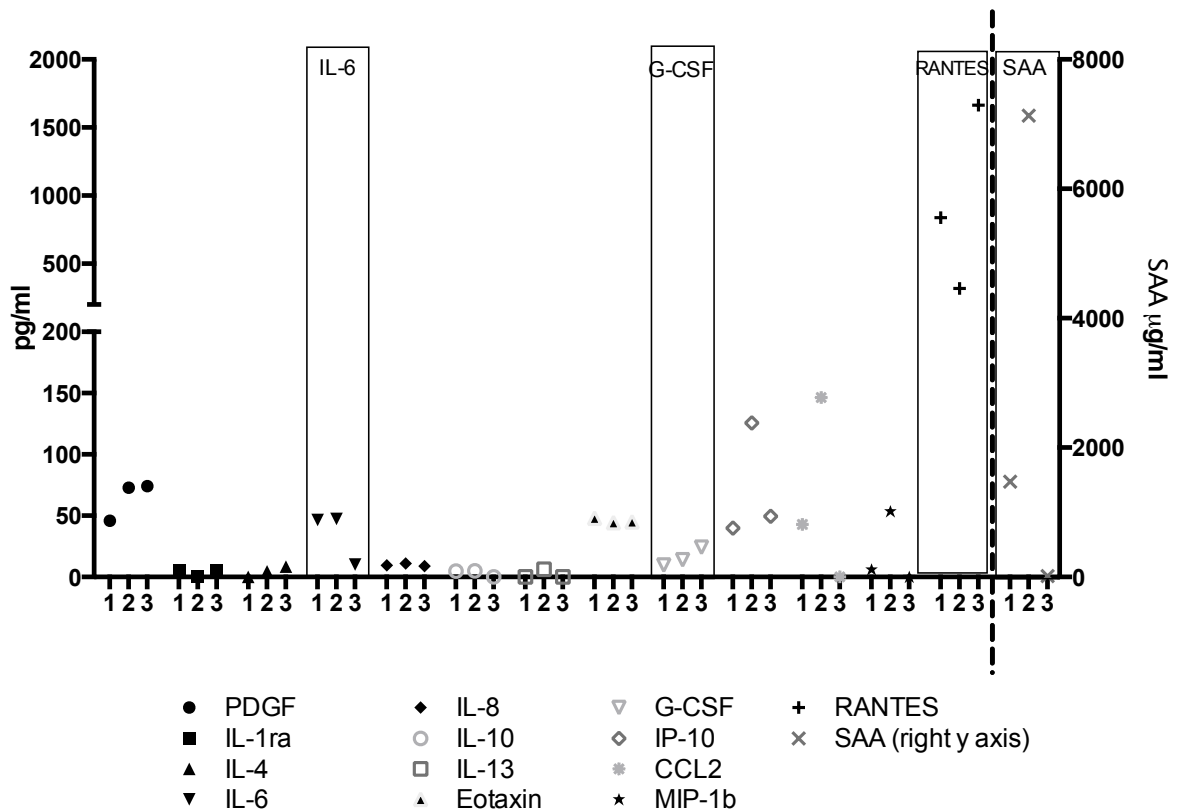


Figure 19: The concentration of cytokines/growth factors detected in the plasma of the three stage IV melanoma patients used in the differentiation assay. Of the 28 tested, 14 are detected. 1, 2 and 3 along the x axis represent patient 1, patient 2 and patient 3. The cytokines/growth factors are each represented by a unique symbol, with the corresponding description in the legend below. The concentration of SAA is shown on the right y axis. The concentrations of all other cytokines/growth factors are shown on the left y axis. Highlighted in boxes are factors implicated in neutrophil and myeloid cell differentiation, as well as the factor of interest, SAA.

3.16 Analysis of low-density granulocytes in melanoma patients treated with anti-CTLA-4 or anti-PD-1 antibodies

Although responses to anti-CTLA-4 (including complete response, partial response and stable disease) can be seen in around 30% of treated patients, it remains that a large proportion of melanoma patients continue to progress (Hodi et al., 2010). Previous studies, analysing the T cell compartment in ipilimumab-treated patients, have revealed that a lower baseline frequency of proliferating CD8+ T cells is associated with relapse (Wang et al., 2012). Given that low-density granulocytes with suggested immune-suppressive capacity are enriched

in a subset of patients, I asked whether the efficacy of anti-CTLA-4 was related to frequency of low-density granulocytes in the peripheral blood of patients.

Since starting this study, there have been reports of altered myeloid cells in patients treated with anti-CTLA-4. However, to my knowledge altered frequency of grMDSC, as defined as total low-density CD15⁺ CD11b⁺ cells, during anti-CTLA-4 treatment has only been reported in one study. The analysis by Pico de Coaña et al. (2013) however, only includes five patients and reports a decrease in the frequency of grMDSC after the first cycle of anti-CTLA-4, which is maintained nine weeks after the start of treatment.

Given the findings that low-density CD15⁺ cells consist of distinct populations, I was particularly interested in distinguishing activated neutrophils from immature neutrophils and assessing the relevance of an enrichment of such populations for patients undergoing treatment with anti-CTLA-4.

At the same time, I asked whether changes could be observed during treatment with another immune-checkpoint inhibitor, anti-PD-1, that like anti-CTLA-4, is effective at inducing objective responses in at least a subset of melanoma patients.

3.16.1 Phenotype of low-density granulocytes during treatment with immunomodulatory antibodies

Analysis of low-density granulocytes during treatment with anti-PD-1 or anti-CTLA-4 antibodies, reveals the presence of activated and immature granulocytes which phenotypically resemble those cells observed before treatment, in terms of CD10 and CD33 expression (figure 20), as well as CD16 and CD11b (data not shown).

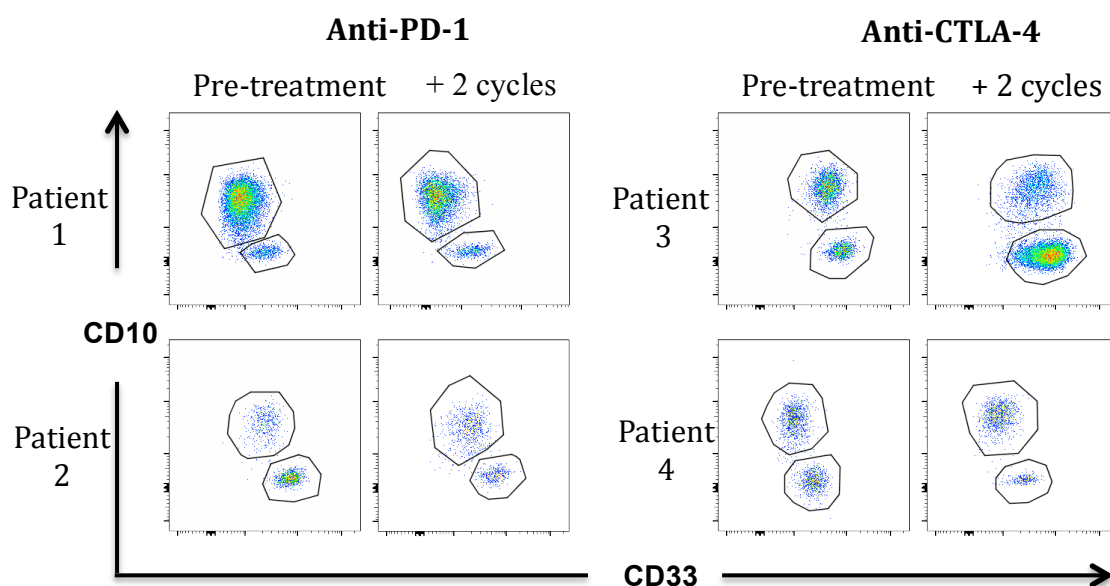


Figure 20: Phenotype of activated and immature neutrophils during anti-CTLA-4 or anti-PD-1 treatment. There are no differences in the phenotype of activated or immature neutrophils following the first two cycles of anti-PD-1 or anti-CTLA-4 treatment. Low-density CD15+ granulocytes still consist of two distinct populations in terms of CD10 and CD33 expression. Example stainings in four patients undergoing immunotherapy, who have both populations in their PBMCs, are shown. Blood samples were taken on the day of treatment and three weeks after the second cycle of treatment.

3.16.2 Implications of an enrichment of activated or immature neutrophils for response to treatment with anti-CTLA-4 or anti-PD-1 antibodies

Blood samples were collected on the first day of treatment with anti-CTLA-4 (prior to the start of treatment). The frequency of CD10+ activated neutrophils was determined and the best overall response within six months of the start of treatment determined. Those patients with a best overall response of progressive disease have a higher frequency of activated neutrophils in their peripheral blood, compared to those patients whose best overall response in six months is non-progressive disease. While the distribution of CD10+ cells is significantly different between patients with progressive and non-progressive disease, this is not true for CD10- immature neutrophils (figure 21).

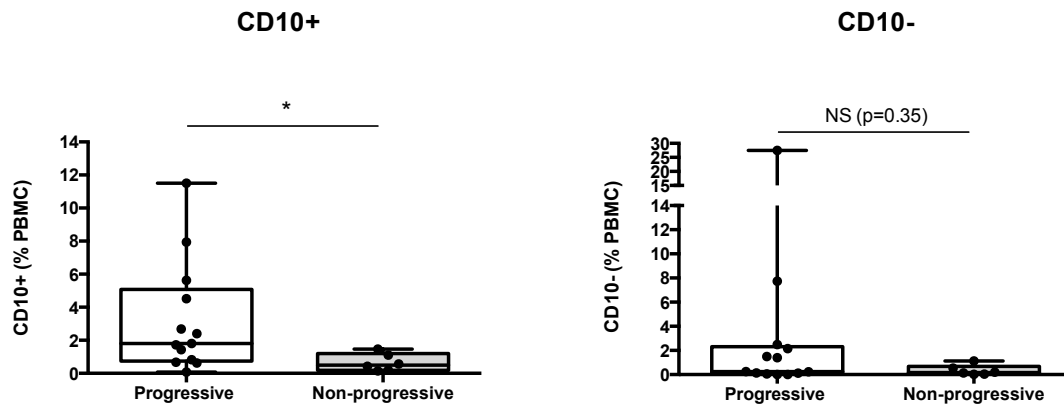


Figure 21: Pre-treatment frequency of low-density neutrophils in the peripheral blood of stage IV melanoma patients treated with anti-CTLA-4. Blood samples were collected immediately prior to the start of treatment and frequency of activated (CD10+) (left panel) and immature (CD10-) neutrophils (right panel) determined. Patient responses were monitored and investigator-assessed response defined as best overall response in six months. Patients were grouped into 'progressive' (best response: progressive disease) and 'non-progressive' (best response: stable disease or partial response). The median frequency and interquartile range are shown. The difference in distribution was determined by the Mann-Whitney U test. * $p < 0.05$, NS: non-significant.

As well as collecting a blood sample prior to the start of treatment, a blood sample was taken from a subset of the patients in figure 21 three weeks after the second cycle of anti-CTLA-4. The frequency of activated neutrophils before and during treatment was determined and shown for those patients whose best response within six months of the start of treatment was progressive disease (red lines in left panel of figure 22A) and those patients with non-progressive disease (blue lines in right panel of figure 22A). Five of the six patients with progressive disease show a decrease in the frequency of activated neutrophils after two cycles of anti-CTLA-4. The sixth patient shows a marginal increase. All four patients with non-progressive disease show an increase in the frequency of activated neutrophils after two cycles of anti-CTLA-4.

The difference between progressive and non-progressive patients is also confirmed when limiting the analysis to patients with a similar basal frequency of activated neutrophils and expressing the data as fold change in activated neutrophil frequency after two cycles of anti-CTLA-4. As indicated by the data in figure 22A, the fold change in those patients with non-progressive disease, is

significantly higher than those with progressive disease and all but one of the patients with a best response of progressive disease have a fold change below 1 (figure 22B).

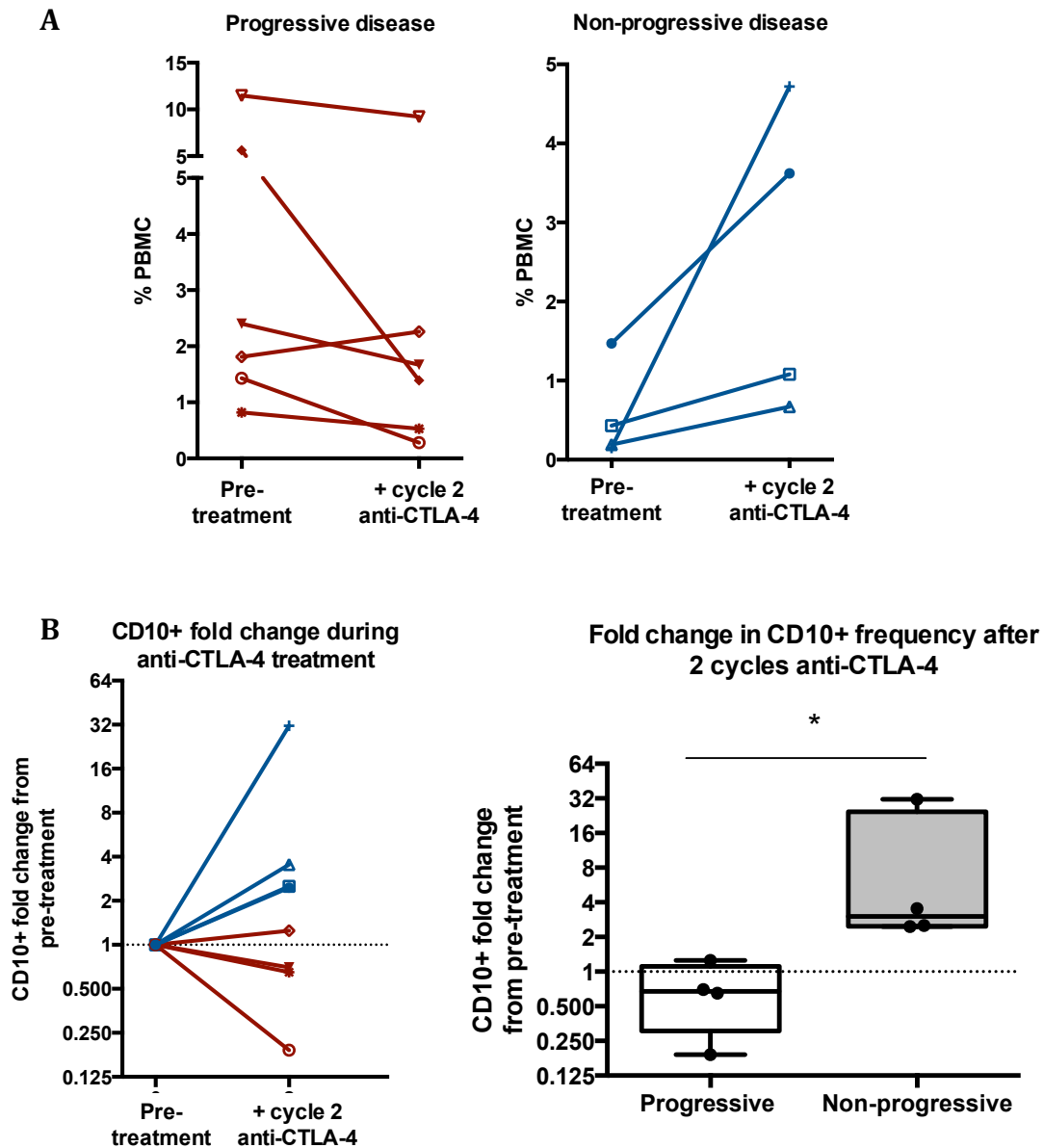


Figure 22: The frequency of activated (CD10+) neutrophils changes during treatment with anti-CTLA-4. A: Of the ten patients whose frequency of activated neutrophils was determined before and after two cycles of anti-CTLA-4, six had a best response in six months of progressive disease (red lines) and four had non-progressive disease (blue lines). B: The fold change in frequency is shown for the eight patients with a pre-treatment CD10+ frequency of $\leq 2.5\%$ (patients with progressive disease shown by red lines and non-progressive by blue lines in left panel). The median fold change after two cycles of anti-CTLA-4 and interquartile range are shown in the right panel. The difference in distribution was determined by the Mann-Whitney U test. * $p < 0.05$

Blood samples were also collected from patients prior to treatment with anti-PD-1 and the frequency of activated and immature neutrophils determined. Unlike anti-CTLA-4, there is no statistically significant difference in the distribution of activated neutrophils in patients with a best response in six months of progressive disease and non-progressive disease (figure 23 left panel). This is also true for immature CD10- neutrophils (figure 23 right panel)

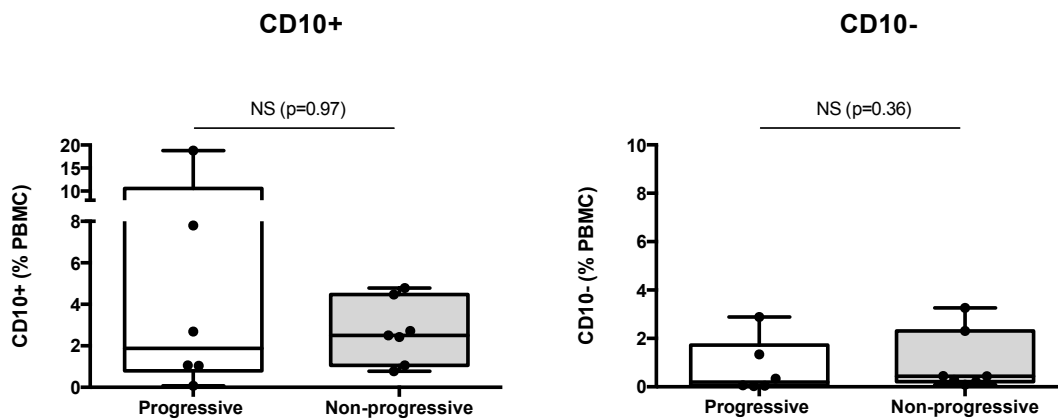


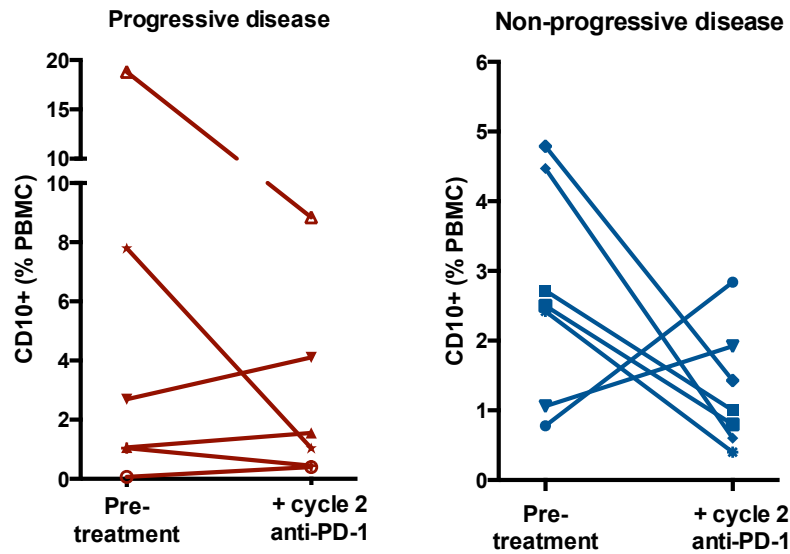
Figure 23: Figure: Pre-treatment frequency of low-density neutrophils in the peripheral blood of stage IV melanoma patients treated with anti-PD-1. Blood samples were collected immediately prior to the start of treatment and frequency of activated (CD10+) and immature (CD10-) neutrophils determined. Patient responses were monitored and investigator-assessed response defined as best overall response in six months. Patients were grouped into 'progressive' (best response: progressive disease) and 'non-progressive' (best response: stable disease or partial response). The median frequency and interquartile range are shown. The difference in distribution was determined by the Mann-Whitney U test. NS: non-significant.

As well collecting a blood sample prior to the start of treatment, a blood sample was taken from the patients in figure 23 after the second cycle of anti-PD-1. Patients were grouped into those with a best response in six months of progressive disease (red lines in left panel of figure 24A) and those with a best response of non-progressive disease (blue lines in right panel of figure 24A). Three patients with progressive disease show a decrease in the frequency of activated neutrophils following two cycles of anti-PD-1 and three patients show an increase. Of those patients with a best response of non-progressive disease,

five patients show a decrease in the frequency of activated neutrophils, and two an increase, following two cycles of anti-PD-1.

For those patients with a similar basal frequency of activated neutrophils, this data is also expressed as fold change in activated neutrophil frequency after two cycles of anti-PD-1 (figure 24B). Of those patients with progressive disease, three have a fold change greater than one, and one patient a fold change less than one. Of those with non-progressive disease, two patients have a fold change greater than one and two patients a fold change less than one. Unlike those patients treated with anti-CTLA-4, when the fold changes of progressive and non-progressive patients treated with anti-PD-1 are compared, statistically there is no difference in the distribution between the two groups (figure 24B right panel).

A



B

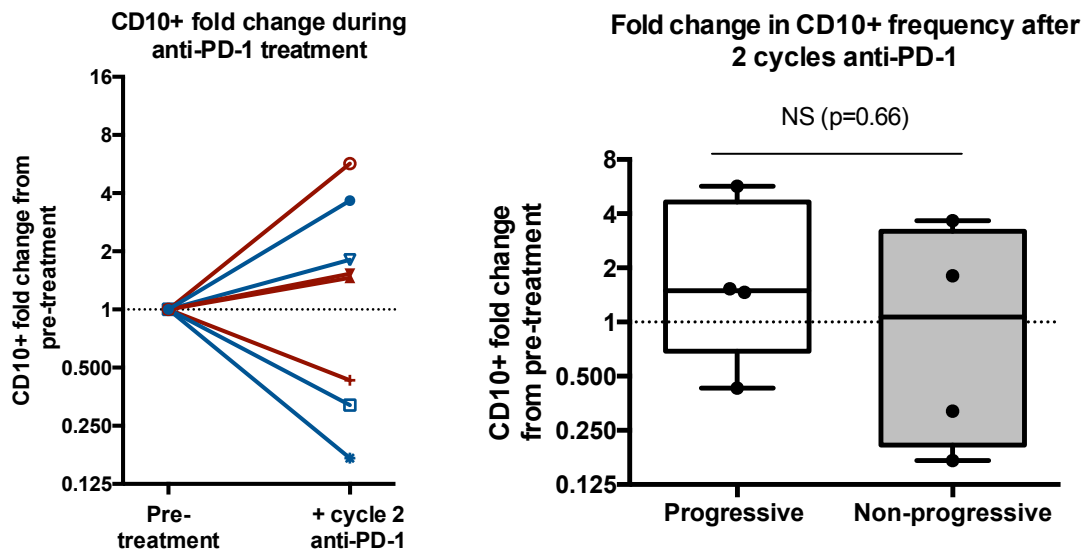


Figure 24: The frequency of activated (CD10+) neutrophils changes during treatment with anti-PD-1. A: Of the 13 patients whose frequency of activated neutrophils was determined before and after two cycles of anti-PD-1, six had a best response in six months of progressive disease (red lines) and seven had non-progressive disease (blue lines). B: The fold change in frequency is shown for the eight patients with a pre-treatment CD10+ frequency of $\leq 2.5\%$ (patients with progressive disease shown by red lines and non-progressive by blue lines in left panel). The median fold change after two cycles of anti-PD-1 and interquartile range are shown in the right panel. The difference in distribution was determined by the Mann-Whitney U test. * $p < 0.05$. NS: non-significant.

While the number of patients treated with anti-CTLA-4 or anti-PD-1 is fairly small, when a larger group of stage IV melanoma patients are analysed for survival, the median overall survival is greater in those patients who have a lower frequency of CD10+ activated neutrophils in their peripheral blood (see appendix A1.4).

3.17 Discussion

The experiments in this chapter have highlighted the following points:

- There is an enrichment of phenotypically distinct 'activated' and 'immature' neutrophil populations in a subset of patients with stage IV melanoma (figure 10). The individuals with the highest frequencies of both neutrophil populations have M1c stage IV disease suggesting in part a link to the degree of metastasis (figure 12)
- Elevated plasma concentration of distinct cytokines/growth factors correlates with the frequency of these neutrophil populations (correlogram – figure 14); for example elevated plasma IL-8 positively correlates with the frequency of activated neutrophils and elevated plasma SAA positively correlates with the frequency of immature neutrophils (figure 15A). Furthermore, there is inter-patient variation in the frequency of these two subsets further supporting the notion that distinct inflammatory axes exist within stage IV melanoma patients
- Although the plasma concentration of SAA positively correlates with the frequency of immature and mature neutrophils (figure 17), SAA does not appear to promote differentiation of CD34+ progenitors into CD15+ cells (figure 18B)
- The phenotype of activated and immature cells does not change during treatment with anti-CTLA-4 or anti-PD-1 antibodies however patients with a best response in six months of progressive disease are more likely to have a higher frequency of activated neutrophils before anti-CTLA-4 treatment compared to those with non-progressive disease (figure 21)

- The frequency of activated neutrophils changes during treatment with anti-CTLA-4. All but one of the patients with progressive disease show a reduction in the frequency of activated neutrophils following two cycles of anti-CTLA-4. However, those with non-progressive disease show an increase in the frequency of activated neutrophils during treatment (figure 22)

3.17.1 Detection of phenotypically distinct ‘activated’ and ‘immature’ neutrophil populations in a subset of patients with stage IV melanoma

Contrary to previous descriptions, suggesting that low-density granulocytes consist of immature or activated neutrophils, I found the presence of cells with two distinct phenotypes; activated neutrophils (CD15+ CD62L- CD10+ CD33int CD16+ CD11b hi CXCR1+ CXCR2+) as well as cells with the phenotype of immature neutrophils (CD15+ CD62L- CD10- CD33hi CD16- CD11b +/- CXCR4+).

While I used a range of markers to characterise CD10- CD33hi low-density neutrophils, without comparing the staining with different neutrophil populations within the bone marrow, it is not possible to precisely define the stage of differentiation these immature, low-density neutrophils represent. It is also possible that if a neutrophil is prematurely released from the bone marrow, it may be phenotypically different (in terms of the markers I’ve chosen to analyse) once it enters the periphery. The cells I have defined as immature neutrophils lack both CD62L and CD16. This phenotype however does not exclusively define immature neutrophils. Loss of these markers also occurs on apoptotic neutrophils (Hart et al., 2000). However, concomitant loss of CD15 is also observed in this instance, and when CD15 expression is analysed on cells defined as immature in the peripheral blood, the MFI of CD15 is slightly higher on CD62L- CD10- CD33hi CD16- cells than on mature and activated neutrophils, supporting an immature rather than apoptotic phenotype. Furthermore, these cells express CXCR4 and the ARG-1 gene in fitting with them being immature neutrophils.

During neutrophil differentiation, the nucleus undergoes a series of distinct changes in morphology, resulting in mature neutrophils with a segmented nucleus consisting of 3-4 distinct nuclear lobes (Sanchez and Wang, 1999). In diseases such as acute myeloblastic leukaemia (AML), in which there is a failure at an early stage of neutrophil differentiation, and in systemic bacterial infections, in which there is premature release of neutrophils from the bone marrow, it is possible to detect myeloblasts (with failed nuclear segmentation) and bands (with indented nuclei) respectively, in the peripheral blood *via* their distinct nuclear morphology (Sanchez and Wang, 1999). As neutrophil precursors are physically different to mature neutrophils, it would be interesting to examine the nuclear morphology of the low-density granulocytes found in the peripheral blood of stage IV melanoma patients, which may help in understanding which stage/s of immature, CD10- CD33hi cells, are present.

Low-density CD15+ cells that express the mature neutrophil marker CD10 have a phenotype which suggests they are activated neutrophils, such as a lack of surface CD62L and more intense staining for cell surface CD11b. The fact that CD15+ CD10+ CD33int cells purify alongside lower-density PBMCs, unlike the bulk CD15+ CD10+ CD33int mature neutrophil population, suggests a loss of granular contents, in fitting with neutrophil activation. Furthermore, they express lower amounts of CXCR1 and CXCR2 compared to mature neutrophils, and express the proinflammatory gene IL-8, fitting with an activated neutrophil phenotype.

As mentioned, different stimuli may polarise neutrophils towards different phenotypes as evidenced by the fact neutrophils can have both pro- or anti-inflammatory, and pro- or anti-tumour, functions (Mantovani et al., 2011). While attempts to understand the function (particularly immunosuppressive capacity) were limited by a failure to sort sufficient cell numbers, genome-wide expression analysis, which can now be carried out on very small cell numbers including single cells, may help in understanding what these cells are capable of and the relevance of an enrichment of these cells in a subset of patients with stage IV melanoma.

It is possible that neutrophils with an activated phenotype in the peripheral blood of stage IV melanoma patients represent cells activated in the circulation due to the presence of cytokines such as IL-8 or it is possible that they could consist of cells that have been activated at tumour sites and have subsequently trafficked back to the peripheral blood. There is evidence in Zebrafish that neutrophils actively traffic bidirectionally between wounds and circulation (Mathias et al., 2006). Retrograde trafficking/reverse migration into the circulation is thought to represent a mechanism, along with neutrophil apoptosis, that may be important for the resolution of infection (Mathias et al., 2006).

There is evidence that human neutrophils are also capable of retrograde trafficking. In an *in vitro* assay, human neutrophils were able to reverse migrate across an endothelial cell monolayer stimulated with TNF- α and these cells were phenotypically distinct, in that they had increased expression of CD54 (ICAM-1) compared to unstimulated neutrophils or those that had been stimulated by fMLP but had not undergone reverse migration (Buckley et al., 2006). Cells with this phenotype also expressed lower CD62L, CXCR1 and CXCR2 compared to unstimulated neutrophils and were positive for CD16. They were also enriched in the circulation of patients with rheumatoid arthritis (RA) and atherosclerosis. It would be interesting to analyse the expression of CD54 on the surface of CD10⁺ CD33^{int} activated neutrophils in patients with stage IV melanoma. It may be that certain factors within the tumour microenvironment promote retrograde transport of neutrophils into the peripheral blood and, depending on the nature of the intratumoural neutrophils (whether they have polarised towards a pro- or anti-tumour phenotype), this may have important implications for the capacity of the immune system to mount an effective anti-tumour response.

While functional analysis of these populations was hindered by lack of cells, it is evident that there is inter-patient variation in the frequency of these populations. Although there is an enrichment of activated neutrophils in the PBMCs of patients compared to healthy controls, the difference in distribution of

immature neutrophils did not quite reach significance. However, there is clearly a subset of patients with a greater frequency of activated or immature neutrophils, and a subset of patients with a frequency of low-density granulocytes that does not differ from healthy controls.

3.17.2 Enrichment of low-density-granulocyte populations

While the frequency of activated neutrophils is significantly increased in patients compared to healthy controls, the difference in distribution for immature neutrophils does not reach significance. While the frequency of low-density granulocytes was measured in the peripheral blood of a greater number of healthy controls (and were barely detectable – data not shown), the frequency of immune cells is known to change with age (notably an increase in myeloid-bias haematopoietic stem cells with age) and therefore it was important that only age-matched healthy donors were compared. Unfortunately, I was only able to obtain blood samples from ten such donors and the failure to detect a significant difference in the distribution of immature neutrophils between patients and healthy controls may reflect the need to increase the sample size of the healthy control population. Clearly, for both low-density neutrophil populations, there are patients with a much high frequency of cells compared to healthy controls, and those with barely detectable cells.

Although statistically there is no difference between the overall distribution of activated or immature neutrophils between the M1a/b and M1c subclasses, it is evident that patients with the highest frequencies of activated or immature neutrophils fall within the M1c subclass. However, even when combining the M1a and M1b data, the M1c subclass sample size is still much larger. In order to determine whether there is any difference between these two groups, increasing M1a and M1b sample size is necessary. It is also clear however that there are patients within the M1c subclass that have very few activated or immature neutrophils in their peripheral blood. Clearly, other factors besides advanced disease staging, must contribute.

3.17.3 The concentration of distinct cytokines correlates with the frequency of distinct low-density granulocyte populations

BioPlex assay and ELISA were used to investigate whether any soluble factors were associated with an enrichment of either activated or immature neutrophils. The frequency of immature neutrophils in the peripheral blood positively correlated with the concentration of plasma SAA. SAA did not correlate with the frequency of activated neutrophils and, of the other parameters analysed, SAA only correlated with the concentration of plasma IL-6 and the frequency of total low-density granulocytes.

On the other hand, the frequency of activated neutrophils positively correlated with the abundance of a range of factors within the plasma. The strongest correlations (that are distinct for activated neutrophils) were observed between activated neutrophil frequency and the concentration of IL-8, TNF- α , VEGF, IP-10, IL-4 and IL-1ra. Weaker, but still statistically significant, correlations are observed between activated neutrophil frequency and the concentration of IFN- γ , IL-7, IL-12p70 and PDGF. Such factors have a range of potential sources.

IL-8 and TNF- α for example, can be produced by activated neutrophils themselves as well as monocytes and macrophages (Strieter et al., 1992) (Ribeiro et al., 2003) (Cassatella et al., 2005) (Agarwal et al., 1995) and thus an enrichment of activated neutrophils in the periphery may contribute to raised IL-8 and TNF- α plasma concentration.

When DCs recognise pathogen-associated molecules *via* PRRs they transition from an immature to mature state (Jong et al., 2005). As well as upregulating costimulatory molecules on their surface, which along with antigen presentation on MHC molecules enables them to activate antigen-specific T cells, mature DCs also secrete cytokines (Jong et al., 2005). One cytokine that is readily expressed by DCs following stimulation is IL-12p70. When naïve T cells recognise antigen

in an environment that is rich in IL-12p70, they polarise towards a type 1 (T_H1) phenotype (Jong et al., 2005). A type 1 response typically occurs in response to intracellular pathogens, such as viruses, and is characterised by T cells that produce cytokines including IFN- γ and IL-2 (Jong et al., 2005). IP-10 or IFN- γ inducible protein (CXCL10) is a chemokine which is produced by a range of cells activated with stimuli that include IFN- γ (Dufour et al., 2002). It is often detected in type-1 inflammation and indeed there is a positive correlation between the concentration of IP-10 and the concentration of IFN- γ in this cohort of patients. This data suggests that in patients with an enrichment of activated neutrophils, there is a type 1 inflammatory environment. However, it should be noted that there is also an association between the frequency of activated neutrophils and the concentration of IL-4, IL-10 and IL-1ra. IL-4 is cytokine, along with IL-5 and IL-13, that is produced by T cells polarised towards a type 2 phenotype. Typically this occurs in response to infections with pathogens such as helminths (Jong et al., 2005). IL-10 and IL-1ra are molecules with anti-inflammatory properties. IL-1ra is produced by a range of cells including monocytes, macrophages and neutrophils in a response to a range of stimuli, including pathogen-derived molecules such as LPS, as well as cytokines, such as IL-4, GM-CSF and IFN- γ (Arend et al., 1998). IL-1ra binds to IL-1 receptors blocking the action of the proinflammatory cytokine, IL-1 β (Kline et al., 1995) (Arend et al., 1998). IL-10 has a range of properties, including induction of regulatory T cells, and is produced by a variety of immune cells in response to inflammatory stimuli (discussed further in chapters 1 and 5) (Jong et al., 2005).

Induction of an immune response is known to not only induce factors that drive an anti-microbial or anti-tumour response, but also to induce factors, such as IL-10, that are important in regulating an immune response in order to prevent immunopathology. Therefore, it is perhaps not surprising that there is an enrichment of factors with opposing functions.

Other factors that positively correlate with the frequency of activated neutrophils are PDGF and VEGF. These factors have been implicated in tumour angiogenesis, with the degree of melanoma vascularisation shown to correlate

with the expression of PDGF and VEGF, along with IL-8 and bFGF (Westphal et al., 2000). Although these factors can derive from other cells such as platelets and smooth muscles cells, it is a possible that the degree of angiogenesis and metastasis contributes to the elevated plasma concentration of these factors. The association of these factors with the frequency of activated neutrophils may to some extent explain why patients with the highest frequency of activated neutrophils have M1c stage IV melanoma (although this is not especially clear given that the patients with the highest frequency of immature neutrophils, which do not correlate with these factors, also fall within the M1c subclass).

In terms of distinction between the two neutrophil populations, importantly, no correlation was observed between the concentration of IL-8 and SAA in plasma and no correlation was observed between the concentration of IL-8 and the frequency of immature neutrophils or the concentration of SAA and the frequency of activated neutrophils. This implies that distinct types of inflammation exist in the context of melanoma, and that different neutrophil populations associate with enrichment of different cytokines/growth factors.

It may also be that particular mutations present within an individual's melanoma cells contribute to the development of a distinct inflammatory environment which is associated with altered neutrophil biology. Although I saw no difference in the frequency of immature or activated neutrophils in the peripheral blood of patients with or without the BRAF V600E mutation, it could be that mutations besides this contribute to a particular inflammatory environment. Melanomas are known to possess a high frequency of variable mutations, including those that directly drive the progression of melanoma, such as BRAF V600E, and passenger mutations that are the result of UV-light-induced high mutational load (Hodis et al., 2012). Perhaps factors produced by melanoma cells themselves, or other immune cells that recognise mutated antigens, could drive the activation of neutrophils or drive their premature release from the bone marrow.

3.17.4 No evidence that SAA can promote the differentiation of neutrophils *in vitro*

The trend towards higher plasma SAA in patients with more immature and mature neutrophils, suggested that SAA may be driving a process known as emergency/reactive granulopoiesis. This occurs during systemic infection/inflammation and enhances neutrophil production in the bone marrow through increased proliferation of myeloid progenitors (Manz & Boettcher, 2014). Enhanced neutrophil production occurs at the expense of lymphopoiesis and functions to replenish neutrophils rapidly being used up in the periphery (Manz & Boettcher, 2014). This process can be stimulated by direct recognition of pathogens by progenitors in the bone marrow or indirectly, by cytokines and growth factors (including G-CSF, GM-CSF, M-CSF and IL-6) produced in response to pathogen detection by other cell types, such as monocytes and macrophages (Manz & Boettcher, 2014). A direct effect of SAA on neutrophil production is not known.

There are four isoforms of the SAA protein. SAA-1 and SAA-2 encode inducible forms that are predominantly produced by hepatocytes and their concentration can increase dramatically during infection and inflammation (Christenson et al., 2013). SAA-3 is not expressed and SAA-4 encodes a constitutively expressed form (Christenson et al., 2013). SAA has been shown to opsonise bacteria and has the capacity to stimulate cytokine production by macrophages and neutrophils *via* the TLR-2 and FPR-2 receptors (Christenson et al., 2013) (De Santo et al., 2013).

In order to address a direct role for SAA in inducing granulopoiesis, and bearing in mind that recombinant SAA has been suggested as a more potent proinflammatory stimulus than physiological SAA (Christenson et al., 2013), the effects of both recombinant and physiological SAA (in the form of patient plasma) on neutrophil differentiation were compared. While there is no increase when the differentiation cocktail was supplemented with recombinant SAA-1 up to a concentration of 1µg/ml, an increase was observed with supplementation with patient plasma. However, this is not specific to SAA concentration, with patient plasma sample 3, containing undetectable SAA, able to induce as much

CD15+ differentiation as patient plasma sample 1. This contradicts the *in vivo* correlation between plasma SAA and immature and mature neutrophil frequency. The plasma from patient 2 has the highest SAA concentration of the three samples and the PBMC from this patient contain 39% immature neutrophils. Patient 1 also has a high SAA concentration but is lower than that of patient 2. Patient 1 has 3% immature neutrophils in their PBMCs. Patient 3, with undetectable plasma SAA, has barely detectable immature neutrophils (0.2%) in their PBMCs.

Further analysis of the plasma samples used revealed that the plasma sample with undetectable SAA has the highest concentration of the cytokine RANTES. An increased concentration of RANTES has been observed in the haematopoietic stem cell (HSC) environment in the bone marrow of ageing mice (Ergen et al., 2012). Through a series of transplantation experiments between young and old mice, involving overexpression of RANTES, *ex vivo* treatment with RANTES and knock-out RANTES mice, the authors show a role for RANTES in myeloid skewing of haematopoietic stem cells in the bone marrow of ageing mice (Ergen et al., 2012). The presence of RANTES in plasma sample 3 could offer a potential explanation for the high frequency of CD15+ cells in these cultures.

As only 28 cytokines/growth factors were analysed in the patient plasma samples, it could be that many others are present which could have a positive or negative effect on the differentiation of haematopoietic progenitor cells. Blocking antibodies against the receptors for SAA, TLR-2 and FPR-2, could be used alongside plasma samples to confirm a role for physiological SAA. However, recombinant SAA-1, suggested as more potent than physiological SAA, does not increase CD15+ differentiation suggesting that this molecule does not influence the differentiation of progenitors *in vitro*.

It could be that *in vivo* SAA stimulates production of another factor and that this factor can stimulate differentiation of haematopoietic progenitors towards a neutrophil fate, but that SAA itself does not act upon progenitors in this *in vitro* assay.

3.17.5 Immune-checkpoint inhibitors and frequency of activated and immature neutrophils

The fact that there is a range of distinct low-density granulocytes in peripheral blood, and that low-density granulocytes have previously been described as possessing immunosuppressive properties, clinically this variation may be relevant, especially given the development of immunomodulatory therapies that have shown success in certain melanoma patients.

Activated neutrophils are associated with numerous inflammatory cytokines and likely reflect how inflamed (in terms of at least of one type of possible inflammatory axis) stage IV melanoma patients are. The fact that patients treated with anti-CTLA-4 who show a best response in six months of non-progressive disease have a lower frequency of activated neutrophils prior to treatment and show an increase in the frequency of the cells after the first two cycles, suggests that anti-CTLA-4 increases inflammation in patients who previously were not strongly inflamed and that this increase is associated with clinical benefit. It may be that in those patients whose tumours have not elicited a strong inflammatory response, and who hence have a lower frequency of activated neutrophils, that the capacity of anti-CTLA-4 to expand T cells and increase infiltration of T cells into the tumour, enables tumour growth to be controlled/tumour burden to at least partially decrease. In those who already have an inflammatory response (but who have failed to control tumour growth and hence the requirement for anti-CTLA-4 treatment) or in those patients in which anti-CTLA-4 fails to increase the frequency of activated neutrophils and associated inflammation, treatment with anti-CTLA-4 is not effective.

While it is necessary to increase the sample size and determine whether this result holds true in a larger cohort of patients, monitoring the frequency of activated neutrophils before and during anti-CTLA-4 treatment could potentially be used to determine likely outcome. As patients with a high frequency of activated neutrophils (> 1.5%) are likely to progress on anti-CTLA-4, it may be

more beneficial for these patients to try alternative treatments. While patients with a lower frequency of activated neutrophils ($< 1.5\%$) may or may not progress on anti-CTLA-4, monitoring the frequency of activated neutrophils after the second cycle of treatment, could identify those unlikely to see a benefit (those whose activated neutrophil frequency does not increase) and thus instead of completing all cycles of anti-CTLA-4/waiting for a response, it may be better to start an alternative treatment sooner. Therefore, the activated neutrophil frequency has the potential to act as a biomarker for anti-CTLA-4 treatment response.

While the frequency of activated neutrophils may merely represent a feature of a particular type of inflammation, it is possible that the abundance of activated neutrophils directly impacts on efficacy of anti-CTLA-4 treatment due to particular functional properties of this cell type. Although, I was unable to prove an immune-suppressive phenotype of activated neutrophils and only analysed the frequency of low-density granulocytes during anti-CTLA-4 treatment, another study has revealed that there is lower expression of PD-L1 on the surface of low-density granulocytes (albeit total CD15⁺ CD11b⁺ cells) in patients who respond to anti-CTLA-4 compared to non-responders, and that after the first cycle of anti-CTLA-4 treatment, there is a reduction in PD-L1 expression (Gebhardt et al., 2015). Through interaction with PD-1 on the surface of T cells, this represents a mechanism by which cells could contribute to suppression of T cell effector function and reveals that it may be important to consider altered functional properties of low-density granulocytes alongside altered frequency.

Unlike treatment with anti-CTLA-4, neither pre-treatment frequency of activated neutrophils nor fold change of activated neutrophils during treatment predicts best response in patients treated with anti-PD-1. Although both drugs work by blocking inhibitory receptors, the mechanism of action of CTLA-4 and PD-1 differ and may account for the different results. A major function of anti-CTLA-4 is to promote CD28 interaction with B7 molecules on the surface of activated APCs, thus promoting the activation and proliferation of T cells in lymphoid tissue, and increasing infiltration of T cells into tumours (Intlekofer & Thompson, 2013)

(Wu et al. 2012). Blockade of anti-CTLA-4 may be particularly important for lowering the threshold of activation of low-affinity T cells, a characteristic of tumour-specific T cells (Intlekofer & Thompson, 2013). The major function of PD-1, *via* interaction with its ligands in non-haematopoietic tissue including tumours, is to limit T cell effector function in peripheral tissue (Intlekofer & Thompson, 2013). Anti-PD-1 removes the PD-1/PD-L1 block on T cell effector functions in the tumour. In fact analysis of the T cell compartment in melanoma patients treated with anti-PD-1 has revealed that responding patients have a higher frequency of CD8+ T cells, as well as a higher frequency of PD-1 and PD-L1-expressing cells, within the tumour prior to treatment (Tumeh et al., 2014).

3.17.6 Summary and Future Work

In summary, in at least a subset of patients with stage IV melanoma, there is an enrichment of distinct low-density neutrophil populations in the peripheral blood and the frequency of these subsets correlates with an enrichment of distinct cytokines/growth factors in the plasma. The presence of such factors may directly act in processes that result in enrichment of these neutrophil populations or merely indicate that distinct types of inflammation exist in certain melanoma patients. There is evidence that low-density neutrophils can act to suppress T cell responses, which may have important implications for anti-tumour T cell immunity. Although I was unable to address whether one population rather than another is capable of immune suppression, there is evidence that the frequency of low-density granulocytes can change in response to immune-modulatory therapy (Pico de Coana et al., 2013). This was a small study and one which did not distinguish between the two low-density granulocytic populations. As there is no correlation between activated and immature neutrophils, it was of interest to analyse the frequency of these populations independently during treatment. These preliminary findings suggest that the pre-treatment frequency of activated neutrophils and fold change during treatment can help to predict outcome and could have implications for treatment with anti-CTLA-4.

The fact distinct neutrophil populations associate with an enrichment of distinct cytokines/growth factors suggests that different inflammatory axes exist with differing effects on neutrophil activation and differentiation. It may be that this is reflected in other components of the immune system. As a successful immune response to melanoma likely depends on the ability to mount an effective T cell response, the inflammatory environment in which a T cell responds to antigen may influence the efficacy of the response. In the next chapter, I will look beyond neutrophils, at monocytic subsets and particularly the T cell compartment.

Chapter 4: Analysis of monocytes and T cells in patients with melanoma

4.1 Introduction

The frequency of activated neutrophils in the peripheral blood of stage IV melanoma patients may reflect the inflammatory status of such patients and is linked to the capacity to respond to anti-CTLA-4 therapy. I was interested in what extent an enrichment of activated neutrophils relates to the immune status of patients. As certain monocyte subsets have pro-tumour functions, I was interested in whether changes in their frequency were associated with changes in neutrophil activation. Given the critical role of T cells in an effective anti-tumour immune response, I was particularly interested in whether a change in neutrophil activation was linked to changes in the frequency and activation of T cell subsets. Given that I also observed an enrichment of neutrophils with an immature phenotype in the peripheral blood of a subset of stage IV melanoma patients and that, unlike activated neutrophils, their enrichment correlated with increased plasma SAA, I was keen to establish whether their enrichment was associated with other features of the immune system, which may help to support the idea that different inflammatory axes can exist in melanoma patients.

In this chapter I use flow cytometry to identify and quantify monocyte subsets using differential expression of CD14 and CD16 and different T cell populations using a range of phenotypic markers, including expression of chemokine and homing receptors as well as activation markers. I analyse the association of monocyte and T cell subsets with the concentration of plasma cytokines/growth factors as well as their association with distinct low-density neutrophil populations. I also attempt preliminary characterisation a CD8+ T cell population and further explore the phenotype of tumour-specific T cells within the blood and tumour.

4.2 Identification of monocyte populations in the peripheral blood of patients with stage IV melanoma

As well as identifying and quantifying different neutrophil subsets, I also analysed another myeloid population, monocytes. Monocytes are a heterogeneous population of cells and as well as differing in functionality (see Chapter 1), subsets also differ in cell surface phenotype. Classical monocytes have been defined as CD14⁺ CD16⁻, inflammatory/intermediate monocytes as CD14⁺ CD16⁺ and patrolling/non-classical monocytes as CD14^{lo} CD16⁺⁺ (Wong et al., 2012). Furthermore, immature monocytic cells that lack expression of the MHC class II molecule, HLA-DR have been shown, in cancers including melanoma, to possess immunosuppressive properties and are thus referred to as moMDSC (Filipazzi et al., 2007).

Based on differential expression of CD14, CD16 and HLA-DR, I identified these different monocytic subsets (figure 1).

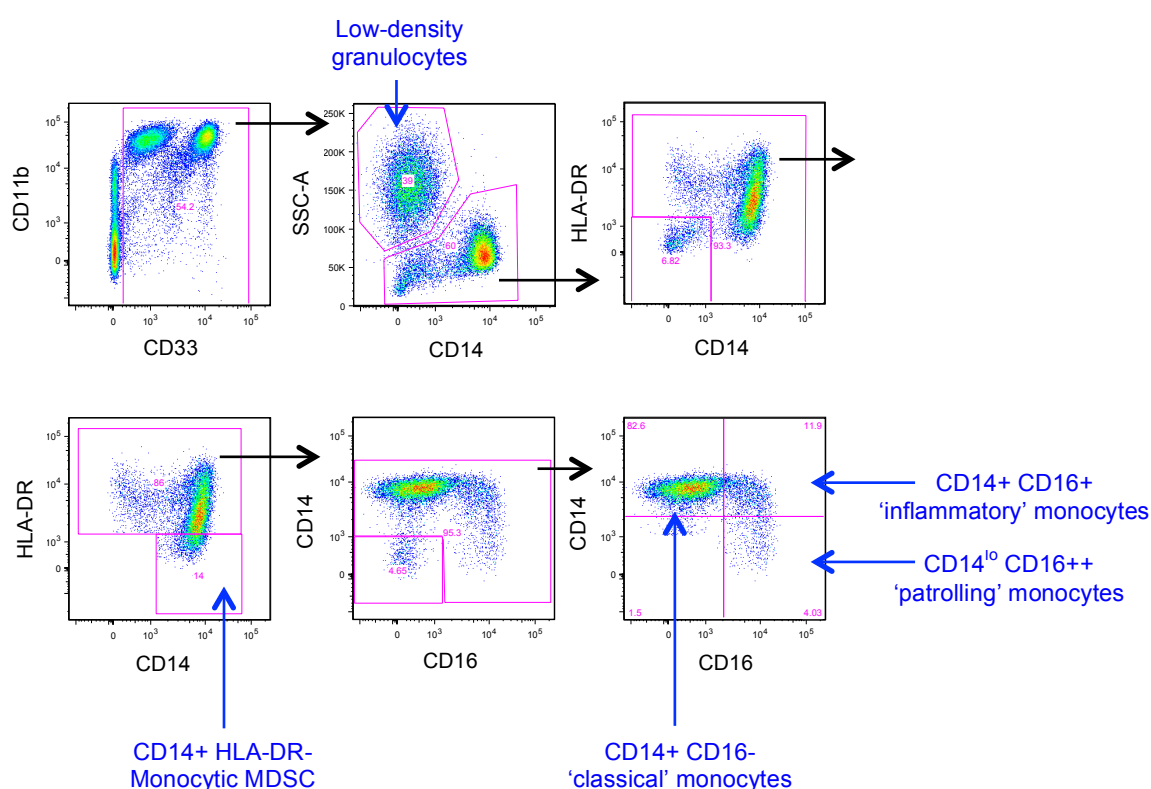


Figure 1: FACS analysis of PBMCs to identify different monocyte populations within the PBMCs of patients with stage IV melanoma. Gating strategy used to identify monocytic populations. Initial gating on live, singlets not shown. CD33⁺ myeloid cells were gated upon, granulocytic cells were excluded. Monocytic populations were identified based on differential expression of CD14, CD16 and HLA-DR - MDSC (CD14⁺ HLA-DR⁻), classical monocytes (CD14⁺ HLA-DR⁺ CD16⁻), inflammatory monocytes (CD14⁺, CD16⁺) and patrolling monocytes (CD14^{lo} HLA-DR⁺ CD16⁺⁺).

4.3 Frequency of monocytic populations in the peripheral blood of patients with stage IV melanoma

Analysis of the frequency of these different populations in stage IV melanoma patients and healthy controls, revealed that while certain patients have a higher proportion of monocytic cells with an moMDSC or inflammatory phenotype, and a lower proportion of classical and patrolling monocytes, the distribution of these populations in patients is not significantly different to healthy controls. While certain studies have found significant increases in the frequency of moMDSC in patients with stage IV melanoma (Filipazzi et al., 2007), other studies failed to observe this (Chavan et al., 2014). Chavan et al. (2014) however, report a significant drop in the proportion of circulating classical monocytes, and while overall not significant, it is true that I observe a decrease in the proportion of classical monocytes in certain patients within this cohort (figure 2). This is likely contributed to by the observable increases in moMDSC and inflammatory monocytes in certain patients.

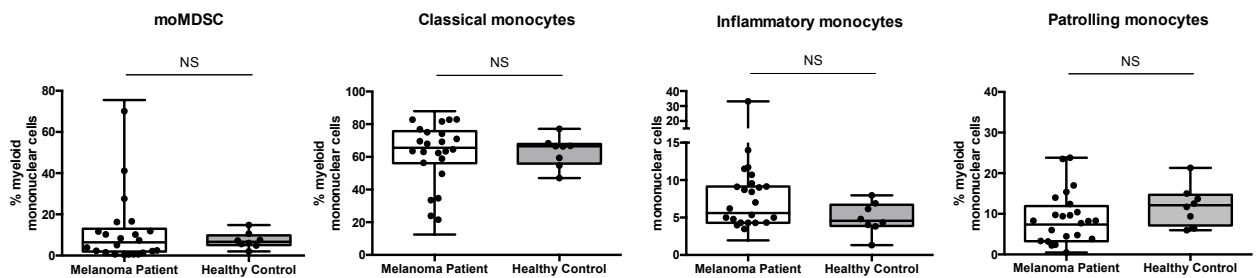


Figure 2: Frequency of monocyte populations in the peripheral blood of patients with stage IV melanoma compared to healthy controls. Values are expressed as a percentage of myeloid mononuclear cells. The median and interquartile range is highlighted and the difference in distribution was determined using the Mann-Whitney U test. NS: non-significant.

4.4 Identification and quantification of T cell subsets and activation status

T cells play a major role in tumour immunity. They are essential in preventing or keeping tumour growth under control, and are capable of eliciting strong anti-

tumour immunity in the context of cancer immunotherapy. At the same time distinct T cell subsets have been described as exerting a pro-tumoural effect (Nishikawa & Sakaguchi, 2010). Understanding how T cell function, phenotype and frequency are modulated in melanoma patients could not only provide a better understanding of the interplay between cancer and the immune system, but also provide insights into how to target the immune system in immunotherapy in order to produce effective anti-tumour immune responses.

I used flow cytometry to investigate whether changes in myeloid cells were associated with altered frequency or activation of distinct T cell subsets. I designed different antibody panels in order to identify distinct T cell subsets, define their activation status and their migratory properties and stained frozen PBMCs from stage IV melanoma patients and healthy controls. These panels included markers of specific T cells subsets such as TCR- $\gamma\delta$, migratory markers such as CCR4 and CXCR3 and the activation markers HLA-DR and CD38.

4.4.1 Identification and quantification of TCR- $\gamma\delta$ T cells and invariant natural killer T cells (iNKT) cells

$\gamma\delta$ T cells possess important anti-tumour properties, such as their ability to lyse tumour cells and to produce numerous pro-inflammatory cytokines upon recognition of phosphoantigens or stress-inducible molecules (Pietschmann et al., 2009) (Silva-Santos et al., 2015). However, as mentioned in the introduction, they may also possess pro-tumour functionality, such as the ability to induce suppressive neutrophils *via* production of IL-17 (Coffelt et al. 2015) (Silva-Santos et al. 2015). Furthermore, it has been shown that the frequency and functionality of $\gamma\delta$ T cells is altered in patients with primary cutaneous melanoma (Argentati et al., 2009). In order to determine whether there were differences in $\gamma\delta$ T cells in this cohort of stage IV melanoma patients, that may be linked to differential neutrophil activation or differentiation, firstly the frequency and activation status of $\gamma\delta$ T cell populations in the peripheral blood were determined (figure 3 and 4). When staining with a pan $\gamma\delta$ TCR antibody, there are two populations with differential staining intensity (figure 3). As the

major population of $\gamma\delta$ T cells in the peripheral blood are V γ 9V δ 2 T cells, it is likely that the population of cells with intermediate staining ('GD int' in figure 3) represents this population. V δ 1 (which can be paired with various V γ genes) T cells make up the minor $\gamma\delta$ T cell population in the peripheral blood (Pietschmann et al., 2009). This population is likely represented by the smaller population of cells that stain brightly with the pan $\gamma\delta$ TCR antibody ('GD hi' in figure 3).

As well as analysing the frequency of $\gamma\delta$ T cells, the activation status of such cells was determined. Upregulation and coexpression of the cell surface molecules CD38 and HLA-DR is associated with activation of T cells, including $\gamma\delta$ T cells and therefore the proportion of CD38⁺ HLA-DR⁺ $\gamma\delta$ T cells was determined in both stage IV melanoma patients and healthy controls (figure 3) (Li et al., 2014) (Kovacsovics-Bankowski et al., 2014) (Raziuddin et al., 1992).

I was also interested in the frequency of iNKT cells in this cohort of melanoma patients. iNKT cells have the capacity to influence the anti-tumour immune response *via* their ability to produce a range of cytokines, to mature antigen-presenting cells, and to directly kill cancer cells *via* perforin-mediated cytotoxicity (McEwen-Smith et al., 2015). Furthermore, decreased number and function of iNKT cells has been reported in patients with numerous types of cancer, including prostate cancer, breast cancer and melanoma (McEwen-Smith et al., 2015) (Crough et al., 2004). The sponge-derived lipid α -GalCer is recognised by iNKT cells and it was *via* α -GalCer CD1d tetramer staining that iNKT cells were identified in the peripheral blood (figure 3).

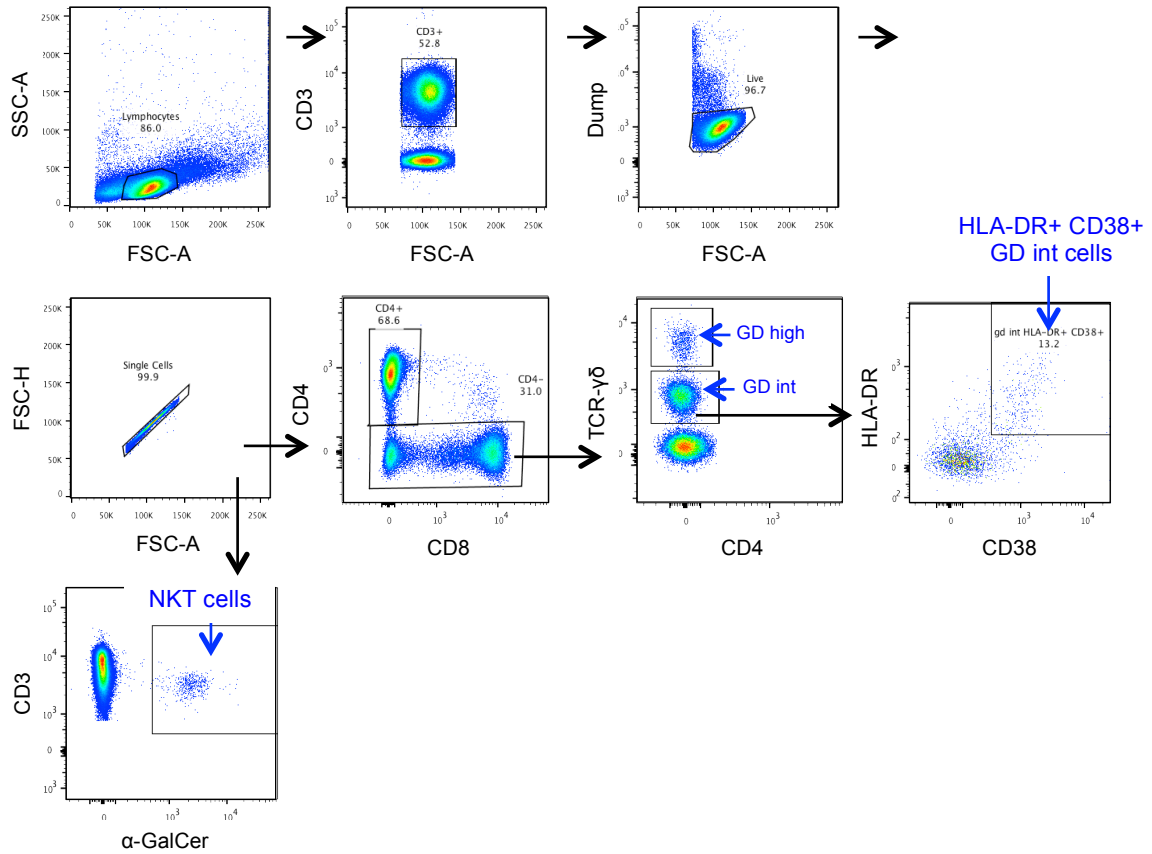


Figure 3: FACS analysis of PBMCs to identify TCR- $\gamma\delta$ T cells and invariant natural killer T cells (iNKT) cells. Live, singlet, CD3+ cells were gated upon. NKT cells were detected by positive α -GalCer CD1d tetramer staining. Within the CD4- fraction TCR- $\gamma\delta$ T cells were identified by positive staining with the TCR- $\gamma\delta$ antibody. Two populations of TCR- $\gamma\delta$ T are detected, those that stain brightly with the TCR- $\gamma\delta$ antibody ('GD high') and those with intermediate staining ('GD int'). T cell populations were analysed for their activation status by analysis of HLA-DR and CD38 positivity.

The frequency of the two $\gamma\delta$ T cell populations and iNKT cells are shown in figure 4A. The distribution in frequencies in this cohort of patients, for all three populations, is not significantly different to healthy controls. While the frequency of V γ 9V δ 2 T cells has been shown to be reduced in the peripheral blood of patients with cutaneous primary non-metastatic melanoma (Argentati et al., 2003), if anything the median frequency of this population (likely represented by TCR- $\gamma\delta$ -int cells) is increased in this group of stage IV metastatic melanoma patients, suggesting that changes in the frequency of such cells may be dependent on disease progression.

Although not reaching statistical significance, the decrease in the median frequency of iNKT cells in this cohort of patients, fits with the pattern of reduced frequency that has previously been observed in melanoma patients (Crough et al., 2004).

With regards to activation of the two $\gamma\delta$ T cell populations, through analysis of the proportion of CD38⁺ HLA-DR⁺ cells, the difference in distribution between patients and healthy controls does not reach significance (figure 4B). It seems evident however, particularly with regards to the TCR- $\gamma\delta$ -int population, that while activated $\gamma\delta$ T cells are barely detectable in healthy controls and a proportion of melanoma patients, that there are a number patients with a higher frequency of activated cells. This may reflect heterogeneity among patients, and it may be that patients with more activated $\gamma\delta$ T cells, have an enrichment of a particular neutrophil population or differ in plasma concentration of certain cytokines/growth factors.

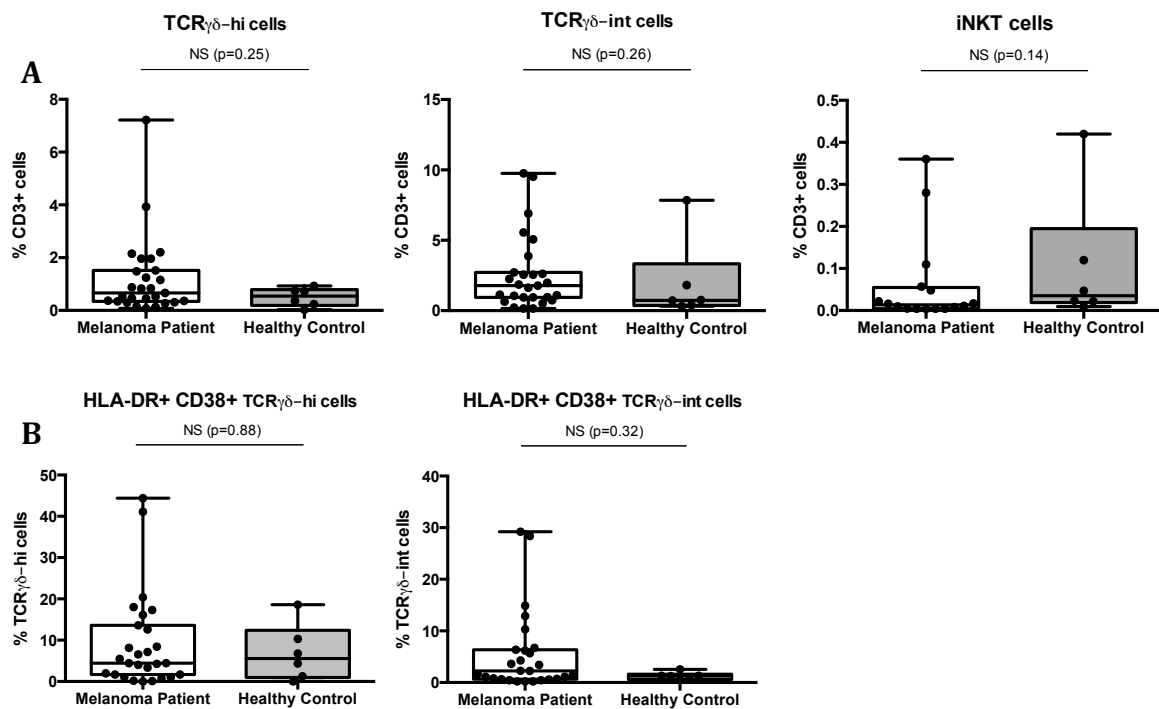


Figure 4: Comparison of TCR- $\gamma\delta$ T cells and iNKT cells in the peripheral blood of stage IV melanoma patients versus healthy controls. A: FACS staining of PBMCs was used to determine the frequency of TCR- $\gamma\delta$ T cells and iNKT cells. NKT cells were identified based on positive staining with α -GalCer tetramer. B: Activated TCR- $\gamma\delta$ T cells were identified by expression of HLA-DR and CD38. The median and interquartile range are highlighted. Although there are certain patients with higher frequencies of activated TCR- $\gamma\delta$ T, compared to healthy controls, statistically the distribution is not significantly different between patients and healthy controls when the Mann Whitney-U test is applied.

4.4.2 Identification and quantification of CD4+ helper T cells using differential cell surface expression of chemokine receptors

Naïve and antigen-experienced T cells can be distinguished from one another based on differential expression of numerous cell surface markers, including the combination of CD45RA and CCR7 (Broere et al., 2011). CD45 is a tyrosine phosphatase important in TCR signalling and exists as different isoforms (Ordonez et al., 2009). While naïve T cells express the CD45RA isoform, this is lacking from antigen-experienced central memory (T_{cm}) and effector memory (T_{em}) cells (Ordonez et al., 2009). CCR7, a chemokine receptor important in

homing of T cells to lymphoid tissue, is also expressed by naïve T cells as well as T_{cm} cells but is lacking from the surface of effector T cells and T_{em} cells which home to peripheral tissue (Broere et al., 2011).

After a naïve CD4⁺ T cell recognises its cognate antigen, its subsequent differentiation/polarisation (and thus subsequent functional properties) is determined by the environment in which activation occurs. CD4⁺ T cells can differentiate into several subsets including T_H1, T_H2, T_H17 and Tregs (Lazarevic et al., 2013). In an environment rich in IL-12 and IFN- γ , CD4 T cells will be polarised towards a T_H1 phenotype, resulting in cells capable of, among other functions, production of IFN- γ . On the other hand, activation of CD4 T cells in an environment rich in IL-4 results in generation of T_H2 cells, capable of IL-4, IL-5 and IL-13 production. T_H17 cells (capable of IL-17, IL-21 and IL-22) and Tregs (capable of producing cytokines with suppressive properties such as IL-10 and TGF- β) are generated in environments rich in IL-1 β , IL-6, TGF- β , IL-21 and IL-22 or TGF- β and IL-2 respectively (Lazarevic et al., 2013).

Such subsets differ in expression of master regulators of transcription, such as T-bet, GATA-3 and ROR- γ t, which result in the generation of cells with distinct functional properties, and as well as differing in cytokine production, such subsets of CD4 T cells also show differential expression of certain chemokine receptors, including CCR4, CXCR3 and CCR6 (Lazarevic et al., 2013) (Mahnke et al., 2013). It is *via* this difference that I identified T_H1, T_H2 and T_H17 cells and by expression of the master regulator of Treg differentiation, FoxP3, and the IL-2 receptor α chain, CD25, that I identified Tregs (figure 5).

I also examined the expression of cutaneous lymphocyte antigen (CLA) on circulating T cell populations. Upon recognition of antigen in skin-draining lymph nodes, T cells upregulate molecules enabling them to home back to the skin (Clark 2010). One such molecule is CLA which binds to E-selectin on endothelial cells of inflamed skin (Clark 2010). Given that I'm analysing the T cell compartment in patients with melanoma, I was interested to see if there were

differences in the proportions of circulating CLA+ T cells. CLA staining is shown in figure 5.

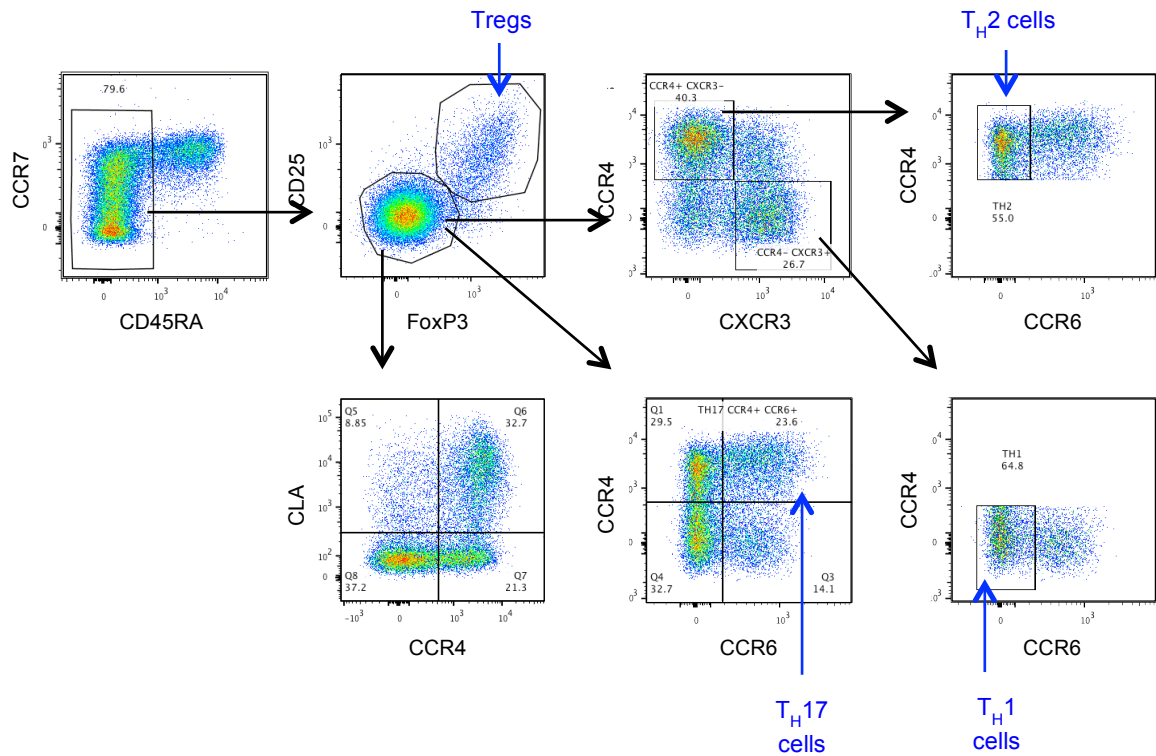


Figure 5: Identification of Helper T cell subsets. Live, singlet, CD3+ CD4+ cells were gated upon, as in figure 3. Antigen-experienced (CD45RA-) cells were analysed for expression of CD25 and FoxP3 and CD25+ FoxP3+ cells defined as Tregs. CD25- cells were analysed for expression of the chemokine receptors CCR4, CXCR3 and CCR6. TH1 cells were defined as CCR4- CXCR3+ CCR6-, TH2 cells as CCR4+ CXCR3- CCR6- and TH17 as CCR4+ CCR6+. Expression of the skin-homing marker cutaneous leukocyte-associated antigen receptor (CLA) was also analysed on T cell subsets.

Figure 6 shows the frequencies of the different T cell populations. Figure 6A and 6B reveal an increase in the proportion of CD4 effector memory cells with a TH2 phenotype in stage IV melanoma patients. The increased proportion of TH2 cells likely depends on an accumulation of TH2 cells compared to an imbalance in the ratio between the different TH subsets, since the frequency of TH2 cells also increases within the CD3+ compartment. This is in keeping with previous reports of TH2 polarisation in patients with malignant melanoma (Lauerova et al., 2002) (Nevala et al., 2009). While there are certain individuals with stage IV

melanoma that have an abundance of Tregs, overall there is no difference in the frequency of Tregs in patients compared to healthy controls.

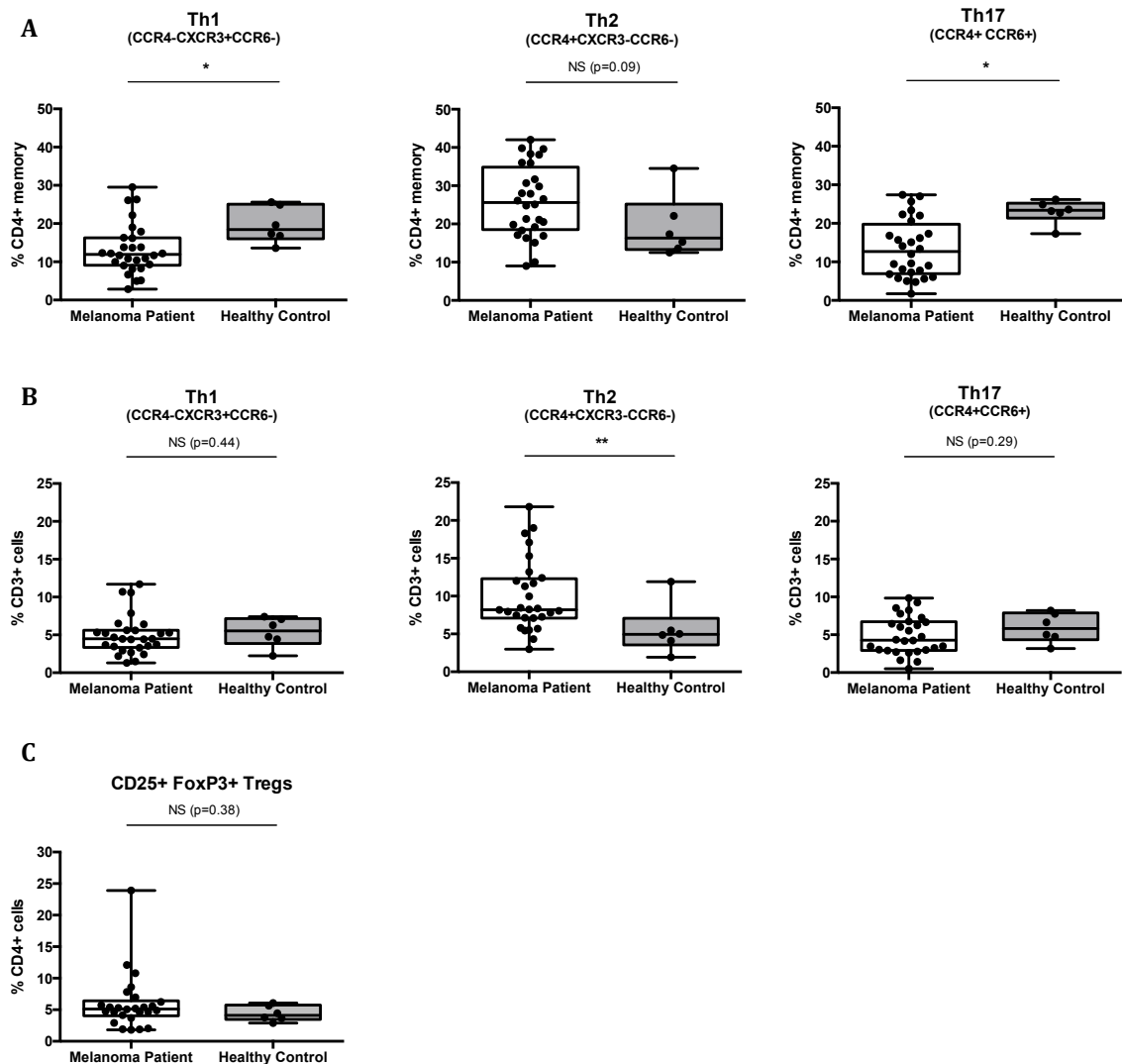


Figure 6: Frequency of CD4+ T cell subsets in the peripheral blood of stage IV melanoma patients versus healthy controls. A: Frequency of T_H1 , T_H2 and T_H17 cells expressed as a percentage of CD4+ memory T cells. There is a decrease in the percentage of CD4+ memory T cells with a T_H1 and T_H17 phenotype in patients compared to healthy controls. The frequency of T_H2 cells appears increased in a proportion of patients but overall this increase does not reach significance. B: Frequency of T_H1 , T_H2 and T_H17 cells expressed as percentage of total CD3+ T cells. There is a statistically significant increase in the percentage of cells with a T_H2 phenotype. Although the median frequency of T_H1 and T_H17 cells decreases in patients compared to healthy controls, this does not reach significance. C: There is also no difference in the frequency of CD25+ FoxP3+ Tregs in patients compared to healthy controls. The median and interquartile range are highlighted. Difference in distribution was determined using the Mann Whitney-U test.

Expression of CLA on selected T cell subsets are shown in appendix A2.1. Of the T cell subsets analysed, there is no difference in the proportion of circulating cells in melanoma patients versus healthy controls that express CLA.

4.4.3 Identification and quantification of CD8+ T cell subsets using differential cell surface expression of chemokine receptors

As well as CD4+ T cells, CD8+ T cells are known to polarise into different subsets upon activation (Annunziato et al., 2015). The Tc1 population of CD8+ T cells is characterised by the expression of the chemokine receptor CXCR3 and production of the type 1 cytokine, IFN- γ . Tc2 cells on the other hand, are characterised by the expression of CCR4, production of type 2 cytokines including IL-4, IL-5 and IL-13 and reduced cytotoxicity compared to Tc1 cells. Tc17 cells, capable of IL-17, IL-21, and IL-22, have also been described (Annunziato et al., 2015) (Inaoki et al., 2003).

As a T_H2 bias has previously been observed in melanoma patients, including this cohort of stage IV patients, I was interested in assessing whether a type 2 bias was also observed in CD8+ T cells. To this end CD8+ T cells were analysed for the expression of CCR4 and CXCR3 (figure 7). While there is clearly a population of CD45RA- memory CCR4+ CXCR3- CD8+ T cells, that may represent Tc2 cells, and a population of CCR4- cells of which a subset expresses CXCR3 (likely Tc1 cells), there is also a population that expresses both CCR4 and CXCR3.

To my knowledge, CD8+ T cells co-expressing CCR4 and CXCR3 have not been described before. Whether this population represents a distinct lineage of CD8+ T cell, or whether they are CCR4+ cells that have transiently upregulated CXCR3, or CXCR3+ cells that have transiently upregulated CCR4, remains to be determined. However, in the context of this analysis I considered CCR4+ CXCR3- and CCR4+CXCR3+ cells as separate subsets and evaluated whether their frequency is independently altered in melanoma patients.

Analysis of the frequency of the two CCR4+ expressing populations, reveals a significant reduction in the frequency of CCR4+ CXCR3+ cells in patients compared to healthy controls, however similarly to low-density neutrophils, a range in frequencies is observed (figure 8). While there is also a range of CCR4+ CXCR3- frequencies in melanoma patients, the distribution is not significantly different to healthy controls.

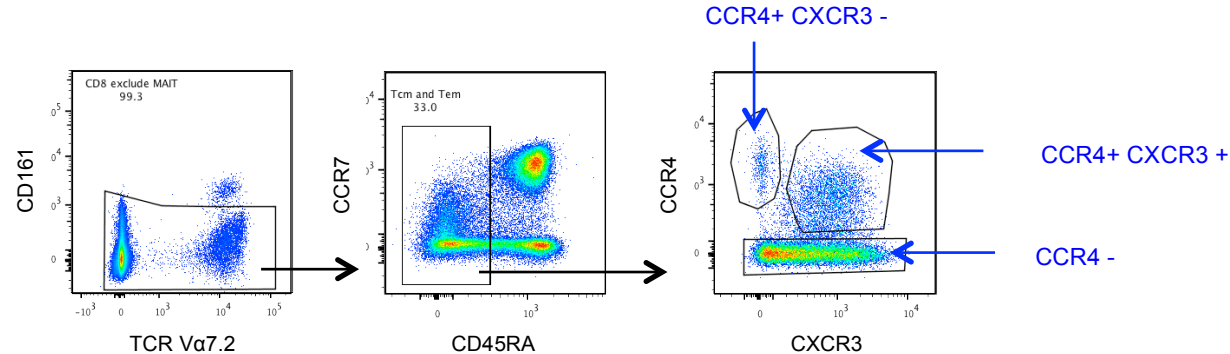


Figure 7: Identification of CD8+ T cell subsets. Live, singlet, CD3+ cells were gated upon, as in figure 3. CD8+ cells were selected (gating not shown). CD161+ TCR Va7.2+ MAIT cells were excluded and CD45RA- antigen-experienced cells were selected. Three CD8+ T cell populations were identified based on differential expression of the chemokine receptors CCR4 and CXCR3: CCR4+ CXCR3-, CCR4+ CXCR3+ and CCR4- cells.

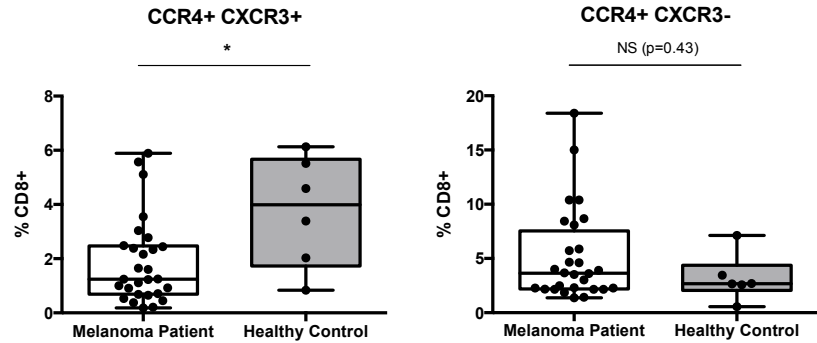


Figure 8: Frequency of memory CD8+ T cell populations in the peripheral blood of patients with stage IV melanoma versus healthy controls. The frequency of CCR4+ CXCR3+ effector memory CD8+ T cells is decreased in patients compared to healthy controls whereas there is no significant difference in the distribution of CCR4+ CXCR3- effector memory CD8+ T cells between groups. The median and interquartile range is highlighted and the Mann Whitney-U test was applied.

4.4.4. Identification and quantification of eomesodermin (EOMES) positive terminally differentiated effector memory RA (TEMRA) CD8+ T cells

While central and effector memory T cells are characterised by their lack of cell surface expression of CD45RA and differential expression of CCR7, and naïve T cells by their expression of both molecules, another CD8+ T cell subset exists that expresses CD45RA but lacks CCR7 (Sallusto et al., 1999). This population has been referred to as the TEMRA subset and is thought to represent the most differentiated effector memory subset. Such cells are thought to be particularly cytotoxic due to high expression of perforin and Fas ligand (Sallusto et al., 1999) (D'Asaro et al., 1996). These cells are also capable of production of cytokines such as IFN- γ (Sallusto et al., 1999) (Willinger et al., 2005). The transcription factor, EOMES, is highly expressed in effector memory and TEMRA CD8+ populations and along with T-bet is a key master regulator of CD8+ T cell differentiation (Willinger et al., 2005) (Gang et al., 2013). TEMRA were identified by expression of CD8, CD45RA and EOMES (figure 9A). The frequency of TEMRA is significantly increased in patients with melanoma compared to healthy controls (figure 9B).

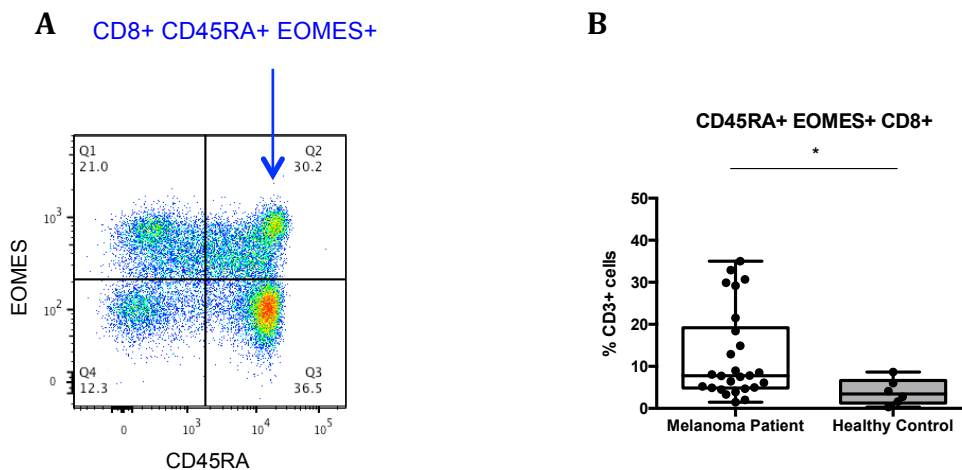


Figure 9: Identification TEMRA CD8+ T cells. A: Gating strategy used to identify TEMRA. Previous gating on live, singlets, CD3+ CD8+ cells not shown. TEMRA were defined as CD45RA+ EOMES+. These cells are CCR7- CD45RA+ (not shown) B: The frequency of TEMRA is increased in the peripheral blood of stage IV melanoma patients compared to healthy controls. The median and interquartile range is highlighted and the Mann Whitney-U test was applied.

4.4.5 Stratification of melanoma patients according to prior treatment with anti-CTLA-4

Anti-CTLA-4 treatment promotes T cell proliferation and this cohort of stage IV melanoma patients included some patients who donated blood prior to treatment with anti-PD-1 (following progression on anti-CTLA-4). To assess whether prior treatment with anti-CTLA-4 impacts on the frequency of certain T cell subsets, I stratified patients accordingly.

Certain differences were observed between melanoma patients who had not previously received anti-CTLA-4 ('MP' in figure 10) and those who had received such treatment ('MP prior α -CTLA-4' in figure 10). For example, there is a significant increase in the frequency of T_H2 cells (expressed as a percentage of CD4+ memory T cells) in melanoma patients previously treated with anti-CTLA-4 compared to healthy controls, however the difference in distribution of T_H2 cell frequency between melanoma patients without prior anti-CTLA-4 does not reach significance (figure 10). While both groups of melanoma patients have a higher frequency of T_H2 cells (expressed as a percentage of CD3+ cells) compared to healthy controls, the median of the group previously treated with anti-CTLA-4 is greater. It is also true that the frequency of Tregs is greater in patients who have previously received anti-CTLA-4 and although not reaching significance, the median frequency of activated TCR- $\gamma\delta$ -int cells (HLA-DR+ CD38+) also appears increased in this group of patients.

It is difficult to understand whether the differences observed between melanoma patients with or without prior treatment is due to a lasting effect of anti-CTLA-4 or a reflection of the fact that this group of patients, having previously failed anti-CTLA-4 (and in all cases at least one other therapy) may have a greater tumour burden. Analysis of the frequency of T cell subsets during anti-CTLA-4 may help to answer this question.

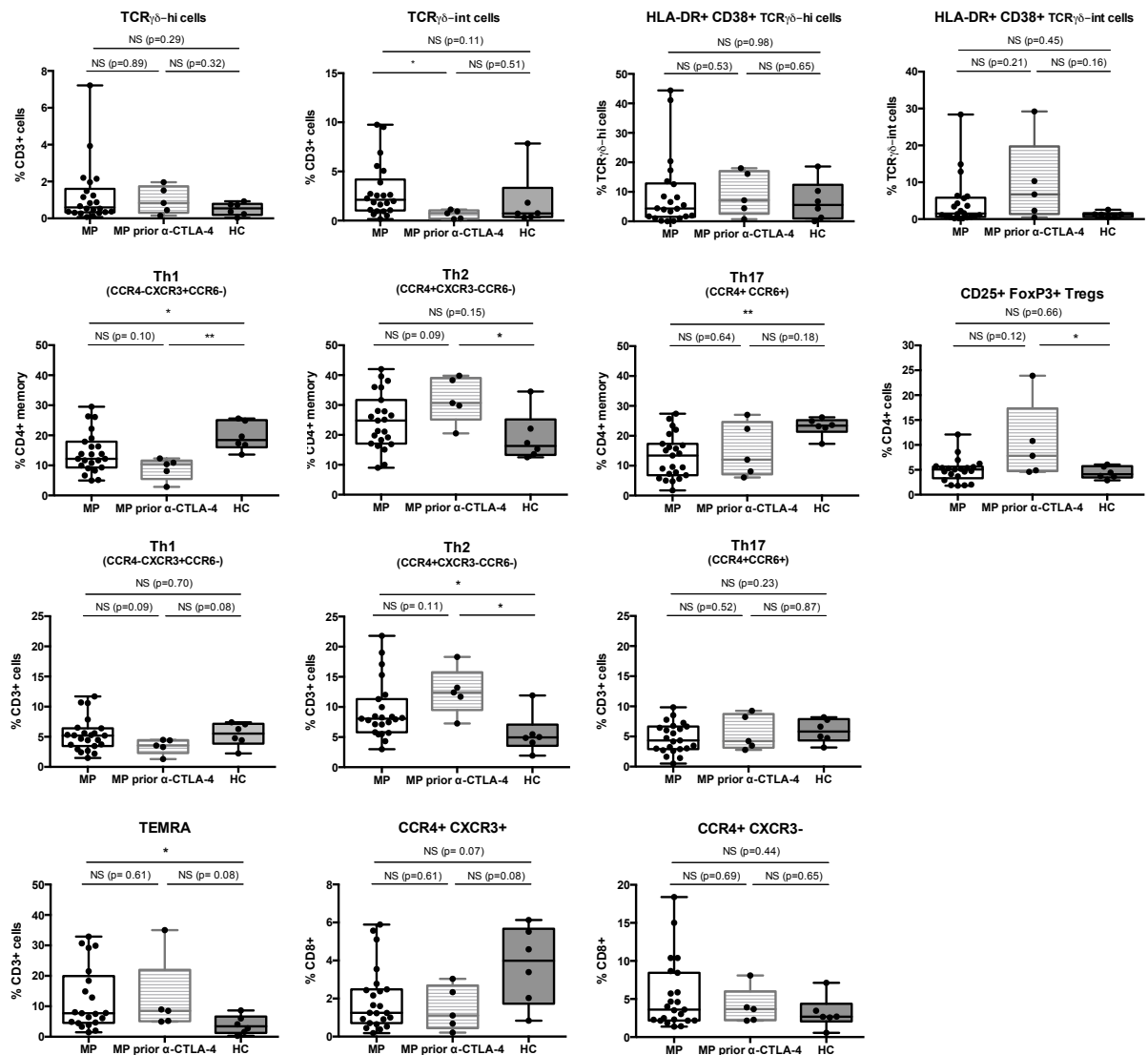


Figure 10: Analysis of the frequency of T cell populations when patient samples are stratified according to previous immunotherapy. T cell populations as in figures 4-9 however patients are divided into those who have never received anti-CTLA-4 treatment (MP) and those who have previously been treated with anti-CTLA-4 (MP prior α -CTLA-4). The frequency in these groups was compared to the frequency of T cell population in healthy controls (HC). The median and interquartile range are highlighted and the difference in distribution between the different groups was determined by the Mann Whitney-U test.

4.5 Correlation of frequency and activation status of T cell populations with certain plasma cytokines, low-density granulocytes and monocytic populations

This cohort might be underpowered to rule out differences between healthy controls and melanoma patients that do not reach statistical significance. However the wide range of frequencies observed in the different T cell subsets might be biologically relevant and associated with distinct inflammatory patterns in melanoma patients. Thus I used RStudio software to create a correlogram in order to identify correlations between the frequency of distinct T cell subsets, myeloid cells and cytokine/growth factors in the peripheral blood of stage IV melanoma patients (Figure 11). This correlogram revealed distinct correlation clusters associated with activated neutrophils and immature neutrophils.

Activated neutrophils ("CD10pos" in figure 11) positively correlate with the frequency of inflammatory monocytes ("CD14pos.CD16pos") and activated TCR- $\gamma\delta$ cells (those with intermediate expression of TCR- $\gamma\delta$) ("GDint.CD38.DR), and negatively correlate with the frequency of CCR4+ CXCR3+ CD8+ T cells. Activated neutrophils can be found in the correlogram in close association with activated TCR- $\gamma\delta$ cells and the cytokines IL-8 and IP-10 (highlighted in the upper black outlined box in figure 11).

Interestingly, a negative correlation between the frequency of activated neutrophils and the frequency of CD8+ T cells expressing both the CCR4 and CXCR3 chemokine receptors was observed. It may be that the inflammatory environment that exists in patients with a higher frequency of activated neutrophils, results in greater infiltration of this population into the tumour microenvironment, and the co-expression of CCR4 and CXCR3 may contribute to such infiltration. It should be noted that as well as negatively correlating with the plasma concentration of IL-8, this population negatively correlates with the plasma concentration of MIP-1 β (CCL4). MIP-1 β signals through a number of chemokine receptors, including CCR4. While the plasma concentration of the CCR4 ligands CCL17 and CCL22 were not measured, it may be that raised plasma MIP-1 β in certain patients reflects high intratumoural concentration of this

cytokine, which may function in recruiting CCR4+ CXCR3+ CD8+ cells into the tumour.

In contrast to activated neutrophils, immature neutrophils are found in close association with the plasma concentration of IL-6 and SAA and the frequency of T_H2 cells, positively correlating with all three variables (highlighted in the lower black outlined box in figure 11). T_H2 polarisation in patients with malignant melanoma has previously been described (Lauerova et al., 2002) (Nevala et al., 2009), as has a relationship between T_H2 cells and MDSCs in inflammation (Delano et al., 2007) (Jeisy-Scott et al., 2011). Whether the positive correlation between the frequency of immature neutrophils and T_H2 cells in stage IV melanoma patients is due to a direct link between immature neutrophils and the T_H2 bias, in that T_H2 cells are directly involved in driving the premature release/expansion of immature neutrophils or that immature neutrophils can skew naïve T cell polarisation in melanoma patients is unclear. However, in the previously described T_H2/MDSC associations, the MDSCs consisted of immature myeloid cells with a predominantly monocytic phenotype unlike that of the immature CD10- CD33^{hi} neutrophils which correlate with T_H2 cell frequency in the context of melanoma. In this cohort of patients, I did not observe a correlation between the frequency of moMDSC (defined as CD14+ HLA-DR- and thought to represent immature monocytic cells) and the frequency of T_H2 cells.

That activated and immature neutrophils correlate with distinct T cell and monocyte populations, with certain T cell and monocyte populations also correlating with the abundance of certain cytokines/growth factors, suggests that differences in the inflammatory profiles that can exist within the context of stage IV melanoma extend beyond differences in neutrophil activation/differentiation. This reinforces the existence of different inflammatory axes in stage IV melanoma, as suggested by data in chapter 3.

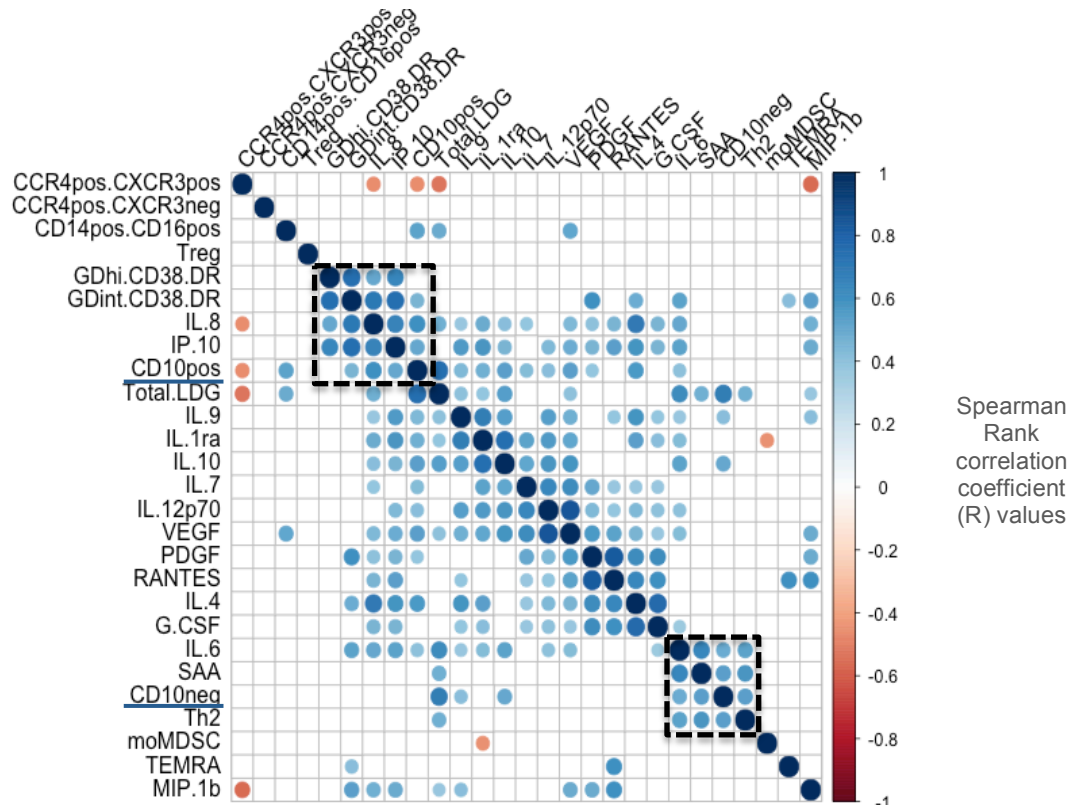


Figure 11: Correlogram highlighting correlations that exist between the frequency of activated and immature neutrophils and the frequency of certain T cell populations and cytokines/growth factors, in the peripheral blood of patients with stage IV melanoma. Correlations were determined using Spearman Rank correlation coefficient (R). Positive correlations are in blue and negative correlations in red, with the colour intensity and size of the circles positively correlating with the strength of the correlation. Only significant ($p < 0.05$) correlations are shown and empty boxes reflect non-significant correlations between two factors. Underlined in blue are activated (CD10pos) and immature (CD10neg) neutrophils. In black-dashed boxes are clusters of immune cell populations and cytokines including either activated or immature neutrophils. Abbreviations - CCR4pos.CXCR3pos: CCR4+ CXCR3+ CD8+ T cells, CCR4pos.CXCR3neg: CCR4+ CXCR3- CD8+, CD14pos.CD16pos: CD14+ CD16+ monocytes, GDhi.CD38.DR: CD38+ HLA-DR+ TCR- $\gamma\delta$ hi, GDint.CD38.DR: CD38+ HLA-DR+ TCR- $\gamma\delta$ int, CD10pos: CD10+ CD33int neutrophils, CD10neg: CD10- CD33hi neutrophils, Total.LDG: total low-density granulocytes, MIP.1b: MIP-1 β . Variables were subject to hierarchical clustering using the hclust function on RStudio software.

4.6 Further characterisation of the phenotype of CCR4+ CD8+ T cells

Analysis of the frequency of CCR4+ CD8+ T cell subsets in melanoma patients led to the observation that CCR4+CXCR3+ T cells but not CCR4+ CXCR3- T cells are

reduced in melanoma patients, and that this reduction is associated with an increased frequency of inflammatory markers such as activated neutrophils and the inflammatory chemokine MIP-1 β (figure 11). CCR4 has been proposed as a therapeutic target for cancer immunotherapy, mostly for its role in mediating the migration of Tregs into the tumour microenvironment (Sugiyama et al., 2013). However the role of CCR4-expressing CD8⁺ T cells has been poorly characterised, and in particular the presence of two distinct subsets of CCR4⁺ cells, to my knowledge, has not been described before. This led me to further explore the nature of CCR4⁺CXCR3⁺ cells and their relationship with tumour immunity.

4.6.1 Negative correlation between the frequency of CCR4⁺ CXCR3⁺ CD8⁺ effector memory cells and the frequency of activated neutrophils in the peripheral blood

The raw data revealing the negative correlation between the frequency of CCR4⁺ CXCR3⁺ CD8⁺ T cells and activated neutrophils, and the lack of correlation between CCR4⁺ CXCR3⁻ CD8⁺ T cells, is shown in figure 12.

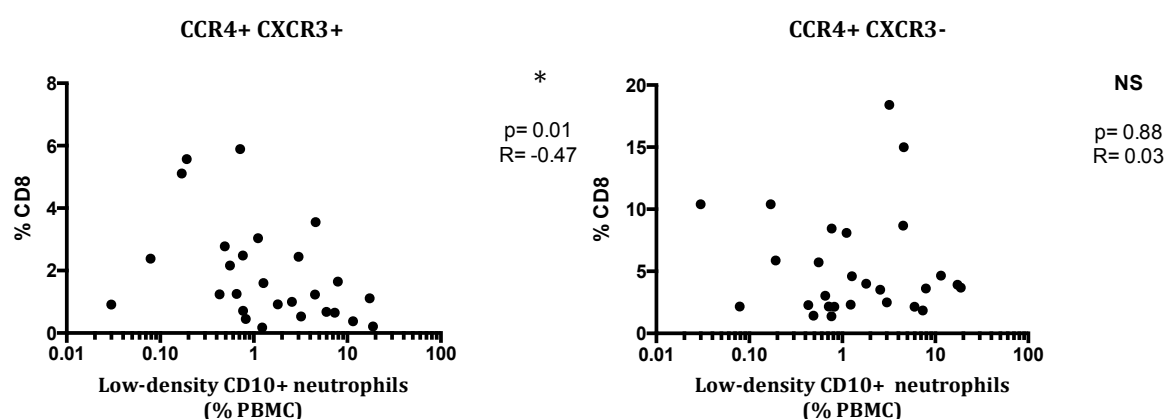


Figure 12: Association of activated neutrophil frequency and CCR4⁺ CXCR3⁺ CD8⁺ memory T cell frequency. A negative correlation is observed between the frequency of activated neutrophils and the frequency of CCR4⁺ CXCR3⁺ CD8⁺ effector memory cells in patients with stage IV melanoma. A correlation between the frequency of activated neutrophils and CCR4⁺ CXCR3⁻ CD8⁺ cells is not observed. The Spearman Rank correlation coefficient (R) was determined.

4.6.2 Maintenance of CCR4+ CXCR3+ phenotype following expansion

While it is possible that CCR4+ CXCR3+ CD8+ T cells represent a distinct, epigenetically imprinted, lineage of CD8+ T cell, such cells might also represent a subset of CCR4+ CXCR3- T cells that have transiently upregulated CXCR3, or indeed a subset of CCR4- CXCR3+ cells that have transiently upregulated CCR4. In an attempt to understand this, I asked whether the CCR4+ CXCR3+ phenotype can be maintained after *in vitro* expansion. Thus CCR4+CXCR3-, CCR4+CXCR3+ and CCR4- CD8+ T cells were sorted from the peripheral blood, expanded *in vitro* with different polyclonal stimuli (either PHA or anti-CD3/28 coated beads), and CCR4 and CXCR3 expression assessed. All expansion was carried out in the presence of IL-2, a potent T cell growth factor. Expression of CCR4 and CXCR3 were analysed on days 7 and 21 following expansion with PHA/IL-2 (figure 13). By day 21, a large proportion CCR4+ CXCR3+ cells (40%) retained CCR4 and CXCR3 expression.

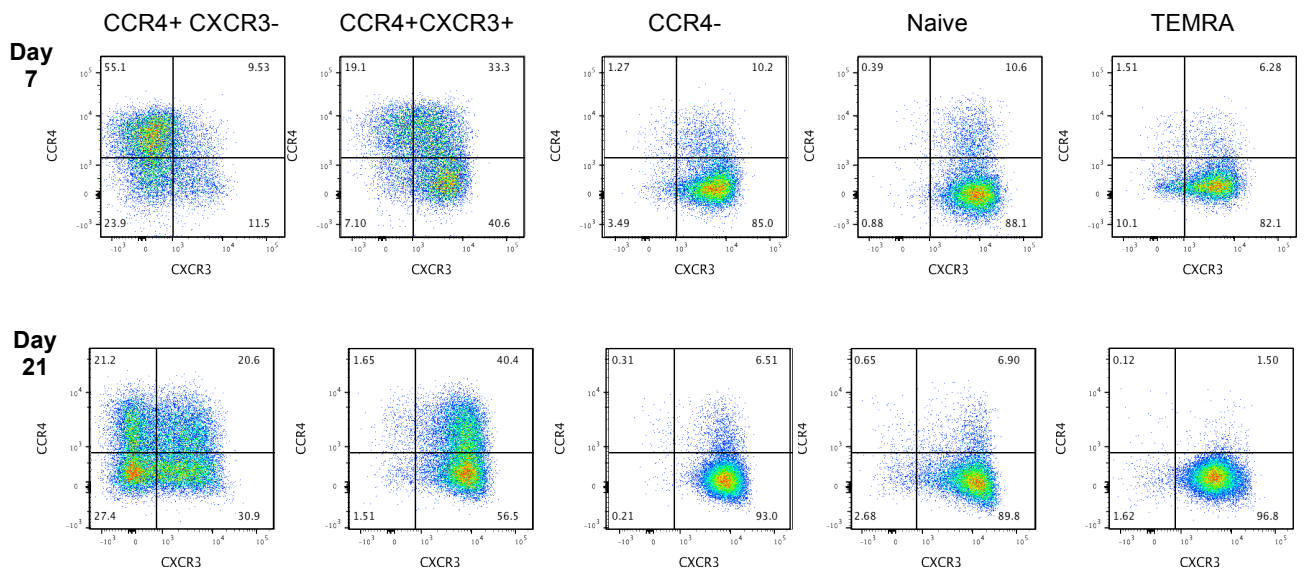


Figure 13: Analysis of CCR4 and CXCR3 expression on sorted CD8+ effector memory T cells following expansion with PHA/IL-2. Cells were sorted from the peripheral blood of a healthy donor according to initial CCR4 and CXCR3 expression (as per gating in figure 7A). Naïve cells were sorted as CCR7+ CD45RA+, and TEMRA as CCR7- CD45RA+. Expression of CCR4 and CXCR3 was analysed on day 7 and 21.

A large proportion of cells (just under 50%) also retained CCR4 and CXCR3 expression when initially stimulated with anti-CD3/28 coated beads, followed by expansion with IL-2 (figure 14).

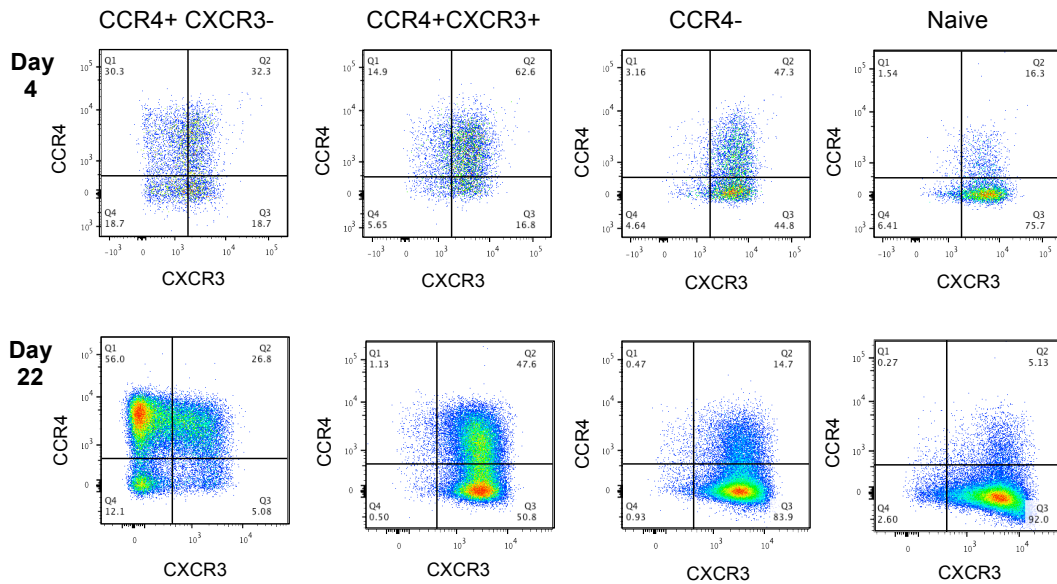


Figure 14: Analysis of CCR4 and CXCR3 expression on sorted CD8+ effector memory T cells following expansion with anti-CD3/28 coated beads and IL-2. Cells were sorted from the peripheral blood of a healthy donor according to initial CCR4 and CXCR3 expression (as per gating in figure 7A). Expression of CCR4 and CXCR3 was analysed on day 4 and 22.

The ability of a large proportion of CCR4+ CXCR3+ cells to maintain expression of both CCR4 and CXCR3 over the course of three weeks, suggests that this phenotype, in at least a subset of CCR4+ CXCR3+ cells, is stably maintained.

In both types of stimulation, there are fluctuations in terms of CCR4 and CXCR3 expression in the different populations compared to the initial phenotype at the time of sorting. A proportion of cells initially sorted as CCR4+ CXCR3- or CCR4- express CCR4 and CXCR3 over the course of the expansion, while a fraction of CCR4+CXCR3+ cells lose the expression of CCR4. This might depend either on contamination of the sorted population, or on the flexibility of the different cell

subsets, which in the presence of cytokines released during proliferation, might upregulate or downregulate the different chemokine receptors.

Although not conclusive, these experiments show that after several rounds of proliferation a large proportion of the sorted populations, including the CCR4+ CXCR3+ subset, still maintain the expression of specific combinations of CCR4 and CXCR3, suggesting that their expression might be epigenetically imprinted and associated with specific features of these cells.

4.6.3 Cytokine expression

4.6.3a Differential expression of cytokines in CD8+ T cell populations at the level of RNA

In order to assess whether the co-expression of CCR4 and CXCR3 is associated with specific functional properties of the CXCR3+CCR4+ subset, compared to the CCR4+ CXCR3- and CCR4- subsets, I analysed the expression of genes encoding a selection of cytokines two hours post stimulation using a nanostring gene expression panel. To this aim I sorted CD8+ T cells from the peripheral blood of two healthy donors based on the expression of CCR4 and CXCR3, as well as naïve cells and the TEMRA subset for comparison, and then stimulated with PMA/ionomycin. (figure 15).

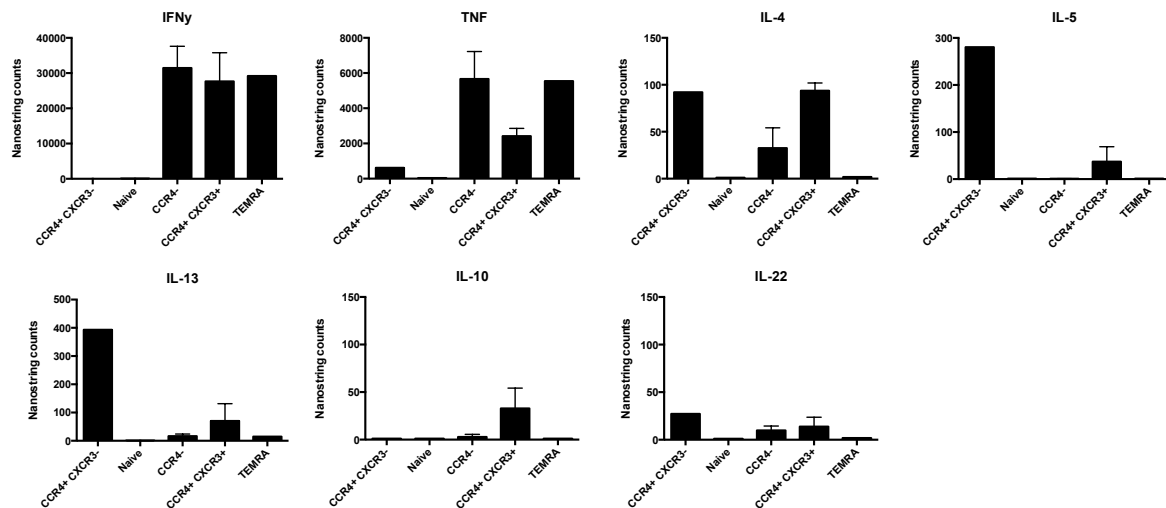


Figure 15: Expression of cytokine genes in distinct CD8+ T cell populations. Expression of 7 cytokines were assessed as part of a nanostring gene expression panel. Cells were sorted according to CCR4 and CXCR3 expression. Naïve T cells were defined as CCR7+ CD45RA+ and TEMRA as CCR7- CD45RA+. All 7 genes were undetectable in unstimulated cells (data not shown). Stimulation with PMA/ionomycin for 2 hours resulted in differential upregulation of these genes in certain T cell populations. Mean and standard deviation for two healthy donors shown.

Following stimulation with PMA/ionomycin, there is similar upregulation of the IFN- γ gene in the CCR4-, TEMRA and CCR4+ CXCR3+ populations (figure 15). This is also true for TNF- α expression, however, greater induction is observed in the CCR4- and TEMRA compared to CCR4+ CXCR3+ cells. TNF- α is also detected in CCR4+ CXCR3- cells, although at a lower amount. With regards to IL-4 expression, the greatest amounts of RNA are detected in the CCR4+ populations, and while low amounts of IL-4 RNA was detected in the CCR4- cells, this was only in one donor.

While the gene encoding the type 2 cytokine IL-4 is expressed at comparable amounts in the two CCR4+ populations, expression of the IL-5 and IL-13 genes, also encoding type 2 cytokines, is much greater in the CCR4+ CXCR3- population. Both populations therefore have the capacity to express IL-4, however while CCR4+ CXCR3+ cells express IFN- γ , CCR4+ CXCR3- cells express IL-5 and IL-13.

There is also evidence of low expression of the IL-22 gene in the CCR4⁺ and CCR4⁻ populations, and IL-10 expression in at least one of the donor's CCR4⁺ CXCR3⁺ cells. It is difficult to know the relevance of these results given the low number of RNA molecules detected being just over the background threshold.

4.6.3b Intracellular cytokine staining

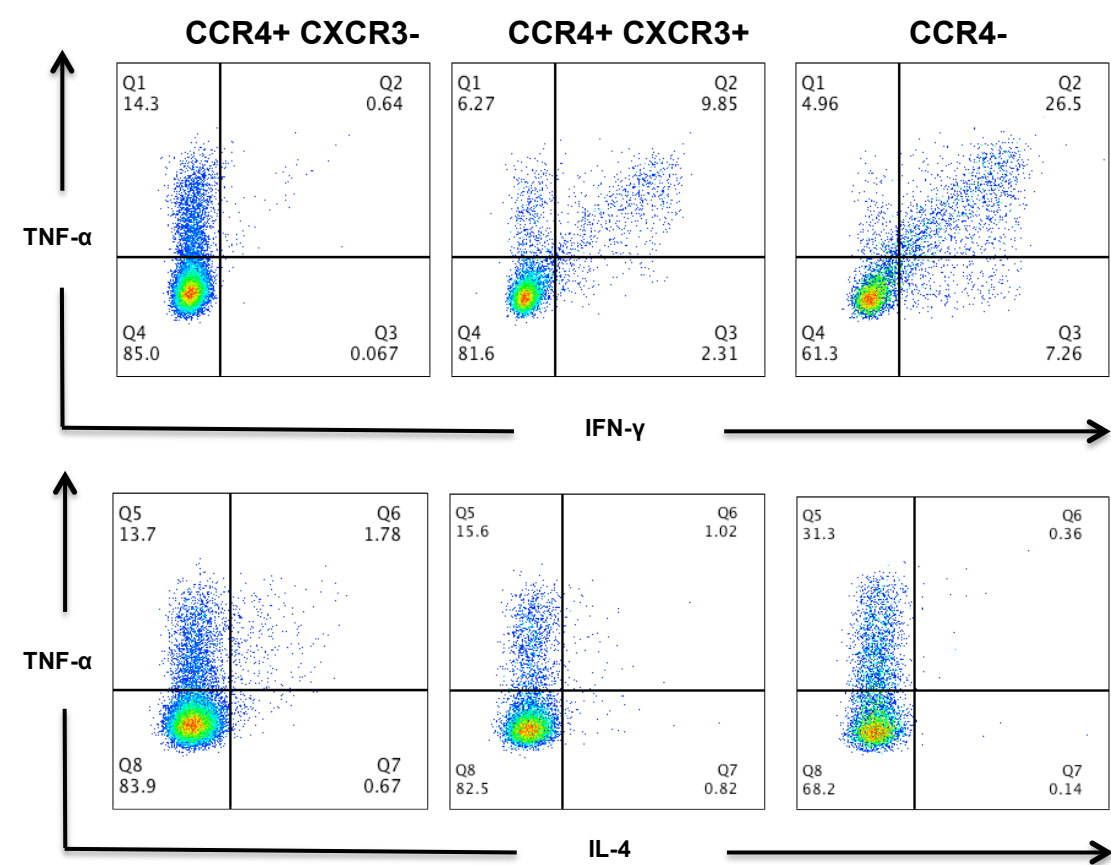
It is evident from figure 15 that the CCR4⁺ CXCR3⁺ population expresses IFN- γ , TNF- α and IL-4. As IFN- γ is considered a type 1 cytokine, and IL-4 a type 2 cytokine, I was interested in whether these two opposing cytokines were expressed by the same cells or by different sub-populations. RNA analysis of a bulk population does not enable detection of heterogeneity in a population. Furthermore, it does not reveal whether the cells have the potential to produce these cytokines at the protein level, which is clearly necessary for functional relevance. Therefore, I sorted cells, stimulated with PMA/ionomycin and assessed protein production by intracellular cytokine staining (figure 16).

Within the CCR4⁻ population (of which a subset expresses the Tc1-associated CXCR3 receptor), although there are some single IFN- γ and single TNF- α producing cells, the responding cells are predominantly IFN- γ + TNF- α + (figure 16A and B). These cells however do not produce IL-4. Unlike the CCR4⁻ cells, very few CCR4⁺ CXCR3⁻ cells make both IFN- γ and TNF- α . While certain cells can produce both IL-4 and TNF- α , and certain cells only IL-4, the majority of responding cells make only TNF- α in response to stimulation with PMA and ionomycin. The fact that the majority of responding CCR4⁻ cells produce the type 1 cytokine IFN- γ , and the fact that IFN- γ -producing cells are barely detectable in the CCR4⁺ CXCR3⁻ fraction, suggests that these populations represent the Tc1 and Tc2 populations of CD8⁺ T cells. While CCR4⁻ and CCR4⁺ CXCR3⁻ cells seem distinct from one another in terms of cytokine production, CCR4⁺ CXCR3⁺ cells show characteristics of both populations. Like CCR4⁻ cells, over half of the responding cells are IFN- γ + TNF- α + (figure 16A and B). However, a significant proportion of cells produce only TNF- α and a small but detectable proportion

produce only IFN- γ , both TNF- α and IL-4 and only IL-4. This suggests that the CCR4+ CXCR3+ population is heterogenous in nature.

While IFN- γ and IL-4 are typically expressed by distinct type 1 and type 2 T cell subsets respectively, TNF- α , like GM-CSF and IL-3, can be produced by both type 1 and type 2 cells (Lattime & Gerton, 2002) (McGhee 2005).

A



B

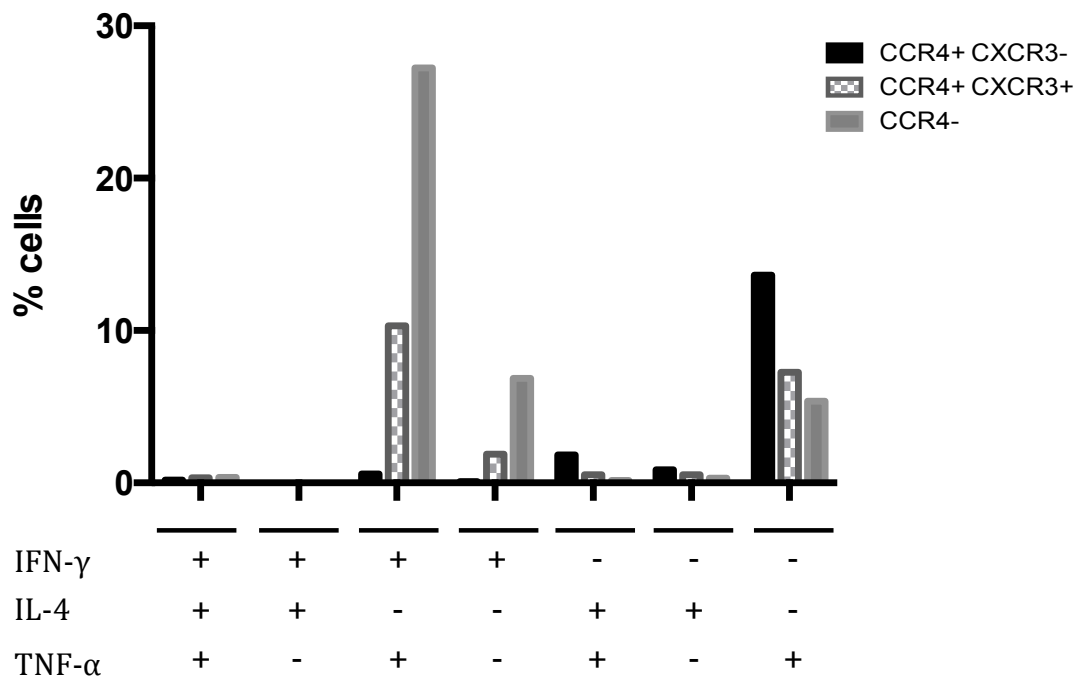


Figure 16: Intracellular cytokine staining of sorted CD8⁺ T cell populations. A: Example FACS plots of cells stimulated with PMA/ionomycin for five hours in the presence of brefeldin A, followed by staining with antibodies against IFN- γ , TNF- α and IL-4. Prior to stimulation, cells defined by CCR4 and CXCR3 expression were sorted as per the gating in figure 7A. B: Quantification of cytokine-producing cells determined by intracellular cytokine staining of sorted CD8⁺ T cell populations.

4.6.4 Analysis of GATA-3 and T-bet transcription factor expression

Differentiation of distinct T cell subsets is dependent on the expression of specific master regulators/lineage-determining transcription factors (Lazarevic et al., 2013). These transcription factors drive distinct gene expression profiles resulting in cells with a specific set of functional properties. It is known that T-bet is a key master regulator of T_H1 and Tc1 differentiation and that its expression is induced in response to TCR, IFN- γ receptor and IL-12 receptor signalling, resulting in cells capable of, among other things, secretion of IFN- γ

(Lazarevic et al., 2013) (Annunziato et al., 2015). T_H2 and Tc2 differentiation however, is dependent on expression of the transcription factor, GATA-3, which is induced upon activation of T cells in an environment rich in IL-4, resulting in cells capable of IL-4, IL-5 and IL-13 secretion (Pai et al., 2004) (Lazarevic et al., 2013).

Expression of the chemokine receptor CXCR3 is associated with type 1 T cells, whereas expression of CCR4 is associated with type 2 T cells (Annunziato et al., 2015). Gene expression and intracellular cytokine analysis revealed that the CCR4⁺ CXCR3⁺ T cell subset can produce both type 1 and type 2 cytokines (figure 15 and 16). Intracellular cytokine staining however shows that those able to produce IFN- γ do not produce IL-4 (and vice versa), suggesting that this is a heterogenous population. In order to see whether this was reflected in expression of type 1 and type 2-associated transcription factors, nuclear staining for T-bet and GATA-3 was carried out (figure 17).

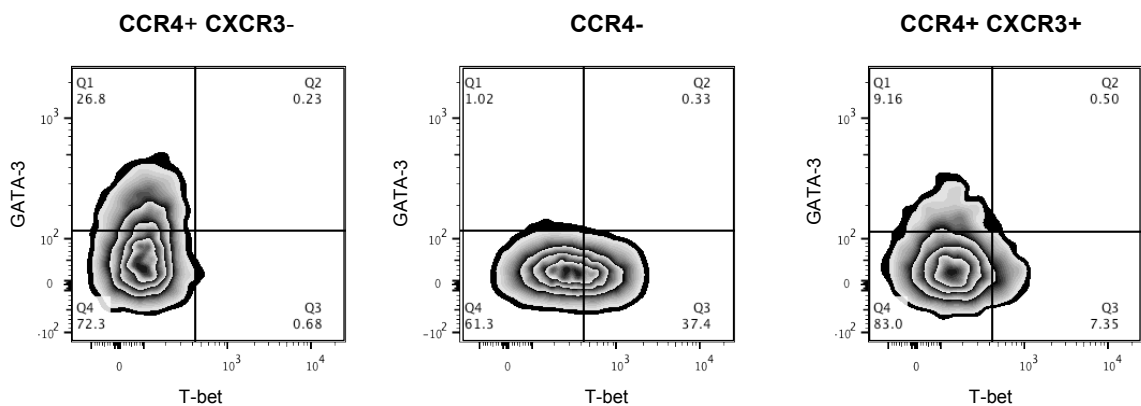


Figure 17: Comparison of CCR4⁺ and CCR4⁻ CD8⁺ populations for expression of T-bet and GATA-3. There is expression of GATA-3 within the CCR4⁺ CXCR3⁻ population and expression of T-bet within the CCR4⁻ population. Within the CCR4⁺ CXCR3⁺ population there are both GATA-3⁺ T-bet⁻ cells and Tbet⁺ GATA-3⁻ cells.

Indeed it can be seen that while expression of GATA-3 is observed in the CCR4⁺ CXCR3⁻ population and T-bet in the CCR4⁻ population, a proportion of cells in the CCR4⁺ CXCR3⁺ population are GATA-3⁺ T-bet⁻ and a proportion of cells GATA-3⁻ T-bet⁺, again supporting the notion that distinct subsets of CD8⁺ T cells reside within the CCR4⁺ CXCR3⁺ population (figure 17).

4.6.5 Activation status of CCR4+ CD8+ T cells

To address the activation status of the two CCR4+ expressing populations I analysed the expression of ICOS and Programmed cell death-1 (PD-1) (figure 18). Both ICOS and PD-1 are members of the CD28/CTLA-4 family. ICOS and CD28, through binding to ICOS-L and CD80/86 on APCs (and in the case of ICOS-L, non-haematopoietic cells during inflammation), respectively, act to promote T cell activation, proliferation and survival (Dong et al., 2001) (Fife and Bluestone, 2008). Conversely, CTLA-4 and PD-1 are inhibitory receptors, important in regulating inflammatory responses and functioning in peripheral tolerance (Dong et al., 2001) (Fife and Bluestone, 2008). Both ICOS and PD-1 are upregulated on activated T cells however, in contrast to an acute infection in which there is resolution and generation of memory T cells, chronic stimulation results in T cell exhaustion, often a failure to generate memory cells, and is associated with high expression of PD-1 (along with many other inhibitory receptors) and T cell dysfunction (Wherry et al., 2007). Chronic stimulation and associated T cell exhaustion is a feature not only of chronic infections but also of cancer and hence the rationale behind antibody therapies to block inhibitory receptors including PD-1 and CTLA-4 (Pauken and Wherry, 2015). In figure 18 it can be seen that a large proportion of CCR4+ CXCR3- cells express ICOS but there are few PD-1+ cells within this population. Conversely, a large proportion of CCR4+ CXCR3+ cells express PD-1 but few are ICOS+.

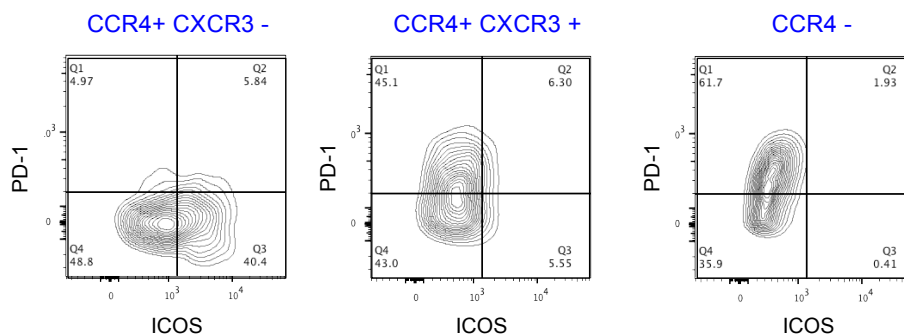


Figure 18: PD-1 and ICOS expression on CD45RA- effector memory CD8+ T cells. There is differential expression of PD-1 and ICOS by the two CCR4+ expressing populations.

The fact that of the CCR4+ CD8+ cells, it is the CXCR3 expressing subset that is enriched in PD-1+ cells, could suggest the presence of tumour-specific T cells within this compartment. A role for CCR4 in recruitment of T cells (including regulatory T cells) into tumours *via* recognition of ligands CCL22 and CCL17, and a role for CXCR3 in recruitment of T cells towards CXCL10/IP-10 in the tumour microenvironment is known (Ondondo et al., 2013). Although PD-1+ cells are also present within the CCR4- population, the co-expression of CCR4 and CXCR3, may result in more effective migration into the inflammatory tumour microenvironment and may account for the reduced frequency of this population in patients with an inflammatory profile associated with neutrophil activation and an enrichment of inflammatory cytokines, including IL-8.

4.7 Functional analysis of CCR4+ CD8+ T cells in blood: detection of tumour-specific cells within distinct CCR4+ populations

Although at this stage I have not been able to demonstrate that CCR4+CXCR3+ CD8+ T cells have distinct functional properties compared to CCR4+ CXCR3- and CCR4- cells, the frequency of this subset is altered in cancer patients. Given that the CCR4+ CXCR3+ population is enriched in PD-1+ cells compared to the CCR4+ CXCR3- population I questioned whether this was a reflection of an enrichment of tumour-specific T cells within this compartment. To investigate the presence of tumour-specific T cells, I used a panel of HLA-A*02-restricted peptides (summarised in table 1 – full sequences can be found in Chapter 2: Materials and Methods).

Table 1: HLA-A*02-restricted peptides used to detect tumour-specific T cells in stage IV melanoma patients. Peptides were chosen from a panel of 145 melanoma-associated CD8+ epitopes described by Kvistborg et al., 2014. The chosen peptides had a detectable response in at least one of the melanoma patients previously tested.

Tumour Antigen	Number of peptides in pool
gp100	7
LAGE-1	2
NY-ESO1	1
MAGE-A1	1
MAGE-A10	1
MAGE-A2	1
MAGE-A3	3
MAGE-C2	3
MART-1	1
Meloe-1	1
PRDX5	1
RAGE-1	1
SSX2	1
TRP2	1
Tyrosinase	1
BING4	1
GnTV	1

I sorted cells from the peripheral blood of HLA-A*02+ patients with stage IV melanoma and expanded tumour-specific T cells with the pool of peptides described in table 1. After expansion, cells were re-stimulated with the tumour peptide pool and responses were measured by intracellular cytokine staining.

Although it was only possible to analyse T cells from three patients, it appears that antigen-specific T cells exist within the different CCR4/CXCR3-expressing populations, and the population in which the strongest responses are observed, at least in terms of IFN- γ and TNF- α production, differs between patients (figure 19A). In patient 1 for example, the strongest response is observed in the CCR4+ CXCR3+ population, with the cells responding predominantly *via* production of TNF- α (figure 19A). While cells within this population have the capacity to produce both TNF- α and IFN- γ (as shown by PMA/ionomycin stimulation in figure 19B), the cells responding to this particular tumour peptide/s do so primarily by TNF- α production.

However, in patient 2, the dominant tumour peptide response observed is by T cells originating from the CCR4⁺ CXCR3⁻ population. This strong TNF- α response is mirrored in the “whole PBMC” response (in which total PBMC, not subjected to sorting, were directly stimulated with the tumour peptide pool). Production of TNF- α following recognition of tumour peptide/s is reflected in the PMA/ionomycin stimulation, in which the predominant response is TNF- α + IFN- γ -.

With regards to patient 3, there is a weak tumour-specific response, detectable in the ‘whole PBMC’ fraction but it is not clear where the dominant response resides in terms of CCR4 and CXCR3 expression.

While it is difficult to draw conclusions from experiments in such a small number of patients, it is evident that tumour-specific T cells can reside within all compartments analysed. As well as tumour-specific cells in the CCR4⁺ CXCR3⁺ and CCR4⁺ CXCR3⁻ populations, tumour-specific T cells were also found in the CCR4⁻ and naïve compartments. Detection of a relatively large response by naïve CD8⁺ T cells, particularly in patient 1 was surprising and it is possible that this response is due to the presence of memory stem T cells (T_{SCM}). These cells are thought to represent the earliest, least differentiated memory subset and account for 2-4% of peripheral blood CD4⁺ and CD8⁺ T cells (Gattinoni et al., 2011) (Flynn & Gorry, 2014). While this population is considered a memory subset, T_{SCM} cells, like naïve T cells, express both CCR7 and CD45RA (Gattinoni et al., 2011).

Interestingly, the cytokine-producing capacity of tumour-specific cells was different depending on which subset the cells were initially expanded from, revealing notable heterogeneity in the functional features of tumour-specific T cells. The location of tumour-specific T cells, in terms of CCR4 and CXCR3 expression may differ among patients and/or may differ depending on the tumour antigens recognised. Therefore as well increasing the number of patients analysed, it would be interesting to look at responses to individual peptides as well as the pool.

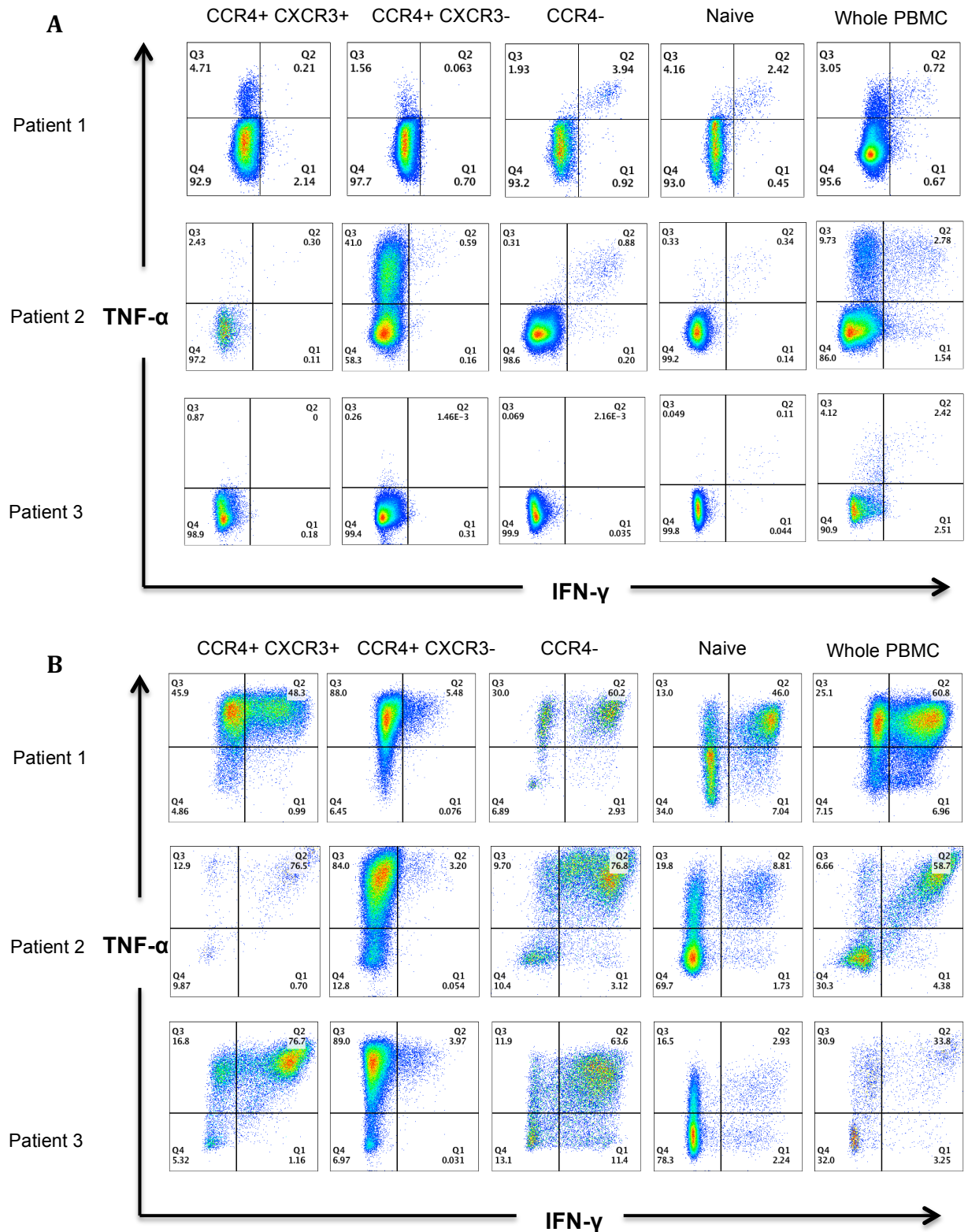


Figure 19: Detection of tumour-specific CD8+ T cells within different T cell populations. Effector memory CD8+ T cells were sorted for differential expression of CCR4 and CXCR3. Cells were stimulated with tumour peptide pool and IL-2 (A). After 14 days cells were restimulated with the pool and stained for IFN- γ and TNF- α production. Naïve CD8+ T cells (CD45RA+ CCR7+) and whole PBMCs (gated on CD3+ CD8+ cells) were also compared. Cells were also stimulated with

PMA/ionomycin as a positive control (B). Cells stimulated with DMSO (negative control) did not produce detectable IFN- γ or TNF- α (data not shown)

4.8 Detection of CCR4+ CD8+ T cells in melanoma tumours

Given that patients with an inflammatory profile associated with neutrophil activation have fewer CCR4+ CXCR3+ T cells in their peripheral blood, a possibility is that such cells leave the circulation in these patients as a result of recruitment to tumours. Furthermore, these cells in the blood are enriched in PD-1+ cells, and intratumoural tumour-specific T cells express PD-1 (Ahmadzadeh et al., 2009). Therefore, I decided to analyse the expression of CCR4 on tumour-infiltrating T cells, and asked whether CCR4 expression is associated with tumour-specific T cells or with specific functional features of tumour-infiltrating T cells. Metastatic melanoma tumour samples, dissociated by Jane Maraka (Cerundolo lab, WIMM), were stained for the expression of CCR4 (figure 20A) and PD-1 (figure 20B) on infiltrating CD8+ T cells.

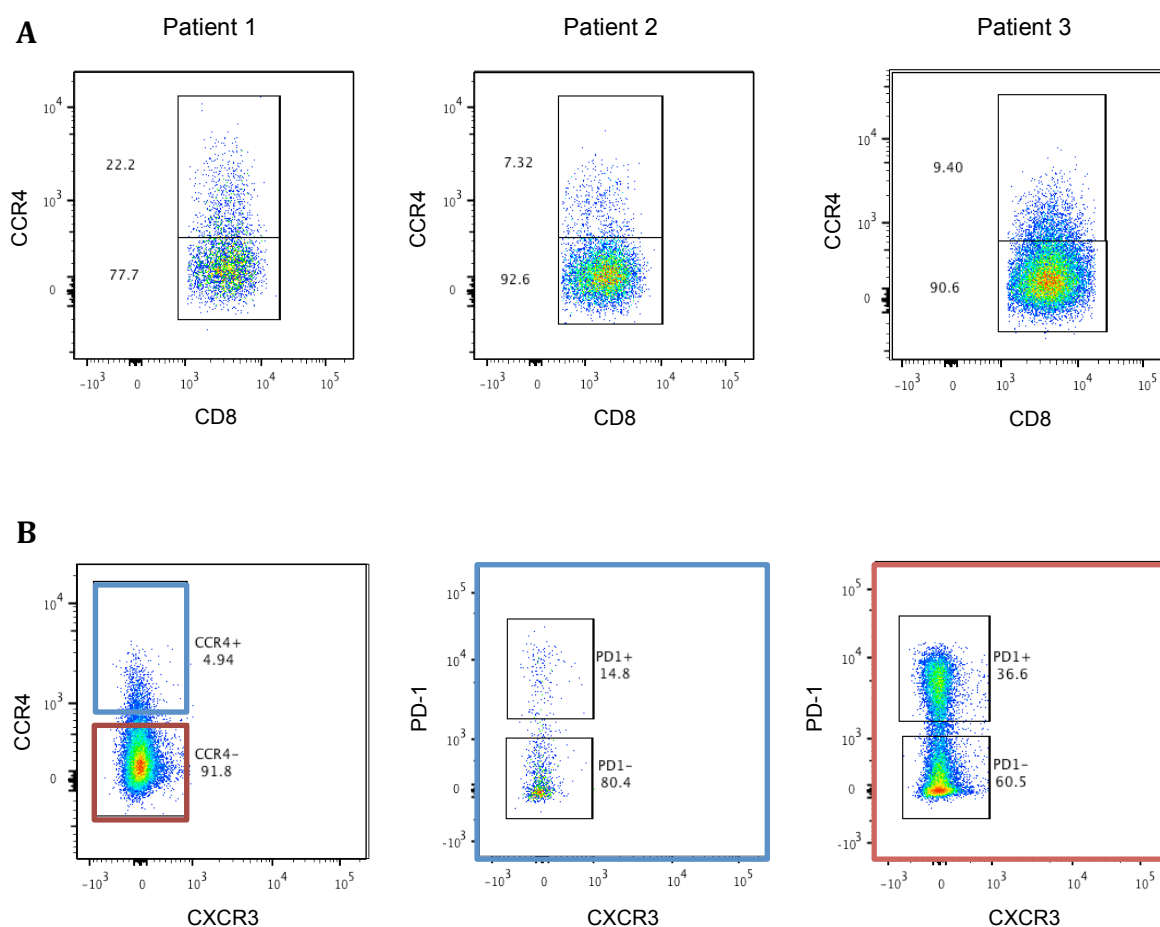


Figure 20: Identification of CCR4+ CD8+ T cells in metastatic melanoma tumours. A: FACS staining of CD8+ T cells following tumour dissociation reveals the presence of CCR4+ cells in tumours. Previous gating on live, singlets, CD45+ CD3+ CCR7- CD45RA- cells not shown. B: CXCR3 expression is low on CD8+ T cells in the tumour (left panel), however CCR4+ T cells (blue box) and CCR4- cells (red box) consist of both PD-1+ and PD-1- cells.

As observed in figure 20A, CCR4+ T cells are present in the tumours of the three samples analysed, with the proportion of CCR4+ CD8+ cells varying among patients. However, as illustrated in figure 20B (left panel), there is very weak expression of CXCR3 on CD8+ T cells isolated from the tumour. It has previously been observed, in the context of human mesenchymal stem cells, that CXCR3 is sensitive to enzymatic digestion with trypsin (Chamberlain et al., 2008). In order to test whether it was possible to recover CXCR3 expression, cell surface expression of CXCR3 was examined on freshly isolated tumour-infiltrating T cells, compared to those left to rest overnight at 37°C. It is evident from figure A2.3 in the appendix that resting overnight results in upregulation of CXCR3 on

CD8+ T cells. However, it was observed that this also results in a reduction in the percentage of PD-1+ cells. As PD-1+ cells are of interest, as they are likely enriched for tumour-specific T cells, resting overnight is not optimal.

As there are both CCR4+ and CCR4- T cells infiltrating melanoma tumours, I was interested in whether there was a difference in the functionality of these cells or the abundance of tumour-specific T cells between these compartments. If the reason behind the lower frequency of CCR4+ CXCR3+ T cells in the peripheral blood of patients with more activated neutrophils is linked to greater tumour infiltration of this T cell population, it may be that the CCR4+ PD-1+ T cells in the tumour are enriched in CCR4+ CXCR3+ cells (which have a greater proportion of PD-1+ cells in circulation compared to CCR4+ CXCR3- T cells) and the CCR4+ PD-1- cells may be enriched in the CCR4+ CXCR3- compartment (which have few PD-1+ cells in circulation). This however remains theoretical.

4.9 Analysis of tumour-infiltrating CD8+ cells

4.9.1 Functional properties of tumour-infiltrating CD8+ effector memory T cells, in terms of cytokine production in response to PMA/ionomycin stimulation

T cells were isolated from metastatic lymph nodes of three patients based on differential expression of CCR4 and PD-1 and expanded with PHA/IL-2. Following expansion, cells were stimulated with PMA/ionomycin and the capacity to produce IFN- γ , TNF- α and IL-4 was analysed (figure 21A and B). Comparison of the CCR4+ subsets revealed differences in cytokine production of PD-1+ and PD-1- cells. It seems that there is an enrichment of IFN- γ + cells in the subset sorted as CCR4+ PD-1+ (with around 70%, 90% and 94% total IFN- γ + cells in patients 1, 2 and 3 respectively). This compares to around 50%, 68% and 15% total IFN- γ + cells in the CCR4+ PD-1- subset (figure 21A). CCR4+ PD-1- cells have a sizeable population of TNF- α + IFN- γ - cells which is all but absent from the CCR4+ PD-1+ population. Although there is inter-patient variation in IL-4 production (with a greater frequency of IL-4+ cells observed in patient 2), it seems that there is also an enrichment of IL-4+ cells in the CCR4+ PD-1-

population (with 32%, 59% and 68% in CCR4+ PD-1- populations compared to 4%, 43% and 5% in the CCR4+ PD-1+ populations) (figure 21B). CCR4+ PD-1- cells are enriched for TNF- α and IL-4 production, properties that are enriched in the peripheral blood CCR4+ CXCR3- cells, and CCR4+ PD-1+ cells are enriched for IFN- γ production, a feature of peripheral blood CCR4+ CXCR3+ cells. It may be that at least some of the CCR4+ PD-1- population derives from the CCR4+ CXCR3- population in the blood, and at least some of the CCR4+ PD-1+ population derives from the CCR4+ CXCR3+ population in the blood, but this remains theoretical.

It is difficult to understand whether there are properties of CCR4+ cells that are unique from CCR4- cells in the tumour. Analysis of CCR4- cells revealed that the PD-1+ fraction is slightly enriched in IFN- γ + cells compared to PD-1- cells whereas the PD-1- fraction is slightly enriched in cells able to produce TNF- α , either with or without co-expression of IFN- γ (figure 21A). Similarly to CCR4+ cells in the tumour, it is the PD-1- fraction of CCR4- cells that is enriched in IL-4+ cells compared to the PD-1+ fraction (figure 21B). However, when CCR4+ PD-1- and CCR4- PD-1- cells are compared, it is those expressing CCR4 that have a higher frequency of IL-4-producing cells.

Of note is the capacity of certain CD8+ T cells within the tumour to produce both IL-4 and IFN- γ (figure 21B). While T cell subsets, such as T_H1 and T_H2, are thought to be distinct, stable lineages, some flexibility has been observed (Rebhahn et al., 2014). For example, when transferred into a strong T_H2-polarising environment (during helminth infection), a large proportion of T_H1 cells have been shown to acquire IL-4 expression (Panzer et al., 2012). The existence of GATA-3+ T-bet+ cells able to secrete both IL-4 and IFN- γ have also been observed during parasitic infection (Peine et al., 2013). Whether the presence of IFN- γ + IL-4+ cells in the tumour reflects a degree of plasticity between different T cell lineages or reflects the acquisition of a property usually associated with an alternate lineage is unclear. It is possible that if the melanoma tumour microenvironment is rich in type 2 polarising factors that perhaps IFN- γ producing cells, such as Tc1 cells, acquire the capacity to produce IL-4.

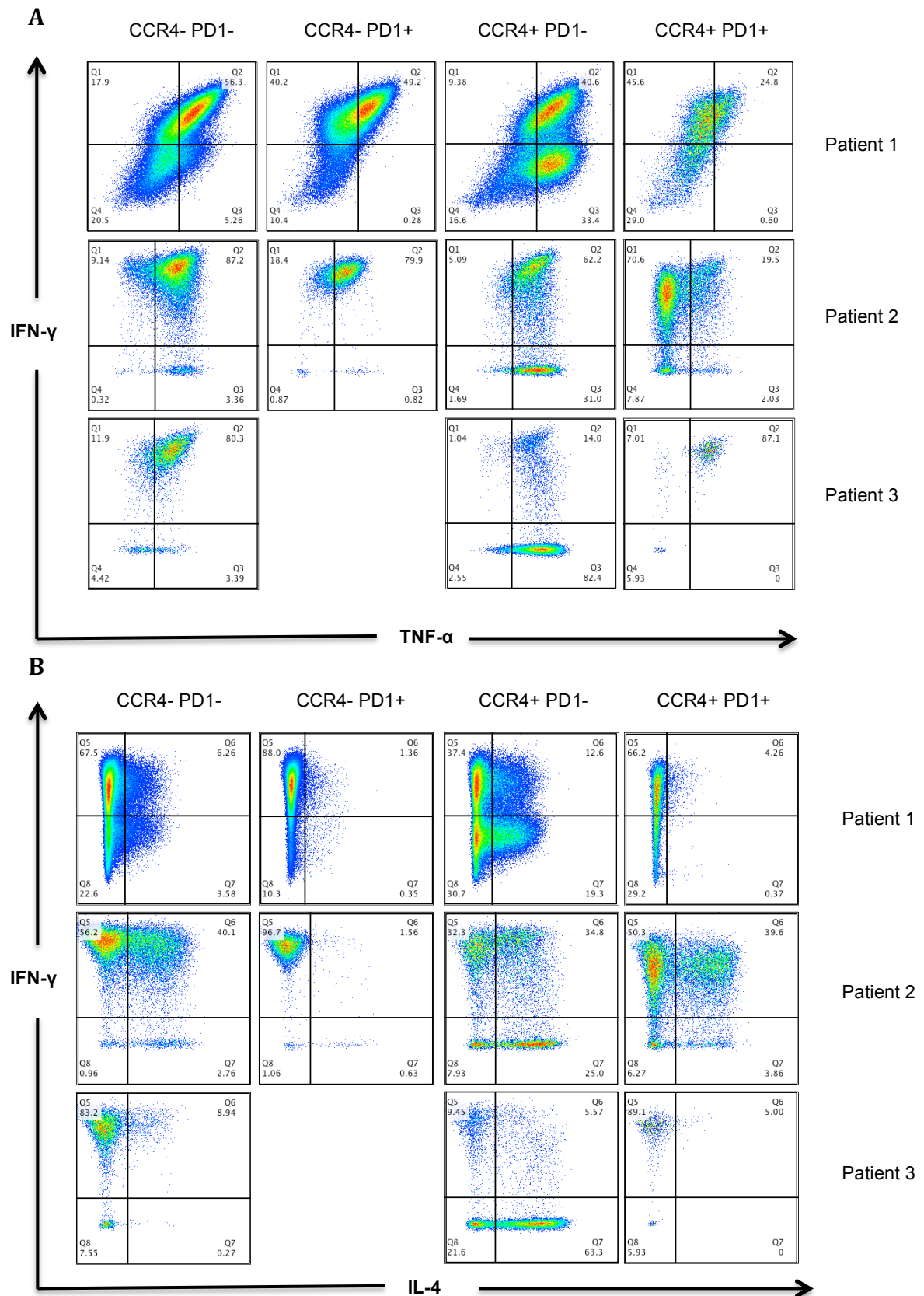


Figure 21: Analysis of cytokine production by intratumoural T cells of three melanoma patients. CD8⁺ T cell subsets were sorted according to expression of CCR4 and PD- 1 and expanded with PHA/IL-2 for three weeks prior to stimulation with PMA/ionomycin for five hours. Cytokine

production was measured by intracellular cytokine staining. A: Analysis of IFN- γ and TNF- α production. B: Analysis of IFN- γ and IL-4 production.

4.9.2 Analysis of tumour-specific T cells within the CCR4+ CD8+ T cell

In order to determine whether there is an enrichment of tumour-specific T cells within a particular compartment, cells with differential expression of CCR4 and PD-1 were isolated from a tumour (and for comparison, cells with differential expression of CCR4 and CXCR3 were isolated from a matched blood sample), cells were expanded with PHA/IL-2 and then stimulated with a pool of tumour HLA-A*02-restricted, melanoma-associated peptides (described earlier in table 1). In the peripheral blood, tumour-specific T cells were found in the CCR4+ CXCR3+ and CCR4- populations (figure 22). As previously described in figure 19, tumour-specific T cells can be observed in the CCR4+ CXCR3- population as well, however in this particular patient's peripheral blood, tumour-specific T cell are undetectable. In the tumour, tumour-specific T cells are enriched in the PD-1+ populations. Interestingly, they were particularly enriched among those PD-1+ cells that express CCR4, with twice as many cells responding by production of IFN- γ than in the CCR4- PD-1+ population (figure 22). While detecting an enrichment of tumour-specific T cells within PD-1+ cells may is not surprising given the knowledge that chronic antigen stimulation results in upregulation of this molecule, enrichment particularly among those cells expressing the chemokine receptor CCR4 is, to my knowledge, novel. Given the functional properties of the CCR4+ PD-1+ population following PMA/ionomycin stimulation and the similarities to the CCR4+ CXCR3+ peripheral blood population, it is possible that certain tumour-specific T cells within the CCR4+ PD-1+ population derive from the CCR4+ CXCR3+ population within the blood.

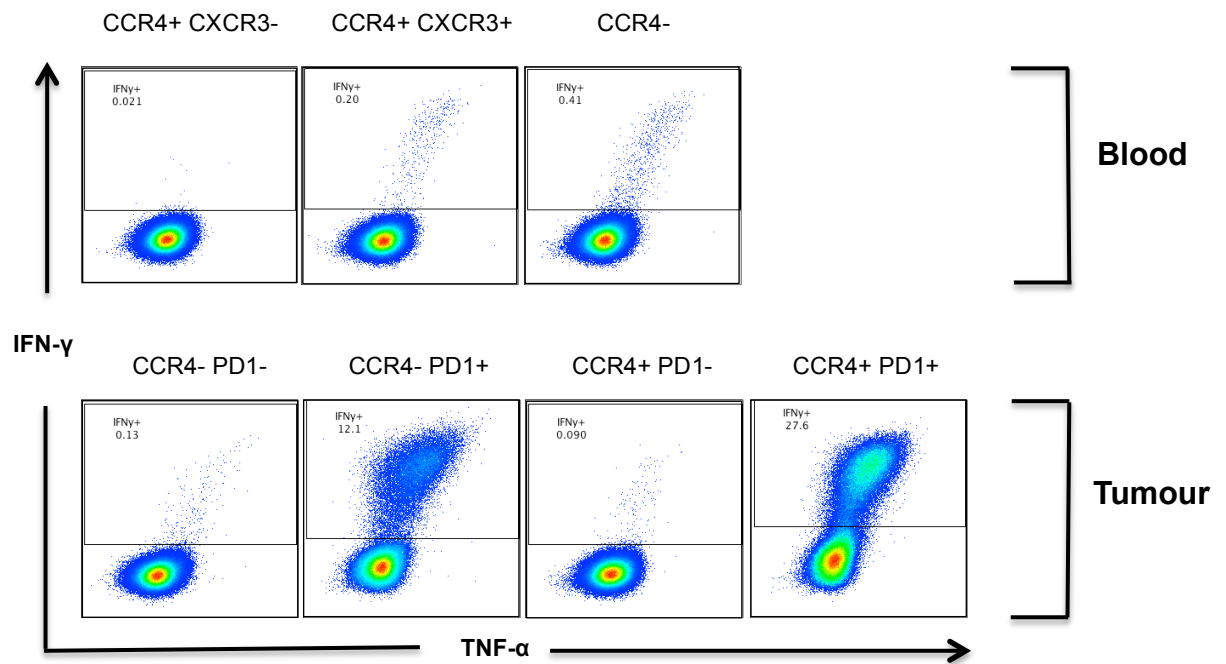


Figure 22: Comparison of tumour-specific T cells within matched blood and tumour samples with regards to CCR4 and CXCR3 expression. A: CD8⁺ T cell subsets were sorted according to expression of CCR4 and CXCR3/PD-1 from the blood or tumour of the same individual. Cells were expanded with PHA/IL-2 for three weeks and then stimulated for five hours with the tumour peptide pool. Response was measured by intracellular cytokine staining.

It has previously been described that tumour-specific TILs isolated from melanoma tumours are enriched in PD-1⁺ cells and that these cells, freshly isolated from tumours, have substantially reduced capacity for production of IFN- γ compared to PD-1⁻ cells (Ahmadzadeh et al., 2009). Following culture of such cells *in vitro* in the presence of IL-2 however, sees a reduction in the expression of PD-1, which is associated with enhanced production of IFN- γ (Inozume et al., 2010). Indeed the tumour-specific T cells found in the tumour in figure 22, after expansion with PHA/IL-2 are capable of responding to *in vitro* stimulation *via* production of IFN- γ .

4.10 Comparison of tumour-specific and microbe-specific T cells within melanoma metastases

Several new approaches are being developed to harness the ability of T cells to destroy cancer cells. These include the adoptive transfer of T cells with an affinity-enhanced TCR and the adoptive transfer of CAR (chimeric antigen receptor)-engineered T cells. Both approaches involve isolation of a patient's own T cells, modification of such cells (with either a TCR specific for a TAA that has been modified to bind with higher affinity, or with an engineered antibody fragment linked to signalling components necessary to trigger activation of the T cell upon antigen recognition) (Kalos et al., 2011) (Miguel et al., 2015). Following expansion, modified cells are reintroduced into the patient. However, the success of such approaches requires infiltration of tumours by the modified T cells.

While tumour-specific T cells were identified among the PD-1⁺ cells within the tumour, a large proportion of infiltrating T cells have not upregulated PD-1 and I was interested in the antigen-specificity of such cells. Different T cell populations (based on differential CCR4 and PD-1 expression as in figure 20B), following expansion with PHA/IL-2, were stimulated with a pool of peptides corresponding to microbial antigens (summarised in table 2 - full sequences can be found in Chapter 2: Materials and Methods). The same cells were also stimulated with the pool of tumour peptides (table 1). In figure 23, it can be seen that microbe-specific T cells (in the IFN- γ ⁺ gate) can be found in the tumour of this patient and are enriched in the PD-1⁻ cells. While the particular microbial antigens recognised by tumour-infiltrating cells in this patient were not determined, it would be interesting to know, in a range of patients, whether the specificity of such T cells is restricted to particular microbial antigens/organisms and whether differences are observed between different tumour types/locations. Such knowledge may enable coupling of therapies that enhance the frequency of particular microbial-specific T cells/preferentially target cells with the capacity to home back to the tumour.

Table 2: HLA-A*02-restricted peptides used to detect microbe-specific T cells in stage IV melanoma patients.

Microbial Antigen	Number of peptides in pool
Influenza A	1
VZV	1
Adenovirus	2
CMV	2
EBV	2
HPV	2
TB	3
HDM	3

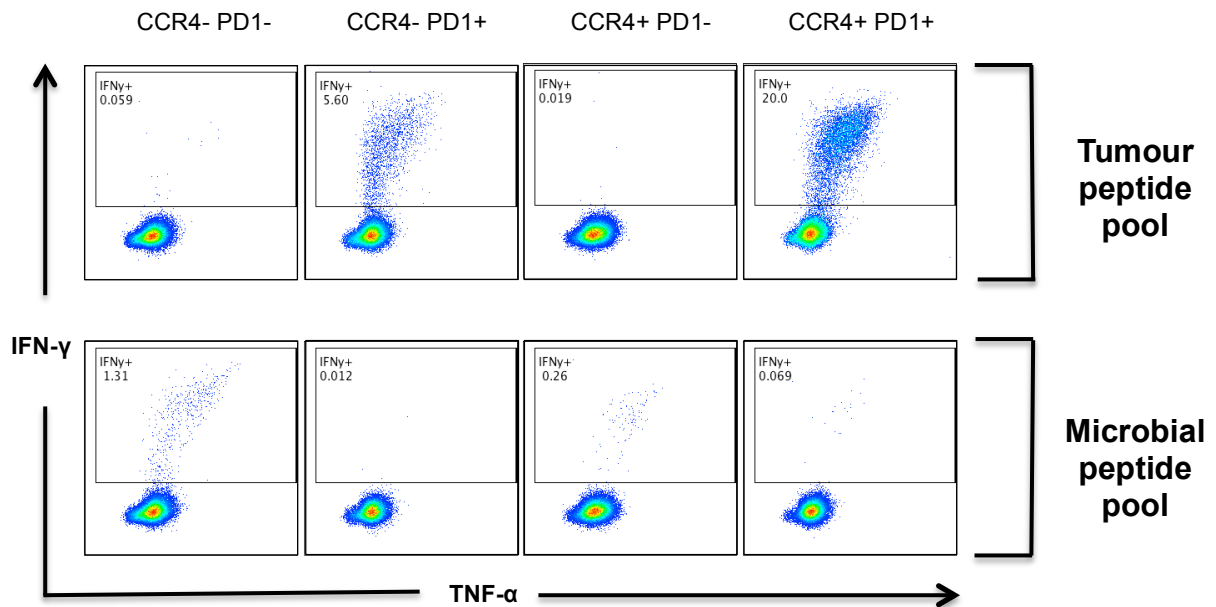


Figure 23: Analysis of tumour and microbe-specific T cells within a melanoma metastasis. Intratumoural tumour-specific T cells are detected in the CCR4- PD1+ and CCR4+ PD1+ compartments, with an enrichment within the CCR4+ PD1+ cells. Microbe-specific T cells are also found within melanoma metastases, although at a lower frequency, however these cells are found within the PD-1 negative fraction of cells (as both CCR4- and CCR4+).

4.11 Discussion

The experiments in this chapter have highlighted the following points:

- As well as correlating with the concentration of certain cytokines/growth factors in the plasma, the frequency of activated and immature neutrophils in the peripheral blood of patients with stage IV melanoma correlates with the frequency of distinct monocyte subsets and T cell subsets/activation status.
- The existence of CD8⁺ T cells with co-expression of CCR4 and CXCR3, the frequency of which is associated with neutrophil activation
- Tumour-specific TILs are enriched among the PD-1⁺ cells, particularly those that express CCR4
- Microbial-specific CD8⁺ T cells can be detected in the tumour among the PD-1⁻ cells.

4.11.1 Association of an enrichment of distinct neutrophil populations with distinct monocyte and T cell populations/properties in patients with stage IV melanoma

While a statistically significant increase in the frequency of CD4⁺ T_H2 cells and CD8⁺ TEMRA in patient samples and a decrease in the frequency of CCR4⁺ CXCR3⁺ CD8⁺ T cells is observed, the distribution of numerous populations, such as activated $\gamma\delta$ T cells and inflammatory monocytes, did not reach significance. However as with the statistically significant different populations, a range of frequencies is observed, akin to low-density neutrophil frequency and the concentration of certain plasma cytokines, and it is possible that statistical significance between patient and healthy donors is not observed due to an insufficient number healthy donor samples.

The heterogeneity in the frequencies of individual cell populations among patient samples, in terms of the range in frequencies observed, is associated with

changes in the frequencies of other immune cell subsets and plasma cytokines/growth factors, supporting the fact that differences among patient samples reflect changes in the inflammatory status of patients.

A positive correlation between the frequency of activated neutrophils and the frequency of inflammatory monocytes and an activated $\gamma\delta$ T cell subset, and a negative correlation between with the frequency of CCR4+ CXCR3+ CD8+ T cells was observed (discussed later) (figure 11). The frequency of immature neutrophils on the other hand, positively correlated with the frequency of T_H2 cells.

Whether there is a causal link between neutrophil activation/differentiation and activation of $\gamma\delta$ T cells, or polarisation of CD4+ T cells, or whether this is indirect and perhaps linked to tumour burden or the presence of particular tumour mutations, is uncertain. However, it seems that the diversity in melanoma patients, in terms of neutrophil phenotype and frequency, extends to other aspects of the immune system and suggests that different inflammatory axes can exist even within stage IV disease.

With regards to $\gamma\delta$ T cells, both V γ 9V δ 2 and V δ 1 T subsets have previously been shown to infiltrate melanomas and to be cytotoxic against melanoma cell lines (Cordova et al., 2012). On the other hand, in certain types of cancer, including pancreatic cancer, the degree of T_H2 cell infiltration is associated with poorer prognosis (De Monte et al., 2011). Such an association may in part be linked to the ability of T_H2 cells to promote polarisation of TAMs towards an M2, tumour-promoting phenotype (DeNardo et al., 2009)

Although difficult to say, given the lack of knowledge regarding the functionality of low-density neutrophils or the degree of tumour-infiltration of immune cell populations in these patients, it may not be entirely implausible to hypothesise that an enrichment of activated neutrophils and $\gamma\delta$ T cells may be more beneficial in terms of tumour control than an abundance of T_H2 cells. Indeed the capacity of anti-CTLA-4 to increase the frequency of activated neutrophils in the

peripheral blood is associated with better response to therapy (chapter 3). However, whether such an increase in activated neutrophils is associated with an increase in $\gamma\delta$ T cell activation during anti-CTLA-4 treatment was not analysed, and perhaps if this is not the case, a higher pre-treatment frequency of activated neutrophils (and activated $\gamma\delta$ T cells) and its association with poorer treatment response reflects the fact this T cell subset may also promote tumour progression.

4.11.2 Limitations of peripheral blood analysis

Limitations of this study include the small number of immune cell subsets analysed. Cell types that may be interesting in terms of the immune response to melanoma, such as dendritic cells (DC) and innate lymphoid cell (ILC) populations, were not studied. I was restricted in the number of colours available on the flow cytometer and therefore it was necessary to use different panels with various combinations of antibodies. The development of mass cytometry (CyTOF) and the ability to analyse a much larger number of parameters, would assist should this study be extended to examine the frequency and properties of a wider range of immune cells.

Furthermore, it is important to note that these differences have only been observed in the peripheral blood of patients. While identification of differences in blood may reflect changes in the tumour, and have the advantage for use as a biomarker should such a difference be useful in predicting disease progression/treatment response, it remains that in this cohort of patients, I do not know whether an enrichment or the degree of tumour infiltration of T_H2 cells or CD8⁺ effector memory subsets for example, or indeed activated or immature neutrophils, varies among patients.

4.11.3 Identification of CD8⁺ T cells with co-expression of CCR4 and CXCR3

Analysis of the CD8⁺ effector memory population revealed the presence of cells co-expressing the chemokine receptors CCR4 and CXCR3 (Figure 7A), which

negatively correlated with the frequency of activated neutrophils in the peripheral blood. I wondered whether such a correlation might reflect greater tumour infiltration of this population, perhaps enhanced by the expression of multiple chemokine receptors.

As mentioned, a role for CCR4 in recruitment of T cells (including regulatory T cells) into tumours *via* recognition of ligands CCL22 and CCL17, and a role for CXCR3 in recruitment of T cells towards CXCL10/IP-10 in the tumour microenvironment is known (Ondondo et al., 2013). Furthermore, CXCL10 enhances the recruitment of CCR4+ cells in response to CCL22 (independent of CXCR3 expression) (Sebastiani et al., 2005). Therefore, this population may be recruited to sites of inflammation, such as tumour, *via* CXCL10 acting upon CXCR3 and the synergistic effect of CCL17/22 and CXCL10 upon the CCR4 receptor.

I have defined this population based on the expression of two chemokine receptors characteristic of type 1 and type 2 T cells, however the expression of other chemokine receptors by this population, and indeed other effector memory CD8+ T cells, was not analysed and it would be interesting to know how these cells differ in terms of other receptors implicated in tumour infiltration.

Further characterisation of CCR4+ CXCR3+ cells revealed that they are relatively stable in terms of CCR4 and CXCR3 after prolonged *in vitro* culture suggesting that they may be a phenotypically distinct population. However, analysis of transcription factor and cytokine expression suggests that this population is heterogeneous in nature. As certain CCR4+ CXCR3- and CCR4- cells can acquire the CCR4+ CXCR3+ phenotype, a possibility is that certain cells within the CCR4+ CXCR3+ population represent Tc2 or Tc1 cells that upregulate chemokine receptors upon activation.

Other functionality, such as capacity for cytotoxicity was not analysed and it would be interesting to further explore this. While a subset of this population may reflect CCR4+ CXCR3- or CCR4- cells that acquire this phenotype, single cell

RNA analysis of this population may help in better understanding what this population consists of, whether there is a unique population of CD8⁺ T cells not previously described, and what such cells are capable of functionally.

It should be noted that while CCR4 is typically associated with type 2 cells, CCR4⁺ CD8⁺ T cells have previously been shown to produce the type 1 cytokine, IFN- γ (Kondo & Takiguchi, 2009). In this study the authors did not consider expression of CXCR3 and perhaps it was cells within the CCR4⁺ CXCR3⁺ population that were responsible for some of the IFN- γ production. However, they did find a sizeable proportion of CCR4⁺ CD45RA⁻ CD8⁺ cells that could produce IFN- γ , TNF- α , IL-2 and IL-4. As PMA/ionomycin stimulation can alter the expression of cell surface markers making it harder to distinguish different populations, I chose to isolate individual populations and then look at cytokine production following stimulation. As Kondo & Takiguchi (2009) were interested in bulk CCR4⁺ cells, it could be that, given the likely heterogeneity of CCR4⁺ cells, the combination of factors produced after stimulating several populations together results in an environment capable of promoting the production of multiple, type 1 and type 2 cytokines. Although PMA/ionomycin is a useful tool for looking at which cytokines potentially stimulated cells can produce, it is of course a non-physiological method of stimulation, bypassing the need for TCR engagement. Whether CD8⁺ T cells exist that typically produce both type 1 and type 2 cytokines, in the context of an infectious disease or tumour-associated inflammation, is unclear.

Given that the percentage of cytokine-producing cells in the experiment in figure 16 are lower than perhaps expected with such a potent method of stimulation (and perhaps a reflection of issues with Brefeldin A or PMA/ionomycin concentration) and while this experiment was repeated in the contexts of controls (including figure 19B) for IFN- γ and TNF- α production, it would be interesting to repeat this experiment including antibodies against IL-4 and IL-17 in a more potent stimulation.

4.11.4 Tumour-specific and microbe-specific CD8+ T cells detected within blood and melanoma metastases

Analysis of the phenotype of intratumoural tumour-specific T cells, revealed an enrichment of these cells within PD-1+ populations, particularly among those that also express CCR4. This data comes from the intratumoural T cells of one melanoma patient and while PD-1 expression amongst tumour-specific T cells is expected, and while CCR4 may contribute to migration of these cells into the tumour, whether an enrichment of tumour-specific T cells is always observed in the CCR4+ population, or whether the distribution differs between different patients (as observed in the blood) is unclear.

It should be noted that the different cytokines produced by intratumoural CD8+ T cells within the same subset can differ between patients. For example, cells from patient 2, when stimulated with PMA/ionomycin (figure 21B), tend to be skewed towards greater IL-4 production, compared to those cells from patient 1 or 3. This is even visible in the CCR4- population. Perhaps factors in the tumour microenvironment of this particular patient promote greater polarisation towards an IL-4-producing phenotype.

Although from my experiments it is difficult to understand whether there are properties of CCR4+ cells that are unique from CCR4- cells in the tumour (and further investigation may require further separation such cells based on other markers as well as CCR4 and PD-1), there are clear differences in the properties of CD8+ T cells in the blood and the location of tumour-specific cells. If there is an enrichment of tumour-specific T cells in a population capable of IFN- γ production, a patient with an enrichment of such cells may have a different outcome compared to a patient with an enrichment of tumour-specific T cells in an IL-4-producing population. In particular, it could be relevant to look at how the frequency of tumour-specific T cells in different subsets changes during immunotherapy and whether there is a correlation with treatment response.

The difficulty in unravelling how CCR4⁺ populations in the peripheral blood relate to CCR4-expressing cells in the tumour is in part due to the lack of CXCR3 expression on freshly isolated intratumoural CD8⁺ T cells. I attempted to enrich for distinct populations using expression of PD-1 and although CCR4⁺ CXCR3⁻ cells, unlike CCR4⁺ CXCR3⁺ cells, lack PD-1 expression in the peripheral blood, this does not mean to say PD-1⁺ CCR4⁺ CXCR3⁻ cells are not found in the tumour. It may be possible, through single cell sorting and RNA analysis, to identify different populations and compare these to CCR4⁺ CD8⁺ T cells in the blood. It may be that other markers, unaffected by tumour digestion, could be used in place of CXCR3 to identify different populations by FACS. Sorting of these populations and analysis of antigen-specificity may help to better understand the relationship between blood and tumour CCR4⁺ CD8⁺ cells.

If CCR4⁺ CD8⁺ T cells can be divided into different populations, with differing functions, this may have important implications for the use of CCR4-blocking antibodies or antagonists as therapeutic strategies. The rationale behind CCR4 blockade relates to the known role of CCR4 in recruitment of Tregs into tumours (Chang et al., 2015). If a patient's tumour-specific CD8⁺ T cells are enriched in a CCR4-expressing population with type 2 cytokine production and reduced cytotoxicity, such as Tc2 cells, then depletion of CCR4⁺ CD8⁺ T cells may, along with blocking of Tregs and perhaps T_H2 cells, contribute to the efficacy of CCR4 blocking therapy. However, if CCR4⁺ CD8⁺ T cells are a heterogeneous population, as suggested by the blood data, it may be that cells with anti-tumour properties may also be prevented from entering tumours, and therefore in these patients, the efficacy of such a therapy may be reduced.

Following isolation and expansion with PHA and IL-2, PD-1⁺ cells can produce cytokines in response to stimulation with tumour peptides or PMA/ionomycin however upregulation of PD-1 is associated with an exhausted phenotype and dysfunction (Ahmadzadeh et al., 2009). While the capacity of these cells to produce cytokines or to participate in other mechanisms such as cytotoxicity *in vitro* may indicate potential *in vivo* function, in order for that function to be realised *in vivo* would likely require the blockade of inhibitory receptors,

including PD-1.

Microbe-specific T cells can also be found within a melanoma tumour. While this is preliminary data, these cells were found within the PD-1⁻ compartment and are likely CD8⁺ memory T cells that may have homed to the tumour due to expression of chemokine receptors specific for factors upregulated in the inflammatory tumour microenvironment. This pool of microbial peptides consists of peptides from a range of microbes including varicella-zoster virus (VZV), house dust mite (HDM), Epstein-Barr virus (EBV) and influenza A virus. Unfortunately, the individual microbial antigens recognised by these tumour-infiltrating cells were not determined and, as expression of specific homing receptors (such as chemokine receptors and integrins) is dependent on the location of the originally encountered antigen, it would be interesting to determine whether CD8⁺ T cells specific for particular microbes home to particular tumour types or tumour locations. If differences can be observed, then it may be possible to exploit this therapeutically. ImmTACs (immune-mobilising monoclonal TCRs against cancer) are bi-specific molecules that consist of a TCR that recognises a TAA (for example, gp100) linked to an anti-CD3 antibody and function by activating intratumoural T cells, regardless of their antigen specificity. As many antigens expressed by tumours are self-antigens, T cells with a high affinity for such antigens are negatively selected in the thymus. As ImmTACs can be generated with high-affinity TCRs, they could overcome this issue and enable activation of intratumoural T cells irrespective of TCR specificity. Although trials are at an early stage, they have shown promising results in the clinic (Oates et al., 2015). As tumour-specific CD8⁺ T cells within the tumour have an exhausted (PD-1⁺) phenotype, responses observed with ImmTACs (used in the absence of immune-checkpoint inhibitors) may be due to activation of microbe-specific PD-1⁻ cells within the tumour. If it is true that the specificity of microbial T cells in tumours is dependent on tumour type or location, such knowledge could potentially be harnessed therapeutically. For example, vaccination strategies to enrich for particular microbial-specific T cells, may result in an enrichment of intratumoural microbe-specific CD8⁺ T cells, which could be targeted to tumour cells with the use of ImmTACs.

Chapter 5: Regulation of IL-10 expression in myeloid cells

5.1 Introduction

As mentioned previously, IL-10 is induced in monocytes/macrophages in response to stimulation with a variety of stimuli, in particular agonists of the pattern recognition receptors TLR4 and TLR2, such as LPS and PamCSK (Saraiva and O'Garra, 2010) (Funderburg et al., 2011). Stimulation with TLR ligands such as LPS results in activation of intracellular signalling pathways leading to nuclear localisation of transcription factors such as NF- κ B (Saraiva and O'Garra, 2010).

The traditional definition of epigenetics relates to heritability of characteristics (independent of gene sequence) by modifications, either directly to the DNA, such as DNA methylation, or to the histone proteins around which DNA is packaged (Bird, 2007). Epigenetics can now be used more broadly to define the biology of chromatin. It may be independent of heritability, and can therefore encompass transient chromatin modifications that are important in determining chromatin properties (Bird, 2007).

Packaging of DNA into chromatin is important for compaction into the nucleus, but this has important implications for gene expression. Assembly of DNA into nucleosomes restricts binding of proteins, such as those involved in transcription. In order for signal-specific/inducible transcription factors to bind to regulatory elements of DNA, such elements must reside within regions of open chromatin (Ghisletti & Natoli, 2013). Packaging of DNA around histone octamers is illustrated in figure 1, as are examples of modifications that are associated with regions of active or inactive chromatin.

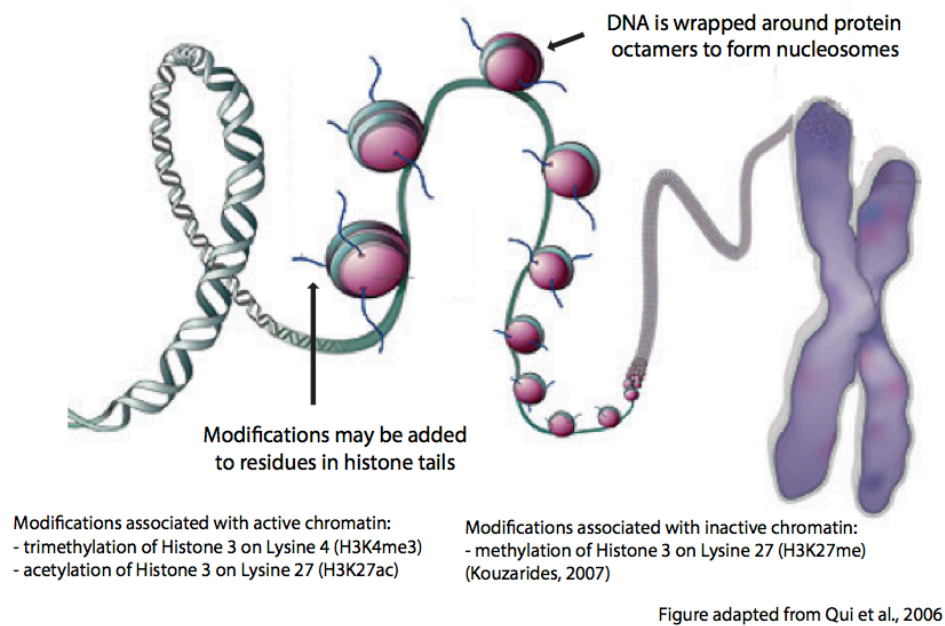


Figure 1: Chromatin structure has important implications for gene expression. DNA is wrapped around protein octamers consisting of two copies of each of the histone proteins, H2A, H2B, H3 and H4 to form nucleosomes, often referred to as the 'beads on a string' conformation (Dong et al., 2012). Various types of modification may be added to numerous histone residues and these modifications are important in determining chromatin properties, such as how open or compact a region of the genome is. They may directly alter nucleosome stability (Williams et al., 2008), or may result in the recruitment of chromatin remodelling complexes that use ATP to temporarily slide or release histones (Kouzarides 2007) (Brown et al., 2011). Remodelling of chromatin is important at regulatory regions of genes, such as the promoter and enhancers, enhancers being regions of DNA that bind transcription factors and positively regulate their target gene. They do so in a distance-independent manner, in that they may be located close to or within the gene they regulate, or far away. Distant enhancers have been shown to interact with the promoter of the gene they regulate via DNA looping (Kagey et al., 2010).

A number of techniques have been developed which enable the identification of potential regulatory regions in chromatin. These techniques can reveal two types of feature of regulatory regions:

- 1) **The accessibility of regions of DNA** - regulatory regions exist in an open conformation and are more accessible than regions of DNA tightly packaged into chromatin. This accessibility means they are preferentially cleaved during enzymatic digestion and can be detected in DNase-

sensitivity (DNase-seq) and transposase-sensitivity (ATAC-seq) assays (Song and Crawford, 2010) (Buenrostro et al., 2013). The physical properties of DNA in nucleosome-free regions means it preferentially purifies in the aqueous phase during phenol-chloroform extraction (in the absence of cross-linking reversal). This forms the basis of the FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) technique (Giresi et al., 2007) and while sequencing approaches enable genome-wide identification of regulatory regions, PCR can be used to study sites of interest.

- 2) **Histone modifications** - the modifications of histones surrounding nucleosome-free regulatory regions are associated with particular chromatin properties. For example, acetylated chromatin (such as H3K27ac) can function to recruit a series of proteins, including Brd4 and the positive transcription elongation factor b (p-TEFb), culminating in phosphorylation of RNA polymerase, which drives the transition to productive elongation (Itzen et al., 2014). Histone modifications can be detected by chromatin immunoprecipitation (ChIP). Sequences found in association with particular histone modifications can be detected by sequencing or targeted PCR.

Both identification of accessible regions of chromatin and detection of histone modifications by ChIP are complementary to one another and help to identify regulatory regions within the genome.

The exact genes that a cell may express are determined during development with the regulatory elements that control their expression being bound by master regulators of transcription and held in an open chromatin conformation (Ghisletti & Natoli, 2013).

PU.1 is one such master regulator of transcription in haematopoietic cells and is important for the differentiation and for defining the enhancer repertoire of monocytes/ macrophages and B cells (Pham et al., 2013). Master regulators, also

known as pioneering transcription factors, are a unique set of transcription factors in that they have the capacity to bind to condensed regions of chromatin, opening these regions up and making them accessible to other transcription factors (Ghisletti & Natoli, 2013). Exactly how they do this is unclear but it is thought that it may include their interaction with chromatin remodelling complexes or competition with nucleosomes for DNA binding (Pham et al., 2013). The binding of PU.1 to a regulatory element, and the opening of the chromatin structure at that location, enables access to cell-specific and signal-specific transcription factors.

Due to the importance of IL-10 in the immune-regulatory mechanisms associated with cancer, I was interested in understanding which regulatory regions within the IL-10 locus might influence how the IL-10 gene is expressed. Using human primary monocytes as a model, I decided to use a combination of FAIRE and ChIP to identify possible regulatory regions within the IL-10 locus in both unstimulated and stimulated cells.

While these techniques are capable of providing correlative data between epigenetic modifications and transcriptional regulation, demonstrating that a potential regulatory region is responsible for the transcriptional regulation of a specific gene has been limited by the difficulty in interfering with specific DNA sequences. However, an accessible and efficient technique for introducing precise genetic modifications has recently been developed. The CRISPR-Cas9 system is based on the prokaryotic adaptive immune system, 'clustered regularly interspaced short palindromic repeats' (CRISPR) (Cong et al., 2013). It is present in many archaea and bacteria and provides protection from previously encountered invading genetic elements such as viruses (Makarova et al., 2011).

The CRISPR-Cas9 system uses a Cas9 nuclease to introduce double-stranded DNA breaks (DSBs), which are then repaired by the cell's DNA repair pathways (Ran et al., 2013i). If the cleaved DNA is repaired by non-homologous end joining (NHEJ) (an error-prone DNA repair mechanism), it is likely that insertion/deletion mutations (indels) will be introduced at the targeted site once

repaired (Ran et al., 2013i). Alternatively, if the cell is provided with a repair template, homology-directed repair can occur, enabling the introduction of precise modifications at targeted sites (Ran et al., 2013i).

The Cas9 nuclease is targeted to the site of interest by a short RNA sequence of 20 nucleotides (guide RNA) *via* complementary base pairing. As only short nucleotide sequences are recognised by Cas9, off-target effects have been described. There are servers available (such as the MIT server provided by the Zhang lab) where the guide sequence is scored based on algorithms developed from experimental data. These algorithms can predict the likelihood of similar sequences being targeted when a guide for a sequence of interest is used. The CRISPR-Cas9 system has also been modified by introduction of a mutation in the Cas9 nuclease which results in an enzyme that can only carry out single strand breaks or nicks (referred to as the Cas9 nickase) (Ran et al., 2013i). In this instance, pairs of off-set guide RNAs and nickases which target opposing strands are used resulting in DSBs at the targeted location but with a higher specificity (Ran et al., 2013i).

In this chapter I use CRISPR-Cas9 technology to modify a potential regulatory region in the IL-10 locus and assess its role in the regulation of the IL-10 gene.

5.2 Primary human monocytes express IL-10 mRNA and protein in response to TLR-4 stimulation

In order to identify which DNA regulatory regions are accessible within the IL-10 gene locus I used human primary monocytes as a model. Primary human monocytes were isolated from the PBMCs of healthy donors using CD14+ positive selection. These cells were stimulated with the TLR-4 agonist, LPS and cells were lysed at various time points over the course of six hours for analysis of IL-10 mRNA production. It can be seen in figure 2A that primary human monocytes express IL-10 mRNA in response to LPS. Expression of IL-10 mRNA appears to be bimodal, in that there is an initial peak in expression 30 minutes post stimulation, which reduces between one and two hours before increasing again at six hours.

Induction of IL-10 mRNA in response to LPS stimulation is also reflected in an increase in the amount of IL-10 protein secreted in the supernatant of stimulated primary human monocytes as seen in figure 2B.

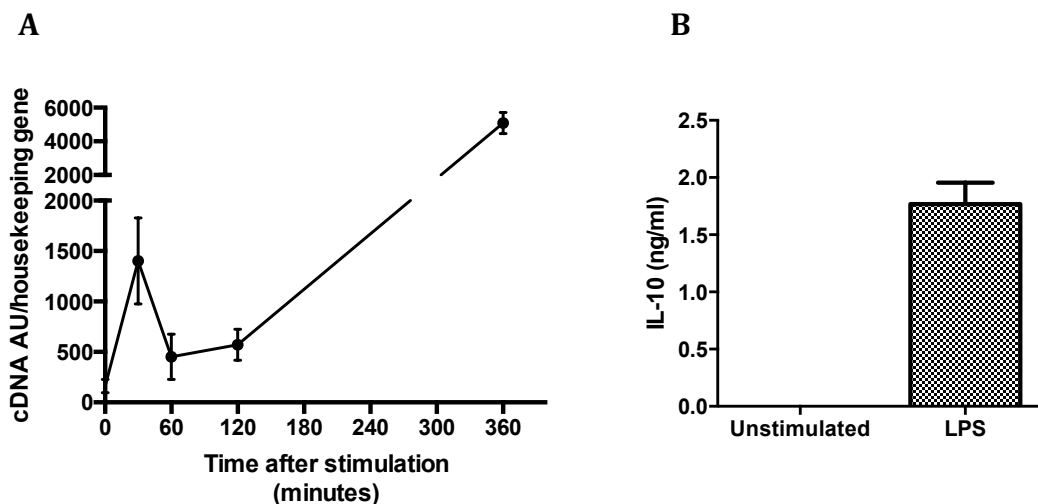


Figure 2: Expression of IL-10 mRNA and protein by primary human monocytes. A: Stimulation with the 1 μ g/ml of the TLR-4 ligand LPS induces expression of IL-10 mRNA as measured by qPCR. Expression increases over the course of six hours. B: LPS induces production of IL-10 protein as measured by ELISA on the supernatant of unstimulated monocytes and monocytes stimulated for 24 hours with 1 μ g/ml LPS. The mean and standard deviation of triplicate values are shown for each time point.

5.3 Identification of regions with open chromatin conformation at the IL-10 locus in primary human monocytes

In order to identify regulatory regions in unstimulated and stimulated primary monocytes I used a combination of FAIRE and ChIP. To select potential regulatory regions in human primary monocytes, the UCSC genome browser was used to design primer pairs encompassing FAIRE positive regions previously characterised in human cell lines as part of the ENCODE project. I used these primers to amplify possible regulatory regions that may be enriched in FAIRE.

Open regulatory regions were detected in monocytes at -1.76kb (1.76kb upstream of the transcriptional start site (TSS)), the TSS itself, +1.2kb downstream of the TSS, as well as a region +5.9kb downstream of the TSS (figure 3A). Other regions identified as accessible in human cell lines, such as -3.9kb and +2.95kb, were not enriched. Although unstimulated CD14⁺ monocytes express detectable IL-10 (as determined by RT-PCR), its expression is further increased upon LPS stimulation (figure 3B). In order to determine whether the enhanced expression of IL-10 with LPS stimulation correlates with an increased accessibility of regulatory regions identified in unstimulated monocytes and/or with chromatin remodelling events that might expose regulatory regions which are not accessible in unstimulated cells, FAIRE was also carried out in monocytes following four hours treatment with LPS. Upon LPS stimulation, there is a slight increase in enrichment at the TSS, +1.2kb and 5.9kb, however none of the closed regions observed in unstimulated cells are exposed following addition of LPS (figure 3A). Of all of the sites tested, the region +1.2kb downstream of the TSS has the greatest FAIRE enrichment, suggesting that this site exists in an open conformation in a higher proportion of monocytes than the other sites tested.

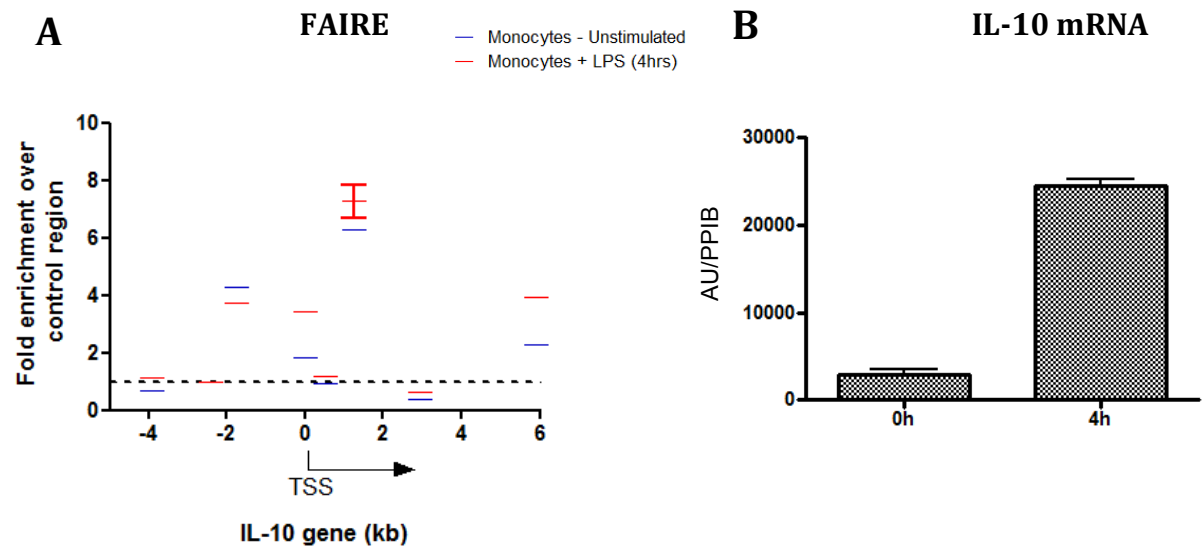


Figure 3: A: Analysis of the IL-10 locus in primary human monocytes by FAIRE, in the presence or absence of stimulation with 1 μ g/ml LPS, reveals sites of open chromatin conformation 1.76kb upstream of the transcriptional start site (TSS) (-1.76kb), the TSS (0), 1.2kb downstream of the TSS (+1.2kb) and 5.9kb downstream of the TSS (+5.9kb). FAIRE was used in conjunction with qPCR, the readout being fold enrichment over an internal negative control. The mean and standard deviation of triplicate values are shown for each region. B: Expression of IL-10 mRNA before and after LPS stimulation. Values normalised to the housekeeping gene, Peptidylprolyl Isomerase B (PPiB).

5.4 The region +1.2kb downstream of the IL-10 promoter is marked by active chromatin histone modifications, H3K27ac and H3K4me3 in primary human monocytes

Transcriptional competence is often associated with specific post-translational modifications at selected residues within the histones distributed across the locus of poised or expressed genes. I looked for the presence of active chromatin modifications across the IL-10 locus in primary human monocytes. The active chromatin modification, H3K4me3, is detected at the IL-10 locus in unstimulated monocytes obtained from three healthy donors (figure 4A). The enrichment is greatest around the TSS, and the region +1.2kb downstream of the TSS, and consistent for the three donors.

H3K27ac, another active chromatin modification, follows a similar pattern to H3K4me3, enrichment being greatest around the TSS and the region +1.2kb downstream of the TSS (figure 4B). Further enrichment is detected 5.9kb downstream of the TSS, matching the FAIRE enrichment at this site, suggesting the presence of a possible regulatory region.

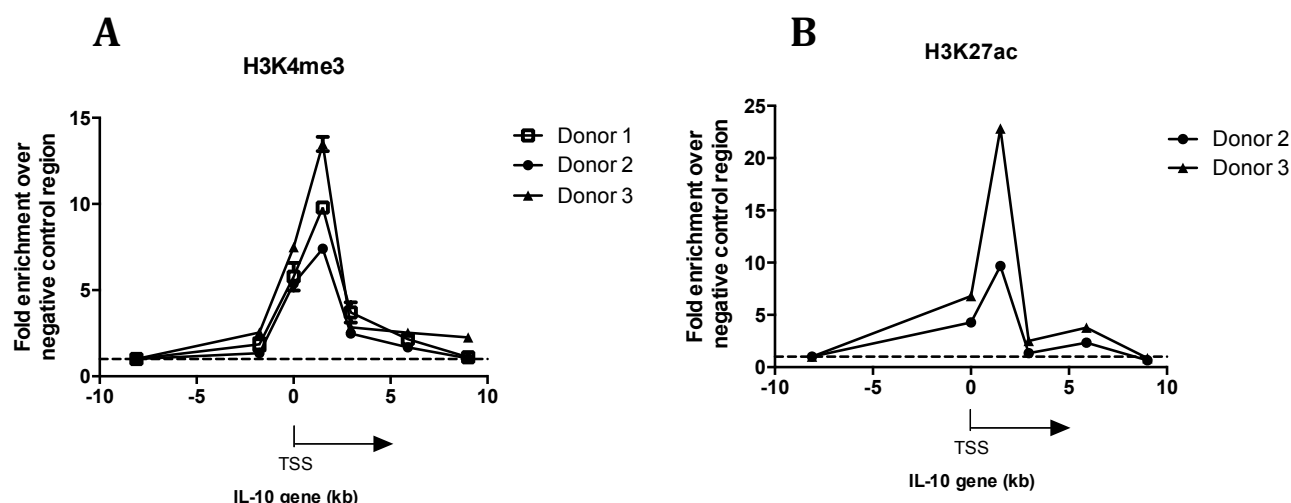


Figure 4: ChIP-qPCR for the histone modifications H3Kme3 and H3K27ac. The site 1.2kb downstream of the TSS is the most highly modified site in terms the active histone modifications, H3K4me3 (A) and H3K27ac (B) across the IL-10 locus in primary human monocytes from multiple donors. These histone modifications can also be detected at the TSS and to a lesser extent the +5.9kb region. Values are expressed as fold enrichment over a negative control region – the dotted line representing a value of 1. The mean and standard deviation of triplicate values are shown for each region.

5.5 In primary human monocytes, the pioneering transcription factor PU.1 binds -1.76kb, +1.2kb and +5.9kb at the IL-10 locus

An enhancer is defined as a regulatory region of DNA that positively regulates transcription and can physically be located upstream of, downstream of or within the target gene. They can also be located far from their target gene (Visel et al., 2009). As almost the entire repertoire of enhancers in monocytes has been shown to be bound by the pioneering transcription factor PU.1 (Ghisletti &

Natoli, 2013), I wished to determine whether these regions of open confirmation were indeed bound by PU.1. Using the UCSC Genome Browser, data from ChIP-seq experiments carried out by Pham et al. (2012) reveal that three of the sites identified as being in an open chromatin conformation by FAIRE, also bind PU.1 (figure 5). This strongly suggests that these regions are regulatory regions in the monocyte genome.

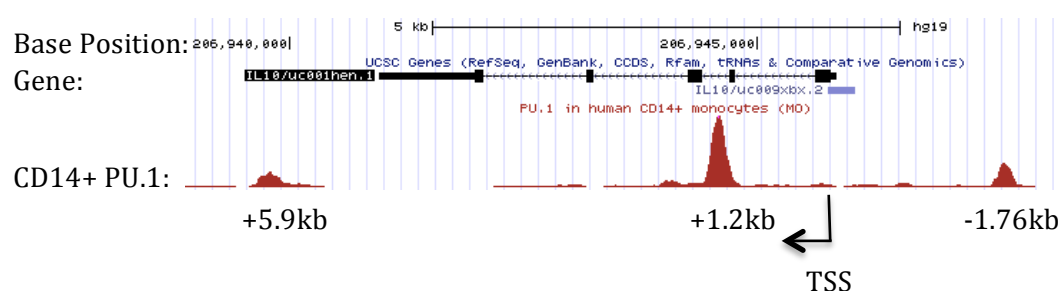


Figure 5: PU.1 ChIP-seq in primary human monocytes. This track was generated from data obtained by Pham et al., 2012, using the UCSC Genome Browser. The sites revealed in FAIRE analysis to have an open chromatin confirmation at the IL-10 locus bind to the pioneering transcription factor/master regulator, PU.1.

5.6 Selecting a monocytic cell line to study the role of the +1.2kb PU.1 binding site in the regulation of IL-10 expression

Based on the FAIRE data in which the +1.2kb site was determined to be the more dominant open region in the IL-10 locus, along with the association of this region with PU.1 and the fact it is heavily acetylated on H3K27 and trimethylated on H3K4, I decided to explore the possibility that this region might play a major role in the modulation of IL-10 expression.

Furthermore, this site has been implicated in an inflammatory disease called Behçet's Disease, which is disease characterised by inflammation of the skin, eyes and orogenital mucosa and is prevalent in the Middle East (Remmers et al.,

2010). It is unknown what causes this disease however genome-wide association studies (GWAS) have identified associations with numerous immune-related genes such as HLA-B*51 as well as SNPs in IL-23R-IL12RB2 (rs924080) and IL-10 (including rs1518111 which is located at the +1.2kb site) (Remmers et al., 2010)

Not only is it possible that these PU.1 binding sites regulate IL-10 expression, but alternatively it is possible that they regulate a neighbouring gene or even a distant gene. The identification of the Behçet's Disease-associated SNP (rs1518111) (and its association with lower IL-10 production in stimulated monocytes) by Remmers et al. (2010) is suggestive of a role for +1.2kb region in regulation of the IL-10 gene.

Allelic imbalance using monocytes from donors heterozygous for rs1518111 reveals that the chromosome containing the Behçet's-associated A allele produces less IL-10 pre-mRNA than the G allele (around 25% of the G allele) (Remmers et al., 2010). This SNP is also associated with reduced IL-10 production at the protein level; cells from healthy donors homozygous for the SNP produce less IL-10 protein following stimulation with LPS, compared to heterozygous individuals, which produce less protein than those homozygous for the wild-type allele. As Behçet's disease is complex and as it is possible to possess this SNP and be healthy, the authors suggest that production of lower amounts of IL-10 in response to inflammatory stimuli, in the presence of other factors, may contribute to the development of this disease.

Whilst the data from Remmers et al. (2010) suggests a role for this site in IL-10 regulation, nobody has directly assessed this by modifying this site, preventing transcription factor binding and showing the effect on IL-10 expression in modified cells versus wild-type.

This data, together with the evidence that the +1.2kb region is the more extensively modified region of the IL-10 locus, hints at an important role for this region in controlling IL-10 expression.

I decided to ask whether the region +1.2kb downstream of the IL-10 TSS regulates expression of the IL-10 gene with the aim of interfering with the PU.1 binding site within this region. The theory behind genetically modifying the DNA sequence that makes up the PU.1 binding site, is that preventing the binding of a pioneering transcription factor/master regulator to this site may result in a closed chromatin conformation which in turn will prevent the binding of other cell and signal-specific transcription factors necessary for the induction of IL-10 expression. If this site does play a positive role in regulation of the IL-10 gene, as suggested by the preliminary data from Remmers et al. (2010) I expect monocytic cells which lack PU.1 at this +1.2kb site to express less IL-10 compared to their wild-type counterparts.

I used the newly described CRISPR-Cas9 technology which has been shown as an easy way to introduce genetic modifications (Ran et al., 2013i). I used the pX330 (with Cas9 nuclease) and pX335 (with Cas9 nickase) plasmids (as described in Chapter 2: Materials and Methods). These plasmids can be used to transiently express the guide RNA and Cas9 enzyme in the cell type of choice. However, due to efficiency of transfection, only a fraction of the cells will be transfected with the CRISPR-Cas9 constructs, and only a fraction of those successfully transfected will carry the modification of interest. Furthermore, as I will use CRISPR-Cas9 in the absence of a template, cleaved DNA will rely on the error-prone NHEJ DNA repair mechanism. This means that different modifications may occur in different cells. In order to obtain monocytic cells with the same genetic modification at the +1.2kb region, I decided to do single cell cloning of the successfully transfected cells. Primary human monocytes do not typically proliferate and therefore an immortalised monocytic cell line was chosen.

5.6.1 Kinetics of IL-10 expression in the THP-1 cell line (with and without differentiation with PMA)

I investigated whether the THP-1 cell line (a cell line derived from an acute monocytic leukaemia patient and which is frequently used as a model for

studying monocytes/macrophages (Qin 2012)) would be a suitable cell type to study the regulation of IL-10 expression.

It is well described that THP-1 cells can be induced to differentiate along the monocytic lineage using phorbol-12-myristate (PMA) or 1,25-dihydroxyvitamin D3 (VD3) (Schwende et al., 1996). Upon differentiation, THP-1 cells have altered properties, such as expression of CD14 and cessation of proliferation (Schwende et al., 1996).

Firstly, I investigated whether the kinetics of IL-10 expression were similar in THP-1 cells compared to monocytes. In the absence of any pre-treatment, THP-1 cells barely express detectable IL-10 mRNA (Figure 6A “-PMA”) over the course of six hours.

As CD14⁺ is expressed on the surface of primary human monocytes, and as it is known to play a role in stimulation of TLR-4 by LPS (Gioannini et al., 2004, Kim & Kim, 2014), I asked whether differentiation of THP-1 cells prior to LPS stimulation may increase expression of IL-10. Pre-treatment with 10nM PMA for 24 hours results in THP-1 cells that are capable of expressing detectable amounts of IL-10 mRNA following LPS stimulation (figure 6A). Expression increases between zero and two hours, plateauing at two hours. However, increasing the differentiation period from 24 to 48 hours, results in THP-1 cells that continue expressing IL-10 up to six hours post-stimulus (figure 6A). This is reminiscent of primary human monocytes, in which IL-10 expression continues to increase between two and six hours.

As primary human monocytes show bimodal expression of IL-10 upon stimulation (figure 2A), I added extra time-points over the six-hour stimulation of 48-hour PMA-differentiated THP-1 cells for a better kinetic study. As in monocytes, there was an early peak in IL-10 expression at 30 minutes post stimulation, which reduces between one and two hours before increasing again at six hours.

As differentiated THP-1 cells express IL-10 in response to LPS stimulation, and as they appear to do so with similar kinetics to monocytes, they may be a suitable model to study IL-10 expression in monocytic cells.

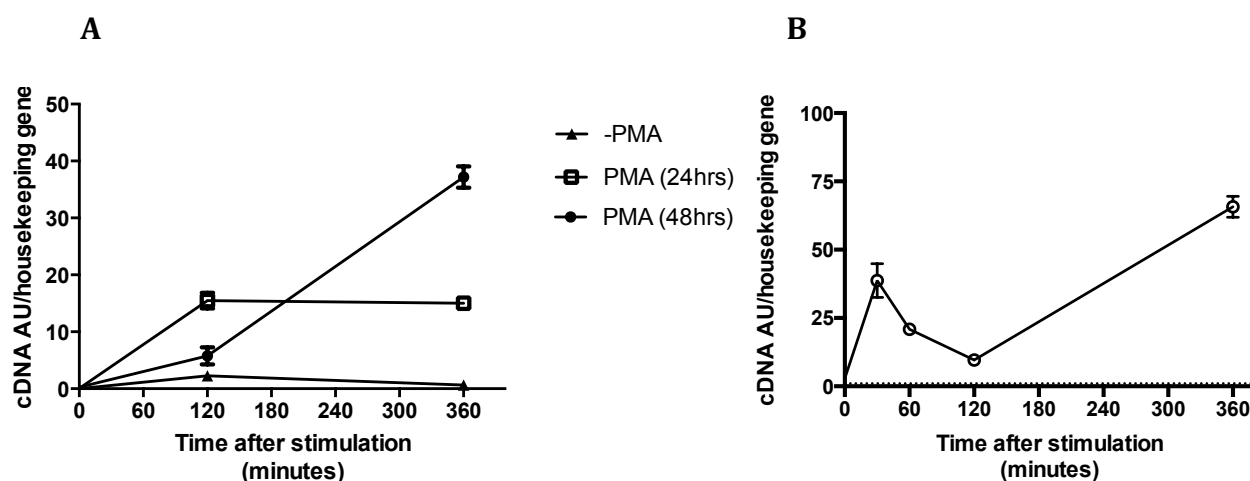


Figure 6: Expression of IL-10 in THP-1 cells in response to LPS stimulation. A: Without differentiation by pre-treatment with 10nM PMA, THP-1 cells express low amounts of IL-10 mRNA in response to stimulation with 1 μ g/ml LPS. Treatment with PMA for 24 hours preceding LPS stimulation, increases IL-10 mRNA production within the first two hours but there is no further increase between two and six hours. Higher mRNA expression is observed with 48 hours pre-treatment with PMA. Expression continues to increase over the course of six hours. B: A closer look at the kinetics of IL-10 expression in THP-1 cells pre-treated with 48 hours PMA reveals an initial early peak in IL-10 mRNA expression at 30 minutes which declines towards two hours before increasing between two and six hours post LPS stimulation. The mean and standard deviation of triplicate values are shown for each time-point.

5.6.2 The region +1.2kb downstream of the IL-10 promoter is also marked by active chromatin histone modifications, H3K4me3 and H3K27ac in the THP-1 cell line

In order to use THP-1 cells as a model for monocytes, it is not only important that the kinetics of IL-10 expression are similar between monocytes and THP-1 cells, but also that the IL-10 locus, in particular the region of interest +1.2kb downstream of the TSS, reflects that of the primary cells.

When the IL-10 locus was analysed by ChIP-qPCR for the presence of H3K4me3 and H3K27ac, the greatest enrichment of these active histone modifications was observed at the site +1.2kb downstream of the TSS. This reflects the IL-10 locus in primary human monocytes (figure 7).

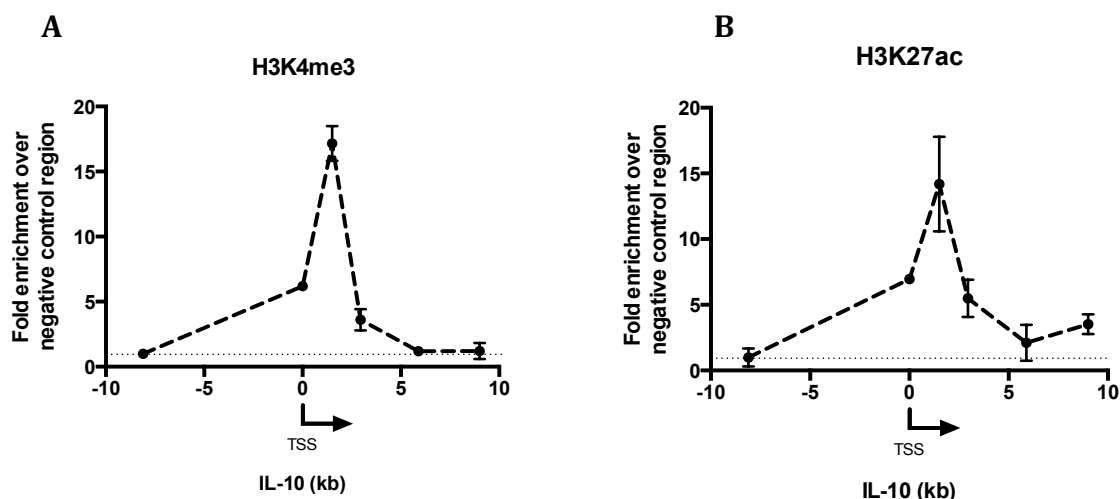


Figure 7: ChIP-qPCR for the histone modifications across the IL-10 locus in THP-1 cells. The site 1.2kb downstream of the TSS is the most highly modified site in terms of the active histone modifications, H3K4me3 (A) and H3K27ac (B). Values are expressed as fold enrichment over a negative control region – the finer dotted line representing a value of 1. The mean and standard deviation of triplicate values are shown for each time-point.

5.6.3 In THP-1 cells, the + 1.2kb site binds the pioneering transcription factor, PU.1

Data from ChIP-seq experiments for PU.1 in THP-1 cells was obtained from Pott et al. (2012) (GEO accession number GSE25426). The data was analysed using SeqMonk software and the track below generated for the UCSC Genome Browser. This data reveals that the same three sites that bind PU.1 in primary human monocytes, also bind PU.1 in THP-1 cells (figure 8). Again, the site +1.2kb downstream of the TSS has the highest peak, suggesting this site is bound by PU.1 in a greater proportion of THP-1 cells than the neighbouring sites. This

supports the use of THP-1 cells as a model for studying the role of the +1.2kb site in monocytic cells.

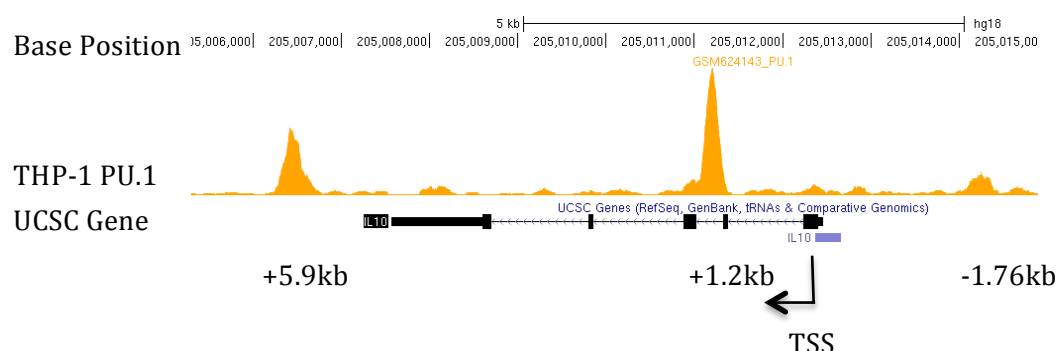


Figure 8: PU.1 ChIP-seq in THP-1 cells. This track was generated from data obtained by Pott et al., 2012, using the UCSC Genome Browser. Sites -1.76kb, +1.2kb and +5.9kb bind the pioneering transcription factor, PU.1 at the IL-10 locus in THP-1 cells.

5.6.4 Confirmation that PU.1 is detected in this THP-1 cell line by ChIP-qPCR

It is known that cell lines can change over time and that prolonged/continuous culturing of cell lines can affect some of their key functions, reducing their reliability and the reproducibility of the results obtained through their usage (Hughes et al., 2007). In order to confirm that the batch of THP-1 cells in my possession also binds PU.1 at the +1.2kb site, I carried out ChIP-qPCR for PU.1 in my THP-1 cell line. The greatest enrichment, as observed by Pott et al. (2012) is also at the region +1.2kb downstream of the TSS (figure 9).

There are however slight differences between my data and that of Pott et al. (2012). They observe greater enrichment at the +5.9kb site compared to -1.76kb site, whereas the opposite is true in my experiment. As the qPCR primers for the different sites were tested and shown to have comparable amplification efficiencies, the differences may reflect changes that have occurred in the cell line over time.

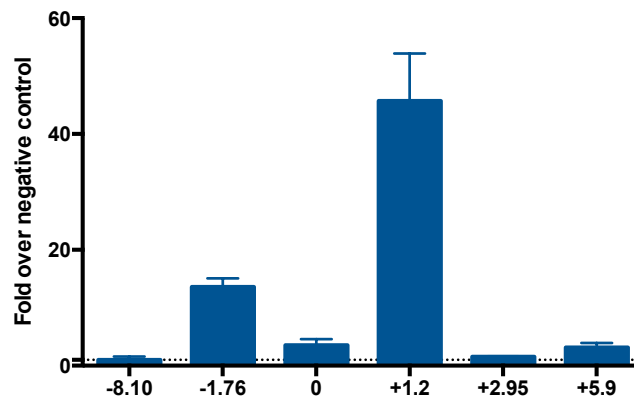


Figure 9: ChIP-qPCR for PU.1 in THP-1 cells. PU.1 binds at the +1.2kb site in the IL-10 locus. It also binds the -1.76kb site and to a much lower extent, the +5.9kb site. It does not bind the negative control regions, -8.1kb and +2.95kb. '0' marks the TSS. The mean and standard deviation of triplicate values are shown for each region.

5.7 Generation and identification of clones with a modified +1.2kb PU.1 binding site

5.7.1 Identifying PU.1 consensus sites within the +1.2kb site and design of CRISPR guides to target these sites

As PU.1 is a master regulator of transcription, the theory is that preventing it from binding to intron 2 of the IL-10 gene, through disruption of the PU.1 binding site, will result in closed conformation of the chromatin and a failure of other cell-specific and signal-induced transcription factors from binding to this site. If this site is involved in regulation of the IL-10 gene, the expectation is that disruption of this site will result in altered IL-10 expression.

The ChIP peak for PU.1 at the +1.2kb site spans the length of intron 2 of the IL-10 gene. In order to disrupt binding using CRISPR/Cas9, it was necessary to pinpoint the sequence to which PU.1 binds. Research into PU.1 binding sites has previously been carried out. In particular, an extensive study by Pham et al. (2013) investigates the binding of PU.1 in cells including primary human

monocytes. Approximately, 3300 individual 12mers make up the PU.1 consensus position weight matrix (PWM) (Pham et al., 2013). Of particular importance is the sequence 5'-GGAA-3' at position 6-9 (although this can also be 5'-AGAA-3'). The authors analysed bound and unbound consensus sequences identified in ChIP-seq experiments, along with *in vitro* analysis of the affinity of recombinant PU.1 to different double-stranded oligonucleotide sequences, and provide a method for scoring potential PU.1 binding sites. An example of the scoring is shown in figure 10.



Figure 10: The PU.1 consensus position weight matrix (PWM) adapted from Pham et al. (2013). Of particular importance is the sequence at position 6-9. This is typically 5'-GGAA-3' but can be 5'-AGAA-3'. The five nucleotides upstream, and the three nucleotides downstream of 5'-G/A GAA-3' are important in influencing the score of the site. The higher the score, the more likely this site is bound by PU.1.

It should be noted that low scoring sequences, representing low affinity motifs, may still be bound *in vivo* but such binding relies on cooperativity with other transcription factors (Pham et al., 2013).

Using the PU.1 PWM I identified six possible PU.1 binding sites within intron 2 and used the method described in Pham et al. (2013) to score these sites.

Pham et al. 2013 describe sites with a score greater than 9 as “high” scoring sites (those most likely to be bound *in vivo*), those with a score of 7-9 as “intermediate” scoring sites, and those with a score of 6 as “low” scoring sites (but which may still bind PU.1 but would likely rely on cooperation with other transcription factors). The sites highlighted in red in figure 11 correspond to the highest scoring sites in intron 2, and score 7.05 and 8.53. These represent the

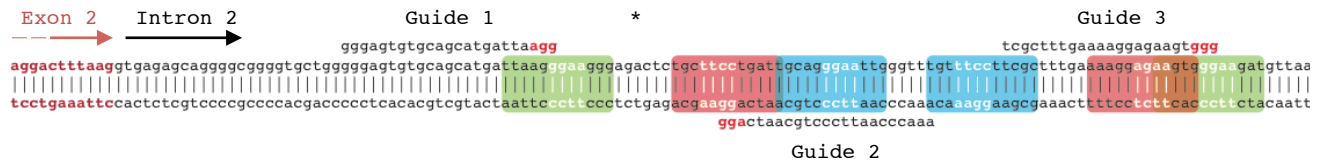
most likely PU.1 binding sites *in vivo*. The site with a score of 7.03 lies near to the Behçet's associated SNP rs1518111, whereas the site with a score of 8.53 is identified on JASPAR (a transcription factor binding profile database) as being the intron 2 PU.1 binding site.

The sites highlighted in green and blue in figure 11, although containing the necessary GGAA sequence, are low to very low in score (the sites highlighted in green represent sites with scores of 6.07 and 4.54 and those in blue represent sites with scores of 2.82 and 3.10). Although it is possible that the site with a score of 6.07 (which lies near to the Behçet's associated SNP rs1518111), binds PU.1 in cooperation with other transcription factors, it is very unlikely that the remaining blue and green highlighted sequences are bound by PU.1.

It should be noted that PU.1 belongs to the ETS family of transcription factors, characterised by a conserved DNA binding domain, referred to as the ETS domain (Cooper et al., 2014). It is known that certain ETS family transcription factors can form homo- and heterodimers (Cooper et al. 2014), and PU.1 itself has been shown to homodimerise (Poon et al., 2012). It can therefore not be ruled out that PU.1 may bind to multiple consensus sites within intron 2.

Thus, the aim was to interfere with the two highest scoring sites (in red in figure 11) and if possible, the 6.07 site (in green and near to the SNP). In order to generate THP-1 cells with a disrupted PU.1 binding site in intron 2, the CRISPR/Cas9 system was used. The position of the guide sequences, designed using the crispr.mit.edu server, are also highlighted in figure 11.

A



B

Binding site score

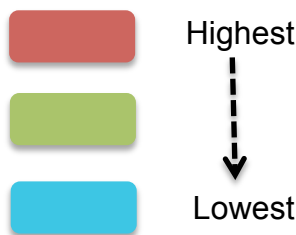


Figure 11: Potential PU.1 binding sites in intron 2 of the IL-10 gene. A: The nucleotide sequence of the end of exon 2 and the start of intron 2, with the potential PU.1 binding sites in intron 2 highlighted in red, blue and green. The 20 nucleotide CRISPR guide sequences are also shown in black. The CRISPR/Cas9 system I will use is based on that of *S. pyogenes*. In this system, the guide sequence must be located 5' of the protospacer- adjacent motif (PAM), the three nucleotides in red at the end of the guide sequence. *Asterisk indicates the location of Behçet's associated SNP rs1518111.

B: The binding sites in Figure 11A are colour-coded based on scored using a method by Pham et al. (2013). The highest scoring sites in the IL-10 locus are in red, the intermediate in green and the lowest scoring sites are highlighted in blue.

5.7.2. Successful generation of clones with a modified +1.2kb PU.1 binding site using the nickase Cas9 (pX335) and guides 2 and 3

After the co-transfection of THP-1 cells with two constructs carrying the nickase Cas9 and guides 2 and 3, and the generation and expansion of 12 THP-1 monoclonal clones, I performed sequencing of the genomic DNA covering the targeted region. This led to the characterisation of the unique mutations introduced at the +1.2kb site. A selection of the DNA sequences at the +1.2kb site in these clones are shown in figure 12. Although certain clones had a deletion of potential PU.1 sites on both alleles, this was only true for the lowest scoring sites (for example, figure 12B). Clones with the highest scoring sites removed only ever occurred on one allele (for example, figure 12C). In this particular clone, there is a small deletion on the other allele in a low scoring site (score 2.82), which increases its score to 5.78.

In certain clones, insertions were observed. In figure D for example, on allele 2, there is an insertion which removes the highest scoring PU.1 site (score 8.53). The insertion however creates a new high scoring site (score 7.53). Analysis of the sequence inserted suggests that the cell used the px335 backbone during DNA repair.

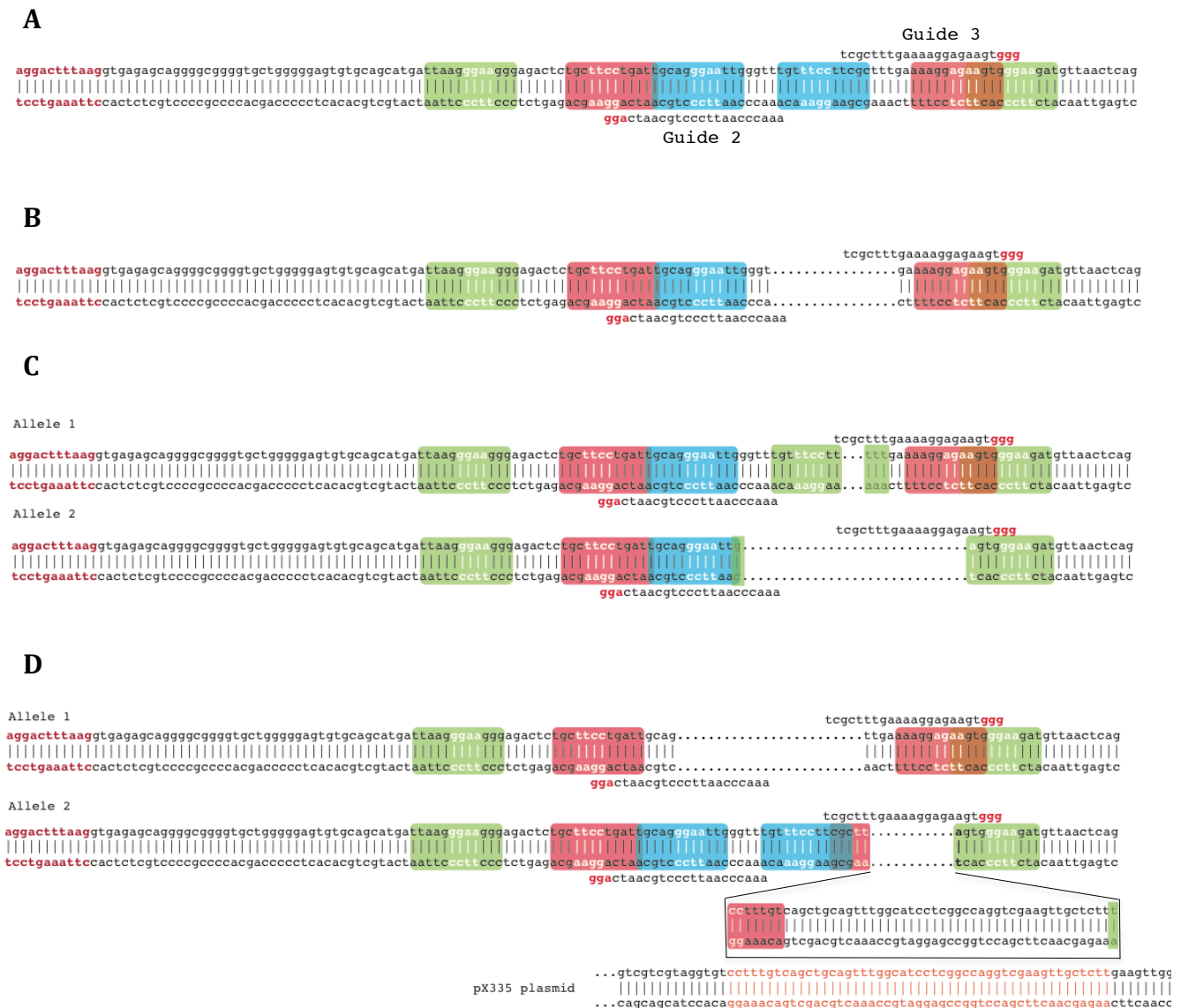


Figure 12: A selection of THP-1 clones generated through use of pX335 CRISPR-Cas9 nickase plasmids. A: A region of DNA from intron 2 of the IL-10 gene of unmodified, wild-type THP-1 cells. The potential PU.1 binding sites and the guide sequences (guide 2 and guide 3) used in conjunction with pX335 are highlighted in black, with the neighbouring PAM in red. B: An example of a THP-1 clone modified using the Cas9 nickase. In this clone, there is removal of a low-scoring PU.1 binding site on both IL-10 alleles. C: A THP-1 clone in which different modifications occur on each allele. On allele 1, there is a small deletion in one of the lowest scoring sites, which increases the score of that site. On allele 2, there is a deletion which removes a low scoring site as well as the highest scoring site. D: A THP-1 clone in which allele-specific modifications are observed. On allele 1, the two lowest scoring sites are removed and on allele 2, a 50 base pair insertion is observed, removing the original high scoring site and creating a new high scoring site. The sequence inserted matches that of the pX335 plasmid.

5.7.3 Successful generation of clones with a modified +1.2kb PU.1 binding site using the nuclease Cas9 (pX330) plasmid and guides 1 and 3

After the co-transfection of THP-1 cells with two constructs carrying the nuclease Cas9 and guides 1 and 3, three THP-1 monoclones with identical modifications were generated. All three clones had the same deletion on both alleles, resulting in the loss of the two highest scoring sites, the two lowest scoring sites and the intermediate site neighbouring the Behçet’s disease-associated SNP.

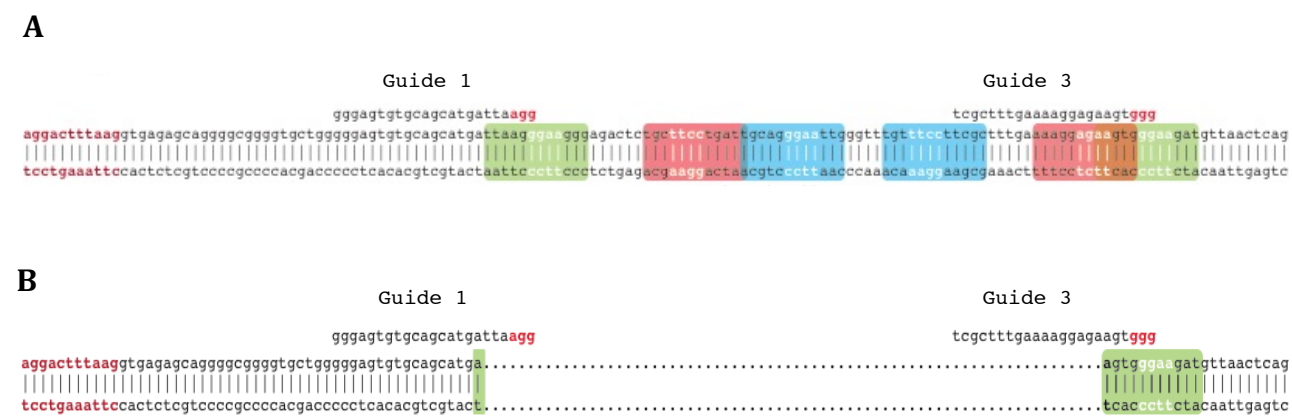


Figure 13: THP-1 clones generated through use of pX330 CRISPR-Cas9 nuclease plasmids. A: A region of DNA from intron 2 of the IL-10 gene of unmodified, wild-type THP-1 cells. The potential PU.1 binding sites and the guide sequences (guide 1 and guide 3) used in conjunction with pX330 are highlighted in black, with the neighbouring PAM in red. B: The three modified THP-1 clones generated through use of pX330 and guides 1 and 3 had the same region of DNA, containing the highest scoring PU.1 sites, removed on both alleles.

As this combination of pX330 nuclease plasmids and guides generated clones that lacked the highest scoring PU.1 sites on both alleles, something that did not occur when using the pX335 nickase, the clones generated with the pX330 plasmids were used for future experiments.

5.7.4 PCR-based protocol for the confirmation of CRISPR/Cas9-induced deletion

The sequence of the THP-1 clones was determined by extraction of genomic DNA, amplification of a section of the IL-10 gene that covered intron 2, Sanger sequencing, and if necessary, sequence deconvolution (as described in figure 5 in Chapter 2: Materials and Methods)

In order to verify that the sequence of the THP-1 clones generated with the Cas9 nuclease had the deletion revealed by sequencing, PCR was carried out on genomic DNA using a forward primer specific for the missing region, and a reverse primer specific for a region of DNA in intron 3 of IL-10 expected to be present in all clones (as described in figure 6 in Chapter 2: Materials and Methods).

The PCR reaction was carried out on DNA from the three modified clones, three clones found to be wild-type and the original THP-1 cells from which the clones derived. When the PCR reaction was ran on a gel, the band of approximately 0.5kb is only present in the wild-type cells, supporting the fact that there is a deletion within this region of intron 2 in the modified clones.

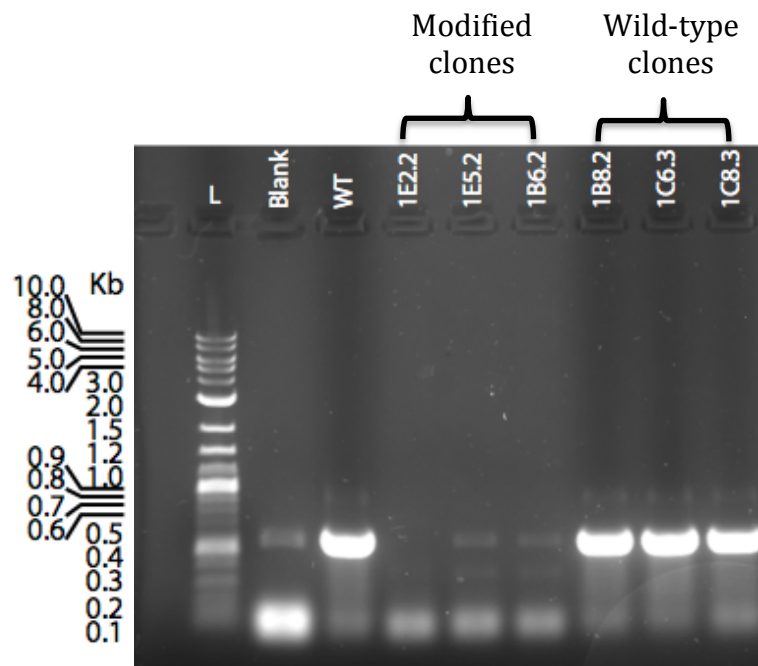


Figure 14: PCR-based detection of CRISPR-Cas9 deletion. PCR using a forward primer that recognises the deleted region and a reverse primer that recognises a region outside of the target site (in intron 3) was carried out in the original wild-type THP-1 cells from which CRISPR clones were generated (WT) as well as in three monoclonal clones shown to be unmodified (Wild-type clones) and three monoclonal clones shown to have a deletion (Modified clones) by Sanger sequencing. In both WT and Wild-type clones, a band ~0.5kb is detected. This band, corresponding to the amplified region, is missing in the three clones shown to be modified by Sanger sequencing and the blank containing water instead of DNA.

5.8 Deletion of PU.1 binding sites leads to an absence of PU.1 protein at +1.2kb as shown by Chromatin immunoprecipitation (ChIP-qPCR)

Having generated THP-1 cells that lack the highest scoring PU.1 sites in intron 2, I wanted to determine whether removal of these sites was sufficient to prevent PU.1 from binding to intron 2.

Genome-wide analysis of PU.1 binding by ChIP-seq is expensive and unnecessary for validation of PU.1 binding at one site. Therefore, ChIP-qPCR, using qPCR primers that recognise +1.2kb region (but outside of the modified area) were

used. In the original wild-type THP-1 cells from which the CRISPR clones were generated, and three wild-type clones for comparison, PU.1 is bound at the +1.2kb region. In the clones with the deletion ('modified clones'), PU.1 is no longer detected.

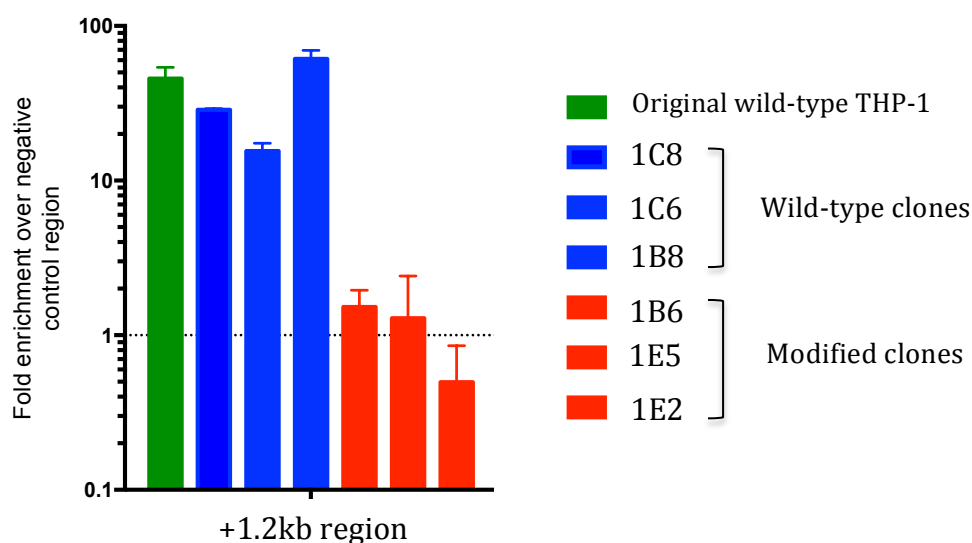


Figure 15: ChIP-qPCR for PU.1 at +1.2kb. Deletion of PU.1 binding sites in intron 2 of the *IL-10* gene using the CRISPR-Cas9 nuclease system results in loss of PU.1 binding to this site ('modified clones'). In clones lacking this deletion (Wild-type clones) and in the original wild-type THP-1 cells from which the clones were derived, PU.1 is detected. Values are expressed as fold enrichment over negative control region. The mean and standard deviation of triplicate values are shown for each cell type.

5.9 The binding of PU.1 to neighbouring -1.76kb and +5.9kb sites is unaffected by the deletion of the PU.1 binding site at +1.2kb.

To assess whether deletion of the PU.1 binding site and prevention of PU.1 binding to intron 2, affects the binding of PU.1 to the surrounding sites, ChIP for PU.1 followed by qPCR for -1.76 and +5.9kb sites was carried out. In modified clones, PU.1 still binds to -1.76 and +5.9kb, even though it is absent from +1.2kb. I also tested the binding of PU.1 to +2.95kb, a region which has been, in numerous cell lines, shown to bind many different transcription factors,

including the B cell transcription factor, Pax5 (data available on the UCSC genome browser) but a site that does not bind PU.1 in primary human monocytes (Pham et al., 2012) or THP-1 cells (Pott et al., 2012). In modified clones, PU.1 does not bind to the +2.95kb region.

ChIP-qPCR was also carried out for the region -8.1kb (8.1kb upstream of the TSS). This site, like +2.95kb, is an internal negative control, except this region has not been shown to bind transcription factors in numerous cell lines (data available on the UCSC genome browser).

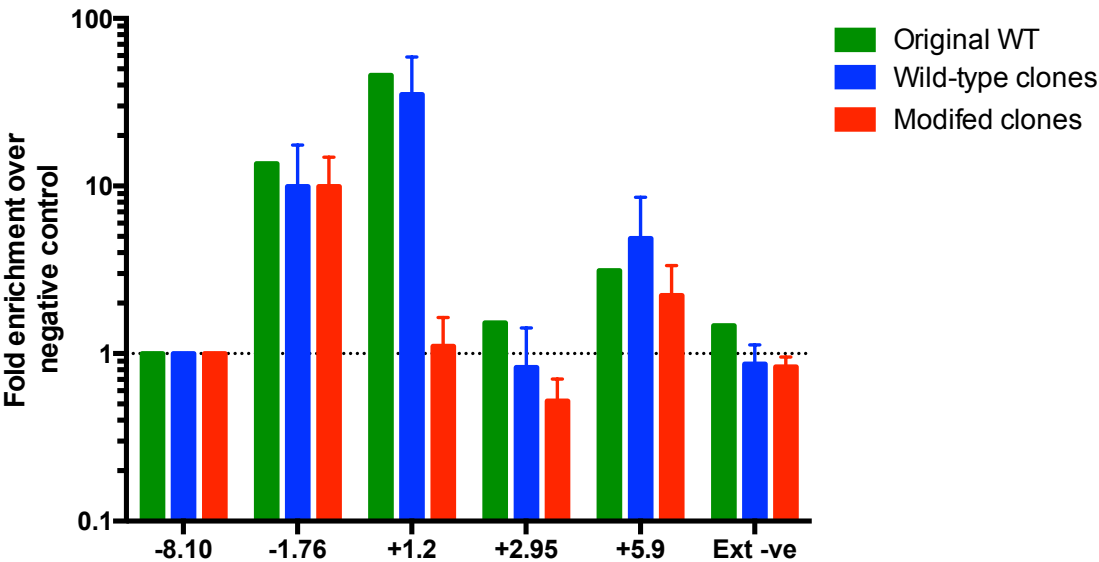


Figure 16: ChIP-qPCR for PU.1 at the IL-10 locus in THP-1 cells. In clones modified at the +1.2kb site (red bars), PU.1 is still binds to -1.76 and +5.9kb sites. It does not bind the negative control regions +2.95kb and -8.1kb, nor does it bind a negative control region outside of the IL-10 locus (Ext -ve). Percentage input is expressed as fold enrichment over -8.1kb. The mean and standard deviation are shown for each region.

5.10 Assessing chromatin conformation at the +1.2kb site in modified clones, using an Assay for Transposase-accessible Chromatin with High - throughput Sequencing (ATAC-seq)

The theory behind deleting the PU.1 binding site in intron 2 of the IL-10 gene and preventing PU.1 from binding to this site, was based on its role as a master

regulator/pioneering transcription factor. The aim being that preventing PU.1 binding would result in a closed chromatin structure at this site and therefore prevent other cell-specific and signal-induced transcription factors from binding.

In order to test whether removing PU.1 from this site affects chromatin structure, ATAC-seq was carried out. ATAC-seq is a relatively new technique to detect regions of open chromatin conformation (Buenrostro et al., 2013). This technique uses the Tn5 transposase, which can both fragment DNA and add sequencing adaptors to the ends of fragmented DNA (Buenrostro et al., 2013). As this fragmentation and addition of sequencing adaptors occurs preferentially at sites with an accessible, open chromatin conformation, sequencing enables the identification of open chromatin regions within the genome, that have potential regulatory roles (Buenrostro et al., 2013).

E. Ballabio and M. Tapia (Milne Lab, WIMM) kindly provided a protocol for ATAC-seq and advice and M. Tapia ran the samples on the sequencer. R. Thorne (Milne Lab, WIMM) kindly analysed the sequencing data.

It can be seen in figure 17, that preventing PU.1 binding at the +1.2kb site, results in a closed chromatin confirmation, as revealed by the absence of a peak at intron 2 in the modified clones.

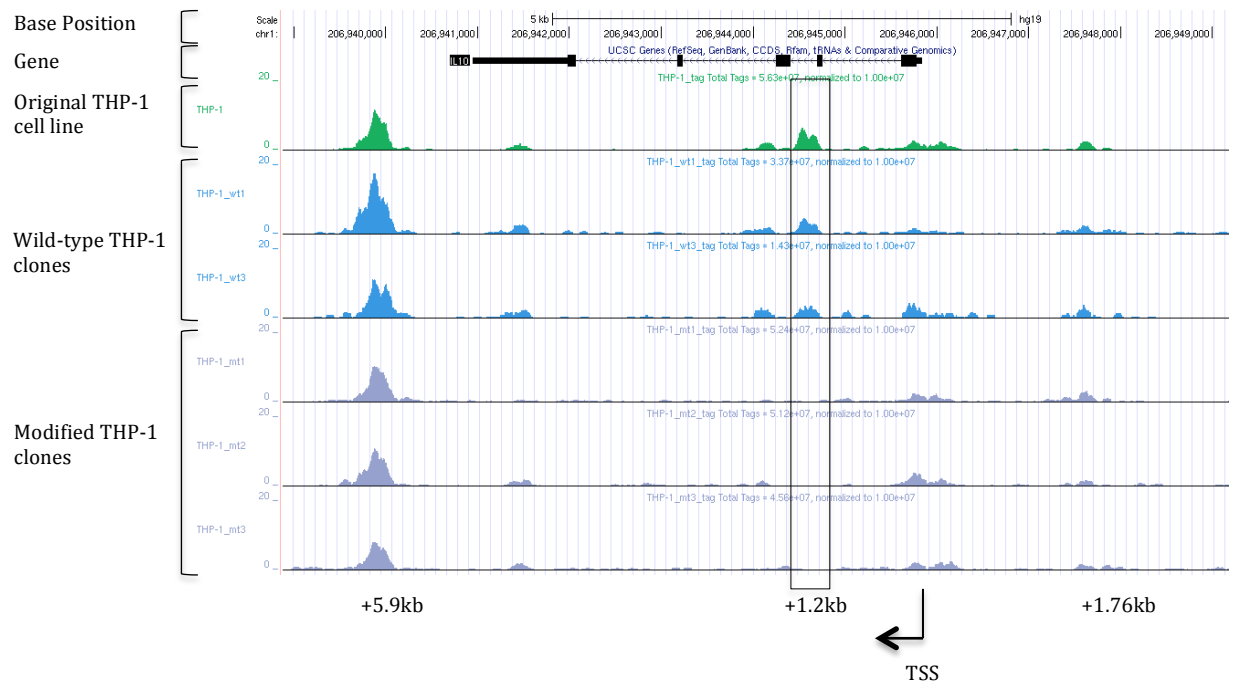


Figure 17: ATAC-seq profile in modified versus unmodified THP-1 cells. Comparison of the original THP-1 cell line from which the clones were derived (green), two of the wild-type clones (blue) and the three modified clones (purple) reveals that removal of the PU.1 binding site, and prevention of PU.1 binding, renders this site inaccessible to transposase suggesting this site now has a closed chromatin conformation.

5.11 Analysis of IL-10 expression following removal of PU.1 from +1.2kb region

If the transcription factor binding site 1.2kb downstream of the IL-10 promoter is involved in positively regulating expression of the IL-10 gene, then it would be expected that removal of the PU.1 binding site and the resulting inaccessible chromatin structure at this site, would result in lower amounts of IL-10 being expressed in response to stimulation.

5.11.1 IL-10 mRNA expression

THP-1 cells were therefore stimulated with LPS and expression of IL-10 mRNA by wild-type and modified THP-1 cells compared over the course of six hours (figure 18). Unexpectedly, by six hours post-stimulation, overall the three

modified clones expressed more IL-10 mRNA than the three wild-type clones. This difference was not dramatic, however small differences in expression are not necessarily unexpected as it is known that several regulatory regions can act together to affect expression.

There was however variation among wild-type and modified clones, in that there was no difference in IL-10 expression at six hours between the wild-type clone 1B8 and the modified clone 1B6. Such variation was also true over the course of six hours, with the wild-type clone 1B8 and the modified clone 1E5 responding more rapidly to LPS stimulation than the remaining clones.

Kinetics of IL-10 expression was also analysed in the original wild-type THP-1 cells from which the wild-type and modified clones were derived (green line on figure 18). The original wild-type cells and the modified clone 1E5 express similar amounts of IL-10 which follows the same bimodal expression pattern, with an early peak in gene expression. Apart from the modified clone 1B6, which also has bimodal expression but expresses much lower amounts of IL-10, the remaining clones do not show this pattern. These differences may reflect heterogeneity among the original wild-type THP-1 population, or it may be possible that the THP-1 cells cultured from single transfected cells, for unknown reasons, have altered properties to the original THP-1 cells.

When attempting to determine whether the deletion introduced at the +1.2kb site has an effect on IL-10 expression, comparison of expression levels in modified clones with wild-type clones that have been through the CRISPR-Cas9 transfection and single cell cloning process may be more accurate than comparison of modified clones with the original wild-type THP-1 cells. The data is suggestive of a role for this site in subtle negative regulation of IL-10 expression but as this is unexpected due to the nature of the IL-10 locus at this site (in terms of active histone modifications, such as H3K27ac, and the association with the Behçet's Disease-associated SNP rs1518111), as well as variation among different clones, it makes it difficult to draw conclusions. Means

by which I could overcome potential variation among wild-type cells and the single cell cloning process are mentioned in the discussion.

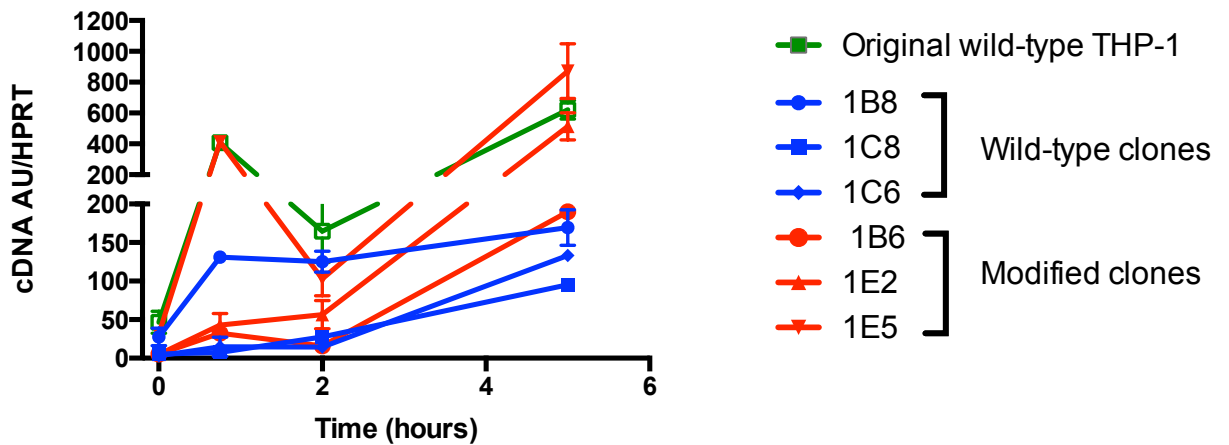


Figure 18: Kinetics of IL-10 expression in modified versus unmodified cells. THP-1 cells were stimulated with 1 μ g/ml LPS and lysed at various times points over 5 hours for analysis of IL-10 mRNA. The original THP-1 cells from which the clones were derived are represented by the green line, modified clones by the red lines and wild-type clones by the blue lines. The mean and standard deviation of triplicate values are shown for each time-point.

5.11.2 IL-10 protein production

I also analysed the kinetics of IL-10 protein production, by ELISA on the supernatant of stimulated cells (figure 19). Although Remmers et al. (2010) saw reduced IL-10 production by cells homozygous for the Behçet's Disease-associated SNP rs1518111, overall I saw higher production of IL-10 by the modified clones. Similarly to IL-10 mRNA expression, there was variation among clones and the original wild-type THP-1 cells produced more similar amounts of protein to the modified clones than the wild-type clones.

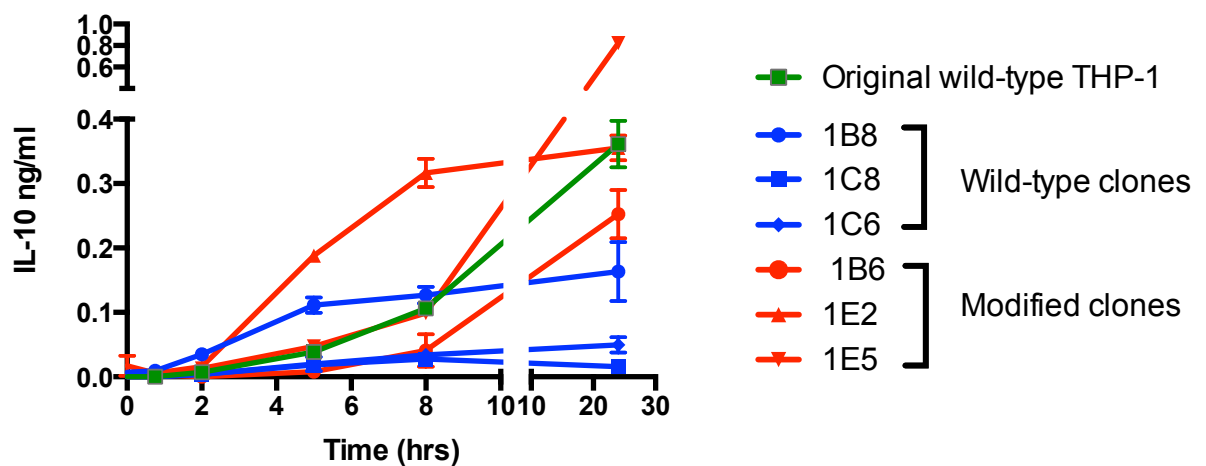


Figure 19: Kinetics of IL-10 protein production in modified versus unmodified cells. THP-1 cells were stimulated with $1\mu\text{g/ml}$ LPS and the supernatant collected at various times points over 24 hours for analysis by ELISA. The original THP-1 cells from which the clones were derived are represented by the green line, modified clones by the red lines and wild-type clones by the blue lines. The mean and standard deviation of triplicate values are shown for each time-point.

5.12 Further analysis of accessible regions of DNA surrounding the IL-10 locus in modified and unmodified cells

The reduction in IL-10 production by wild-type THP-1 clones compared to the polyclonal population suggests that the CRISPR-Cas9 and single cell cloning process might have an adverse effect on THP-1 cell capacity to produce IL-10 in response to stimulation. If this is the case, the increased expression of IL-10 in modified THP-1 clones compared to wild-type THP-1 clones would indicate that the +1.2kb site plays a negative role in IL-10 expression. To test this hypothesis, it would be necessary to compare a greater number of wild-type and modified clones.

However, the evidence that IL-10 is still expressed in the absence of the +1.2kb region does not necessarily rule out a positive role for this site in regulation of IL-10. Redundancy in the role of enhancers in controlling gene expression has been described, and it is possible that this region acts in concert with several other regulatory sites and that clear differences in expression would not be observed until all sites have been inactivated (Xiong et al., 2002).

An alternative possibility is that removal of this PU.1 binding site, affects an enhancer elsewhere in the genome which could compensate for the loss of the +1.2kb site. In order to explore this further, and to gain an understanding of the enhancer repertoire surrounding the IL-10 locus in modified and unmodified THP-1 cells, further analysis of the ATAC-seq data was carried out by R. Thorne. Samples from modified and wild type clones were normalised for library size. ATAC peaks were called in the region 150kb upstream and 150kb downstream of the IL-10 gene and the number of aligned reads that map to each peak were used to calculate fold change between the mean of the modified THP-1 clones and the mean of the wild-type clones. If a region of DNA becomes accessible due to changes in chromatin conformation, a new ATAC peak would appear. As fold change was calculated as modified mean/wild-type mean, values greater than one represent increases in modified cells compared to wild-type, whereas values below one represent increases in wild-type compared to modified cells.

I was particularly interested in the appearance of ATAC peaks in modified THP-1 cells and thus fold changes greater than one. In the region 150kb upstream and 150kb downstream of the IL-10 gene, there are 49 ATAC peaks. The majority of peaks enriched in modified THP-1 cells represent small fold changes. However, the top three enriched peaks are 3, 3.4 and 21 fold greater in modified cells compared to wild-type (highlighted by the darkest red lines on figure 20) and are located 71kb, 26kb and 47kb downstream of the modification site, respectively. It is also true that certain peaks became less accessible in modified clones (highlighted by the blue lines on figure 20). The top three regions are located at the +1.2kb site of modification (44 fold decrease) and at 77kb and 64kb downstream of the modified site which show a 6.9 and 3.5 fold reduction in modified cells compared to wild-type.

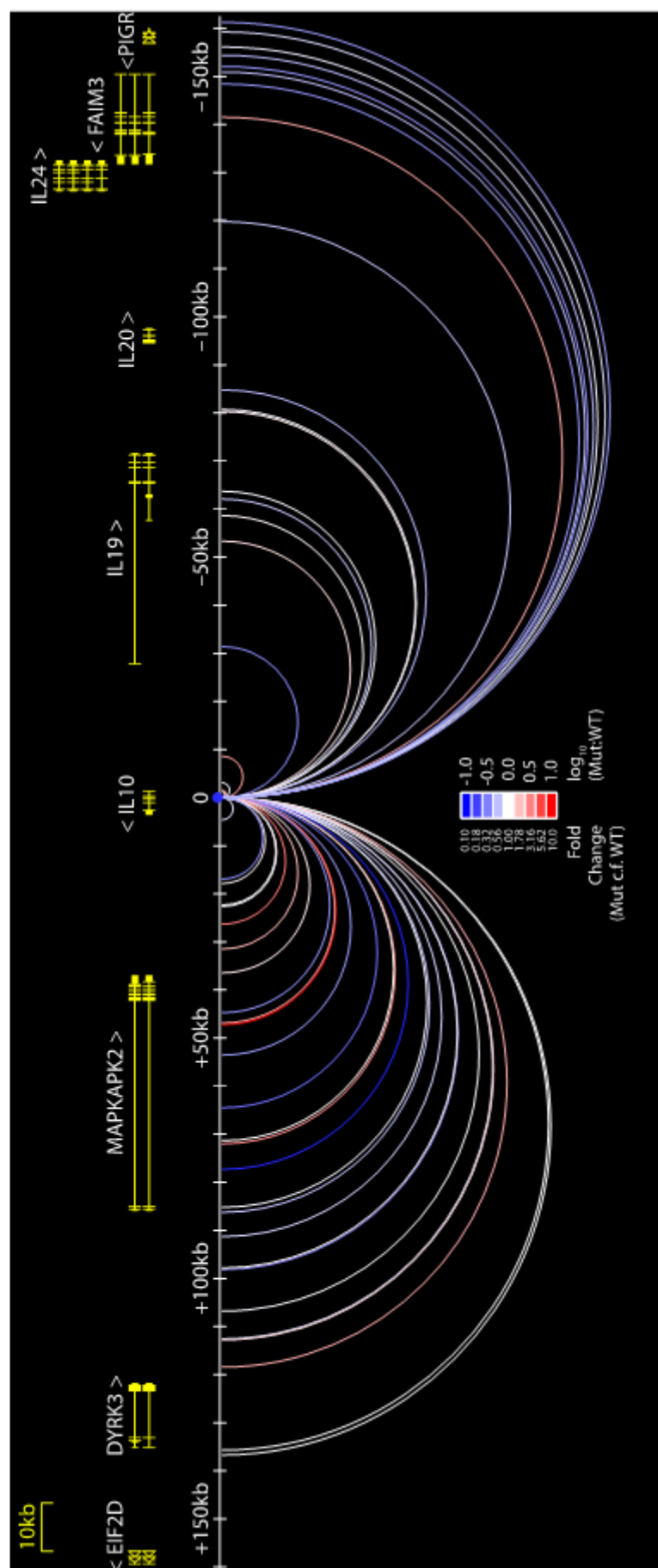


Figure 20: Fold change in ATAC peaks in the 300kb surrounding the IL-10 gene. Curved lines link the ATAC peak to the IL-10 modification site and the colour of the line reflects the fold change of that peak. Peaks enriched in modified cells are coloured in red, those unchanged in white and those enriched in wild-type cells are in blue. Gene sequences are highlighted in yellow at the top of the diagram.

The raw data (in the form of tracks uploaded to the UCSC genome browser) for the top three enriched peaks are shown in figure 21. The original wild-type THP-1 cell line is also shown for comparison. Although it is evident that there are differences in peak size at these particular genomic locations in modified clones versus wild-type clones, when the original wild-type THP-1 cell line is also compared, it does not differ from the modified cells. Unexpectedly (but perhaps not surprising given the mRNA and protein expression data), it's as though, at these sites, wild-type clones are the most different of the three populations.

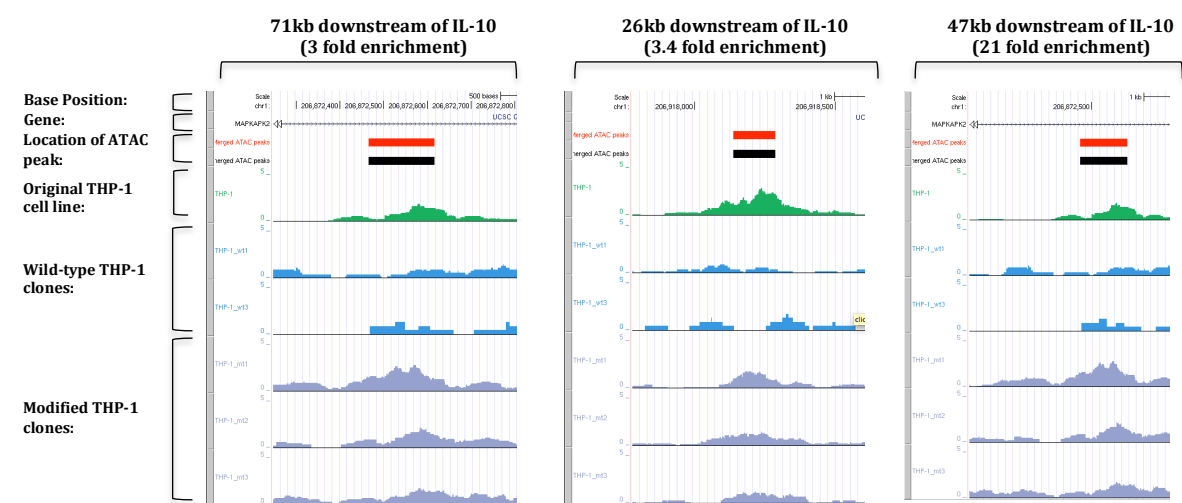


Figure 21: ATAC-seq tracks corresponding to the top three enriched peaks. Comparison of the ATAC peaks at the top three enriched sites in modified THP-1 clones (purple) compared to wild-type THP-1 clones (blue) and the original THP-1 cell line (green).

It is difficult to know whether this is because of heterogeneity among the THP-1 population and the possibility that the wild-type and modified clones derive from cells with different properties, or that that the CRISPR process and single cell cloning results in changes to both modified and unmodified cells, meaning comparison to the original THP-1 cell line is unfair.

5.13 Discussion

In this chapter I have attempted to determine the role of a transcription factor binding site in the regulation of the IL-10 gene. This site, +1.2kb downstream of the IL-10 TSS, binds the master regulator PU.1 and a SNP (rs1518111) within this region is associated with the inflammatory disease, Behçet's Disease. Monocytes with this particular SNP produce lower amounts of IL-10 RNA and protein upon stimulation. However, to my knowledge, no one has directly studied the role of this site in regulating IL-10. The presence of this SNP and the fact the histones surrounding this region are heavily modified with active histone modifications suggested a role for this site in positive regulation of IL-10 expression.

5.13.1 CRISPR-Cas9 considerations and alternative modifications

I used the CRISPR-Cas9 system to modify this site in the monocytic THP-1 cell line. Whilst this is a fairly simple and efficient method for introducing genetic modifications, there is a limit to the number of sequences that can be targeted by the 20 nucleotide guide sequence. The region to be targeted must immediately be followed by a Protospacer Adjacent Motif (PAM), which in the case of Cas9 from *S. pyogenes*, is 5' NGG 3' (Ran et al., 2013i). Although guide sequences for use with the Cas9 nuclease can be scored on the likelihood of off-target effects, it is possible to increase specificity of the target site by targeting two Cas9 nickases to two neighbouring 20 nucleotide sequences on opposite strands of the target site. In order to efficiently introduce indels by the NHEJ DNA repair pathway, it has been shown the offset between the two guide sequences should be between 4-20 base pairs (Ran et al., 2013ii). Furthermore, the efficiency of creating genetic modifications using the double-nickase approach is much greater when guide RNA pairs create a 5' overhang (Ran et al., 2013ii).

Within the +1.2kb region of interest, there were a limited number of guide RNA sequences to choose from. Furthermore, I was unsure of the exact PU.1 site to target. The highest scoring site (8.53) is more than 30bp away from the second

highest scoring site (7.05). This 7.05 site resides within a few base pairs of the Behçet's associated SNP. Furthermore, the relatively low scoring site (6.07), also within a few base pairs of the SNP, and which could potentially bind PU.1 through cooperation with other factors, is around 50bp from the highest scoring site.

While the position of the guides used with the nickases generated clones with a range of genetic modifications, unfortunately only the lowest scoring sites were removed on both alleles. I also used a combination of two nucleases and two guide sequences. As each nuclease and guide sequence was on a separate plasmid, there was the possibility that only one plasmid (i.e. one guide) would successfully transfect one THP-1 cell and thus there would only be a modification at the region of DNA nearest that guide sequence. However, in all clones generated, the mutations were identical and the same section of DNA was removed on both alleles, corresponding to breaking of the DNA at exactly the predicted sites of both guide/Cas9 complexes. This nuclease and guide combination resulted in three clones lacking the two highest scoring sites, the two lowest scoring sites and the intermediate site neighbouring the Behçet's Disease-associated SNP. This deletion occurred on both alleles. As I do not know whether multiple PU.1 molecules bind +1.2kb, deletion of all sites was favourable.

Instead of deleting a section of DNA, an alternative approach would be to precisely modify the potential PU.1 binding sites within this region. It has been shown that if the Cas9 enzyme and guide RNA are provided to a cell in conjunction with an oligonucleotide sequence of one's choice as a repair template flanked by homology arms (i.e. the sequence at either end is homologous to the region surrounding the double-stranded break), then it is possible to create precise mutations or inserts by relying upon the cell's homology-directed repair mechanism (HDR) (Yang et al., 2013) (Zheng et al., 2014). One could therefore supply a synthetic oligonucleotide corresponding to the PU.1 binding region, but that contained mutations in the critical 5' GGAA 3' nucleotides of each consensus site. It may be possible therefore to create precise

genetic modifications just at the potential PU.1 sites, without removing a section of the gene.

By ChIP-qPCR, I have shown that deletion of the predicted PU.1 sites results in clones that lack PU.1 binding to the +1.2kb region. As they have a deletion of the two highest scoring sites and the region around the SNP, it is not possible to precisely pinpoint the exact PU.1 binding site. However, prevention of PU.1 from binding to this region, results in this region being inaccessible to transposase tagmentation, shown by ATAC-seq, which implies a closed chromatin structure, suggesting inaccessibility of this site to other cell and signal-specific transcription factors. It should also be noted that upon removal of the +1.2kb PU.1 binding site, the +1.5kb region becomes inaccessible (figure 17) suggesting that there may be degree of cooperation or interaction between these sites.

5.13.2 Considerations when deleting regulatory regions

It is important to note that regulation of genes *via* cis-acting elements such as enhancers is complex. Many sites may interact with the promoter of a target, through looping of DNA, to regulate expression of that gene (Doyle et al., 2014). It may be that deletion of only one regulatory element results in a small or undetectable change in gene expression due to compensation by other regulatory elements (Xiong et al., 2002) (Spensberger et al., 2012). It may be that it is necessary to delete all enhancers for example in order to see an effect. Whilst this +1.2kb site has features that suggest a role for positive regulation of this gene, it would be of interest to gain a more complete view of the sites involved in IL-10 regulation. This includes those sites that may be distant to the IL-10 gene. A technique known as chromatin conformation capture would enable the detection of distal sites that interact with the IL-10 promoter (Naumova et al., 2012). Sites identified with this approach could then be validated by targeted disruption with CRISPR-Cas9 technology.

5.13.3 Is this site involved in negative regulation or in LPS-independent induction of IL-10?

In response to LPS stimulation, and contrary to expectations, the three modified clones produced more IL-10 than wild-type clones. This was true when cells were stimulated with 1µg/ml (shown in figure 18 and 19) but also when the LPS concentration was reduced to 200ng/ml and 40ng/ml (Appendix A3.1). However, this difference in IL-10 production was only small. Although this may not be unexpected following deletion of a single regulatory region, in which redundancy between sites may mask an effect, IL-10 induction in the original wild-type THP-1 cell line was greater than in the wild-type clones. If it is the case that the CRISPR-Cas9 transfection procedure and single cell cloning process have an adverse effect on the capacity of THP-1 cells to produce IL-10, this region may be involved in negative regulation of this gene.

A role in negative regulation was unexpected given the Behçet's-associated SNP and acetylation of histones surrounding this site. An alternative possibility is that this site may not play a role in LPS-mediated induction of IL-10. As described in the introduction, there are many stimuli, receptors and signalling pathways that may result in induction of the IL-10 gene and it may be that certain transcription factors bind to this site but only in response to certain stimuli. As Behçet's Disease is a sterile inflammatory disease, I was interested in whether there was a difference in expression when cells were stimulated with the acute-phase protein, SAA, which can induce IL-10 *via* TLR-2 and FPR2. While there was robust expression of IL-10 mRNA and production of IL-10 protein in primary human monocytes (with protein concentration continuing to increase to 12 hours and remaining as high at 24 hours post stimulation) there was poor induction in THP-1 cells stimulated with SAA (Appendix A3.2) (Lee et al., 2006). This highlights that there are differences between primary cells and cell lines and thus any findings in cell lines should be, as much as possible, verified in primary cells.

5.13.4 Bimodal expression

When the kinetics of IL-10 expression in the bulk population is examined (figure 2A and figure 18), it seems that there is bimodal expression, with an early peak at 30 minutes post-stimulation. This is only observed in the modified clone 1E5, and to a much lesser extent, the modified clone 1B6. While it is unclear why there is an initial peak in expression of IL-10 in the original wild-type THP-1 cell line, expression of TNF- α in monocytic cells stimulated with LPS is bimodal (Nakayama et al., 2013). As well as inducing TNF- α , LPS has also been shown to upregulate IL-32- γ expression and data from experiments in which IL-32- γ is blocked by RNA interference suggest that LPS-induced IL-32- γ is responsible for the second peak in TNF- α expression (Nakayama et al., 2013).

With regards to IL-10 expression, the first peak occurs much earlier than that of TNF- α , at 30 minutes post-stimulation. Studies into IL-10 expression do not seem to look prior to one hour post stimulation and I can find no evidence of bimodal expression of IL-10 in the literature. However, it has been shown in bone marrow-derived macrophages that induction of IL-10 is dependent on the sequential induction of type I interferons (IFNs) and IL-27 (Iyer et al., 2010).

(Iyer et al., 2010). It has been proposed that LPS signalling *via* TLR-4 results in activation of the TRIF-dependent signalling pathway leading to expression of type I IFNs (IFN- α and IFN- β). Upon binding to the type I IFN receptor, they induce expression of the IL-27 gene. IL-27 (composed of the p27 and EBI3 subunits), upon binding to the IL-27 receptor, then directly induces IL-10 expression in a STAT1 and STAT3 dependent manner (Iyer et al., 2010). Therefore, it may be possible that the two peaks in expression are based on the activation of different signalling pathways, with the latter peak a result of type I IFN/IL-27-induced stimulation of the IL-10 gene. In order to see whether this may be a possibility, I carried out a preliminary experiment to look at the kinetics of type I IFNs, IL-27 and IL-10 induction in human THP-1 cells in response to LPS. The data is shown in Appendix A3.3. There is an early increase in expression of IFN- β that precedes expression of IL-10. There is also an increase in expression of the gene encoding the IL-27 subunit, EBI3, and this increase precedes the second peak in IL-10 expression. This data fits with the possibility that the second peak in IL-10 expression is a result of prior signalling

via these molecules. On the other hand, the IL-27 p28 subunit does not increase in expression between zero and two hours however I only measured RNA amounts at 2 hours and 5 hours post stimulation and it may be necessary to look at further time points in between to determine whether there is an increase in IL-27 p28 prior to the second peak in IL-10 expression. Note that only mRNA was analysed, and as it would be the protein that would ultimately be binding to its receptor to bring about such induction, it would also be interesting to look at protein levels. Further investigation could involve blocking the effects of type I IFNs or IL-27, perhaps through the use of blocking antibodies against their receptors.

Perhaps the lack of bimodal expression in the majority of clones indicates that the CRISPR-Cas9 and single cell cloning process has had an adverse effect on the capacity of THP-1 to produce IL-10.

5.13.5 Final remarks

When considering the induction of IL-10 expression in modified clones, wild-type clones and the original wild-type THP-1 cells, the differences observed between the cell lines tested means drawing conclusions about the role of this transcription factor binding site in regulating the IL-10 gene are difficult.

It may be that deletion of this PU.1 site has no effect on IL-10 production or deletion may in fact enhance IL-10 production. Alternatively, it could be that THP-1 cells are too heterogenous a population to draw conclusions and deletion of this region in the bulk population, avoiding a single cell cloning approach (which may also impact on the capacity of such cells to produce IL-10), would be a more desirable way to answer this question. This may be achieved through the use of the lentiviral CRISPR-Cas9 system. This is a more efficient approach for delivering the guide RNA and Cas9 enzyme to THP-1 cells which are particularly difficult to transfect transiently. Another possibility would be to use an inducible CRISPR-Cas9 system which following transduction of a large number of cells, where the expression of the Cas9 enzyme and/or guide could be induced. It

could then be possible to study the role of regulatory sites in the bulk modified population (in which the Cas9 and guide expression is induced) and bulk wild-type population (uninduced). This should remove the effect of heterogeneity and should enable rapid elucidation of the relevance of regulatory sites as there would be no requirement for single cell cloning. This also means there is the possibility of carrying out these experiments with primary human monocytes.

Chapter 6: Discussion and concluding remarks

6.1 Analysis of low-density neutrophils in patients with melanoma

While the immune system can control tumour growth, immune regulatory mechanisms can contribute to tumour progression and result in a failure to eliminate immunogenic tumours such as melanoma. To identify how the inflammatory response can influence tumour biology and be targeted in immunotherapy it's necessary to understand which aspects of the inflammatory response are associated with accumulation of immune-regulatory populations and how these inflammatory responses impact on the frequency and activity of cells involved in tumour control.

Low-density granulocytes with a phenotype suggestive of activated neutrophils (CD15+ CD10+ CD33int CD11b++ CD62L-) and granulocytes with a phenotype suggestive of immature neutrophils (CD15+ CD10- CD33hi CD11b+/- CD62L-) were detected in the peripheral blood of patients with stage IV melanoma. The accumulation of activated neutrophils correlated with an increase in the plasma concentration of certain cytokines/growth factors including IL-8. It is unclear if the presence of such cells in the peripheral blood of patients is a result of activation of neutrophils in the circulation by inflammatory cytokines, such as IL-8, or a result of reverse trafficking of neutrophils from tumours. The accumulation of immature neutrophils correlated with an increased plasma concentration of SAA. Appearance of immature neutrophils in the peripheral blood occurs during systemic infection as a result of emergency granulopoiesis. G-CSF is a factor implicated in driving this process, however there was no association with plasma concentration and immature neutrophil frequency. IL-6 however was associated with an enrichment of immature neutrophils, as was SAA.

Marked inter-patient variation in the frequency of these low-density neutrophil subsets was observed and given that these subsets associated with an accumulation of distinct cytokines in the plasma, suggested the existence of distinct inflammatory axes, one associated with activation of the immune system

and potentially deployment of effector function, and another associated with emergency granulopoiesis. Using the frequency of activated and immature neutrophils as potential markers of distinct inflammatory axes, analysis of patients undergoing anti-CTLA-4 treatment revealed that a lower frequency of activated neutrophils before treatment and an increase in frequency during treatment were associated with better treatment response. This is consistent with the hypothesis that anti-CTLA-4 treatment might be successful in breaking immune tolerance against tumour-associated antigens, but fails in those patients who already have an on-going immune response unable to control tumour growth. The presence of activated neutrophils might be a biomarker of the immune response against the tumour, with a low frequency in patients with less immunogenic tumours perhaps, with an increase after treatment in those patients where anti-CTLA-4 is breaking tolerance and inducing an immune response. As this study involved a small cohort of patients it is important to confirm these findings in a larger number of patients however, there is potential for monitoring of activated neutrophil frequency as a predictive biomarker of anti-CTLA-4 response.

6.2 Analysis of monocytes and T cells in patients with melanoma

The neutrophil data suggested the existence of distinct inflammatory axes, one associated with activation of the immune system, the other associated with emergency granulopoiesis. Given the association of activated neutrophil frequency with anti-CTLA-4 response, I was interested in whether a change in neutrophil activation was associated with wider changes in the immune system and therefore analysed monocyte and T cell populations in patients. I was also interested in whether there were wider changes associated with neutrophil differentiation.

I analysed the frequency of monocyte subsets and a number of T cell subsets, the expression of markers of activation (CD38, HLA-DR and ICOS), homing (CLA) and exhaustion (PD-1) on such subsets and have shown there to be a difference in the distribution of certain populations in the peripheral blood of patients

compared to healthy controls. Furthermore, the range of frequencies observed among patients correlates with changes in the frequency of low-density neutrophil populations and plasma cytokines/growth factors supporting the idea that an enrichment of certain neutrophil populations is a reflection of greater changes in the immune system and that different inflammatory axes may exist in stage IV melanoma patients. Exactly why different inflammatory axes may exist is unclear however it is possible that particular mutations in melanoma cells contribute to a particular inflammatory environment that combined with tumour burden (given the fact the highest frequencies of either neutrophil population are observed in M1c disease) impacts on immune cell biology. Sequencing of tumour cells from patients with an abundance of either population may help in answering this.

Analysis of the CD8⁺ T cell compartment revealed the presence of a CD8⁺ T cell population co-expressing CCR4 and CXCR3, which negatively correlated with the frequency of activated neutrophils in melanoma patients. This negative correlation might suggest that this population is recruited into the tumour during the inflammatory response, and might play a role in anti-tumoural immune responses. Indeed tumour-specific T cells could be found in the CCR4⁺ CXCR3⁺ compartment, as well as in CCR4⁺ CXCR3⁻ and CCR4⁻ populations, and functionality of tumour-specific T cells, in terms of cytokine production, appears to differ depending on their phenotype. However, given the limited number of tumour samples and the issue of reduced CXCR3 expression following tumour dissociation, it was difficult to discriminate whether tumour-infiltrating T cells were enriched in CCR4⁺CXCR3⁺ T cells. Nevertheless expression of CCR4 and CXCR3/PD-1 in peripheral blood/tumour-infiltrating cells appeared to be associated with different functional capacity.

While there is evidence that the CCR4⁺ CXCR3⁺ phenotype is relatively stable and can be maintained over time, this population seems heterogeneous and single-cell sorting may help to determine whether there is a previously unidentified CD8⁺ T cell population with unique properties within CCR4⁺ CXCR3⁺ cells.

Dissecting the heterogeneity of tumour infiltrating T cells, I confirmed that tumour-specific T cells are present in the PD-1+ populations, but I was also able to identify microbe-specific T cells within PD-1- cells. It is possible that the promising results of novel therapies such as ImmTACs could be due to the targeting of intratumoural microbe-specific T cells, which lacking PD-1, should be unexhausted and functionally competent. Whether T cells recognising particular microbes are preferentially recruited to particular tumours, and whether this is dependent on the location the microbe-specific T cells previously encountered antigen, is unclear however the potential to influence the degree of tumour infiltration by functionally-competent cells for use in conjunction with molecules such as ImmTACs warrants further investigation.

6.3 Regulation of IL-10 expression in myeloid cells

IL-10 is a cytokine with important regulatory properties and an inability or a reduced capacity to produce this cytokine is associated with inflammation and immunopathology. It is also an important feature of immunosuppressive cells in cancer, including both T cells and myeloid cells. Focussing on myeloid expression, I analysed the regulation of IL-10 through disruption of a putative enhancer +1.2kb downstream of the IL-10 TSS. As a model of IL-10 expression in myeloid cells the THP-1 cell line was used. The IL-10 locus in THP-1 cells has a similar epigenetic profile as in primary human monocytes, with similar PU.1 binding sites and similar acetylation of histones at the +1.2kb site. Potential PU.1 consensus sites within the +1.2kb region were deleted using CRISPR-Cas9 technology resulting in a failure of this transcription factor to bind and rendering this site closed in conformation.

Contrary to expectations, deletion of this site resulted in slightly greater induction of IL-10 in modified clones compared to wild-type clones suggesting that perhaps this site is involved in negative regulation of the IL-10 gene. However, the increase in expression was only minor and could be dependent on other issues such as heterogeneity of the THP-1 cell population.

As well as raising the issue of heterogeneity, these experiments have raised the possibility that the CRISPR-Cas9 single cell cloning process may have an adverse effect on the capacity of THP-1 cells to produce IL-10. Furthermore, redundancy among regulatory sites may make it difficult to observe differences in expression following deletion of only one site. A more comprehensive analysis of the regulatory regions, considering sites outside of the IL-10 locus (and subsequent deletion alongside the +1.2kb site) in bulk THP-1 cells or even primary human monocytes using the lentiviral CRISPR-Cas9 system, could enable a greater understanding of the regulation of this gene in myeloid cells.

Appendices

Appendix 1

A1.1 Analysis of activated (CD10+) and immature (CD10-) low-density neutrophil frequency and stratification according to last previous treatment

An enrichment in the frequency of activated neutrophils is observed in the peripheral blood of patients compared to healthy controls when analysis is extended to cover a larger number of patients (figure A1.1 (1)). Although the frequency of immature neutrophils does not reach significance, it is clear that at least a subset of patients have a higher frequency of these cells compared to healthy controls (figure A1.1 (1)).

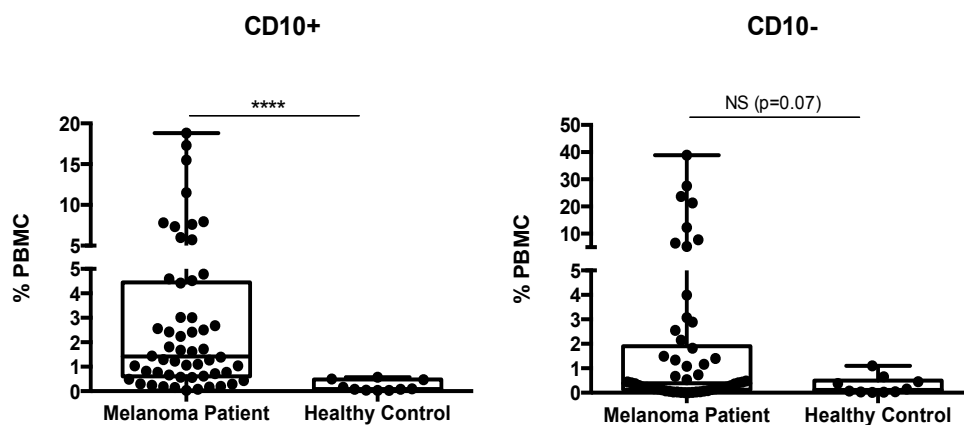


Figure A1.1(1): Analysis of the frequency of activated and immature neutrophils in the peripheral blood of patients with stage IV melanoma (extended to include a larger number of patients without use of unique patient symbols). Median and interquartile range shown. Difference in distribution determined using Mann-Whitney U Test. **** $p < 0.0001$, NS: non-significant.

A large number of patients in this cohort had not received any previous systemic treatment ('naïve'), however others had received chemotherapy, kinase inhibitors, anti-CTLA-4 immunotherapy or other treatment prior to providing a blood sample. Although blood was taken at least one month after cessation of previous treatment, I wished to assess whether previous treatment was responsible for the differences observed in the abundance of neutrophil

populations. Patients were grouped according to last previous treatment and the frequency of neutrophil populations shown in figure A1.1 (2). The corresponding statistical analysis is summarised in table A1.1. While certain groups, such as ‘chemotherapy’, ‘BRAF/MEK/Pan-RAFi’ and ‘other’ contain five or fewer patients and thus make it difficult to draw solid conclusions from, it is clear that there are at least certain patients (excluding activated neutrophils in kinase inhibitor-treated group) with an enrichment of either population over healthy controls. Of interest, is the frequency of activated neutrophils in the anti-CTLA-4-treated group, which is increased compared to systemic treatment naïve patients. It should be noted that all patients previously treated with anti-CTLA-4 had disease progression and were returning to clinic to start treatment with anti-PD-1 at the time of blood collection. Whether the increase in activated neutrophil frequency in this group of patients reflects an effect of this drug on activated neutrophil frequency or whether this reflects the fact that this group of patients, having failed anti-CTLA-4 (and in all cases at least one other therapy), may have a greater tumour burden. It may also be a combination of these factors.

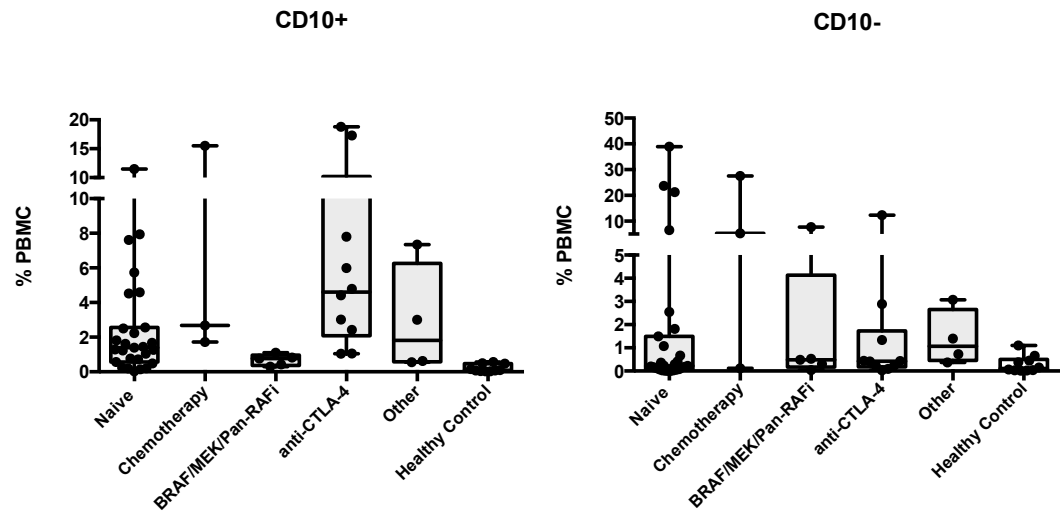


Figure A1.1 (2) Analysis of activated and immature neutrophil frequency following stratification of stage IV melanoma patients according to last previous treatment. Naïve denotes those who have never received prior systemic therapy. BRAF/MEK/Pan-RAFi refers to those patients who have received an inhibitor of any of these three kinases. Healthy control values are also shown. Median values and interquartile ranges are shown. Statistical analysis of the difference in distribution between groups is summarised in tables A1.1 A and B.

Table A1.1: Summary of the statistical differences in distribution between stage IV melanoma patients, grouped according to last previous treatment, along with healthy controls. A: p values for the differences in distribution of activated CD10+ frequency B: p values for the differences in distribution of immature CD10- frequency. Differences in distribution was determined according to the Mann-Whitney U Test

A

Activated CD10+	Naïve	Chemo-therapy	BRAF/MEK/Pan-RAFi	Anti-CTLA-4	Other	Healthy
Naïve						
Chemotherapy	NS (0.10)					
BRAF/MEK/Pan-RAFi	NS (0.12)	*				
Anti-CTLA-4	*	NS (0.71)	**			
Other	NS (0.84)	NS (0.63)	NS (0.41)	*		
Healthy	****	**	*	****	**	

B

Immature CD10-	Naïve	Chemo-therapy	BRAF/MEK/Pan-RAFi	Anti-CTLA-4	Other	Healthy
Naïve						
Chemotherapy	NS (0.28)					
BRAF/MEK/Pan-RAFi	NS (0.50)	NS (0.57)				
Anti-CTLA-4	NS (0.40)	NS (0.37)	NS (0.76)			
Other	NS (0.15)	NS (0.63)	NS (0.41)	NS (0.30)		
Healthy	NS (0.30)	NS (0.11)	NS (0.25)	NS (0.14)	*	

A1.2 Analysis of the immune-suppressive capacity of low-density granulocytes identified from patient with stage IV melanoma

Low-density CD15+ cells, defined as grMDSCs, have previously been identified in patients with stage IV melanoma (Schilling et al., 2013). However, in this instance, the suppressive capacity of total low-density CD15+ cells was tested, rather than separating cells into distinct populations. Furthermore, like numerous publications in the MDSC field, a sub-optimal method was used for determining immune-suppressive capacity of these cells. Frequently, the suppressive capacity of MDSCs is revealed by the capacity of such cells to suppress T cell proliferation. Commonly, T cells are stimulated with anti-CD3

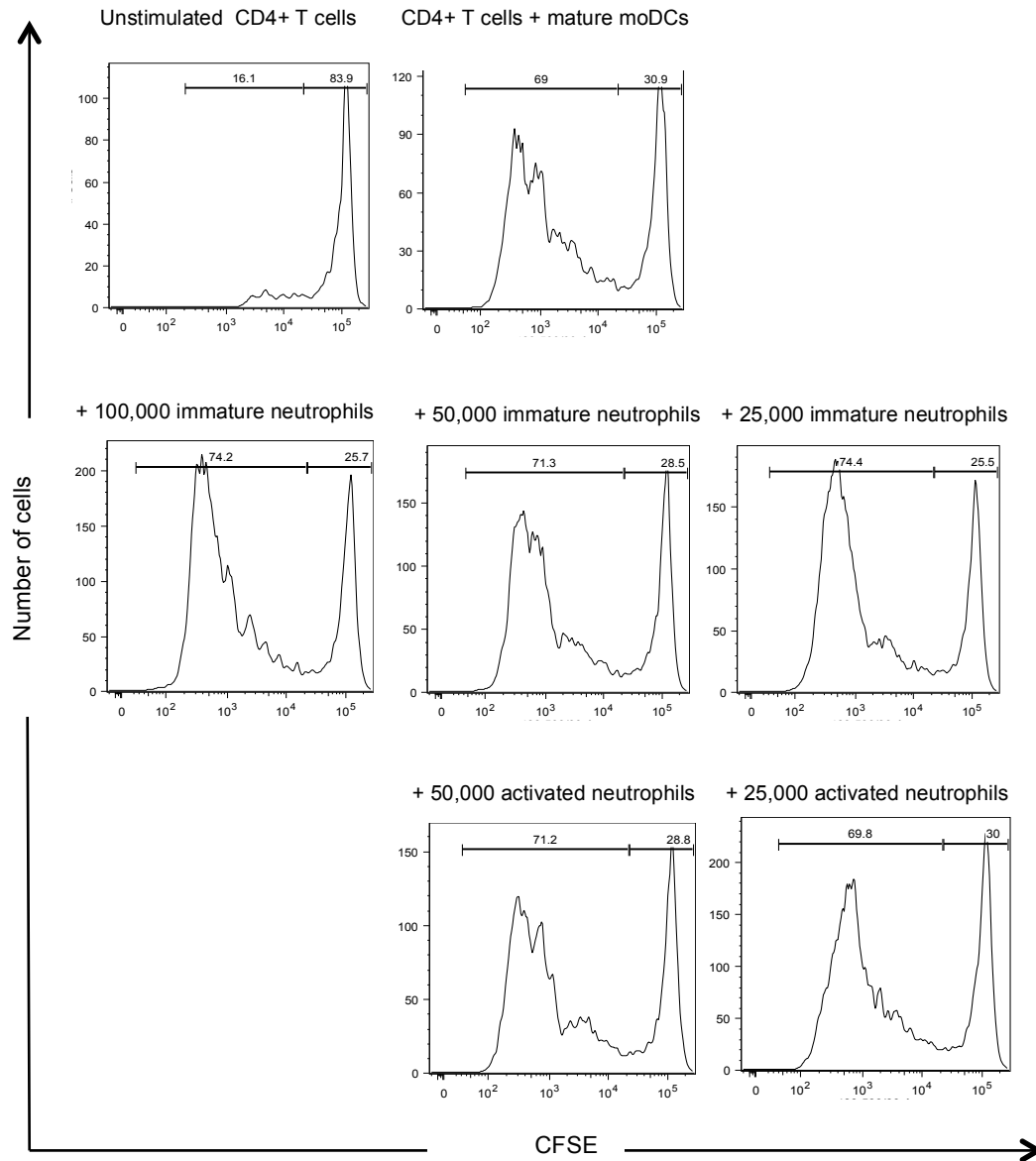
and/or anti CD28 antibodies. However, neutrophils can produce ROS and as ROS have the capacity to alter protein structure, they may affect the binding of such antibodies to T cells and therefore the degree of T cell stimulation. Furthermore, if these antibodies are coated on magnetic beads for example, and such beads are used to stimulate T cell proliferation, it may be that neutrophils, being phagocytic cells, may take up the beads and thus reduce T cell proliferation by reducing the degree of stimulation independent of any T cell suppressive capacity (Hock and McKenzie, 2013).

I stimulated T cells from one donor with DCs from another donor, akin to a mixed leukocyte reaction (MLR) in which MHC mismatch drives T cell proliferation. In vitro generation and maturation of DC from monocytes takes approximately five days. Given the unpredictability of patient samples, I aimed to characterise the capacity of activated and immature neutrophils to suppress the proliferation of T cells stimulated either in an MLR or via anti-CD3/28 antibodies (with controls to confirm that such antibodies were not damaged).

Assay 1: Testing proliferation of CD4⁺ T cells, stimulated with mature moDCs, in the presence or absence of sorted activated or immature neutrophil populations

Proliferation, as measure by CFSE dilution, was measured after 5 days stimulation as shown in figure A1.2 (1). Although there is some unexpected proliferation in the unstimulated T cell population, the proportion of CFSE low cells and the position of the CFSE low peaks reveals that extensive CD4⁺ T cell proliferation has occurred within 5 days of culture with mature moDC. As seen in the FACS histograms and quantification of CFSE low cells in the graph below, T cell proliferation is unaffected by the addition of activated or immature neutrophils at the beginning of the stimulation.

A



B

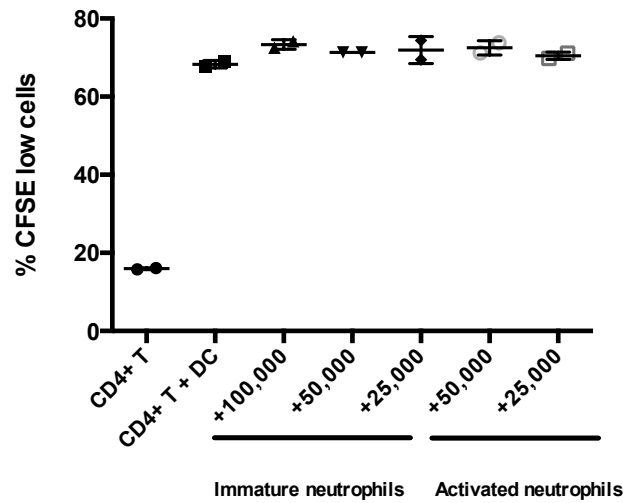


Figure A1.2 (1): Failure to detect suppression of CD4+ T cell proliferation by activated or immature neutrophils. A: Histograms revealing the degree of CFSE dilution in unstimulated CD4+ T cells or CD4+ T cells stimulated in the presence or absence of varying numbers of FACS-sorted activated or immature neutrophils at day 5. B: Quantification of duplicates. 25,000 mature moDCs were used to stimulate 50,000 T cells in the presence or absence of neutrophil populations.

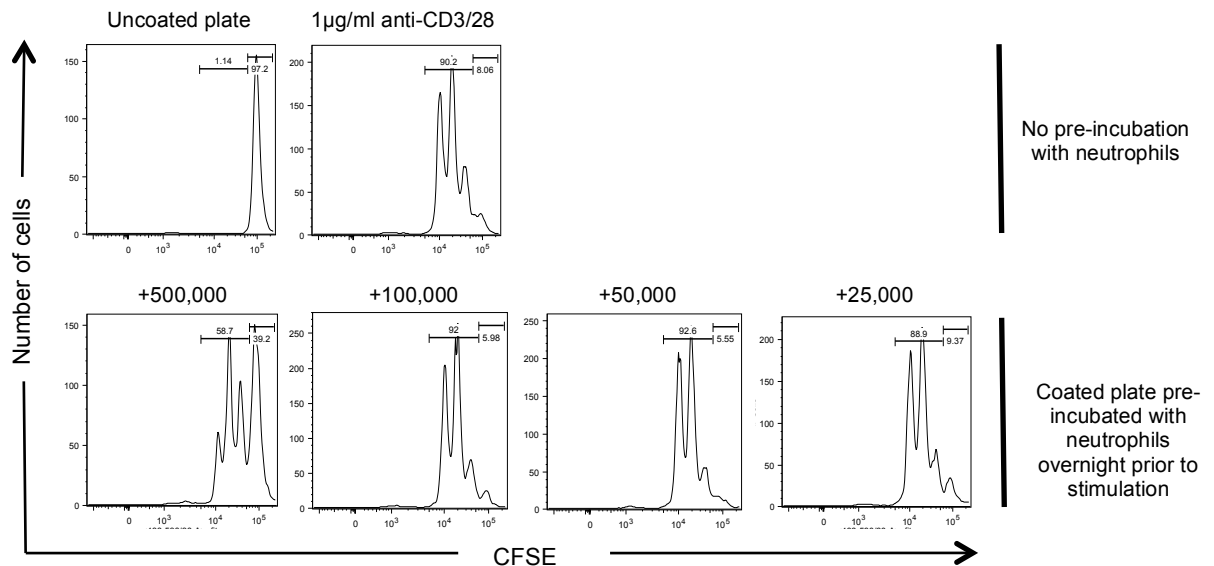
It may be that neither activated nor immature neutrophils are capable of suppressing CD4+ T cell proliferation. However, it is possible that they exert suppression early on in T cell activation but by examining T cell proliferation at day 5, such suppression may not be observed. I therefore attempted to repeat this assay and look at day 3 and 4 post-stimulation. However, a combination of issues with batches of human serum (which I believe resulted in the failure of allogeneic DC-induced T cell proliferation) and the difficulty in obtaining sufficient numbers of activated and immature neutrophils, meant I was unable to successfully test the suppressive capacity of these cells at an earlier time-point in this assay.

In parallel to testing suppression in an MLR, I investigated whether anti-CD3/28-coated plates may be a suitable alternative.

Assay 2: Testing proliferation of T cells on anti-CD3/28-coated plates and whether such plates could be damaged in the presence of neutrophils

Tissue culture plates were coated with 1µg/ml anti-CD3/28 antibodies. Wells were incubated overnight in the presence of varying numbers of mature neutrophils from a healthy donor, or in the presence of medium alone. The following day, wells were washed to remove neutrophils and pan T cells were added. Cells were stimulated for 3 days and proliferation was measured by CFSE dilution, as seen in figure A1.2 (2). Pre-incubation with an excess of neutrophils (500,000 per well) results in a reduction in the capacity of those wells to stimulate T cell proliferation following neutrophil removal, suggesting that neutrophils can indeed damage anti-CD3/28 antibodies. However, pre-incubation with fewer neutrophils (in line with the numbers of cells I would use in a suppression assay) fails to affect the degree of T cell proliferation. However, it would be interesting to see whether this is also true following pre-incubation with 25,000-100,000 stimulated neutrophils or neutrophils from a stage IV melanoma patient.

A



B

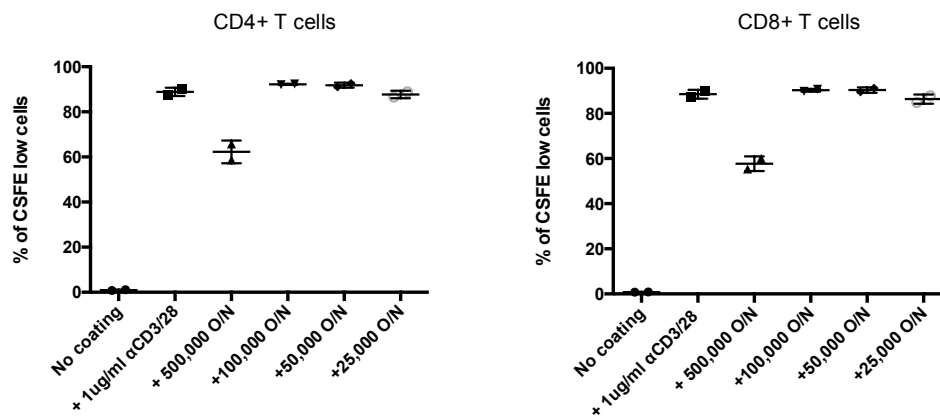


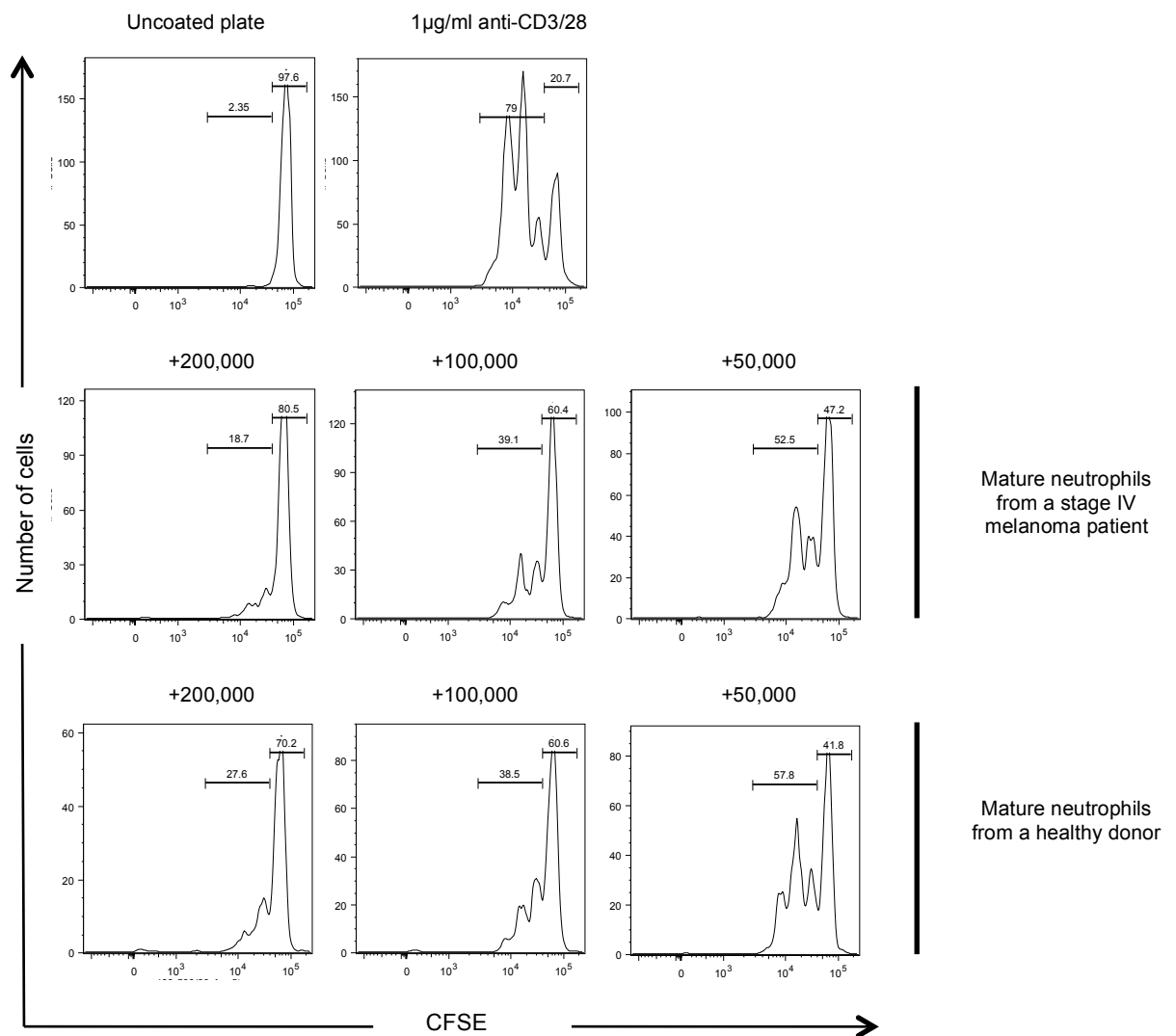
Figure A1.2 (2): Pre-incubation of anti-CD3/28 coated plates with 500,000 neutrophils interferes with the capacity of anti-CD3/28 to stimulate T cell proliferation. Proliferation is unaffected following incubation with lower numbers of neutrophils. A: Pan T cells were stimulated in various conditions and example histograms showing CFSE dilution for CD4+ T cells are shown. **B:** Quantification of CFSE low cells for both CD4+ and CD8+ T cells. 50,000 T cells were stimulated.

Due to questions regarding which assay is appropriate to study the suppressive capacity of neutrophils, it would be useful to have both a positive and negative control for suppression. Mature neutrophils from stage IV melanoma patients have previously been shown to suppress T cells, whereas those from healthy donors do not (De Santo et al., 2010).

Assay 3: Establishing whether anti-CD3/28-coated plates are useful in studying the suppressive capacity of neutrophils, using mature neutrophils isolated from the peripheral blood of stage IV melanoma patients and healthy controls

From figure A1.2 (3)B, in the presence of neutrophils from stage IV melanoma patients or healthy donors, there is suppression of T cell proliferation. This suppression is titratable, with less suppression (and a higher percentage of CFSE low cells) when fewer neutrophils are added to the T cells at the beginning of stimulation.

A



B

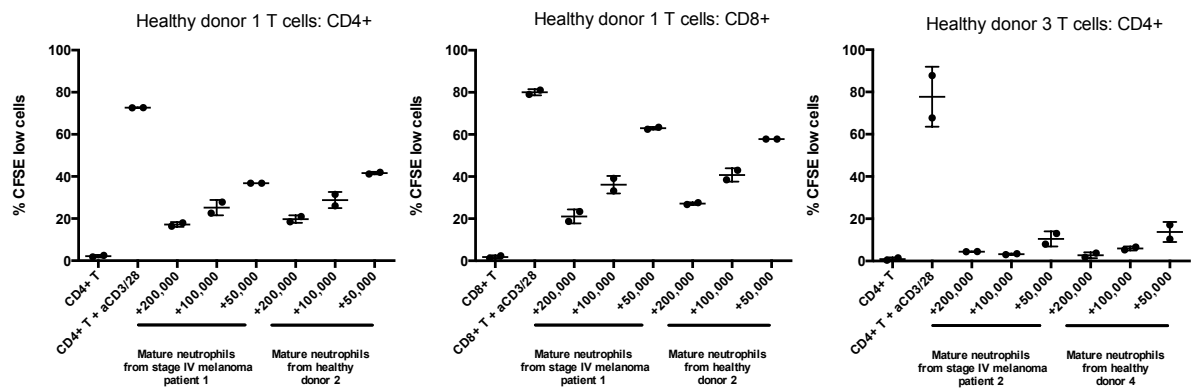


Figure A1.2 (3): Detection of suppression of T cell proliferation by mature neutrophils from both stage IV melanoma patients and healthy controls. A: Example FACS histograms to show the degree of CFSE dilution/proliferation in donor 1's CD8+ T cells when stimulated with 1 μ g/ml CD3/28 (coated plate) compared to unstimulated cells ('uncoated plate'). Proliferation of CD8+ T cells stimulated with 1 μ g/ml CD3/28 (coated plate) in the presence of 200,000, 100,000 and 50,000 mature neutrophils, purified from either a stage IV melanoma patient or healthy donor, are also shown. B: Quantification of proliferation of donor 1's CD4+ and CD8+ T cells stimulated with 1 μ g/ml CD3/28 (coated plate), in the presence or absence of mature neutrophils from a stage IV melanoma or healthy donor. This was repeated using different CD4+ T cells and neutrophils from a different stage IV melanoma patient and healthy control (right panel). 50,000 T cells were stimulated per well.

Suppression by healthy donor neutrophils was unexpected. Neutrophils activated in response to LPS stimulation have previously been shown to possess T cell suppressive function (Pillay et al., 2012). As neutrophils are known to be sensitive to purification, it is possible that during purification, neutrophils were activated and acquired suppressive capacity.

As well as difficulties in obtaining positive and negative controls of T cell suppression. Attempts were also confounded by the inability to sort large number of cells and the unpredictability of cell frequency within blood samples. Although I investigated the use of carrying out MLR type reactions in 384 well plates (data not shown), I was still unable to sort sufficient numbers of neutrophils to determine whether activated or immature neutrophils from the peripheral blood of stage IV melanoma patients are capable of suppressing T cell proliferation.

A1.3 Comparison of plasma cytokine concentrations in patients with BRAF V600E mutation

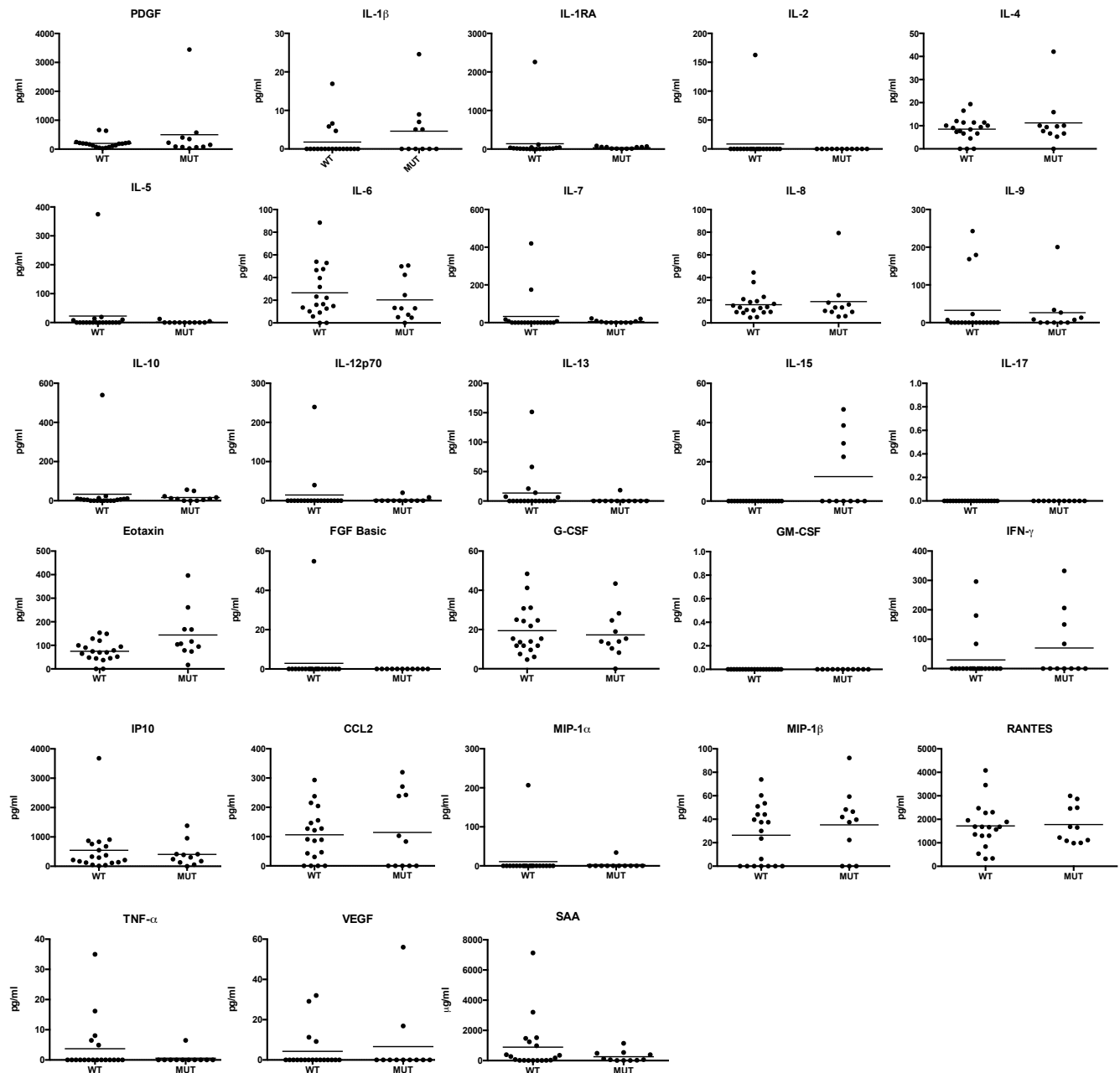


Figure A1.3: Plasma concentration of 28 cytokines and growth factors in wild-type versus BRAF (V600E) mutant stage IV melanoma patients. There is no statistical difference between the mean concentration of cytokine/growth factor in plasma of wild-type versus BRAF mutant patients, except for IL-15 and Eotaxin which are increased in the plasma of patients with the BRAF mutation ($p=0.01$ and $p=0.02$ respectively). Median value is marked. Differences in distribution were determined using the Mann-Whitney U test.

A1.4 Survival of stage IV melanoma patients according to frequency of activated neutrophils in the peripheral blood

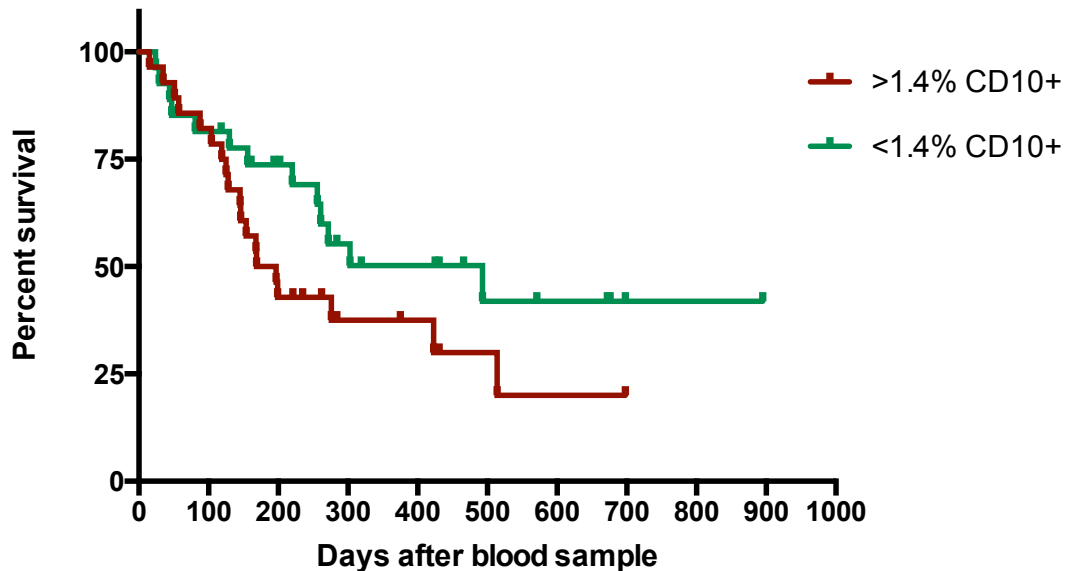


Figure A1.4.1: Kaplan-Meier survival curves for melanoma patients according to frequency of CD10+ low-density (activated) neutrophils. Overall survival is greater in patients with a lower frequency of activated neutrophils at the time of sampling (median survival 398 days) compared to those with a higher frequency of CD10+ neutrophils (median survival 183 days). However, the difference in overall survival curves does not reach significance ($p=0.12$).

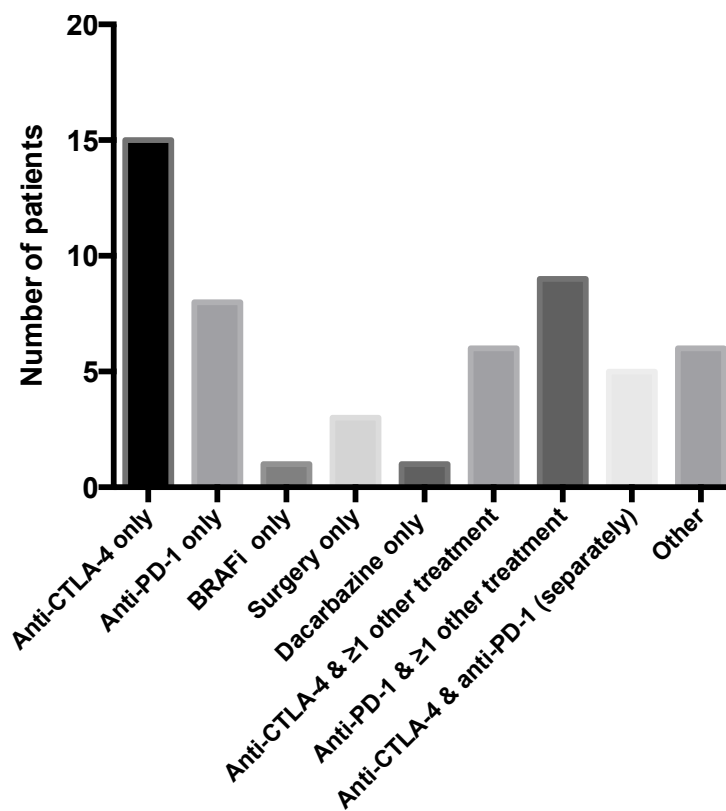


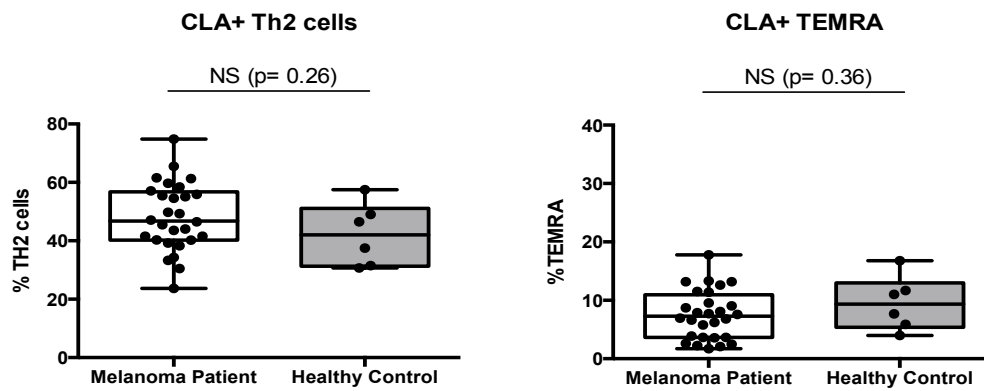
Figure A1.4.2: Treatment following blood sample collection for patients shown in A1.4.1.

Appendix 2

A2.1 Expression of the skin-homing marker cutaneous leukocyte-associated antigen receptor (CLA) on peripheral blood lymphocytes

There is no difference in the percentage of T_H2 or TEMRA cells expressing CLA (figure A2.1A). There is an enrichment of CLA positive cells within the CCR4+ populations of CD8+ T cells (figure A2.1B left panel) however there is no difference in the expression of CLA in CCR4+ CXCR3-, CCR4+ CXCR3+ or CCR4- populations in stage IV melanoma patients compared to healthy controls (figure A2.1B right panel).

A



B

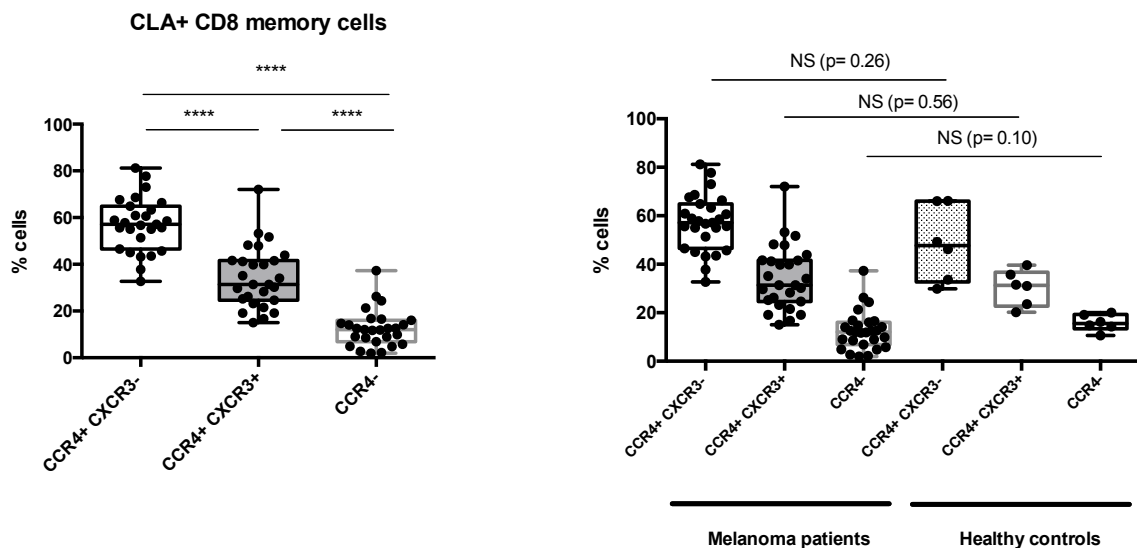


Figure A2.1 Expression of the skin-homing marker cutaneous leukocyte-associated antigen receptor (CLA) on peripheral blood lymphocytes. A: Expression of CLA on CD4+ T_H2 cells and the CD8+ TEMRA subset in stage IV melanoma patients compared to healthy controls. B: Expression of CLA on effector memory CD8+ T cell subsets that differentially express CCR4 and CXCR3. The median and interquartile range are shown and the difference in distribution was determined using the Mann-Whitney U Test. ****: $p < 0.0001$. NS: non-significant.

A2.2 Expression of CCR7 and CD45RA on CD8+ T cells infiltrating metastatic melanoma lymph nodes

Analysis of CCR7 and CD45RA expression of CD8+ T cells within three metastatic melanoma lymph nodes reveals the absence of CCR7+ CD45RA+ (naïve) T cells supporting the notion that these metastatic lymph nodes are no longer functioning as lymph nodes and hence T cells within these metastases should be considered as tumour-infiltrating T cells.

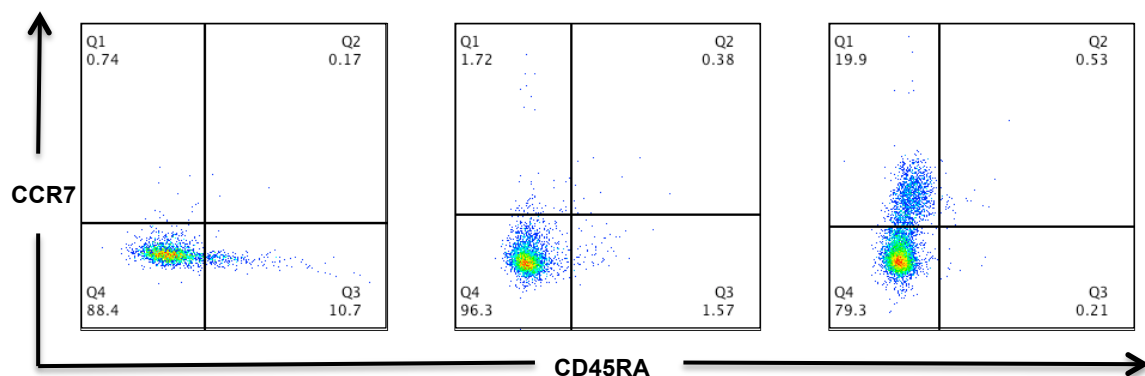


Figure A2.2: Expression of CD45RA and CCR7 on T cells in metastatic lymph nodes from three patients. An absence of CD45RA+ CCR7+ naïve T cells within lymph nodes.

A2.3 Upregulation of CXCR3 on tumour-infiltrating CD8+ T cells after overnight incubation at 37°C

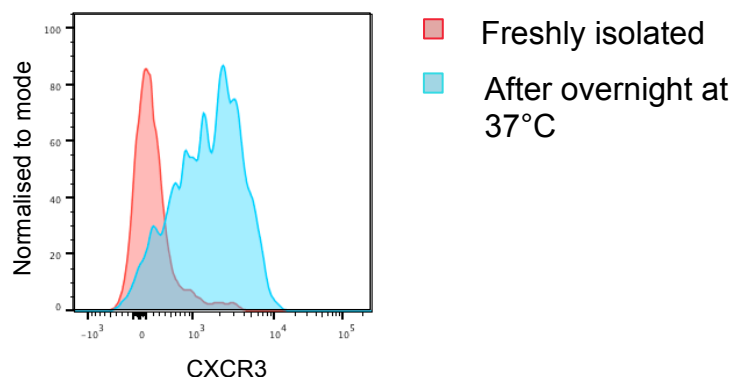


Figure A2.3: CXCR3 expression on CD8+ T cells isolated from melanoma metastases. Upregulation of CXCR3 expression on the surface of CD8+ T cells after overnight incubation at 37°C (turquoise histogram) in comparison to CXCR3 expression on freshly isolated CD8+ T cells (red histogram).

Appendix 3

A3.1 Stimulation of wild-type and modified THP-1 clones with lower concentrations of LPS

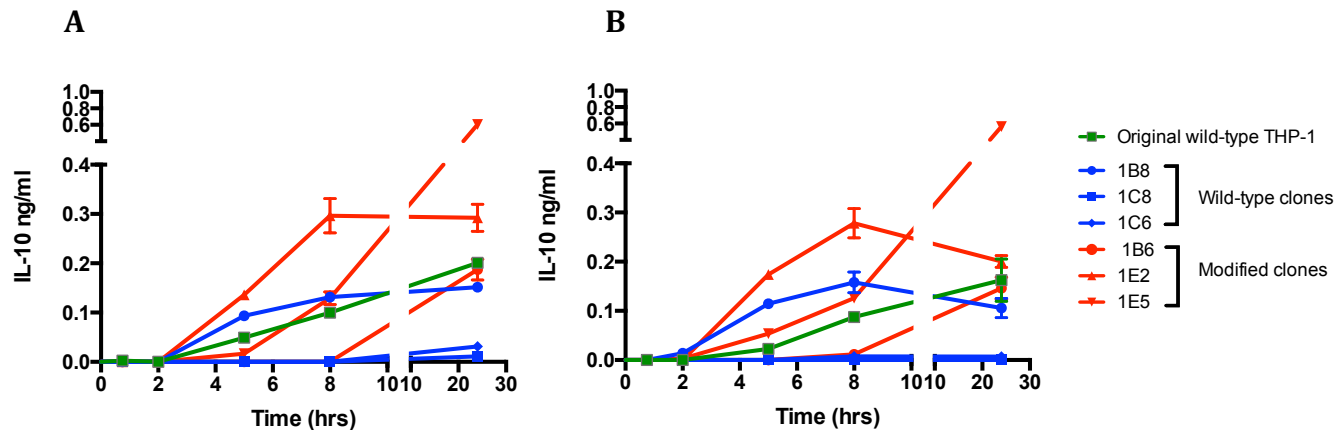


Figure A3.1: A: Analysis of IL-10 protein production by THP-1 cells stimulated with 200ng/ml LPS. B: Analysis of IL-10 protein production by THP-1 cells stimulated with 40ng/ml LPS. Mean and standard deviation of triplicate values shown.

A3.2 Stimulation of wild-type and modified THP-1 cells with SAA

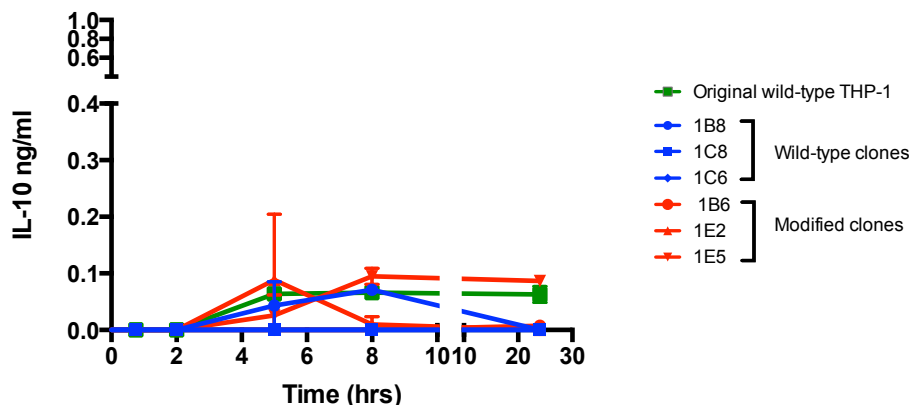


Figure A3.2: Analysis of IL-10 protein production by THP-1 cell stimulated with 1µg/ml SAA. Mean and standard deviation of triplicate values shown.

A3.3 Analysis of IFN- β , IL-27 and IL-10 expression in wild-type THP-1 cells in response to LPS stimulation

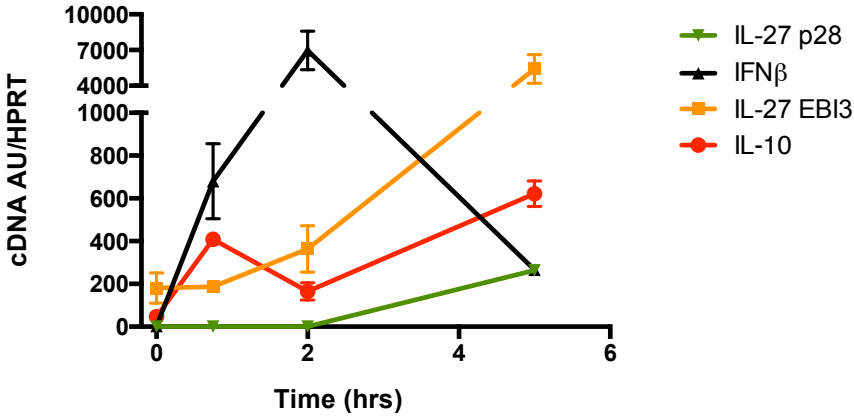


Figure A3.3 Kinetics of expression of IFN- β , IL-27 and IL-10 over the course of five hours following stimulation with 1 μ g/ml LPS, as measured by qPCR.

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